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Departamento de Parasitología

Búsqueda de nuevos fármacos de actividad antiparasitaria frente a *Leishmania* spp y *Trypanosoma cruzi*

TESIS DOCTORAL

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Búsqueda de nuevos fármacos de actividad antiparasitaria frente a *Leishmania* spp y *Trypanosoma cruzi*

Memoria de Tesis Doctoral presentada por
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por la universidad de Granada.

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A mis padres.

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ÍNDICE

1.- RESUMEN.

Actualmente, miles de personas en todo el mundo, están afectadas por las llamadas enfermedades olvidadas (Neglected diseases) para las cuales no se dispone de tratamientos eficaces o adecuados. En su mayoría se trata de enfermedades tropicales infecciosas, causadas por parásitos protozoarios, que afectan fundamentalmente a la población de los países menos desarrollados. Se estima que casi el 50% de la población mundial está expuesta a estas infecciones, y que aproximadamente 500 millones de personas sufren cada año patologías relacionadas con este tipo de enfermedades (http://www.who.int/neglected_diseases/en/). Las enfermedades olvidadas son la leishmaniasis, la oncocercosis, la enfermedad de Chagas, la lepra, la tuberculosis, la esquistosomiasis, la filariasis linfática, la tripanosomiasis africana, y el dengue. (Hunt P. TDR/SDR/SEB/ST/07.2) Muchas enfermedades olvidadas son potencialmente mortales, y otras dan como resultado una elevada discapacidad. Los parásitos que causan estas dolencias son muy conocidos desde los puntos de vista de la biología, inmunología y genética, pero la investigación y desarrollo de herramientas terapéuticas se encuentra en estado embrionario.

La enfermedad de Chagas o Tripanosomiasis Americana, es una de las parasitosis más importantes en humanos, tanto por su prevalencia como por la gravedad de su cuadro clínico. Su agente etiológico es el protozoo flagelado *Trypanosoma cruzi,* descubierto por Carlos Chagas en 1909. Este parásito es endémico de América Latina, donde afecta a aproximadamente 10-12 millones de

personas y mata a más de 15.000 personas cada año. Actualmente, esta enfermedad es considerada como una enfermedad globalizada ya que han aparecido casos en zonas no endémicas, habiendo cientos de miles de personas infectadas en Europa (principalmente España y Portugal), Estados Unidos, Canadá, Japón y Australia. Los afectados suelen ser inmigrantes sudamericanos que a menudo desconocen que están infectados, lo cual tiene importantes implicaciones de salud pública para la gestión de bancos de sangre y prestación de asistencia sanitaria. (Clayton 2010).

La enfermedad de Chagas se transmite a los humanos y a más de 150 especies de animales tanto salvajes como domésticos de forma vectorial por insectos de la subfamilia Triatominae. Aunque se conocen más de 130 especies de triatominos, sólo tres de ellas son vectores de *T. cruzi*: *Triatoma infestans*, *Rhodnius prolixus*, y *Triatoma dimidiata*. *Tri. infestans* es el vector más importante, y es el principal vector en regiones endémicas sub-Amazónicas. *R. prolixus* es el principal vector en el norte de América del Sur y América Central, y *Tri. dimidiata* ocupa un área similar, pero también se extiende más al norte, a México. La enfermedad se transmite vectorialmente en un 80% de los casos y en un 20% por transfusión de sangre contaminada o por vía congénita. (Rassi y col. 2010).

En humanos, la enfermedad presenta dos fases, una aguda que aparece poco tiempo después de la infección. En esta fase aparecen los siguientes síntomas: fiebre, malestar, linfadetis, hepatomegalia y esplenomegalia, y, la otra crónica tras un

periodo silencioso o asintomático que puede durar varios años. Las lesiones de la fase crónica afectan irreversiblemente órganos internos como el corazón, el esófago, el colon y el sistema nervioso periférico. Varios estudios han puesto de manifiesto que un 27% de los individuos infectados desarrollan síntomas cardiacos que los pueden conducir a una muerte súbita, un 6% trastornos digestivos, principalmente megavísceras y un 3% trastornos del sistema nervioso periférico (Charmina y col. 2007).

Los medicamentos que se utilizan para Chagas son compuestos nitroheterocíclicos descubiertos empíricamente hace tres décadas: un nitrofurano, nifurtimox (Lampit® Bayer) y benznidazol, un derivado del nitroimidazol (Rochagan®, Radanil®, Roche) y que son poco eficaces en la fase crónica de la enfermedad.

Las leishmaniasis son otro grupo de importantes enfermedades de las regiones tropicales y subtropicales, producidas por parásitos protozoarios pertenecientes al género *Leishmania*, y transmitida por la picadura de insectos dípteros de los géneros *Phlebotomus*, en el Viejo Mundo y, *Lutzomyia* en el Nuevo Mundo (Laison y Saw 1987, Berman y Wyler 1980).

Para la Organización Mundial de la Salud (OMS), son afecciones cosmopolitas o endémicas que originan importantes problemas en la salud pública, debido a la amplia diversidad en las formas clínicas producidas según la especie de

Leishmania pudiendo tratarse de: lesiones cutáneas, mucosas o viscerales que pueden llegar a ser fatales (Murray y col. 2000). Especialmente relevante es el impacto psicológico en las personas afectadas por la forma mucocutánea que provoca mutilaciones difíciles de tratar (Brazil y Gilbert 1976).

Un gran número de animales, tanto domésticos como silvestres, actúan como hospedadores principales y reservorios de las especies de *Leishmania* que afectan a humanos (Ashford 2000). Entre ellas destaca el reservorio canino cuya sintomatología es frecuentemente a la vez cutánea y visceral (Dereure 1999).

La enfermedad se caracterizaba por malestar general, accesos febriles, anemia grave, atrofia muscular y esplenomegalia.

Más de 350 millones de personas en 88 países distintos padecen esta enfermedad y se estima que entre 1 y 2 millones de personas se infectan al año. (http://www.who.int/leishmaniasis/en/)

En el tratamiento la leishmanaiasis se utilizan desde hace más de 70 años derivados pentavalentes del antimonio como: Estibogluconato sódico (Pentostan) o la Meglumina antimoniato (Glucantime). Otros medicamentos utilizados son: Anfotericina B (AmBiosome®) que se administra durante un máximo de 10 días y no presentan toxicidad pero, es muy caro, la miltefosina es de administración oral pero el tratamiento dura 4 semanas y tiene restricciones de uso para gestantes y niños; la Pentamidina y el Ketoconazol.

Un agente tripanocida y leishmanicida debe ser:

- Selectivo y potente, tanto contra las formas intracelulares como contra las formas extracelulares.

- De acción rápida y completa.

- Efectivo para impedir la evolución de la fase aguda de la infección.

- Inocuo para la persona tratada.

- No debe inducir resistencia del parásito al medicamento.

- Su farmacodinámica debe alcanzar niveles afectivos tripanocidas de concentración de la droga en el plasma sanguíneo, en fluidos biológicos y en tejidos.

- Su modo de acción debe ser estable y, de preferencia, efectivo por vía de administración oral.

2.- JUSTIFICACIÓN Y OBJETIVOS.

Los Tripanosomátidos causan enfermedades muy extendidas en los seres humanos y animales. El tratamiento de muchas de estas enfermedades se ve obstaculizado por la falta de medicamentos eficaces y seguros. Debido a ello, son necesarias nuevas estrategias para el desarrollo de fármacos.

El presente trabajo tiene un objetivo global: la búsqueda de moléculas capaces de ejercer un potente efecto tripanocida y leishmanicida, que sean lo suficientemente selectivas para no causar daño al individuo hospedador.

Los principales puntos de estudio de la investigación planteada se centran en:

1. Selección de nuevos compuestos de síntesis y naturales activos que ofrezcan una alternativa a los fármacos existentes frente a *T. cruzi, L. infantum* y *L. braziliensis.*

2. Realizar pruebas de la actividad tripanocida y leishmanicida de los productos frente a las formas extracelulares e intracelulares.

3. Realizar pruebas de citotoxicidad sobre células vero y macrófagos.

4. Comparar la acción de estos productos con las drogas comerciales “Benznidazol” y “Glucantime”.

5. Estudiar la posible inhibición de la capacidad de invasión celular así como la de multiplicación, la diferenciación de las formas flageladas a formas amastigotas, y la diferenciación de estas últimas a formas flageladas del parásito (esto último en el caso de *T. cruzi*).

6. Estudiar su posible mecanismo de acción, a través del efecto de estos productos sobre las principales vías metabólicas, cambios estructurales y sobre la actividad de la superóxido dismutasa.

7. Estudios *in vivo* en modelo murino de los productos que presentaron buenos resultados frente a *T. cruzi.*

3.- INTRODUCCIÓN.

3.1.- Género *Trypanosoma.*

El género *Trypanosoma* es uno de los más importantes dentro de la familia *Trypanosomatidae* por causar importantes enfermedades.

T. cruzi, es el agente etiológico de la enfermedad de Chagas; *T. brucei rhodesiense* y *T. b. gambiense*, son los agentes etiológicos de la enfermedad del sueño; y *T. b. brucei, T. congolense, T. equiperdum y T. equinum*, causan enfermedades en animales.

3.1.1.- Clasificación taxonómica.

En función del comportamiento del parásito en sus hospedadores y principalmente en el vector, el género *Trypanosoma* se divide en dos grupos: estercoraria y salivaria.

El grupo llamado salivaria, incluye tripanosomas que se desarrollan en el tubo digestivo, posteriormente atraviesan el epitelio digestivo y llegan a las glándulas salivales, donde las formas infectivas son inoculadas mecánicamente. En este grupo se encuentran: *T. brucei, T. congolense.*

En el grupo estercoraria, se incluyen los tripanosomas que se desarrollan en el tubo digestivo del vector, progresando en el sentido de la porción intestinal con

liberación de las formas infectivas con las heces. En este grupo tenemos a *T. cruzi* y a *T. lewisi.*

Aunque estos grupos no presentan estatus taxonómico, fueron creados por Hoare (Hoare, 1972) para separar los tripanosomas de mamífero cuyo desarrollo ocurre exclusivamente en el intestino de los vectores, y son transmitidos por las heces, de los tripanosomas transmitidos por inoculación de formas metacíclicas en la saliva. (Grisard, 2002; De Santa-Izabel y col., 2004)

La clasificación, para *T. cruzi*, acordada por Levine (Levine y col., 1980) es la siguiente:

Reino: Protozoa (Goldfuss 1818)

Filo: Sarcomastigophora (Honigberg and Balaniuth 1963)

Subfilo: Mastigophora (Diesind 1866)

Clase: Zoomastigophorea (Calkins 1909)

Orden: Kinetoplastida (Honigberg 1963)

Familia: Trypanosomatidae (Doflein 1901)

Género: *Trypanosoma* (Gruby 1943)

Especie: *Trypanosoma cruzi*

3.1.2.- Morfología.

Los flagelados Tripanosomátidos tienen la posibilidad de pasar a lo largo de su ciclo vital por varias formas, distinguibles entre sí por la zona del nacimiento del flagelo y la posición relativa entre el núcleo y el kinetoplasto (ADN mitocondrial compuesto de maximinicírculos y minicírculos). Estas formas reciben los nombres de amastigotas, epimastigotas y tripomastigotas.

En el hospedador mamífero, *T. cruzi* aparece en las formas tripomastigotas y amastigotas, mientras que, en el vector se desarrolla como epimastigotas y tripomastigotas metacíclicas.

Tripomastigota sanguíneo. Son pequeñas (15-20 μm), de forma alargada, con el kinetoplasto grande alejado de la parte anterior del nucleo, presenta flagelo y membrana ondulante. Este estadio se encuentra en la sangre y no tiene capacidad de dividirse, pero si la tiene para invadir otras celulas del hospedador vertebrado parasitado. Presenta pleomorfismo con formas delgadas, rechonchas e intermedias.

Amastigota. Se encuentra en el citoplasma de determinadas células (en una vacuola parasitófora), principalmente macrófagos, células musculares, etc., donde se multiplica continuamente por fisión binaria. (Guzman-Marin y col., 1999) Es

esferoidal u oval de pequeño diámetro (2-5 μm). Presentan núcleo, kinetoplasto y un flagelo externo muy corto. (Hoare y Wallace, 1966).

Epimastigota. Es la forma replicativa, no infectiva para el ser humano o mamífero, flagelados anchos, muy móviles, con el kinetoplasto entre el núcleo y el flagelo libre, con 20 a 25 μm de longitud. Este estadio morfológico se multiplica de manera profusa en el intestino de los triatominos para dar lugar a las formas tripomastigotas metacíclicas.

Tripomastigota metacíclica. Es una forma no replicativa pero infectiva para el ser humano u otros mamíferos. Tiene forma alargada y mide unas 20 a 25 μm de longitud. Se distingue por presentar un núcleo vesiculoso y hacia la parte posterior de éste se localiza el kinetoplasto de forma casi siempre esférica. El flagelo, con su membrana ondulante, se observa a lo largo del cuerpo del parásito y surge libremente en el extremo posterior.

3.1.3.- Ciclo biológico.

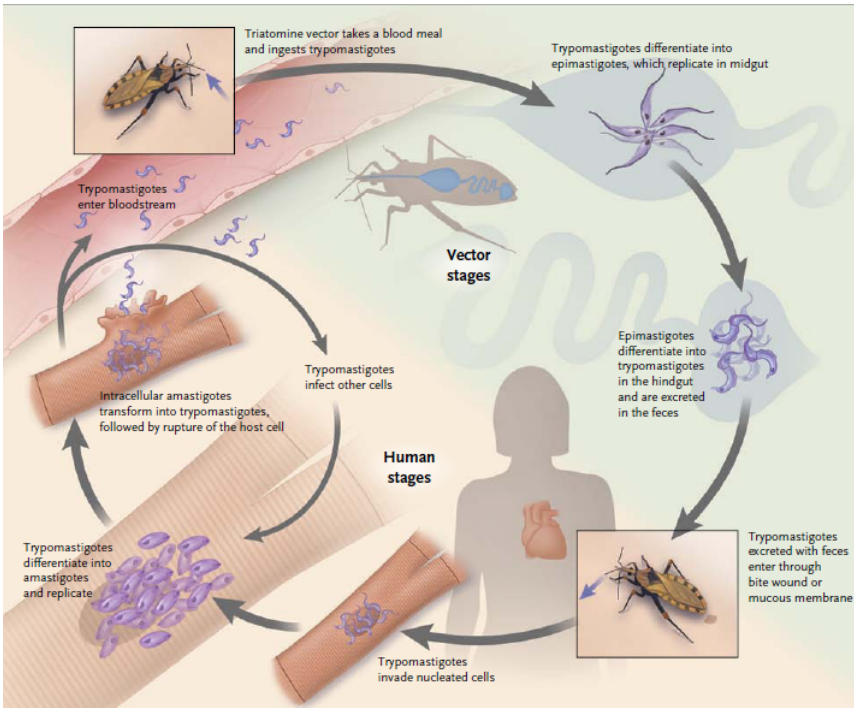
El parásito presenta un ciclo de vida heteroxeno, que requiere la presencia de dos hospedadores: vector (triatomino) y el hospedador definitivo (mamífero).

Los triatominos nacen libres de infección, infectándose al alimentarse de hospedadores infectados. El ciclo de desarrollo puede realizarse en todos los

estadios de ninfas o adulto del vector. Las tripomastigotas migran al intestino medio del insecto, donde se transforman en epimastigotas. Allí se multiplican por fisión binaria un gran número de veces, dando lugar nuevamente a tripomastigotas metacíclicas, que migran al intestino posterior, desde donde son excretados con las heces. (Stevens y col., 1999; 2001).

Los triatominos tras alimentarse suelen defecar, liberando en sus heces las formas tripomastigotas metacíclicas que pueden entrar al hospedador a través de las mucosas o heridas. El mamifero al rascarse inocula las formas tripomastigotas en la herida de la picadura, por lo que las especies de vectores que presentan un retraso en la defecacion tienen menos probabilidad de transmitir la enfermedad de Chagas que los que defecan en el hospedador (de Souza y col., 2010). En el nuevo hospedador, las formas metacíclicas pueden invadir inmediatamente las células en la puerta de entrada (macrófagos, fibroblastos o células epiteliales) o ser diseminados por la circulación linfática hasta otras localizaciones, transformándose en amastigotas. Las amastigotas se multiplican intracelularmente, y sufren una nueva transformación hasta tripomastigotas, que ocasionan la lisis de la célula, permitiendo su salida al torrente sanguíneo. Estas tripomastigotas pueden infectar otras células colindantes, pero carecen de capacidad multiplicativa, ya que la única forma replicativa en el vertebrado es la forma amastigota intracelular.

La infeccion puede adquirirse tambien por via oral cuando la comida o liquidos estan contaminados con *T. cruzi* y son ingeridos por animales o el hombre. (Coura, 2006).



(Antitrypanosomal therapy for chronic Chagas' disease. *N Engl J Med.* **364:** 2527-2534.)

3.1.4.- Diagnóstico.

El diagnóstico de la Enfermedad de Chagas, se realiza mediante varios métodos:

Examen microscópico. Se lleva a cabo mediante la observación al microscopio óptico de un frotis sanguíneo teñido de una muestra de la capa leucocitaria o de tejidos como el corazón. Las formas tripomastigotas sanguíneas son alargadas y en las preparaciones teñidas tienen forma de C. Las formas amastigotas están en el interior celular y son redondas u ovoides. El problema de esta técnica es que es difícil diferenciar morfológicamente a *T. cruzi* de otras especies no patógenas como *T. rangeli*. Además, las formas sanguíneas sólo se pueden observar cuando la parasitemia es alta, en la fase aguda o de reactivación (CFSPH, 2009).

Aislamiento del agente. *T. cruzi* puede ser cultivado a partir de muestras de sangre o tejidos, y suele ser más exitoso cuando se toma la muestra en la fase aguda. El organismo va a encontrarse en el miocardio pero también se puede aislar de otros órganos como los ganglios linfáticos, hígado, tracto gastrointestinal, cerebro, líquido cefalorraquídeo. También se usa el xenodiagnóstico para su aislamiento, inoculando sangre sospechosa de estar infectada en ratones o ratas; este método es el más usado en América del Sur, aunque suele llevar bastante tiempo (de 1 a 6 meses) y personal especializado (CFSPH, 2009).

La sensibilidad del xenodiagnóstico varía del 9% al 87,5%, y de los hemocultivos del 0% al 94% (Barbosa y col., 1983; Luz y col., 1994).

Pruebas moleculares. La PCR es una técnica altamente especifica pero cuya sensibilidad varía mucho (del 45% al 96,5%), sobre todo en pacientes en fase crónica y con una serología no concluyente. La PCR ha sido valiosa para evaluar el nivel parasitario y el seguimiento del tratamiento.

Pruebas serológicas. La más usada es la IFI, pero también se utiliza la ELISA. En *T. cruzi* se presenta el problema de la reacción cruzada con otros tripanosomátidos. El diagnóstico serológico es el único que permite obtener diferentes resultados según el tipo de antígeno usado, la fase de la enfermedad y la clase de Ig (IgG o IgM).

Es importante recordar que en estudios epidemiológicos se deben preferir las pruebas con alta sensibilidad, para disminuir la tasa de falsos negativos.

3.1.5.- Tratamiento.

Las drogas usadas en la actualidad para el tratamiento de la enfermedad de Chagas son el nitrofurano nifurtimox (NFX, Lampit®, Bayer) y el nitroimidazol benznidazol (BNZ, Rochagan®, Radanil®, Roche), cuya actividad anti-T. cruzi fue descubierta empíricamente hace más de tres décadas. Estas drogas son especialmente eficaces para curar la infección en la fase inicial y aguda, con tasas de curación de hasta el 80%. Los medicamentos son considerados menos eficaces en

pacientes que han sido infectados de forma crónica durante más de 10 años. Benznidazol y nifurtimox no son ideales, ya que sus efectos secundarios pueden ser graves y pueden llevar a la resistencia. En consecuencia, muchos médicos prefieren no recetar estos medicamentos (Clayton, 2010).

La eficacia del producto también varía dependiendo del área geográfica, probablemente debido a las diferencias en la sensibilidad a las drogas de las distintas cepas de *T. cruzi*.(Urbina, 2010).

El Nifurtimox se suele administrar en dosis de 8-10 mg/Kg/día, administrado en tres o cuatro tomas, durante 90-120 días y está disponible en comprimidos de 30 y 120 mg. El Benznidazol se dosifica generalmente de 5 a 7,5 mg/Kg/día, en dos o tres tomas, durante 30-60 días y está disponible en comprimidos de 100 mg. (Ministerio de Sanidad y Política Social, 2009).

El conocimiento de la bioquímica del parásito ha llevado a desarrollar fármacos y a comprender su modo de acción.

El Nifurtimox y Benznidazol actúan a través de la generación de radicales libres. Nifurtimox actúa produciendo radicales libres y el Benznidazol inhibe la síntesis de macromoléculas.

Blancos terapéuticos potenciales, pueden ser la ruta de la biosíntesis del glutatión, de la tripanotiona, tioles de bajo peso molecular que se encuentran

exclusivamente en los tripanosomátidos. Estos tioles eliminan los radicales libres y participan en la conjugación y desintoxicación de numerosos medicamentos. La inhibición de estos blancos podría hacer al parásito mucho más susceptible a la acción tóxica de fármacos como el Nifortimox y el Benznidazol.

Alopurinol y análogos de las purinas inhiben el transporte de las purinas en *T. cruzi*. Este parásito no puede sintetizar purinas de novo.

Derivados del nitroimidazol, itraconazol, ketoconazol, fluconazol y posaconazol, inhiben el metabolismo de los esteroles, inhiben la síntesis del ergosterol. *T. cruzi* tiene ergosterol y los antimicóticos impiden su síntesis sin afectar al hospedador humano que tiene colesterol. (Maya y col., 2007; Apt y Zulantay, 2011)

Posaconazol: es un fármaco antifúngico que puede erradicar las formas amastigotas intracelulares de *T. cruzi* y es más efectivo que el Benznidazol en la reducción de *T. cruzi* en modelos animales experimentales. También se ha probado su efectividad en un paciente en Barcelona (Clayton 2010; Urbina, 2010).

TAK-187 es un derivado triazol de acción prolongada con un amplio espectro de actividad fungicida, que también presenta una potente actividad anti T. cruzi. Es capaz de curar las infecciones agudas y crónicas en modelos murinos. Causa menos daño cardiaco que el Benznidazol. Ha completado la fase I (Clayton 2010; Urbina, 2010).

El primer medicamento cuyo blanco terapéutico es la cruzipaina, una catepsina L-cistein proteasa similar responsable de las actividades proteolíticas de todos los estados evolutivos de *T. cruzi,* es el K777. Este medicamento está en fase I de seguridad. Existen otros inhibidores de las cistein proteasas pero K777 ha sido el más eficaz en estudios con roedores, perros y primates.(Clayton 2010)

La droga antiarrítmica más usada en pacientes crónicos de Chagas que presentan problemas cardiacos es Amiodarome (Rassi y col, 2010). Este medicamento actúa alterando la homeostasis del calcio del parásito e inhibiendo la síntesis del ergosterol. (Clayton 2010)

La enzima Superóxido dismutasa (SOD), puede actuar también como un posible blanco terapéutico.

En las células de mamíferos, el estrés oxidativo se evita por cuatro enzimas antioxidantes principalmente, y dos de ellas son: Cu/Zn-SOD, la enzima responsable de la conversión del anión superóxido en peróxido de hidrógeno, que se encuentra en el citoplasma, y la Mn-SOD, con la misma acción que la Cu/Zn-SOD pero sólo se encuentra en la matriz mitocondrial.

Tanto *Trypanosoma* como *Leishmania* presentan diferencias importantes en términos de defensa antioxidante en comparación con el hospedador mamífero. Por ejemplo, los tripanosomátidos contienen la isoforma Fe-SOD, que normalmente sólo se encuentra en las bacterias, y está ausente en las células eucariotas. (Turrens

2004). Por lo que los organismos que carecen de ella muestran una menor proporción de crecimiento, un promedio de vida menor, hipersensibilidad frente a los compuestos producidos en el ciclo oxido-reductor e incremento de la mutagénesis espontánea y del índice de mortalidad (Salas y col. 2001).

Durante las décadas pasadas se han probado centenares de productos experimentales con diferentes estructuras químicas para el tratamiento de las infecciones producidas por *T. cruzi,* pero sólo algunos de ellos han completado la fase preclínica con éxito relativo. Serios efectos adversos evitaron su uso.

En la actualidad, hay muchos fármacos alternativos que se están estudiando para tratar esta enfermedad.

Diamidinas: Las diamidinas aromáticas, muestran una excelente actividad antimicrobiana contra hongos, bacterias y protozoos, y se asocian de modo no covalente y no intercalante al surco menor del ADN, en sitios ricos en AT. La pentamidina, primera diamidina sintetizada, se utiliza en el tratamiento de infecciones causadas por *T. brucei* y la leishmaniasis. Para superar las limitaciones que presenta esta droga, se ha realizado la búsqueda de nuevos compuestos catiónicos aromáticos, incluyendo la síntesis de prodrogas que son convertidas enzimáticamente en diamidinas. La metamidoxima (DB289), prodroga de furamidina (DB75) actualmente se encuentra en ensayos clínicos de fase III para la tripanosomiasis africana. Desafortunadamente, a pesar de la baja toxicidad

presentada inicialmente en África, el Cáucaso y en poblaciones hispanas, estudios recientes han llevado a la retirada de esta droga de ensayos en humanos.(Soeiro y de Castro 2011).

El DB75 y su análogo fenila DB569, son activos frente a *T. cruzi* y, aunque ambas diamidinas tienen capacidades similares de conexión al ADN, el análogo presenta mayor actividad sobre el parásito. DB569 aumenta la supervivencia de ratones infectados, reduce la carga parasitaria, y produce la reversión de alteraciones electrocardiográficas y del número de células TCD8+ en la inflamación cardiaca en las fases aguda y crónica. (Soeiro y de Castro 2011).

DB1362 presenta una potente actividad frente las formas amastigotas y tripomastigotas de *T. cruzi,* además de alteraciones notables en los núcleos y las mitocondrias de los parásitos en sangre. Estos daños se han observado en otro tipo de diamidinas y amidinas inversas. Además en estudios en modelo murino, DB1362 reduce la parasitemia y la inflamación cardiaca. (da Silva y col. 2008).

La arilimidamida (amidina inversa) DB1470 presenta una mayor actividad antitripanosomátida. La mayor actividad de las amidinas inversas con respecto a las amidinas clásicas puede deberse a las diferencias en sus propiedades físicas. (De Souza y col. 2011).

Derivados nitroimidazoles: El etanidazol, un 2-nitroimadazol, presenta actividad tripanocida frente a las formas tripamastigotas y amastigotas de *T. cruzi*

sin afectar a la viabilidad celular de las células del hospedador. El etanidazol es menos potente para eliminar al parásito que el Benznidazol, pero considerando los efectos secundarios, de estudios anteriores se ha comprobado que etanidazol es menos neurotóxico. (Petray y col. 2004).

El megazol 5-nitroimidazol, ha demostrado ser altamente activo *in vitro* e *in vivo* contra *T. cruzi,* incluyendo cepas resistentes al Benznidazol, y se ha convertido en un núcleo estructural para el diseño de nuevas grogas para el tratamiento de la Enfermedad de Chagas. Megazol, se ha descrito como un carroñero tripanotión, el cofactor para la tripanotión reductasa. (Soeiro y de Castro 2011).

Derivados de Indazol: 3-Ciano-2-(4-iodofenil)- 2H-indazol Nóxido, el cual exhibió alta actividad antichagásica sobre las cepas Brener y Tulahuen y las formas epimastigotas y tripomastigotas de *Trypanosoma* y el hecho de la presencia del grupo N-oxido es importante para la actividad antichagásica. Esta investigación reporta por primera vez la actividad antichagásica de 5- nitroindazoles e indazoles N-óxido. (Gerpe y cols. 2006)

Actividad tripanocida de productos naturales: La naturaleza despliega ante nosotros un poderoso arsenal químico, que se manifiesta en las interacciones entre diferentes especies o entre individuos de una misma especie. Los organismos (plantas, insectos) necesitan defenderse de microorganismos y de otros organismos predadores o competidores, y en muchos casos recurren a compuestos químicos

específicamente diseñados para tales fines: los "metabolitos secundarios" o "productos naturales", como más comúnmente se los conoce. Desde la antigüedad, el hombre ha venido utilizando los productos naturales principalmente de plantas terrestres, con fines terapéuticos (medicina tradicional) o de defensa (venenos y toxinas). Este conocimiento derivó en el estudio sistemático de los productos naturales de plantas. A pesar de los grandes avances en el área de la química combinatoria, la mayor parte de las drogas medicinales actualmente en uso siguen siendo productos naturales o análogos basados en ellos. Esto es debido a la enorme diversidad estructural de los productos naturales y a que son naturalmente biosintetizados en forma esteroespecífica, lo cual les confiere propiedades estructurales únicas. Esta diversidad estructural de sustancias es uno de los requerimientos principales para lo que se conoce como "drug discovery". La importancia de los productos naturales como compuestos "cabeza de serie" ha sido comprendida por los laboratorios medicinales, lo cual ha promovido un renovado interés por la preservación de los recursos naturales especialmente en países en vías de desarrollo donde estas enfermedades son endémicas. (Garcia-Barriga, 1986, Rutter, 1990).

Las naftoquinonas, son compuestos que aparecen en distintas familias de plantas. Presentan propiedades redox y están involucradas en los diferentes procesos biológico oxidativos. (Soeiro y de Castro 2011).

2,3-difenil-1,4-naftoquinona (DPNQ), presenta gran actividad a bajas concentraciones frente formas epimastigotas, triponastigotas y amastigotas y no es citotóxico frente a células de mamíferos. Además en modelos murinos, se ha podido observar un retraso significativo en el pico de la parasitemia y una tasa de supervivencia mayor en comparación con el grupo control. DPNQ, es el primer inhibidor competitivo de la lipoamida deshidrogenasa de *T cruzi*. (Ramos y col. 2009) Muchos compuestos, como por ejemplo terpenos, se han aislado de distintas especies y han presentado actividad antitripanosomátida (Mendoza y col. 2003, Cunha y col. 2006).

No sólo las plantas son las responsables de la aportación de compuestos a la terapia antiprotozoaria, muchos de ellos proceden de organismos marinos como por ejemplo las esponjas marinas (Orhan y col. 2010)

3.2.- Género *Leishmania.*

El género *Leishmania,* al igual que el género *Trypanosoma,* pertenece al grupo de protozoos kinetoplástidos. Estos protozoos, son los responsables de la leishmaniasis. Estas parasitosis están presentes en todos los continentes, excepto en la Antártida.

Este género incluye más de 20 especies capaces de infectar a los humanos mediante la picadura del mosquito hembra (Diptera: Phlebotominae) (Scheltema y col. 2010)

La Leishmaniasis puede presentar un amplio rango de síntomas clínicos, dando lugar a la forma cutánea, mucocutánea y visceral. La forma más común es la leishmaniasis cutánea. La leishmaniasis visceral es la más severa.

Leishmaniasis cutánea: Produce úlceras en partes del cuerpo como la cara, brazos, piernas. Puede haber un gran número de lesiones en los individuos afectados. Muchas veces cuando las úlceras sanan quedan cicatrices, que pueden causar prejuicios sociales.

Leishmaniasis mucocutánea. Las lesiones pueden conducir a la destrucción parcial o total de las membranas mucosas de la nariz, boca y garganta. Esta forma al igual que la anterior, puede conducir a la víctima de ser rechazado por la sociedad.

Leismaniasis visceral. También conocida como kala-azar, se caracteriza por episodios de fiebre irregular, pérdida de peso, hinchazón del bazo y del hígado y anemia. Si la enfermedad no se trata, la tasa de mortalidad puede ser de hasta 100% en 2 años. (http://www.who.int/leishmaniasis/en/)

En la siguiente tabla se resumen las distintas especies que producen los diferentes tipos de leismaniasis.

***Leishmania* found in humans**

Subgenus	*L. (Leishmania)*	*L. (Leishmania)*	*L. (Viannia)*	*L. (Viannia)*
Old World	*L. donovani* *L. infantum*	*L. major* *L. tropica* *L. killicki*[a] *L. aethiopica* *L. infantum*		
New World	*L. infantum*	*L. infantum* *L. mexicana* *L. pifanoi*[a] *L. venezuelensis* *L. garnhami*[a] *L. amazonensis*	*L. braziliensis* *L. guyanensis* *L. panamensis* *L. shawi* *L. naïffi* *L. lainsoni* *L. lindenbergi* *L. peruviana* *L. colombiensis*[b]	*L. braziliensis* *L. panamensis*
Principal tropism	Viscerotropic	Dermotropic	Dermotropic	Mucotropic

[a] Species status is under discussion
[b] Taxonomic position is under discussion

(WHO technical 526 reports series 949. 2010.)

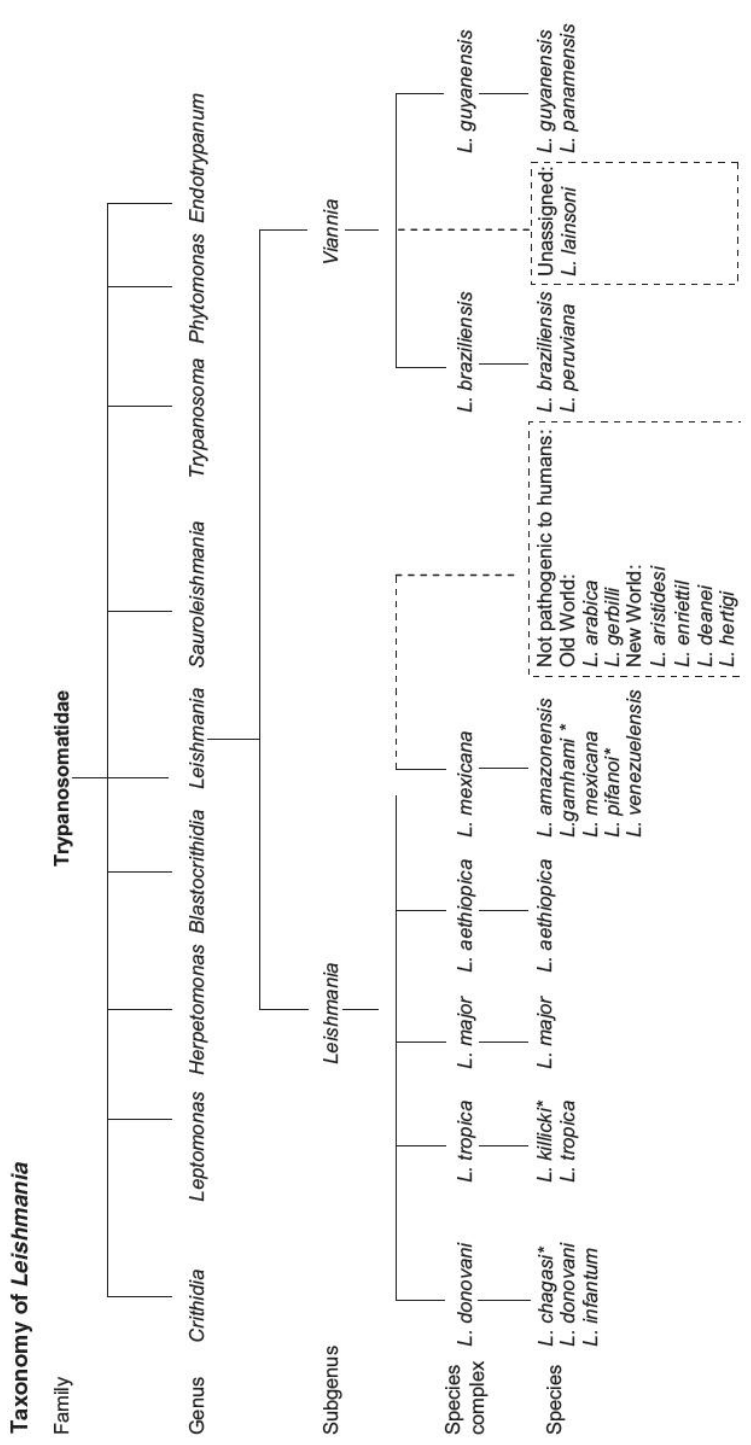
3.2.1.- Clasificación taxonómica.

La clasificación de las especies del género *Leishmania*, se basó en un principio en criterios ecobiológicos tales como vectores, distribución geográfica, tropismo, propiedades antigénicas y manifestación clínica.(Bray 1974, Lumsden 1974, Pratt y David 1981, Lainson y Shaw 1987)

Las diferencias en el desarrollo en el interior del vector son utilizadas para definir los subgéneros. *Leishmania* (*Leishmania*), que lleva a cabo su desarrollo en las partes superior y media del aparato digestivo (desarrollo suprapilórico), mientras

que *Leishmania* (*Viannia*) necesita un desarrollo adicional en la parte final del aparato digestivo (desarrollo peripilórico) (Lainson y Shaw 1987).

La posición taxonómica de las especies descritas en la actualidad en los seres humanos se muestra en la siguiente Tabla.



(WHO technical 526 reports series 949. 2010.)

3.2.2.- Morfología.

La morfología de *Leishmania* está determinada por un ciclo de vida complejo, ya que, los parásitos están expuestos a diferentes ambientes. Estos parásitos son digenéticos o heteroxenos, muestran dos estadíos básicos en su ciclo: uno extracelular con la morfología promastigota, que se desarrolla en el hospedador invertebrado (Phlebotomino) y, el estadío intracelular con la forma amastigota que aparece en el hospedador vertebrado (Trager 1953).

La forma **promastigota** se desarrolla en el aparato digestivo del flebotomo (vector de la enfermedad) y constituye la forma infectiva para el hospedador vertebrado. Tiene forma alargada, de 15 a 20 μm de longitud y presenta un flagelo libre en su parte anterior que puede llegar a medir el doble de la longitud del cuerpo del parásito. Al microscopio electrónico muestra un núcleo central, ribosomas, retículo endoplasmático, aparato de Golgi, una única mitocondria y el kinetoplasto que aparece como un cuerpo electrodenso en la zona anterior (Lofgren 1950; Crowther y col. 1954).

La forma **amastigota** se observa en los tejidos parasitados de los hospedadores vertebrados, generalmente en el interior de las células del sistema mononuclear fagocitario (SMF). En el interior de estas células se pueden encontrar las amastigotas englobadas en una vacuola parasitófora que se forma tras la fusión del lisosoma y el fagosoma una vez que el parásito ha sido internalizado (Chang y

Dwyer, 1978). Las amastigotas son formas ovoides con un tamaño comprendido entre 2 y 5µm de diámetro. Estas formas presentan un núcleo central y un kinetoplasto alargado, pudiéndose apreciar la existencia de bolsillo flagelar y un flagelo vestigial (Chang y Dwyer, 1978).

Durante el desarrollo del parásito en el vector, *Leishmania* adopta varias morfologías intermedias entre la forma flagelada y la aflagelada. Las formas descritas y enumeradas por orden de aparición a lo largo de esta transformación son las promastigotas procíclicas (aparecen entre las 24 y 48 horas tras la ingesta), nectomonas (48 - 72 horas), leptomonas (4 - 7 días), haptomonas (5 - 7 días) y metacíclicas (7 -14 días), estas últimas son los responsables de la transmisión de la infección al vertebrado (Chang y col., 1990; Kamhawi, 2006).

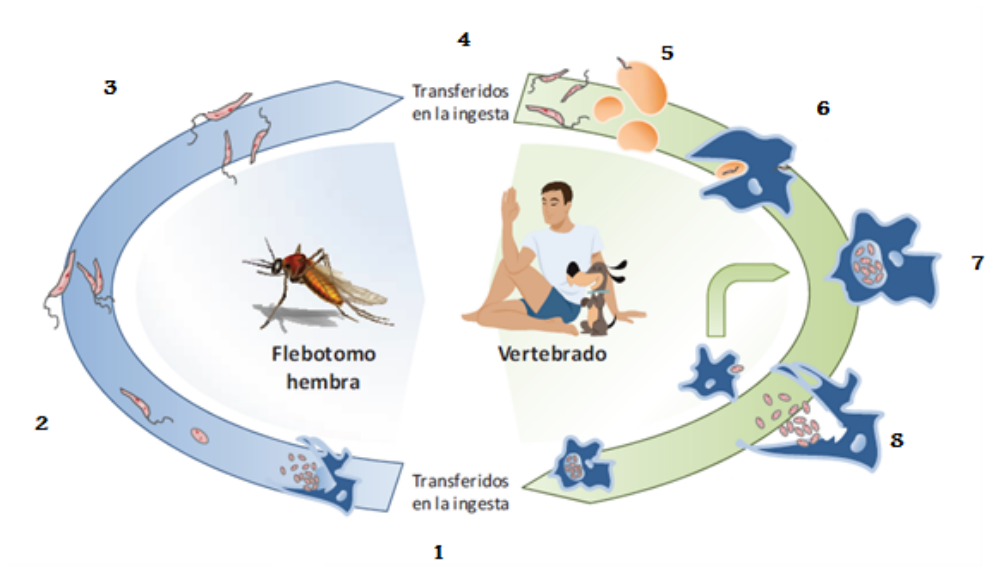
3.2.3.- Ciclo biológico.

Cuando el flebotomo ingiere sangre con macrófagos infectados con amastigotas de un individuo parasitado comienza el desarrollo del parásito en el interior de su aparato digestivo. Durante un periodo inicial de 6 a 9 días (dependiendo de la especie) *Leishmania* se desarrolla diferenciándose en los distintos estadíos hasta convertirse en promastigotas metacíclicas, en un proceso conocido como metaciclogénesis (Kamhawi, 2006).

La sangre de la que se alimenta el vector permanece contenida inicialmente en la región abdominal del aparato digestivo gracias a la membrana peritrófica, al tercer día de desarrollo, los parásitos migran hacia la zona anterior del intestino medio. Una vez en el intestino, las promastigotas avanzan hacia la zona bucal pasando por los distintos estadios del desarrollo (Kamhawi, 2006).

La transmisión de la infección se produce cuando el flebotomo infectado se alimenta sobre un nuevo hospedador vertebrado. Al mismo tiempo que el flebotomo se alimenta de la sangre, las formas infectivas (promastigotas metacíclicas) entran en el huésped vertebrado a través de la trompa. Tradicionalmente se ha considerado que, una vez introducidos en el huésped, las formas promastigotas son fagocitadas directamente por los macrófagos. Sin embargo, publicaciones recientes sugieren que estas formas podrían ser inicialmente fagocitadas por los neutrófilos y éstos a su vez fagocitados por los macrófagos. (Van Zandbergen y col. 2004, Ritter y col. 2009, Jochim y Teixeira 2009, Charmoy y col. 2010)

En los macrófagos, son incluidas en una vacuola parasitófora en la que las promastigotas se transforman en amastigotas. Éstas se multiplican por fisión binaria en el interior del macrófago hasta que es lisado, liberándose al exterior de la célula e infectando nuevas células (Handman, 1999; Cunningham, 2002; Handman y Bullen, 2002).



Ciclo de vida de *Leishmania*

3.2.4.- Diagnóstico.

Debido a la variedad de signos clínicos que presenta la leishmaniasis, el diagnostico es muy difícil, por lo que se han desarrollado varios procedimientos para facilitar esta tarea. Sin embargo es esencial el conocimiento de las bases de cada prueba, sus limitaciones y su interpretación clínica; así como la combinación de más de una prueba diagnóstica.

El **diagnóstico clínico** se basa en los síntomas que van a ser diferentes según la fase de infección y el estado inmunológico del hospedador. Debido al cuadro clínico variado e inespecífico, se recomienda hacer diagnóstico diferencial con otras enfermedades infecciosas y desordenes inmunológicos.

La **observación directa** trata de demostrar que el paciente está albergando *Leishmania*, mediante la visualización, en el frotis o en la histopatología, de formas amastigotas en los tejidos infectados utilizando muestras biológicas de ganglio, médula, piel o sangre. Estos métodos siguen siendo técnicas estándar por su gran especificidad y sencillez.

También puede inocularse la muestra en un medio de cultivo apropiado e incubar hasta que los parásitos se multipliquen. La realización de un frotis permite la observación inmediata de la muestra mientras que el cultivo necesita varios días para permitir la diferenciación y crecimiento de los parásitos. Sin embargo, la observación directa podría conducir a diagnosticar un falso negativo si la muestra que se toma no es representativa o no se realiza una tinción adecuada (Barrouin-Melo y col., 2004; Reithinger y Dujardin, 2006).

Los **métodos inmunológicos** se basan en la detección de la enfermedad mediante la respuesta inmune celular, y/o respuesta inmune humoral a través de anticuerpos específicos desarrollados como consecuencia de la enfermedad. Las técnicas más utilizadas son el IFI, la ELISA y el Western-blot.

El IFI es la técnica más utilizada por su rapidez, sencillez y posibilidad de titular ofreciendo un grado relativo de la enfermedad. Sigue considerándose la técnica estándar a pesar de no ser la más sensible, pues ciertos estudios han

demostrado que un 38% de los resultados pueden ser falsos negativos (Reale y col., 1999).

La ELISA se empezó a usar en el diagnóstico de la leishmaniasis en los años 70 y presenta valores de sensibilidad que oscilan entre el 85% y el 96%, y de especificidad del 86% al 98%. Sin embargo, la utilizacion de antígenos purificados específicos de *Leishmania spp* están contribuyendo a mejorar estos resultados (Braz y col., 2002).

El Western-blot permite conocer qué anticuerpos están presentes en la muestra problema o la detección de antígenos circulantes. Esta técnica es más utilizada en investigación que en diagnóstico clínico.

Las **pruebas moleculares** se centran en la detección de ADN del parásito mediante PCR, PCR anidada o PCR en tiempo real. Estas técnicas son las más específicas y sensibles; sin embargo resultan más costosas y necesitan un equipo técnico más amplio y personal técnico más preparado. (Piarroux y col., 1993).

3.2.5.- Tratamiento.

El tratamiento farmacológico de las leishmaniasis ha estado disponible desde el comienzo del siglo XX, pero sólo unos pocos medicamentos son utilizados. Aunque se han observado diferencias a la susceptibilidad a las drogas, y las

manifestaciones de la enfermedad entre las diferentes especies de *Leishmania* del Viejo Mundo y la leishmaniasis del Nuevo Mundo, los mismos medicamentos se usan para el tratamiento. Aunque la OMS ha proporcionado una recomendación para el tratamiento de la leishmaniasis, existen diferentes pautas terapéuticas en diversos países y regiones del mundo. Dado que, además, nos enfrentamos a un aumento en la resistencia a los medicamentos disponibles en algunas regiones, es necesaria una discusión a nivel mundial para mejorar el uso de los medicamentos disponibles y promover el desarrollo de nuevos fármacos.

A día de hoy, existen nueve tipos de fármacos en distintas fases de aplicación para combatir esta enfermedad.

Antimoniales pentavalentes (SbV): La primera opción para el tratamiento de la Leishmaniasis. En 1912 se trató en Brasil el primer caso de leishmaniasis con una forma de antimonial trivalente (SbIII). A partir de 1920 se dispuso de los antimoniales pentavalentes (SbV) y desde 1945 del estibogluconato sódico (Ramos y Segovia, 1997).

Existen dos formulaciones en el mercado que contienen la forma pentavalente del compuesto: antimoniato de meglumina, comercializado con el nombre de Glucantime® (Aventis, Francia), que contiene 85 mg de SbV/ml y el estibogluconato de sodio, conocido como Pentostam® (GlaxoSmithKline, Reino Unido) con 100mg de SbV/ml (Croft y Yardley, 2002).

Se administran por vía parenteral durante un periodo de 10 a 30 días. Estos compuestos son efectivos, pero bastante tóxicos atendiendo a la relación eficacia/cantidad de SbV administrado, dañando el corazón del enfermo hasta el punto de alcanzar una mortalidad del 3-6%. Al problema de la toxicidad hay que añadir el hecho de que distintas especies de *Leishmania* presenten resistencias a estos fármacos en diversas zonas del Viejo y Nuevo Mundo a lo largo de los últimos 20 años. (Croft y col. 2006)

El mecanismo de acción de los compuestos antimoniales se basa en su capacidad para inhibir selectivamente enzimas necesarios para la glicolisis y la oxidación de los ácidos grasos del parásito (Baneth y Shaw, 2002).

En los últimos años se ha incrementado el estudio de los mecanismos que conducen a la resistencia. (Croft y col. 2006, Aït-Oudhia y col. 2011).

Pentamidina: Es uno de los fármacos utilizados como segunda opción si los antimoniales no resultan efectivos. La pentamidina es el nombre común del isotionato de Pentamidina, una diamidina aromática.

La formulación de isotionato de Pentamidina, administrada por vía parenteral, ha sido comercializada con el nombre de Pentacarinat®. La Pentamidina también ha sido comercializada en forma de dimetasulfonato de Pentamidina con el

nombre de Lomidine®. (Méndez y Alunda 2001, Baneth y Shaw 2002, Guerin y col. 2002).

Parece ser que actúa inhibiendo la biosíntesis de las poliaminas, se une al surco menor del ADN y afecta al potencial de membrana mitocondrial. (Basselin y col. 2002, Bray y col. 2003, Mukherjee y col. 2006) La pentamidina se ha utilizado con éxito para tratar la leishmaniasis cutánea.

Anfotericina B. (AnB). Se trata de un polieno antibiótico y fungicida que se utiliza contra la leishmaniasis desde 1959. En algunas partes del mundo, es el fármaco de elección. Su acción se basa en su capacidad para unirse al ergosterol formando poros en la membrana celular de *Leishmania*, resultando en la pérdida de iones y produciendo la muerte celular. Cuatro fórmulas de medicamentos están disponibles: anfotericina B deoxicolato, anfotericina liposomal, el colesterol anfotericina de dispersión y lípidos complejos anfotericina. (Goto y col. 2010)

Todas las formulaciones comparten una eficacia similar, sin embargo, las diferencias se han observado en relación con los efectos secundarios de las formulaciones, con efectos secundarios más intensos asociados con anfotericina B desoxicolato, que puede provocar daño renal.

Miltefosina (MIL): Fosfolípido utilizado como un medicamento contra el cáncer.

En el año 2002 se aprobó un nuevo fármaco de primera opción para tratar la leishmaniasis visceral en la India, la hexadecilfosfocolina o miltefosina, comercializada bajo el nombre de Impavido.(Croft y Coombs 2003)

Es activo por vía oral. Estudios con animales han demostrado que tiene cierta toxicidad reproductiva y por tanto, está contraindicado en el embarazo y debe usarse con precaución en mujeres en edad reproductiva. Los efectos secundarios gastrointestinales parecen ser comunes, pero rara vez lo suficientemente grave como para justificar la interrupción del tratamiento. (Moore y Lockwood, 2010).

Como tratamiento para la leishmaniasis, la miltefosina ejerce efectos tóxicos directos sobre los parásitos. También Miltefosina modula las células inmunes como los macrófagos, que conduce a la eliminación del parásito a través de radicales oxidativos. Estudios indican que a diferencia de los antimoniales pentavalentes, la miltefosina funciona con independencia del sistema inmunitario principalmente a través de la toxicidad directa contra el parásito (Griewank y col. 2010).

Su gran ventaja radica en que es el primer fármaco que puede ser administrado oralmente.

Paromomicina (PM) o Aminosidina: Es un antibiótico aminoglucósido producido por *Streptomyces rimosus*, usado para el tratamiento de enfermedades bacterianas y con demostrada capacidad antiparasitaria (Baneth y Shaw, 2002).

Las formulaciones inyectables se usan contra la leishmaniasis visceral mientras que las formulaciones tópicas han sido utilizadas contra formas cutáneas de la enfermedad (Croft y Coombs 2003).

Se han observado pequeños signos subclínicos de ototoxicidad y nefrotoxicidad. Hay algunas cepas de *Leishmania* en algunas zonas de África que se han mostrado resistentes a este fármaco.

Azoles: Son compuestos que se utilizan para tratar la infección por hongos, pero se han utilizado para tratar la leishmaniasis. Algunos informes han demostrado la eficacia del fluconazol, ketoconazol, itraconazol para tratar la leishmaniasis. Los compuestos azoles consiguen lisar el parásito al alterar la composición de su membrana lo que aumenta su permeabilidad. (Goto y col. 2010). Afectan la ruta biosintética del ergosterol al inhibir el citocromo P450.

Recientemente, se ha realizado un estudio con posaconazol, 400 mg por vía oral dos veces al día, a una paciente que era reacia a someterse a un tratamiento de administración diaria parenteral y estaba preocupada por los efectos adversos. En este estudio, se ha visto que la lesión se resolvió rápidamente y el tratamiento finalizó a los 14 días y no aparecieron signos recurrentes después de un año.

La rápida resolución de la lesión sugiere que el posaconazol es una prometedora alternativa terapéutica para el tratamiento de las leishmaniasis.(Paniz Mondolfi y col. 2011).

Sitamaquina: Anteriormente conocida como WR6026, la sitamaquina es un derivado de la 8-aminoquinolina con un amplio espectro de actividad antiprotozoaria. (Yeates 2002)

Su mecanismo de acción está basado en su capacidad para provocar un rápido colapso del potencial de membrana de la mitocondria de *Leishmania* (Taylor y col. 1991) Datos obtenidos en humanos han mostrado que la Sitamaquina es, en general, bien tolerada aunque pueden producirse vómitos, dolor abdominal, cefaleas o trastornos renales. (López Martin y col. 2008)

Imiquimod o imidazoquinolona: Es un compuesto antivírico utilizado originalmente contra los papilomavirus que causan verrugas genitales. Este principio activo es el ingrediente principal de la crema comercializada como Aldara (3M Pharmaceuticals) usada contra queratosis, carcinoma de células basales o verrugas genitales. (Croft y Coombs 2003, Davis y Kedzierski 2005)

El mecanismo de acción del imiquimod se basa en su capacidad para inducir la producción de citoquinas (IFNg, TNFh, IL1, IL6 o IL8) que activan la producción de óxido nítrico en los macrófagos, favoreciendo la destrucción de los amastigotes intracelulares. (Testerman y col. 1995; Buates y Matlashewski 1999)

Alopurinol: Es un análogo de purina (hipoxantina) utilizado por primera vez contra la leishmaniasis en los años 80. Su mecanismo de acción se basa en la

inhibición de enzimas de la ruta de las purinas como la xantina oxidasa. Este compuesto es metabolizado por *Leishmania* produciendo un análogo de inosina inactivo, frenando así el metabolismo de las purinas (Nelson y col. 1979).

La mayor ventaja que ofrece el Alopurinol es que no suele generar efectos secundarios, por lo que se recomienda su uso en casos con nefritis crónica debida a la leishmaniasis. (Plevraki y col. 2006)

Compuestos experimentales: La investigación en el campo de la quimioterapia continúa en la actualidad puesto que no se ha desarrollado un compuesto que produzca la curación total de la enfermedad.

Hasta el momento se han ensayado gran cantidad de compuestos con actividad leishmanicida in vitro con resultados prometedores, siendo algunos de ellos seleccionados para ser evaluados in vivo.

La capacidad leishmanicida de compuestos aislados de plantas se ha estudiado en gran detalle.

La tendencia actual en la terapia de la leishmaniasis es la combinación de fármacos con actividad leishmanicida. Animales tratados con la combinación de Ketoconazol (50 mg/Kg, 5 días) + miltefosina (5 mg/Kg, 5 días), presentaron un aumento en la eficacia contra el parásito comparado con animales tratados con dichos fármacos por separado. Además al añadir a la combinación un

inmunomodulador la eficacia aumentó aun más. (Shakya y col. 2011). Aunque, se están realizando estudios para comprobar la eficacia de nuevas moléculas.

4.-PUBLICACIONES.

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In Vivo Trypanosomicidal Activity of Imidazole- or Pyrazole-Based Benzo[*g*]phthalazine Derivatives against Acute and Chronic Phases of Chagas Disease

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The *in vivo* trypanosomicidal activity of the imidazole-based benzo[*g*]phthalazine derivatives **1**−**4** and of the new related pyrazole-based compounds **5** and **6** has been studied in both the acute and chronic phases of Chagas disease. As a rule, compounds **1**−**6** were more active and less toxic than benznidazole in the two stages of the disease, and the monosubstituted derivatives **2**, **4**, and **6** were more effective than their disubstituted analogs. Feasible mechanisms of action of compounds **1**−**6** against the parasite have been explored by considering their inhibitory effect on the Fe-SOD enzyme, the nature of the excreted metabolites and the ultrastructural alterations produced. A complementary histopathological analysis has confirmed that the monosubstituted derivatives are less toxic than the reference drug, with the behavior of the imidazole-based compound **4** being especially noteworthy.

Introduction

Tropical and subtropical diseases caused by protozoal parasites remain a major public health problem in many less developed countries, because of the lack of effective drugs or because of increasing resistance against the few affordable drugs.[1,2] American trypanosomiasis, also known as Chagas disease, is one of the most devastating. It is caused by the kinetoplastid protozoan *Trypanosoma cruzi*, which is vectorially transmitted by a bug depositing feces on and subsequently biting the skin surface. Other routes of contamination are infected blood transfusions, organ transplantation, and even mother-to-child transmission during pregnancy or breastfeeding.[3] Chagas disease takes the form of an acute infection, during which most patients do not know that they are infected, with further evolution to a chronic and systemic stage severely affecting the heart, esophagus, and colon. It is endemic throughout all Latin America and is classed by the WHO[a] as the third most widespread tropical disease after malaria and schistosomiasis.[4] It is estimated that about 100 million people are at risk of infection, that from 15 to 20 million people are infected, and that 50000 die annually as a result of infection by this parasite.

Unfortunately, current treatment for this disease is very limited, and no successful vaccine has been developed.[5] The drugs available are mainly nitroheterocyclic compounds like the nitrofurane nifurtimox or the nitroimidazole derivative benznidazole, but they are only effective against the acute phase, exhibiting very limited efficacy in the chronic stage.[6] Furthermore, they are quite toxic and cause severe side effects like pancreatitis and cardiac toxicity.[7] The search for more effective drugs is mainly centered on their potential action against essential and exclusive components of the trypanosomatids. Targets under study are 6-phosphogluconate dehydrogenase or dihydrofolate reductase, a key enzyme for DNA synthesis.[8] Another promising target is the iron superoxide dismutase (Fe-SOD). This enzyme is found exclusively in protozoa and plays an essential antioxidant role in the survival of the parasite, due to its scavenging ability for the superoxide anion.[9] The action against enzymes involved in the biosynthesis of trypanothione, which also represents an important defense barrier against oxidative damage, is being investigated.[10] This is the case with ornithine decarboxylase (ODC), *S*-adenosylmethionine decarboxylase (AdoMet-DC), spermidine synthase (SpdS), trypanothione synthase (TryS), and trypanothione reductase (TryR).

In close connection with this matter, our research group has been preparing heteroaromatic compounds containing the 1-alkylamino- or 1,4-bisalkylaminobenzo[*g*]-phthalazine system, which exhibit interesting transition metal-complexing properties and have shown remarkable *in vitro* antiparasitic activity[11] (Figure 1). We have described in a recent study that the introduction of imidazole units in the flexible side chains of the benzo[*g*]phthalazine system (Figure 2, compounds **1**−**4**)

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[a]Abbreviations: SOD, superoxide dismutase; WHO, World Health Organization; ODC, ornithine decarboxylase; AdoMet-DC, adenosylmethionine decarboxylase; SpdS, spermidine synthase; TryS, trypanothione synthase; TryR, trypanothione reductase; BZN, benznidazole; TEM, transmission electron microscopy; HMQC, heteronuclear single quantum coherence experiment; HMBC, heteronuclear multiple bond coherence experiment; dpi, days postinfection; TLC, thin-layer chromatography; TMS, tetramethylsilane, MEM, minimal essential medium; NAD(P)H, nicotinamide adenine dinucleotide phosphate.

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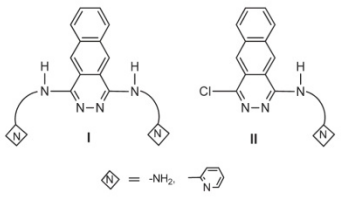


Figure 1. Models for the design of benzo[*g*]phthalazine derivatives with potential antiparasitic activity.

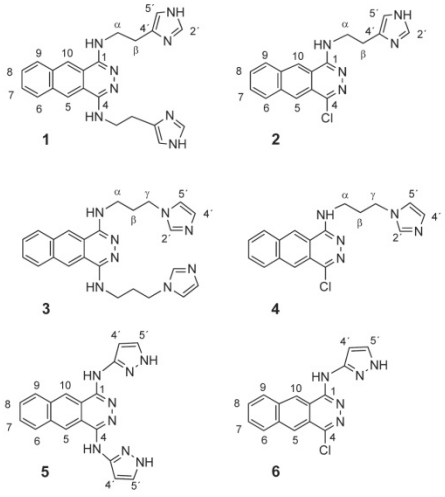


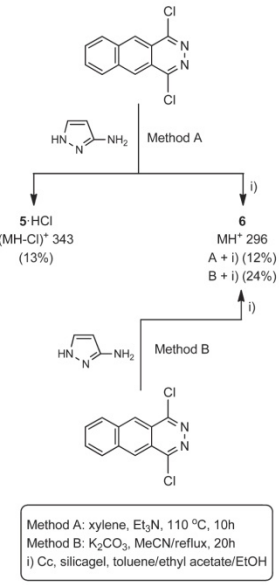
Figure 2. Imidazole- (**1**−**4**) and pyrazole-based (**5**,**6**) structures tested *in vivo* against *T. cruzi*.

enhances their *in vitro* trypanosomicidal activity against *T. cruzi* and also show the ability to inhibit the crucial antioxidant Fe-SOD enzyme.[12]

Even more interestingly, these compounds also exhibit very low *in vitro* toxicity against Vero cells with respect to a typical antiparasitic drug like benznidazole. Taking into account the considerable complexing ability toward metal ions shown by our compounds,[11] we think that competitive complexation for the metal ion of SOD could be an effective way of deactivating the antioxidant protective effect of the enzyme,[13] which will affect both the growth and survival of parasitic cells.

These results prompted us to design two new related compounds lacking the flexible methylene groups at the side chains, in which the imidazole units have been replaced by pyrazole rings (Figure 2, compounds **5** and **6**). Although pyrazoles are rarely found in nature, they are present in many pharmaceuticals with a wide range of biological activities.[14] Even more, pyrazoles are effective tools in supramolecular chemistry, and their conjugate bases have been found to bind metals in a variety of coordination modes and are considered robust bridging ligands.[15,16] The increased system rigidity and the presence of a heterocyclic ring with well-known complexing abilities could allow a cooperative action of both the

Scheme 1. Synthesis of the New Pyrazole-Based Derivatives



pyridazine and pyrazole nitrogens in metal complexation and modify Fe-SOD inhibition.

In the first part of this study, we describe the synthesis and identification of compounds **5** and **6**, as well as their *in vitro* antiparasitic activity, toxicity, and SOD inhibition indexes in relation to those found previously for compounds **1**−**4** and benznidazole (BZN).[12] Considering the promising *in vitro* results obtained for compounds **1**−**6**, herewith we report a detailed study on the *in vivo* antiparasitic activity and toxicity of these compounds, both in the acute and in the chronic phases. Furthermore, we also include a NMR study concerning the nature and percentage of the excretion metabolites in order to obtain information on the inhibitory effect of our compounds on the glycolytic pathway, since it represents the prime source of energy for the parasite. The effects of compounds **1**−**6** on the ultrastructure of *Trypanosoma cruzi* are also considered on the basis of transmission electronic microscopy (TEM) experiments. Finally, a histopathological analysis is performed in search of better insight into compound toxicity.

Results and Discussion

Chemistry. The preparation of compounds **5** and **6** (Figure 2) was performed from 1,4-dichlorobenzo[*g*]phthalazine as shown in Scheme 1, using similar experimental conditions to those reported by our group for the synthesis of **1**−**4**[12] and other analogs.[11]

Thus, nucleophilic substitution at the C-1 and C-4 positions of the starting compound with 3-aminopyrazole by heating in xylene at 110 °C for 10 h (method A) afforded the bis- and monoalkylamination products **5**·HCl (mp 286 °C) and **6** (mp 235 °C) in 13% and 12% yield, respectively.

Isolation of the disubstituted compound **5**, which was obtained in the hydrochloride form, was straightforwardly achieved by recovering the precipitate (a pure reddish solid). The monosubstituted compound **6** was isolated in the free form from the remaining organic phase after elimination of the solvent under vacuum and purification of the residue by flash chromatography on silica gel with a toluene/ethyl acetate/ethanol mixture of increasing polarity. Alternatively, **6** could be obtained in a higher yield (24%) by refluxing the starting compounds in acetonitrile for 20 h in the presence of potassium carbonate (method B).

Both compounds were unequivocally identified on the basis of their ESI mass spectra, analytical data, and IR, ^{1}H NMR, and ^{13}C NMR spectroscopy. Assignment of the NMR signals could be achieved using bidimensional techniques (gHMQC and gHMBC). The mono- and bis-alkylamination products could be easily differentiated in both ^{1}H and ^{13}C spectra by comparing the H_5/H_{10} and C_1/C_4 signals at rings B and A of the benzo[*g*]phthalazine moiety. Disubstituted compound **5**·HCl exhibited a unique signal for C_1 and C_4 and also for H_5 and H_{10} as a singlet. On the contrary, C_1 and C_4 gave clearly different signals in its monosubstituted analog, being the carbon linked to the chlorine atom shielded about 8 ppm. In a similar way, H_5 and H_{10} appeared as two singlets separated by 1.34 ppm, and the proton in the neighborhood of the chlorine was also shielded with respect to the one coplanar with nitrogen. Neat differences were also observed among the hydrogens at ring C in both derivatives, with the disubstituted compound exhibiting the expected symmetrical pattern. It should be noted that the pyrazole NH hydrogens give very low-field signals in the ^{1}H NMR spectra (12.2 ppm and 12.7 ppm for **5**·HCl and **6**, respectively). This fact probably indicates the presence of strong N···H···N inter- or intramolecular bonding in both molecules.

Finally, the two ESI mass spectra (positive mode) exhibited molecular ions corresponding to the proposed structures (*m*/*e* 343 $[MH - Cl]^+$ and 296 $[MH^+]$ for **5** and **6**, respectively).

***In Vitro* Anti-*T. cruzi* Activity.** In a first step, the inhibitory effect of the new pyrazole compounds **5** and **6** on the *in vitro* growth of. *T. cruzi* epimastigotes was measured at concentrations ranging from 1 to 100 μM, and compared with those of the previously synthesized imidazole analogs **1**−**4**.[12] IC_{50} values obtained after 72 h of exposure are shown in Table 1 including benznidazole as the reference drug. The trypanosomicidal activity of the two pyrazole derivatives was similar (monosubstituted **6**, $IC_{50} = 17.5$ μM) or even slightly higher (disubstituted **5**, $IC_{50} = 32.8$ μM) than that found for benznidazole (15.8 μM), although better results were obtained in the case of the imidazole compounds. An evaluation of cytotoxicity using mammalian Vero cells as the cellular model (Table 1) showed that the two pyrazole derivatives were much less toxic than benznidazole and that, as already observed for the imidazole derivatives, the monoalkylamine products were less toxic than the bisalkylamine analogs. On the other hand, the selectivity index calculated for the pyrazole derivatives was about three times (**5**) and nine times (**6**) higher than that of BZN. However, SI values were again significantly lower than those found for the imidazole analogs.

In most activity assays of new compounds against parasites, forms that develop in vectors are used (epimastigotes in the case of *T. cruzi*), because they are easier to handle *in vitro*.

Table 1. *In Vitro* Activity, Toxicity and Selectivity Index Found for the Imidazole-Based (**1**−**4**) and Pyrazole-Based (**5**,**6**) Benzo[*g*]phthalazine Derivatives on Epimastigote Forms of *T. cruzi*[a]

compound	activity IC_{50} (μM)	toxicity IC_{50}[b] (μM)	SI[d]
benznidazole	15.83	13.56	0.85
1	14.2[c]	88.7[c]	6.24
2	< 0.3[c]	213.0[c]	710.0
3	< 0.2[c]	69.3[c]	346.5
4	13.7[c]	145.8[c]	10.64
5	32.8	98.3	2.99
6	17.5	132.6	7.58

[a] Note: Average of three separate determinations. [b] On Vero cells after 72 h of culture. IC_{50} = the concentration required to give 50% inhibition, calculated by linear regression analysis from the Kc values at concentrations employed (2.5, 25, and 125 μM for compounds **1**-**4**; 10, 25, and 50 μM for **5** and **6**). [c] Published in *J. Med. Chem.*[12] [d] Selectivity index = IC_{50} Vero cells/IC_{50} epimastigote.

However, as reported previously for **1**−**4**, we have opted here for including the effect caused by our compounds on the forms that are developed in the host (amastigotes and trypomastigotes), since they are highly informative in determining how the host is really affected.[17]

Figure 3 displays results obtained concerning *T. cruzi* propagation in Vero cells for compounds **5** and **6**. In these assays, we used the IC_{25} of each product as the test dosage, with BZN as the reference drug. When 1×10^5 Vero cells were incubated for 2 days and then infected with 1×10^6 metacyclic forms, obtained using methods described in the Experimental Section (control experiment; Figure 3A), the parasites invaded the cells and underwent morphological conversion to amastigotes within 1 day after infection.

On day 10, the rate of host-cell infection reached its maximum. When compound **5** or **6** was added to the infected Vero cells with *T. cruzi* metacyclic forms (IC_{25} concentration), the infection rate significantly decreased with respect to the control, reaching 63% (**5**) and 68% (**6**) on day 10 of the experiment. The decrease in infection rate found for these compounds was substantially higher than the one measured for BZN (39%).

On the other hand (Figure 3B), the average number of amastigotes per infected cell increased to 39.6 amastigotes/cell in the control experiment on day 6, decreasing to a value of 30.6 on day 8 and increasing again to 35.0 on day 10. Those fluctuations in the number of amastigotes could be explained considering that they leave the cell and invade it again in a cyclic way, thus originating new replication and variations in the number of amastigotes per cell. When the pyrazole compounds were tested, both of them inhibited *T. cruzi* amastigote replication in Vero cells. Thus, the addition of a concentration equivalent to the IC_{25} of these compounds produced a remarkable decrease in the amastigote number per infected cell, reaching on day 10 a 56% reduction for **6** and 40% for **5** with respect to the control experiment. These results are striking when compared with those obtained for BZN, which only reached a 15% reduction in the amastigote number.

Rupture of the Vero cell membrane implies liberation of amastigotes and further transformation in trypomastigotes. Therefore, we have also measured the variation in the trypomastigote number in the culture medium (Figure 3C). The control experiment had a trypomastigote number of 4.4×10^4 on day 10, and reductions of 53% and 63% were found for compounds **5** and **6**, respectively. These reductions were more profound than that obtained for BZN (43%).

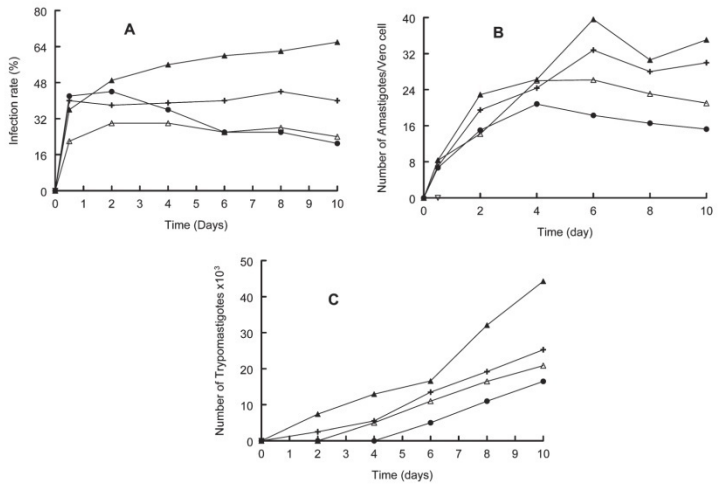


Figure 3. Effect of compounds **5** and **6** on the infection rate and *T. cruzi* growth: (A) rate of infection; (B) mean number of amastigotes per infected Vero cell; (C) number of trypomastigotes in the culture medium; (▲) control; (+) BZN; (△) compound **5**; (●) compound **6**. Measured at IC_{25} concentration. Values are means of three separate experiments.

From the data described above and from those reported previously on *T. cruzi* propagation in the presence of **1**−**4** in Vero cells,[12] it can be concluded that the monoalkylamino-substituted compounds **2**, **4**, and **6** are considerably more active than their disubstituted analogs and also that the six compounds tested are more effective than BZN.

***In Vivo* Anti-*T. cruzi* Activity.** The good results obtained in the *in vitro* tests performed on both the imidazole- and pyrazole-based derivatives prompted us to study their *in vivo* activity in mice. We decided to evaluate their impact on the two significant stages of Chagas disease: the acute phase, considered as the first 40 days postinfection, and the chronic phase, from 80 days postinfection. It has been recently published that the intravenous doping route results in high mortality rates,[18] so we opted for the intraperitoneal route, using a concentration of 5 mg/kg, which did not result in any animal mortality. Female Swiss mice were inoculated intraperitoneally with 3000 metacyclic trypomastigotes, and treatment began 7 days postinfection by the ip route with 1 mg/(kg·day) of each compound for 5 days. Administration was performed with a saline solution. A group treated in the same manner with vehicle (control) was included. During the study of the acute phase activity, the level of parasitemia was determined every 2 days (Figure 4A,B). None of the animals treated with either the control or compounds **1**−**6** died during treatment, whereas the survival percentage of the mice treated with BZN was 80%.

A comparison of the data represented in Figure 4A,B showed that, with respect to control mice, the six compounds tested were able to diminish the trypomastigote number on the day of maximum parasitic burden, which was day 25 postinfection. On day 40 pi, a significant reduction of the parasitemia was found for the monoalkylamino-substituted derivatives **2**, **4**, and **6** with respect to the disubstituted derivatives **1**, **3**, and **5**. Additionally, the imidazole-based compound **4** was especially efficient among all the tested

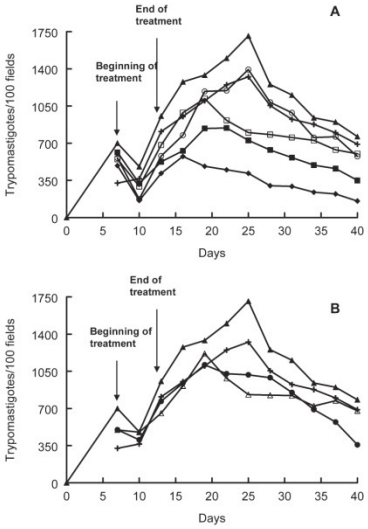


Figure 4. Parasitemia in the murine model of acute Chagas disease: (A) control (▲) and receiving 5 mg/kg doses of BZN (+); compound **1** (○); compound **2** (■); compound **3** (□); compound **4** (◆); (B) receiving 5 mg/kg doses of BZN (+); compound **5** (Δ); compound **6** (●).

compounds. From these data, the following order for *in vivo* activity could be established: **4** > **2** ≈ **6** > **1** ≈ **3** > **5** > BZN.

Concerning the activity in the chronic phase, serological tests were performed 40 and 120 days postinfection (Table 2).

Table 2. Differences in the Level of anti-*T. cruzi* Antibodies between Day 40 and Day 120 Post-infection for Compounds **1**−**6** and BZN, Expressed in Absorbance Units (abs)

compounds[a]	ΔA[b]
control (untreated)	0.146
benznidazole	0.110
1	0.044
2	−0.076
3	0.266
4	−0.151
5	0.138
6	−0.228

[a] Dose of 1 mg/(kg·day), administered by the intraperitoneal route for five days (see Experimental Section). [b] ΔA = (absorbance at 490 nm on day 120 p.i.) − (absorbance at 490 nm on day 40 p.i.).

None of the animals treated with compounds **1-6** or with the control drug BNZ showed negative anti-*T. cruzi* serology. However, the monosubstituted compounds **6**, **4**, and **2** and, to a much lesser extent, the disubstituted analog **1**, decreased antibody levels between days 40 and 120, showing higher performance than BNZ in this assay. Differences in the level of anti-*T. cruzi* antibodies were in agreement with the parasitemia findings.

In summary, it can be concluded that the monoalkylamino-substituted imidazole- or pyrazole-based compounds **4**, **6**, and **2** are remarkably active against *T. cruzi in vitro* and also *in vivo*, both in the acute and in the chronic phase of the infection.

Studies on the Mechanism of Action. In order to get some information about the mechanisms of action of compounds **1**−**6** on the parasite, we performed the following experiments:

(a). Inhibitory Effect on *T. cruzi* Fe-SOD Enzyme. Previous results concerning the inhibitory activity of compounds **1**−**4** on the essential antioxidant parasite enzyme Fe-SOD[12] prompted us to compare the effect of the whole group of compounds at a range of concentrations from 1 to 100 μM. We used epimastigote forms from the Maracay strain of *T. cruzi*, which excrete Fe-SOD when cultured in a medium lacking fetal calf serum.[19] Results obtained are displayed in Figure 5A, and the corresponding IC_{50} values have been calculated and included in the same figure. Significant inhibition values of the enzyme activity were found for the six tested compounds, with the monoalkylamino imidazole derivatives **2** and **4** being especially effective at both the lower and higher doses. Compounds **2** and **4** exhibited IC_{50} values of 9.4 and 1.3 μM, respectively. The activity of the pyrazole-based **6** was lower at a concentration of 50 μM, with a IC_{50} of 17.2 μM, although it was more active than the three disubstituted compounds.

In any case, a high degree of activity against the parasitic Fe-SOD would be of no value if the same pattern was found for human SOD. Therefore, we also compared the effect of compounds **1**−**6** over CuZn-SOD from human erythrocytes (Figure 5B). The results show that the degree of inhibition of human CuZn-SOD was very small at both higher and lower doses, with IC_{50} values reaching 103.3, 129.3, and 786.9 μM for **6**, **4**, and **2**, respectively, in sharp contrast with those calculated in Figure 5A. These results enhance the potential antiparasitic interest of the alkylamino-benzo[*g*]phthalazine derivatives studied in this work and could support a competition for the metal ion of SOD as one of the feasible mechanisms involved.

In order to obtain more information on the higher activity shown by compounds **2** and **4** over Fe-SOD, we performed a tentative molecular modeling study on the mode of interaction of the mono- and disubstituted imidazole-based compounds with the enzyme. The *T. cruzi* Fe-SOD structure was obtained from the Brookhaven protein data bank (2gcp). In a first stage, we docked molecules **1**−**4** with the enzyme using the AutoDock 4.2 program.[20] It was found that the preferred location for compounds **2** and **4** was inside the enzyme cavity, although with a poor stabilization energy (1.5 kcal/mol). Interestingly, the disubstituted compounds **1** and **3** were not able to enter the enzyme cavity. The presence of the second alkylamino chain should yield stronger interactions with external amino acids preventing entrance into the cavity. In a further approach, the ability for iron complexation was tested by selecting only one strand of the enzyme and using the AMBER forcefield implemented in Hyperchem 8.0.[21] In all cases, burial of the lateral chain into the protein involved better stabilization energies (15−33 kcal/mol) and considerable shortening of the preferred Fe−N distances to 0.4−0.5 nm.[22] However, a 0.1 nm separation of the two strands was required for allowing compounds **1** and **3** to enter the cavity, and additional destabilization energies of about 40 kcal/mol were found. As shown in Figure 6, the most effective interaction of compound **4** seemed to take place through the sp^2 imidazole nitrogens and not with the phthalazine heteroatoms. Although molecular modeling is only an orientative tool, we think that these theoretical results could contribute to explaining the activity differences found between the mono- and disubstituted compounds.

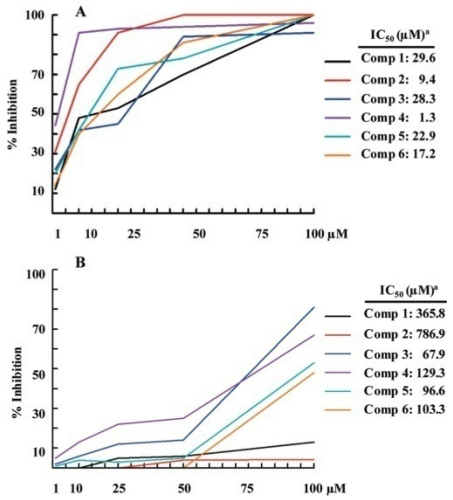


Figure 5. (A) *In vitro* inhibition (%) of Fe-SOD in *T. cruzi* epimastigotes for compounds **1**−**6** (activity 20.77 ± 3.18 U/mg). (B) *In vitro* inhibition of CuZn-SOD in human erythrocytes for compounds **1**−**6** (activity 23.36 ± 14.21 U/mg). Values are the average of five separate determinations. Differences between the activities of the control homogenate and those incubated with compounds **1**−**6** were obtained according to the Newman−Keuls test. IC_{50} (the concentration required to give 50% inhibition) was calculated by linear regression analysis from the K_c values at the concentrations employed (1, 10, 25, 50, and 100 μM).

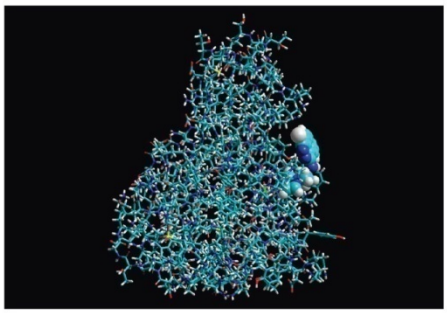


Figure 6. Interaction of the imidazole-based monosubstituted compound **4** with one strand of the *T. cruzi* Fe-SOD enzyme (Fe marked in green).

(b). Metabolite Excretion Effect. As far as it is currently known, none of the studied trypanosomatids are capable of completely degrading glucose to CO_2 under aerobic conditions, and they excrete parts of their carbon skeleton into the medium as fermented metabolites, which differ depending on the species considered.[23] *T. cruzi* consumes glucose at a high rate, thereby acidifying the culture medium due to incomplete oxidation to acids. ^{1}H NMR spectra enabled us to determine the fermented metabolites excreted by trypanosomatid in culture. One of the major metabolites excreted by *T. cruzi* is succinate, the main role of which is to probably maintain the glycosomal redox balance. It is thought that succinate provides two glycosomal oxidoreductase enzymes that allow for reoxidation of NADH, produced by glyceraldehyde-3-phosphate dehydrogenase in the glycolytic pathway. Succinic fermentation offers the significant advantage of requiring only half of the phosphoenolpyruvate (PEP) produced to maintain the NAD^+/NADH balance. The remaining PEP is converted into acetate, L-lactate, L-alanine, or ethanol, depending on the species considered.

In order to obtain information on the effect of compounds **1**−**6** on metabolite excretion, ^{1}H NMR spectra obtained from the trypanosomatids treated with **1**−**4** and **5** and **6** (Figures 1S and 2S, respectively, located in the Supporting Information) were recorded and compared with those obtained from cell-free medium (control) 4 days after inoculation with the *T. cruzi* strain. In the control experiment, *T. cruzi* excreted acetate and succinate as the major metabolites and, at a lower level, L-alanine, in agreement with previously reported data.[24] When trypanosomatids were treated with compounds **1**−**6**, the excretion of some of these catabolites was clearly altered at the dosage employed. The variation in the height of the peaks corresponding to the significant catabolites is shown in Table 3. It can be seen that the excretion of succinate and also of acetate was inhibited by compounds **1**−**4**. The inhibition of acetate and succinate excretion could explain the observed increase in L-alanine production, considering that the imidazole-based compounds operate at the electronic chain level, preventing reducing power recharge, or even that they act on the mitochondria and, consequently, oxidative phosphorylation is affected. The pyrazole-based compounds **5** and **6** exhibited a different behavior. Succinate was the only one of the abovementioned metabolites that was inhibited by compound **5**, and a new peak, which was identified as malate appeared. This could be explained on the basis of its action at the fumarate reductase level, leading to inhibition of succinate formation and excretion of malate. On the other hand, compound **6** could inhibit any of the enzymes involved in the formation of acetate from pyruvate (i.e., succinate dehydrogenase). Both **5** and **6** strongly inhibited lactate excretion, a fact that could be explained by a direct action on the lactic fermentation enzyme. It should be noted that we did not find any significant alteration of fuel metabolism in the presence of benznidazole.

Table 3. Variation in the Height of the Peaks Corresponding to Catabolites Excreted by *T. cruzi* Epimastigotes in the Presence of Compounds **1**−**6** with Respect to the Control Test[a]

compound	Ala	A	S	Lac	Mal
1	+35%	−30%	−39%	=	ud
2	+22%	−18%	−37%	=	ud
3	+43%	−22%	−27%	=	ud
4	+61%	−26%	−49%	=	ud
5	=	=	−24%	−55%	+100%
6	=	−36%	+27%	−61%	ud

[a] Ala, L-alanine; A, acetate; S, succinate; Lac, lactate; Mal, malate; (−) peak inhibition; (+) peak increasing; (=) no difference detected; (ud) peak undetected.

(c). Ultrastructural Alterations. We found by transmission electron microscopy (TEM) that morphological alterations are important in *T. cruzi* epimastigotes treated with compounds **1**, **2**, **4**, **5**, and **6** as compared with controls cells (Figure 7). Significant damage was found in the case of compound **1**. Many parasites were dead, and alterations were evident in most of the survivors. Especially significant was the presence of a much higher number of glycosomes than in the control (panel control of Figure 7, panel 1 of Figure 7). An extensive vacuolization was also found in many cases. Vacuolization was also the most characteristic feature observed in parasites treated with compound **2**, together with the presence of cytoplasms containing empty vacuoles and the absence of cytoplasmic organelles (panel 2 of Figure 7).

The most extensive damage was found in parasites treated with compound **4** (panel 4 of Figure 7). The shape of many parasites was so distorted that their morphology was completely unrecognizable, and neither the nucleus not the cytoplasmic organelles could be observed. Many others were dead, and a great number of flagella could be observed inside the supernatant liquid. The few recognizable parasites always presented with empty or lipidic vacuoles. Modifications related to those found with **4** were observed after treatment of the epimastigotes with compound **5** (panel 5 of Figure 7). Most of the cytoplasms were electron dense and exhibited bulk vacuoles, nucleus and shape were also unrecognizable, and both kinetoplasts and mitochondria were inflated. The presence of **6** (panel 6 of Figure 7) also resulted in distorted shapes and strong vacuolization. In summary, a wide range of ultrastructural alterations in epimastigote forms of *T. cruzi* treated with these compounds were found. These alterations, which took place mainly at the mitochondria level, explain the metabolic changes commented above in the production of succinate and acetate and may have been due to disturbances in the activity of enzymes involved in the pyruvate metabolism inside the mitochondria.

Histopathological Analysis. In order to get better insight into the toxicity level induced by our compounds on the liver, a key organ in many vital functions, we performed a

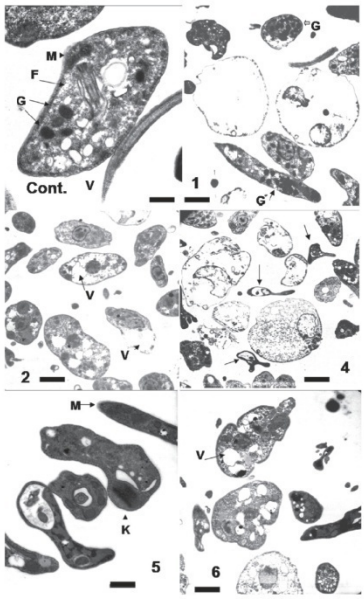


Figure 7. Ultrastructural alterations by TEM in *T. cruzi* treated with compounds **1**, **2**, and **4**−**6**: (Cont) control parasite showing organelles with their characteristic aspects, such as nucleus (N), kinetoplast (K), reservosomes (R) mitochondrion (M), flagellum (F), vacuole (V), and glycosomes (G); bar = 583 μm; (1) epimastigotes of *T. cruzi* treated with compound **1**; bar = 1.00 μm; (2) epimastigotes of *T. cruzi* treated with compound **2**; bar = 1.59 μm; (4) epimastigotes of *T. cruzi* treated with compound **4**; bar = 1.59 μm; (5) epimastigotes of *T. cruzi* treated with compound **5**; bar = 583 μm; (6) epimastigotes of *T. cruzi* treated with compound **6**; bar = 1.00 μm.

histopathological analysis on mice infected with the parasite and treated with the monosubstituted compounds **2**, **4**, and **6** (Figure 8). Those compounds were selected because of their especially good activity results in both the *in vivo* and *in vitro* tests. We observed that mice infected but not treated developed several alterations to the liver after 90 days, undoubtedly due to the action of the parasite during the chronic phase. Among them were lymphocytic infiltration with the formation of many microgranulomas (+++++), delocalized hepatic destruction (+++), and, to a lesser extent, lymphocytic infiltration of the portal tracts (++), and interstitial hemorrhage (++). When parasite-infected mice were treated with the reference drug (BZN), lymphocytic infiltration and the formation of granulomas diminished (++), due to the lower number of parasites remaining after drug treatment. In contrast, a significant increase in delocalized hepatic destruction (++++), very severe hepatic destruction in the portal tracts (+++++), and the presence of necrotic cells in those tracts (+++) were found, caused by the toxicity of BZN.

Treatment of infected mice with the monosubstituted compounds also showed some hepatic damage 90 dpi. However, alterations were never as severe as those ones

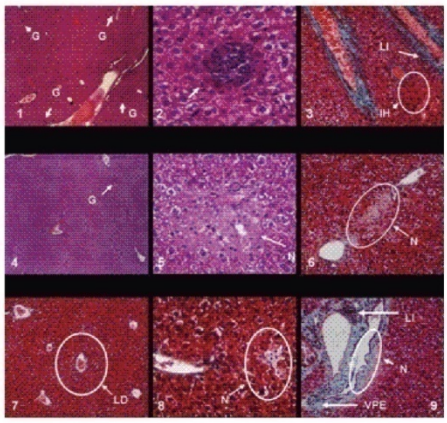


Figure 8. Histopathological analysis of the liver. First line: Mice were infected but left untreated for 90 dpi (1) resulting in microgranulomas (G); (2) detail of a microgranuloma; (3) lymphocytic infiltration (LI) and interstitial hemorrhage (IH). Second line shows mice infected and treated with BZN (5 mg/kg) for 90 dpi resulting in (4) microgranulomas (G) and (5, 6) necrosis (N). Third line shows mice infected and treated with the monosubstituted compounds (5 mg/kg) after 90 dpi: (7) compound **2** shows damage in the portal tract zone (LD); (8) compound **4** shows necrosis (N); (9) compound **6** shows lymphocytic infiltration in the portal tract zone (LI), necrosis (G), and thickening of the blood vessel walls in the portal tracts (VPE).

commented on above for BZN, indicating a lower toxicity for the three compounds tested. Only a very slight formation of granulomas with lymphocytic infiltration (+) and minor delocalized hepatic destruction (+) could be detected after treatment with **2**. In the case of **4**, a light thickening of the blood vessel walls was also observed. Pyrazole-based **6** appeared to be a little more toxic, since the livers presented some lymphocytic infiltration in the portal tracts (++), together with a small interstitial hemorrhage (++). We can conclude that, on the hepatic level, our monosubstituted compounds **2**, **4**, and **6** are much less toxic for the host than BZN, and compound **4** is apparently the most benign among the three of them.

Experimental Section

Chemistry. 3-Aminopyrazole was purchased from a commercial source (Aldrich) and used without further purification. 1,4-Dichlorobenzo[*g*]-phthalazine was obtained from 2,3-naphthalenedicarboxylic acid according to a procedure previously reported by our group.[25] Solvents were dried using standard techniques.[26] All reactions were monitored using thin layer chromatography (TLC) on precoated aluminum sheets of silicagel $60F_{254}$. Compounds were detected with UV light (254 nm). Chromatographic separations were performed on columns in the indicated solvent system using flash chromatography on silicagel (particle size 0.040−0.063 mm). Melting points were determined in a Gallenkampf apparatus and were uncorrected. ^{1}H NMR spectra were recorded on a Bruker 300 and a Variant XL 300 at 300 MHz and ^{13}C NMR spectra at 75 MHz at room temperature employing DMSO-d_6 as the solvent. Chemical shifts are reported in ppm (δ scale) from TMS. All assignments were performed on the basis of ^{1}H−^{13}C heteronuclear multiple

quantum coherence experiments (gHMQC and gHMBC). IR spectra were recorded on a Perkin-Elmer 257 spectrometer (4000−400 cm^{-1} range). Electrospray mass spectra were recorded with a Hewlett-Packard 1100 MSD apparatus. Elemental analyses were performed in a Perkin-Elmer 2400-CHN instrument by the Departamento de Analisis, Centro de Química Orgánica "Manuel Lora-Tamayo", CSIC, Madrid, Spain. Elemental analysis was used to ascertain purity higher than 95% for all the biologically tested compounds.

Synthesis of 1,4-Bis(3′-pyrazolylamino)benzo[*g*]phthalazine (5) and 4-Chloro-1-(3′-pyrazolylamino)benzo[*g*]phthalazine (6) (Method A). A solution of 1,4-dichlorobenzo[*g*]phthalazine (278 mg, 1.12 mmol), 3-aminopyrazole (204 mg, 2.45 mmol), and triethylamine (0.6 mL, 3.60 mmol) in xylene (20 mL) was heated at 110 °C for 10 h. After cooling to room temperature, the reaction mixture afforded a reddish precipitate, which was filtered and dried under vacuum, giving 53 mg (13% yield) of a pure crystalline solid that was identified as **5**·HCl. Mp 286 °C (d). IR (KBr) *ν*: 3500−2500, 3137, 2959, 2925, 2855, 1573, 1484, 1440, 1419, 1124, 1048, 999, 924, 894, 748 cm^{-1}. ^{1}H NMR (DMSO-d_6): *δ* 12.21 (brsg, 2H, NH pyrazole), 9.49 (s, 2H, H-5, H-10), 8.25 (m, 2H, H-6, H-9), 7.87 (m, 2H, H-7, H-8), 7.79 (d, 2H, H-5′), 6.55 (d, 2H, H-4′). ^{13}C NMR (DMSO-d_6): *δ*: 149.15 (C-3′), 145.26 (C-1, C-4), 133.99 (C-5a, C-9a), 130.08 (C-5′), 129.45 (C-7, C-8), 128.97 (C-6, C-9), 125.39 (C-5, C-10), 119.54 (C-4a, C-10a), 97.20 (C-4′) ppm. ESI-MS (positive mode, MeOH) *m*/*z* (%): 343 ([MH − Cl]$^+$, 100). Anal. ($C_{18}H_{14}N_8$·HCl·0.5H_2O) C, H, N, Cl.

The remaining xylene solution was evaporated under vacuum, and the residue was chromatographed on a silicagel supported column using a mixture of increasing polarity of toluene/ethyl acetate/ethanol as the eluent. From the fraction with R_f = 0.58 (toluene/ethyl acetate/ethanol, v/v = 2:1:0.3), 39 mg (12%) of a yellow solid was isolated and identified as corresponding to the free monosubstituted compound **6**. Mp: 235 °C (desc.). IR (KBr) *ν*: 3000−3500, 3330, 3214, 2923, 2854, 1616, 1566, 1439, 1393, 976, 892, 752 cm^{-1}. ^{1}H NMR (DMSO-d_6): *δ* 12.7 (brsg, 1H, NH pyrazole), 10.40 (s, 1H, H-10), 9.01 (s, 1H, H-5), 8.48 (d, 1H, H-5′), 8.43 (m, 1H, H-9), 8.27 (m, 1H, H-6), 7.84 (m, 2H, H-7, H-8), 6.02 (d, 1H, H-4′) ppm. ^{13}C NMR (DMSO-d_6): *δ* 159.54 (C-1), 151.69 (C-4), 150.01 (C-3′), 134.44 (C-5a, C-9a), 131.95 (C-5′), 129.66 (C-10), 129.56, 129.47, 129.36, 129.18 (C-6, C-7, C-8, C-9), 125.45 (C-5), 123.31 (C-10a), 118.06 (C-4a), 98.83 (C-4′) ppm. ESI-MS (positive mode, MeOH) *m*/*z* (%): 296 (MH^+, 100). Anal. ($C_{15}H_{10}N_5Cl$·0.5C_2H_5OH) C, H, N, Cl.

Alternative Synthesis of 4-Chloro-1-(3′-pyrazolylamino)benzo-[*g*]phthalazine (6) (Method B). To a suspension, heated at reflux, of 1,4-dichlororobenzo[*g*]phthalazine (450 mg, 1.80 mmol) and potassium carbonate (2.45 g, 17.75 mmol) in acetonitrile (80 mL), 300 mg (3.61 mmol) of 3-aminopyrazole in 30 mL of acetonitrile was added dropwise, and the mixture was heated under reflux for 20 h. After cooling to room temperature, the orange solid containing the reaction product and potassium carbonate was filtered, dissolved in chloroform, and treated with a 20% NaOH aqueous solution. After the organic layer was washed with water and the solvent was evaporated under vacuum, the residue was chromatographed on a silicagel supported column using a mixture of increasing polarity of toluene/ethyl acetate/ethanol as the eluent. From the fraction with R_f = 0.58 (toluene/ethyl acetate/ethanol, v/v = 2:1:0.3), 124 mg (24%) of a yellow solid was isolated and identified as indicated above as the free monosubstituted compound **6**.

Parasite Strain and Culture. The Maracay strain of *T. cruzi* was isolated at the Institute of Malariology and Environmental Health in Maracay (Venezuela). Epimastigote forms were obtained in biphasic blood-agar NNN medium (Novy−Nicolle−McNeal) supplemented with minimal essential medium (MEM) and 20% inactivated fetal bovine serum and afterward reseeded in a monophasic culture (MTL), following the method of Luque et al.[27]

***In Vitro* Trypanosomicidal Activity Assay.** To obtain the parasite suspension for the trypanosomicidal assay, the epimastigote culture (in the exponential growth phase) was concentrated by centrifugation at 1000*g* for 10 min, and the number of flagellates were counted in a hemocytometric chamber and distributed into aliquots of 2 × 10^6 parasites/mL. The compounds were solved in DMSO (Panreac, Barcelona, Spain) at a concentration of 0.01%, previously assayed as nontoxic and without inhibitory effects on parasite growth. The compounds were dissolved in the culture medium, and the doses used were 100, 50, 25, 10, and 1 *μ*M. The effect of each compound against epimastigote forms, as well as the concentrations (assayed at different concentrations for every drug), were evaluated at 72 h, using a Neubauer hemocytometric chamber.

Cell Culture and Cytotoxicity Tests. Vero cells (Flow) were grown in MEM (Gibco), supplemented with l0% inactivated fetal calf serum and adjusted to pH 7.2, in a humidified 95% air−5% CO_2 atmosphere at 37 °C for 2 days. For the cytotoxicity test, cells were placed in 25 mL colie-based bottles (Sterling) and centrifuged at 100*g* for 5 min. The culture medium was removed, and fresh medium was added to a final concentration of 1 × 10^5 cells/mL. This cell suspension was distributed in a culture tray (with 24 wells) with 100 *μ*L/well and incubated for 2 days at 37 °C in a humid atmosphere enriched with 5% CO_2. The medium was removed, and fresh medium was added together with the product to be studied (at concentrations of 100, 50, 25, 10 and 1 *μ*M). The cultures were incubated for 72 h. The vital stain trypan blue (0.1% in phosphate buffer) was used to determine cell viability. The number of dead cells was recorded, and the percent viability was calculated in comparison to that of the control culture. The IC_{50} values were calculated by linear regression analysis from the K_c values at the concentrations employed.

Transformation of Epimastigotes to Metacyclic Forms. In order to induce metacyclogenesis, parasites were cultured at 28 °C in modified Grace's medium (Gibco) for 12 days as previously described.[28] Twelve days after cultivation at 28 °C, metacyclic forms were counted in order to infect Vero cells. The proportion of metacyclic forms was around 40% at this stage.

Cell ®assay. Vero cells were cultured in MEM medium in a humidified 95% air−5% CO_2 atm at 37 °C. Cells were seeded at a density of 1 × 10^5 cells/well in 24-well microplates (Nunc) with rounded coverslips on the bottom and cultivated for 2 days. Afterward, the cells were infected *in vitro* with metacyclic forms of *T. cruzi*, at a ratio of 10:1. The drugs (IC_{25} concentrations) were added immediately after infection and were incubated for 6 h at 37 °C in a 5% CO_2. The nonphagocytosed parasites and drugs were removed by washing, and then the infected cultures were grown for 10 days in fresh medium. Fresh culture medium was added every 48 h.

The drug activity was determined from the percentage of infected cells and the number of amastigotes per cell infected in treated and untreated cultures in methanol-fixed and Giemsa-stained preparations. The percentage of infected cells and the mean number of amastigotes per infected cell were determined by analyzing more than 100 host cells distributed in randomly chosen microscopic fields. Values are the means of four separate determinations. The number of trypomastigotes in the medium was determined as previously described.[28]

***In Vivo* Trypanocidal Activity Assay.** Groups of five BALB/c female mice (6−8 weeks old; 25−30 g) maintained under standard conditions were infected with 3 × 10^3 bloodstream *T. cruzi* metacyclic forms by the intraperitoneal route. The animals were divided into the following groups: (i) group 1 uninfected (not infected and not treated); (ii) group 2 untreated (infected with *T. cruzi* but not treated); (iii) group 3 uninfected (not infected and treated with 1 mg/kg of body weight/day, for five consecutive days (7−12 days postinfection) by the intraperitoneal route);[18] and (iv) group 4 treated (infected and treated for five consecutive days (7−12 days postinfection) with the compounds and benznidazole).

Five days after infection, the presence of circulating parasites was confirmed by the microhematocrit method. Five microliters of blood drawn from the tail of the treated mice were taken and diluted 1:15 (50 μL of citrate buffer and 20 μL of lysis buffer at pH 7.2), and this vehicle also was employed as a negative control. The counting of parasites was done in a Neubauer chamber. The number of deaths was recorded every two days.

One group of four animals treated with each compound and BZN was included for serologic studies. Treatments were started seven days after animal infection. Compounds were administered in a similar way to the methods described above and at the same concentration.

Quantitative evaluation of circulating anti-*Trypanosoma cruzi* antibodies at days 40 and 120 postinfection was carried out by the use of an enzyme-linked immunoassay. The sera, diluted to 1:100, were reacted with an antigen constituted by a soluble Fe-SOD of *T. cruzi* epimastigotes. The results are expressed as the ratio of the absorbance (Abs) of each serum sample at 490 nm to the cutoff value. The cutoff for each reaction was the mean of the values determined for the negative controls plus three times the standard deviation.

SOD Enzymatic Inhibition in the Presence of Compounds 1−6. The parasites were cultured as described above and suspended (0.5−0.6 g/mL) in 3 mL of buffer 1 (0.25 M sucrose, 25 mM Tris−HCl, 1 M EDTA, pH 7.8) and disrupted by three cycles of sonic disintegration, 30 s each at 60 V. The sonicated homogenate was centrifuged at 1500*g* for 10 min at 4 °C, and the pellet was washed three times with ice-cold STE buffer 1, for a total supernatant fraction of 9 mL. This fraction was centrifuged (2500*g* for 10 min at 4 °C), the supernatant was collected, and solid ammonium sulfate was added. The protein fraction, which precipitated between 35% and 85% salt concentration, was centrifuged (9000*g* for 20 min at 4 °C), redissolved in 2.5 mL of 20 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA (buffer 2), and dialyzed in a Sephadex G-25 column (Pharmacia, PD 10), previously balanced with buffer 2, bringing it to a final volume of 3.5 mL (fraction of the homogenate). The protein concentrations were determined by the Bradford method.

Iron superoxide dismutase (Fe−SOD) activity was determined by NAD(P)H oxidation according to Paoletti and Mocali.[29] One unit was the amount of enzyme required to inhibit the rate of NAD(P)H reduction by 50%. CuZn-SOD from human erythrocytes used in these assays was obtained from Boehringer (Mannheim), while all the coenzymes and substrates came from Sigma Chemical Co. Data obtained were analyzed according to the Newman−Keuls test.

Metabolite Excretion. Cultures of *T. cruzi* epimastigotes (initial concentration 5×10^5 cells/mL) received IC_{25} of the compounds (except for control cultures). After incubation for 72 h at 28 °C, the cells were centrifuged at 400*g* for l0 min. The supernatants were collected to determine excreted metabolites by nuclear magnetic resonance spectroscopy (^{1}H NMR) as previously described by Fernández-Becerra et al.[30] Chemical shifts were expressed in parts per million (ppm), using sodium 2,2-dimethyl-2-silapentane-5-sulfonate as the reference signal and were used to identify the respective metabolites, resulting in findings consistent with those described by Fernández-Becerra et al.[30]

Ultrastructural Alterations. The parasites, at a density of 5×10^6 cells/mL, were cultured in their corresponding medium, containing the drugs at the IC_{25} concentration. After 72 h, the cultures were centrifuged at 400*g* for 10 min, and the pellets were washed in PBS and then fixed with 2% (v/v) formaldehyde−glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4) for 2 h at 4 °C. Pellets were prepared for transmission electron microscopy following the technique of Luque et al.[27]

Histopathological Analysis. At 90 dpi, livers were removed, cut longitudinally, rinsed in ice-cold PBS, and fixed in 10% buffered formalin. The tissues were dehydrated and embedded in paraffin. Sections were cut at a thickness of 4−5 μm and stained with Giemsa, hematoxylin−eosin, and Masson's trichrome. Slides were coded for blinded analysis and histological examinations were performed using a conventional light microscope and were visualized in at least 30 fields (total magnifications, ×40, ×100, ×200, and ×400) for each slide. Histological alterations were given a score of 0 (−) to 5 (+++++), with 0 representing the complete absence of alterations and 5 representing the most severe alterations, respectively.

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Supporting Information Available: Details on combustion analysis and the NMR spectra obtained from the metabolite excretion studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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Supporting information

In vivo Trypanosomicidal Activity of Imidazole- or Pyrazole-Based Benzo[*g*]phthalazine Derivatives against Acute and Chronic Phases of Chagas Disease

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Table of Contents:

Elemental Analysis

Compound	Molecular Formula	Anal. Calculated	Anal. Found
1,4-bis(3´-pyrazolylamino)benzo[*g*]-phthalazine hydrochloride	($C_{18}H_{14}N_8$·HCl·0.5H_2O)	C, 55.75 H, 4.16 N, 28.89 Cl, 9.14	C, 55.48 H, 4.36 N, 28.64 Cl, 8.90
4-chloro-1-(3´-pyrazolylamino)benzo-[*g*]phthalazine	($C_{15}H_{10}N_5Cl$·0.5C_2H_5OH)	C, 60.29 H, 4.11 N, 21.97 Cl, 11.12	C, 60.50 H, 4.28 N, 21.68 Cl, 10.82

In vitro leishmanicidal activity of imidazole –or pirazole- based benzo[g]phthalazine derivatives against *Leishmania infantum* and *Leishmania braziliensis* species. *J Antimicrob Chemother* 2012; 67: 387–97

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In vitro leishmanicidal activity of imidazole- or pyrazole-based benzo[*g*]phthalazine derivatives against *Leishmania infantum* and *Leishmania braziliensis* species

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Objectives: To evaluate the *in vitro* leishmanicidal activity of imidazole-based (**1**–**4**) and pyrazole-based (**5**–**6**) benzo[*g*]phthalazine derivatives against *Leishmania infantum* and *Leishmania braziliensis*.

Methods: The *in vitro* activity of compounds **1**–**6** was assayed on extracellular promastigote and axenic amastigote forms, and on intracellular amastigote forms of the parasites. Infectivity and cytotoxicity tests were performed on J774.2 macrophage cells using meglumine antimoniate (Glucantime) as the reference drug. The mechanisms of action were analysed by iron superoxide dismutase (Fe-SOD) and copper/zinc superoxide dismutase (CuZn-SOD) inhibition, metabolite excretion and transmission electronic microscopy (TEM).

Results: Compounds **1**–**6** were more active and less toxic than meglumine antimoniate. Data on infection rates and amastigote mean numbers showed that **2**, **4** and **6** were more active than **1**, **3** and **5** in both *L. infantum* and *L. braziliensis*. The inhibitory effect of these compounds on the antioxidant enzyme Fe-SOD of promastigote forms of the parasites was remarkable, whereas inhibition of human CuZn-SOD was negligible. The ultrastructural alterations observed in treated promastigote forms confirmed the greater cell damage caused by the most active compounds **2**, **4** and **6**. The modifications observed by ^{1}H-NMR in the nature and amounts of catabolites excreted by the parasites after treatment with **1**–**6** suggested that the catabolic mechanisms could depend on the structure of the side chains linked to the benzo[*g*]phthalazine moiety.

Conclusions: All the compounds assayed were active *in vitro* against the two *Leishmania* species and were less toxic against mammalian cells than the reference drug, but the monosubstituted compounds were significantly more effective and less toxic than their disubstituted counterparts.

Keywords: *L. infantum*, *L. braziliensis*, Fe-SOD, metabolite excretion, ultrastructural alterations in trypanosomatids

Introduction

Leishmaniasis is the name assigned to a group of diseases caused by kinetoplastid protozoan parasites of the genus *Leishmania*. Its impact on public health is evident when considering the fast expansion of endemic zones over recent years. It is now endemic in 98 countries, mainly in the New World, but also in Europe and Asia. More than 350 million people are at risk, and 2 million new cases arise every year, with an annual mortality rate higher than 60000, a number that is only surpassed by malaria among parasitic diseases.[1,2] In fact, leishmaniasis is placed ninth in the WHO global analysis of the most severe infectious diseases. In recent times, it has been reported that coinfection of HIV with leishmaniasis in immunocompromised hosts is a new factor that is increasing the endemic areas all around the world.[3]

Leishmaniasis appears as three major clinical forms in humans: (i) visceral, the most severe and life-threatening form; (ii) cutaneous, originating as nodules and ulcers that may persist for years; and (iii) mucocutaneous, causing permanent lesions in the mouth, nose or genital mucosa.[4] They are produced by multiple and phylogenetically distinct species. *Leishmania infantum* is considered the main aetiological agent of visceral leishmaniasis in southern Europe. It uses dogs as the reservoir and affects mainly children between 1 and 4 years old, although since the advent of HIV infection and

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increased use of immunosuppression for transplants and chemotherapy, nearly half of the new cases are now in adults.[5–7] Another significant species, derived from the subgenus *Viannia*, is *Leishmania braziliensis*, which occurs mainly in the Andean countries and the Amazonian basin and causes cutaneous and mucocutaneous leishmaniasis.[1,2,8] Those two species were selected by us as representative targets for the biological assays performed in the present work.

The design of new drugs active against the different forms of these parasitic diseases, especially visceral leishmaniasis, is an urgent necessity. To date, no effective vaccination is available, and diagnostic tools are not specific due to deficient vector control measures.[9,10] Physicians rely mainly on chemotherapy as the primary weapon to fight all the clinical manifestations mentioned above. However, the drugs in use until now are largely ineffective or cause a multitude of severe toxic side effects and, furthermore, the parasitic organisms rapidly develop strong drug resistance, decreasing their sensitivity to these drugs.[11] For more than 50 years, heavy metal derivatives, mainly pentavalent antimonials, have been used as the standard drugs for the treatment of leishmaniasis. Among them, the most representative are sodium stibogluconate (Pentostam) and meglumine antimoniate (Glucantime). It is supposed, but not verified, that they act by inhibiting ATP synthesis. However, antimonials cause many toxic effects, including nausea, vomiting, diarrhoea, skin eruptions, dizziness, cardiac arrhythmia, hypotension, hepatitis and pancreatitis. Furthermore, drug administration is difficult, low dosages favour resistance in the parasite and higher dosages are disturbingly toxic.[12,13] Other types of drugs have been tested recently. Pentamidines are only effective against cutaneous leishmaniasis and, although better tolerated, they cause diabetes mellitus at high doses. Fluconazole, amphotericin B and miltefosine are not useful against visceral leishmaniasis and, in spite of their milder toxicity, effectiveness against other forms of the disease is limited.[12,13] In summary, more effective, less expensive and less toxic drugs are needed.

Antioxidant enzymes count among the main defence mechanisms for the survival of *Leishmania* parasites. They protect the parasite against the toxic oxygen intermediates and nitrogen species produced during mitochondrial respiration that damage proteins, lipids and DNA, leading to cell death.[14] One of the most important is superoxide dismutase (SOD), which is localized in the mitochondria and is responsible for the dismutation of radical anion peroxide into oxygen peroxide and molecular oxygen. Although there are several types of SOD, they can be differentiated by the nature of the metal ion co-factors (i.e. Cu^{2+} and Zn^{2+} in human SOD). Interestingly, the only type of SOD present in trypanosomatids is iron SOD (Fe-SOD).[15] The enzyme appears as a dimeric protein, and the coordination geometry of the active Fe site is a distorted trigonal bipyramidal arrangement, whose axial ligands are His43 and a solvent molecule, and whose in-plane ligands are His95, Asp195 and His199.[16] Studies performed on the role of this enzyme in *Leishmania* species confirm that it is an essential defence line in host–parasite relationships. Since Fe-SOD is not present in humans, it may be considered an attractive target for leishmaniasis drug therapy.[17]

In recent years our research group has designed new heterocyclic systems with general structures I and II (Figure 1). These compounds contain a nucleus of benzo[*g*]phthalazine

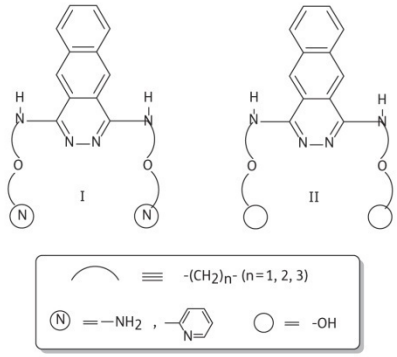

Figure 1. Schematic models of 1,4-bis(alkylamino)benzo[*g*]phthalazine derivatives functionalized with amino, pyridine or hydroxy groups at the end of the side chains.

functionalized in the 1 and 4 positions of the pyridazine ring with flexible alkylamino side chains. The alkylamino substituents vary in nature and length. Series I exhibit terminal sp^3 amino groups or heterocyclic rings endowed with sp^2 nitrogen atoms, whereas series II is characterized by the presence of a terminal OH group. Both models have shown complexing ability for transition metals, but the complex structure strongly depends on the side chain terminal functionalization: the sp^2 or sp^3 nitrogens of podands I are actively involved in complexation, giving rise to monopodal dinuclear complexes, whereas podands II afford tripodal dinuclear complexes in which the terminal hydroxy groups are not involved.[18] The antiparasitic activity of both groups of compounds has been evaluated *in vitro* against *Trypanosoma cruzi* epimastigotes and amastigotes, and good activities were found for the type I compounds, especially in the case of sp^2 nitrogens, while the type II compounds were clearly less active. Furthermore, structures with terminal nitrogens inhibited the action of the Fe-SOD enzyme much better than one with terminal OH groups. Human copper/zinc SOD (CuZn-SOD) inhibition was negligible in any case. We concluded that, in all cases tested, the complexing ability could be related in some way to Fe-SOD inhibition and, consequently, to trypanosomicidal activity.[18]

With the aim of improving the biological results described above, new 1-alkylamino-4-chloro- and 1,4-dialkylaminobenzo[*g*]phthalazine derivatives with general structures III and IV (Figure 2) were prepared.[19,20] The side chains were functionalized with terminal imidazole (compounds **1**–**4**, Figure 3) or pyrazole (compounds **5**–**6**) rings. Both pentagonal heterocycles are endowed with electron-donating sp^2 nitrogens, which suggests excellent complexing properties against transition metals, and the imidazole derivatives had previously been shown to be active against trypanosomatids.[12] Activity tests performed *in vitro* and *in vivo* with *T. cruzi* demonstrated that all compounds **1**–**6** were effective against both the acute and chronic phases of Chagas' disease. Antiparasitic activity *in vitro* was enhanced

with respect to series I and II. Moreover, the new compounds were more active and less toxic than the reference drug (benznidazole). Among them, the monosubstituted derivatives **2**, **4** and **6** gave better results both in terms of activity and the absence of toxicity than their disubstituted analogues. In accordance with our expectations, excellent values of inhibition of Fe-SOD activity were also obtained, whereas the effect on human CuZn-SOD was negligible.[19,20]

Taking into account the essential role played by the Fe-SOD enzyme in both *Leishmania* and *T. cruzi* parasites, we considered it to be of great interest to study the activity of compounds **1**–**6** against *L. infantum* and *L. braziliensis* (promastigote and amastigote forms), as representative species causing visceral and cutaneous leishmaniasis, respectively. In this work, their antiproliferative activity and unspecific mammalian cytotoxicity in the species considered were evaluated *in vitro*, and these measures were complemented by infectivity assays on J774.2 macrophage cells. Furthermore, the treated parasites were submitted to a thorough study of the possible mechanisms of action of the compounds assayed, as follows: (i) inhibition of the parasitic Fe-SOD and human CuZn-SOD enzymes was tested and compared; (ii) an ^{1}H-NMR study concerning the nature and percentage of metabolite excretion was performed in order to obtain information on the inhibitory effect of **1**–**6** on the glycolytic pathway, since it represents the primary source of energy for the parasite; and (iii) alterations caused in the cell ultrastructure of the parasites were recorded using transmission electronic microscopy (TEM).

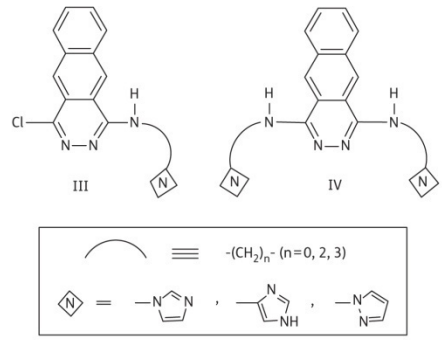

Figure 2. Schematic models of 1-alkylamino-4-chloro- and 1,4-bis(alkylamino)benzo[g]phthalazine derivatives functionalized with imidazole or pyrazole rings at the end of the side chains.

Materials and methods

Chemistry

The starting amines were 2-(imidazol-4-yl)-ethylamine (histamine), 3-(imidazol-1-yl)propylamine and 3-aminopyrazole and were purchased from Sigma-Aldrich and used without further purification. 1,4-Dichlorobenzo[g]phthalazine was obtained from 2,3-naphthalenedicarboxylic acid as previously reported by our group.[21] Solvents were dried using standard techniques.[22] All the reactions were monitored using thin-layer chromatography (TLC) on precoated aluminium sheets of silica gel 60F254. Compounds were detected with UV light (245 nm). Chromatographic separations were performed on columns using flash chromatography on silica gel (particle size 0.040–0.063 mm) or standard techniques on basic aluminium oxide. Melting points were determined in a Reichert–Jung hot-stage microscope. ^{1}H- and ^{13}C-NMR spectra were recorded with Varian Unity XL-300, Varian Unity Inova-400 or Varian Unity 500 spectrometers at room temperature employing $CDCl_3$, CD_3OD or DMSO-d_6 as solvents. Chemical shifts were recorded in ppm from tetramethylsilane (Δ scale). Infrared (IR) spectra were recorded on a Perkin Elmer 681 spectrometer. Electrospray mass spectra were recorded with a Hewlett-Packard 1100 MSD apparatus and fast atomic bombardment mass spectra with a VG Autospec spectrometer using an *m*-nitrobenzyl alcohol matrix. Elemental analyses were provided by the Departamento de Análisis, Centro de Química Orgánica 'Manuel Lora Tamayo', CSIC, Madrid, Spain.

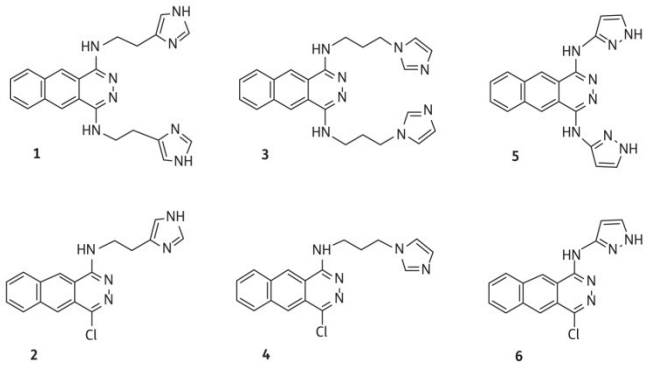

Figure 3. Benzo[g]phthalazine derivatives tested against *Leishmania infantum* and *Leishmania braziliensis* in this study.

The initial preparation and identification of compounds **1**–**6** has been described previously by our group, but was carried out again in sufficient quantities and purity for performing all the biological assays described in this work. The syntheses followed the procedures established by us,[19,20] so only the amounts employed and the yields obtained are indicated below.

Preparation of the imidazole-based compounds 1 and 2

A solution of 1,4-dichlorobenzo[*g*]phthalazine (1.54 g, 6.24 mM), 2-(imidazol-4-yl)ethylamine (2.77 g, 25.0 mM) and triethylamine (1.90 g, 19 mM) in xylene (200 mL) was heated at 120–130°C for 10 h. Standard work up of the reaction mixture[19] afforded 0.52 g (22% yield) of 1,4-bis[2-(imidazol-4-yl)ethylamino]benzo[*g*]phthalazine [**1**, melting point (mp) 124–125°C, mass spectra fast atom bombardment (MS FAB): 399 [M^++1]] and 1.32 g (63% yield) of 1-[2-(imidazol-4-yl)ethylamino]-4-chlorobenzo[*g*]phthalazine (**2**, mp 243–245°C, MS FAB 323 [M^+]).

Preparation of the imidazole-based compounds 3 and 4

A solution of 1,4-dichlorobenzo[*g*]phthalazine (1.60 g, 6.44 mM), 3-(imidazol-1-yl)propylamine (3.10 g, 26.0 mM) and triethylamine (1.90 g, 19 mM) in xylene (200 mL) was heated at 120–130°C for 10 h. Standard work up of the reaction mixture[19] afforded 0.81 g (29% yield) of 1,4-bis[3-(imidazol-1-yl)propylamino]benzo[*g*]phthalazine (**3**, mp 120–123°C, MS FAB: 427 [M^++1]) and 1.01 g (49% yield) of 1-[3-(imidazol-1-yl)propylamino]-4-chloro-benzo[*g*]phthalazine (**4**, mp 215–217°C, MS FAB 337 [M^+]).

Preparation of the pyrazole-based compounds 5 and 6

A solution of 1,4-dichlorobenzo[*g*]phthalazine (2.24 g, 8.96 mM), 3-aminopyrazole (1.64 g, 19.60 mM) and triethylamine (4.80 g, 48 mM) in xylene (250 mL) was heated at 120–130°C for 10 h. Standard work up of the reaction mixture[20] afforded 0.60 g (18% yield) of 1,4-bis(3′-pyrazolylamino)benzo[*g*]phthalazine in the monohydrochloride form [**5**·HCl, mp 284–286°C, electrospray ionization mass spectra (ESI-MS) positive mode: 343 [MH-Cl]$^+$] and 0.40 g (16% yield) of free 4-chloro-1-(3′-pyrazolylamino)benzo[*g*]phthalazine (**6**, mp 232–234°C, ESI-MS positive mode 296 [MH]$^+$).

Parasite strain and culture

L. infantum (MCAN/ES/2001/UCM-10) and *L. braziliensis* (MHOM/BR/1975/M2904) were cultured *in vitro* in medium trypanosomes liquid (MTL) together with 10% inactive fetal calf serum (FCS) kept in an air atmosphere at 28°C, in Roux flasks (Corning, USA) with a surface area of 75 cm^2, according to the methodology described by González *et al.*[23]

SOD enzymatic inhibition

Parasites cultured as described above were suspended (0.5–0.6 g/mL) in 3 mL of STE buffer 1 (0.25 M sucrose, 25 mM Tris–HCl, 1 M EDTA, pH 7.8) and disrupted by three cycles of sonic disintegration, for 30 s each at 60 V. The sonicated homogenate was centrifuged at 1500 **g** for 10 min at 4°C, and the pellet was washed three times with ice-cold STE buffer 1, giving a total supernatant fraction of 9 mL. This fraction was centrifuged (2500 **g** for 10 min at 4°C), the supernatant was collected and solid ammonium sulphate was added. The protein fraction, which precipitated between 35% and 85% salt concentration, was centrifuged (9000 **g** for 20 min at 4°C), redissolved in 2.5 mL of 20 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA (buffer 2) and dialysed on a Sephadex G-25 column (Pharmacia, PD 10), previously balanced with buffer 2, bringing it to a final volume of 3.5 mL (fraction of the homogenate). The protein concentrations were determined by the Bradford method.

Fe-SOD activity was determined by NAD(P)H oxidation according to Paoletti and Mocali.[24] One unit was the amount of enzyme required to inhibit the rate of NAD(P)H reduction by 50%. The CuZn-SOD from human erythrocytes used in these assays was obtained from Boehringer (Mannheim), while all the coenzymes and substrates came from Sigma Chemical Co. Data obtained were analysed according to the Newman–Keuls test.

In vitro activity assays: extracellular forms

Promastigote assay

Compounds **1**–**6** were dissolved in DMSO (Panreac, Barcelona, Spain) at a concentration of 0.1% and were assayed as non-toxic and without inhibitory effects on parasite growth, according to González *et al.*[23] The compounds were dissolved in the culture medium at concentrations of 100, 50, 25, 10 and 1 μM. The effects of each compound against the promastigote forms and its concentrations were tested at 72 h using a Neubauer haemocytometric chamber. The leishmanicidal effect was expressed as the IC_{50} value, i.e. the concentration required to result in 50% inhibition, calculated by linear regression analysis from the K_c values of the concentrations employed.

Cell culture and cytotoxicity tests

The macrophage line J774.2 [European collection of cell cultures (ECACC) number 91051511] was derived in 1968 from a tumour in a female BALB/c mouse. The cytotoxicity test on macrophages was performed according to the method described by González *et al.*[23] After 72 h of treatment, cell viability was determined by flow cytometry. Thus, 100 μL/well of propidium iodide solution (100 μg/mL) were added and incubated for 10 min at 28°C in darkness. Afterwards, 100 μL/well of fluorescein diacetate (100 ng/mL) was added and incubation was performed under the conditions described above. Finally, the cells were recovered by centrifugation at 400 **g** for 10 min and the precipitate was washed with PBS. Flow cytometric analysis was performed with a FACSVantage™ flow cytometer (Becton Dickinson). The percentage viability was calculated with respect to the control culture. The IC_{50} was calculated using linear regression analysis from the K_c values of the concentrations employed.

Infectivity assay

J774.2 macrophage cells were grown in minimal essential medium (MEM) plus glutamine (2 mM) and 20% inactive FCS, in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Cells were seeded at a density of 1×10^4 cells/well in 24-well microplates (Nunc) with rounded coverslips on the bottom and cultured for 2 days. The cells were then infected *in vitro* with promastigote forms of *L. infantum* or *L. braziliensis* at a ratio of 10:1. The drugs (IC_{25} concentrations) were added immediately after infection and incubation was performed for 12 h at 37°C in 5% CO_2. Non-phagocytosed parasites and drugs were removed by washing, and then the infected cultures were grown for 10 days in fresh medium. Cultures were washed every 48 h and fresh culture medium was added. Drug activity was determined on the basis of both the percentage of infected cells and the number of amastigotes per infected cell in treated and untreated cultures in methanol-fixed and Giemsa-stained preparations. The percentage of infected cells and the mean number of amastigotes per infected cell were determined by analysing more than 200 host cells distributed in randomly chosen microscopic fields. Values are the means of three separate determinations.

In vitro activity assays: intracellular forms

Axenic amastigote assay

Axenic amastigote forms of *Leishmania* spp. were cultured following the methodology described previously by Moreno *et al.*[25] Thus, promastigote transformation to amastigotes was achieved after 3 days of culture in M199 medium (Invitrogen, Leiden, The Netherlands) supplemented with 10% heat-inactivated FCS, 1 g/L β-alanine, 100 mg/L L-asparagine, 200 mg/L sucrose, 50 mg/L sodium pyruvate, 320 mg/L malic acid, 40 mg/L fumaric acid, 70 mg/L succinic acid, 200 mg/L α-ketoglutaric acid, 300 mg/L citric acid, 1.1 g/L sodium bicarbonate, 5 g/L 2-(N-morpholino)ethanesulfonic acid (MES), 0.4 mg/L haemin and 10 mg/L gentamicin, pH 5.4, at 37°C. The effect of each compound against axenic amastigote forms was tested at 48 h using a Neubauer haemocytometer.

Amastigote assay

Adherent macrophage cells were infected with promastigotes in the stationary growth phase of *Leishmania* spp. at a ratio of 10:1 and maintained for 24 h at 37°C in air+5% CO_2. Non-phagocytosed parasites were removed by washing, and the infected cultures were incubated with the compounds (1, 10, 25, 50 and 100 μM) and then cultured for 72 h in MEM plus glutamine (2 mM) and 20% inactive FCS. Compound activity was determined from the percentage reductions in amastigote number in treated and untreated cultures in methanol-fixed and Giemsa-stained preparations. Values are the means of three separate determinations.[23]

Metabolite excretion

Cultures of *L. infantum* and *L. braziliensis* promastigotes (initial concentration 5×10^5 cells/mL) received the IC_{25} of the compounds (except for control cultures). After incubation for 96 h at 28°C the cells were centrifuged at 400 **g** for l0 min. The supernatants were collected to determine the excreted metabolites by ^{1}H-NMR, and chemical shifts were expressed in ppm, using sodium 2,2-dimethyl-2-silapentane-5-sulphonate as the reference signal. The chemical displacements used to identify the respective metabolites were consistent with those described by Fernández-Becerra *et al.*[26]

Ultrastructural alterations

The parasites were cultured at a density of 5×10^5 cells/mL in the corresponding medium, each of which contained the compounds tested at the IC_{25} concentration. After 96 h, the cultures were centrifuged at 400 **g** for 10 min, and the pellets produced were washed in PBS and then incubated with 2% (v/v) *p*-formaldehyde/glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4) for 2 h at 4°C. The pellets were then prepared for TEM employing the technique of González *et al.*[23]

Results and discussion

As explained above, previous studies have indicated that benzo[g]phthalazine derivatives **1**–**6** may be considered to be prospective chemotherapeutic drugs in the treatment of diseases caused by members of the Trypanosomatidae.[19,20] We comment now on the results obtained concerning the toxic activity of these compounds against two species of *Leishmania* (*L. infantum* and *L. braziliensis*).

SOD enzymatic inhibition in parasites and human erythrocytes

The marked inhibitory activity of compounds **1**–**6** on the essential antioxidant enzyme Fe-SOD of *T. cruzi*[20,21] prompted us to assay their effect on the two *Leishmania* species at a range of concentrations from 1 to 100 μM. We used promastigote forms of *L. infantum* and *L. braziliensis*, which excrete Fe-SOD when cultured in a medium lacking inactive FCS.[24] Inhibition data obtained are shown in Figure 4(a and b), and the corresponding IC_{50} values are included in order to make the interpretation of results easier. For comparison, Figure 4(c) shows the effect of the same compounds on CuZn-SOD obtained from human erythrocytes. The most remarkable result was that Fe-SOD activity was significantly inhibited by the six tested compounds, whereas inhibition of human CuZn-SOD was consistently lower, with IC_{50} values ranging from 120.0 to 350.3 μM. Different

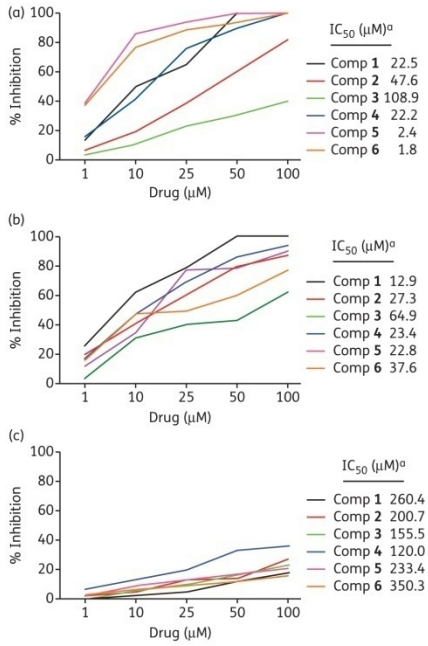

Figure 4. *In vitro* inhibition (%) of Fe-SOD in (a) *Leishmania infantum* and (b) *Leishmania braziliensis* promastigotes for compounds **1**–**6**. (c) *In vitro* inhibition of CuZn-SOD in human erythrocytes for compounds **1**–**6**. Values are means of three separate determinations. Differences between the activities of the control homogenate and those incubated with compounds **1**–**6** were analysed with the Newman–Keuls test. [a]IC_{50} was calculated by linear regression analysis from the K_c values at the concentrations employed (1, 10, 25, 50 and 100 μM).

behaviour was observed between the two *Leishmania* species assayed. The pyrazole-based derivatives **5** and **6** were much more effective against *L. infantum* than the imidazole-based compounds, with **6** about 12-fold more inhibitory than **1** and **4**, and about 25- and 60-fold more inhibitory than **2** and **3**, respectively. The negligible inhibitory effect of the monosubstituted derivative **6** against human SOD should also be noted (IC_{50} 350.3 μM).

In the case of *L. braziliensis*, differences in the activity of the six tested compounds were much less marked, since the most effective compound (**1**) was only about 5-fold more inhibitory than the least active one (**3**). The disubstituted derivative **3** had the worst IC_{50} against both L. *infantum* and *L. braziliensis*. We conclude that the IC_{50} values could support some kind of interaction of these benzo[*g*]phthalazine derivatives with Fe-SOD active sites, especially on the basis of the results described for *L. infantum*: the increased system rigidity in compounds **5** and **6** and the presence of pyrazole rings with well-known complexing ability should allow a cooperative action of both the pyridazine and pyrazole nitrogens in metal complexation and should favour enzyme inhibition.

In vitro antileishmanial evaluation

Most studies on the *in vitro* biological activity of new compounds against *Leishmania* spp. are performed on promastigote forms because it is much easier to work with these forms *in vitro*. However, since extracellular forms are not the developed forms of the parasite in vertebrate hosts, evaluations made with extracellular forms are merely indicative of the potential leishmanicidal activity of the compounds tested. Consequently, a preliminary test using extracellular promastigote forms should always be complemented by a subsequent evaluation using intracellular forms (amastigotes in vertebrate host cells) for a better understanding of the activity results obtained.[23] Besides extracellular promastigotes, we also prepared extracellular axenic amastigote forms of both parasites according to the procedure described by Moreno *et al*.[25] Intracellular assays were performed by infecting macrophage cells with promastigotes, which transformed into amastigotes within 1 day after infection. Tables 1 and 2 show the IC_{50} values obtained after 72 h of exposure when compounds **1**–**6** were assayed in promastigote forms, axenic amastigote forms and intracellular amastigote forms of *L. infantum* and *L. braziliensis*. Values for the reference drug, meglumine antimoniate, are included in all cases for comparison.

If we consider now the results displayed in Table 1 for activity against *L. infantum*, the leishmanicidal activity in both extra- and intracellular forms was similar or, in most cases, higher than that found for meglumine antimoniate, with the best results those of compounds **2** and **6**. Only the imidazole derivative **4** exhibited lower activity than meglumine antimoniate in the three forms assayed. More interesting are the toxicity data, since all six compounds tested were found to be much less toxic for macrophages than the reference drug. Thus, compound **2** was 31-fold less toxic than meglumine antimoniate, and even the most toxic among them, the disubstituted derivative **3**, was 6.5-fold more benign. Toxicity values substantially influence the more informative selectivity index (SI) data, and best values were again obtained for the monosubstituted compounds **2** and **6**, with SI exceeding those of the reference drugs by 46-, 66- and 38-fold in the case of **2**, and by 37-, 52- and 25-fold for **6.** It should be noted that, when comparing the SI of every pair of compounds with the same type of side chain (i.e. **1** and **2**, **3** and **4**, **5** and **6**), the monosubstituted derivatives always gave better results than their disubstituted analogues, as happened in the *T. cruzi* tests.[20] The same behaviour was found when considering the macrophage toxicity of the three pairs, since the monosubstituted compounds were always less toxic than their counterparts.

Very similar conclusions can be extracted from the *L. braziliensis* results shown in Table 2. The monosubstituted

Table 1. *In vitro* activity, toxicity and selectivity index for imidazole-based (**1**–**4**) and pyrazole-based (**5**–**6**) benzo[*g*]phthalazine derivatives in extra- and intracellular forms of *L. infantum*

	Activity IC_{50} (μM)[a]				Selectivity index[b]		
Compounds	Promastigote forms	Axenic amastigote forms	Intracellular amastigote forms	[c]Macrophage toxicity IC_{50}	Promastigote forms	Axenic amastigote forms	Intracellular amastigote forms
Glucantime	18.0±3.1	30.0±2.7	24.2±2.6	15.20±1.3	0.8	0.5	0.6
1	15.3±1.7	16.2±3.0	24.2±2.6	306.9±16.7	20 (25)	19 (38)	13 (22)
2	12.6±1.8	14.3±1.4	20.4±1.1	467.8±20.5	37 (46)	33 (66)	23 (38)
3	15.4±3.3	17.7±2.0	21.2±3.6	98.9±8.9	6 (7)	6 (12)	5 (8)
4	20.1±3.2	31.3±4.2	46.9±6.6	289.7±11.4	14 (17)	9 (18)	6 (10)
5	17.3±2.2	28.4±1.5	26.5±2.9	269.8±13.7	16 (20)	9 (18)	10 (17)
6	10.0±0.7	11.5±0.9	20.3±1.2	299.9±17.9	30 (37)	26 (52)	15 (25)

Results are means of three separate determinations.
[a]IC_{50} was calculated by linear regression analysis from the K_c values at concentrations employed (1, 10, 25, 50 and 100 μM).
[b]Selectivity index (SI) is IC_{50} macrophage toxicity/IC_{50} activity in extracellular or intracellular forms of the parasite. The figure shown in parentheses is the fold increase in the SI of the compound compared with that of the reference drug.
[c]Against J774.2 macrophages after 72 h of culture.

compounds **2** and **6** again gave the best SI results in the three assays performed, with values exceeding those of the reference drugs 72-, 76- and 36-fold in the case of **2**, and 38-, 28- and 30-fold for **6**. It can also be seen that the three monosubstituted compounds **2**, **4** and **6** had a better SI than their respective disubstituted pairs **1**, **3** and **5**. The imidazole-based derivative **2** had the lowest toxicity and highest SI values in both *L. infantum* and *L. braziliensis*.

Going one step further in the activity study, the effects of the compounds on the infectivity and intracellular replication of the amastigote forms were determined. Macrophages were cultured and infected with promastigotes in the stationary phase. The parasites invaded the cells and underwent morphological conversion to amastigotes within 1 day after infection. On day 10, the rate of host cell infection reached its maximum (control experiment).

For these assays we used the IC_{25} of each product as the test concentration, with meglumine antimoniate as the reference drug. As shown in Figure 5, when the imidazole compounds **1**–**4** (Figure 5a) and the pyrazole derivatives **5** and **6** (Figure 5b) were added to macrophages infected with *L. infantum* promastigotes, the infection rate significantly decreased with respect to the control in all the compounds tested, and five of them were also more effective than meglumine antimoniate, with the disubstituted **3** as the only exception (56% compared with 60% for the reference drug). The three monosubstituted derivatives **2**, **4** and **6** (81%, 78% and 76%, respectively, in terms of infectivity decrease) were substantially more active than their disubstituted analogues. On the other hand, data on the mean number of amastigotes per infected macrophage cells (Figure 5c and d) led to similar but even more compelling conclusions: all six compounds were much more effective than meglumine antimoniate (only a 22% decrease), and the three monosubstituted compounds were clearly more active than the disubstituted ones (67%, 61% and 47% for **2**, **4** and **6** compared with 32%, 46% and 32% for **1**, **3** and **5**, respectively).

The same experiment was performed with *L. braziliensis*, and the results obtained concerning infection rates (Figure 6a and b) and amastigote numbers (Figure 6c and d) are shown in Figure 6. In both cases, all six compounds were more effective than meglumine antimoniate, and also in both cases the three monosubstituted compounds were found to be more active than the disubstituted ones. The infectivity rates calculated from Figure 6 were **2** (82%)>**4** (78%)>**6** (70%)≫**1** (57%)>**3** (51%)>**5** (47.13%)>meglumine antimoniate (40%), and the decreases in amastigote number were **2** (74%)>**6** (65%)>**4** (64%)>**3** (60%)>**1** (57%)>**5** (51%)>meglumine antimoniate (47%).

If we now compare the results obtained for both *Leishmania* species, it can be concluded that, as happened with *T. cruzi*, the monosubstituted derivatives were clearly the most active, and they were also much less toxic against the host than meglumine antimoniate. The imidazole-based compounds **2** and **4** seemed to be more effective than the pyrazole-based **6**, with **2** as the most active and also the least toxic of all.

Ultrastructural alterations

The remarkable leishmanicidal activity shown by compounds **1**–**6** should induce important damage to parasite cells. Therefore, we performed a TEM study on promastigote forms of *L. infantum* and *L. braziliensis* cultured in medium containing the compounds tested at their IC_{25} concentrations. As expected, significant morphological alterations were observed compared with untreated control cells. Figure 7 shows the structural features obtained from control and treated cells of *L. infantum*. Treatment with meglumine antimoniate resulted in morphological alterations in the promastigotes, which exhibited reduced size and shape abnormalities, presenting in many

Table 2. *In vitro* activity, toxicity and selectivity index for imidazole-based (**1**–**4**) and pyrazole-based (**5**–**6**) benzo[g]phthalazine derivatives in extra- and intracellular forms of *L. braziliensis*

	Activity IC_{50} (μM)[a]				Selectivity index[b]		
Compounds	Promastigote forms	Axenic amastigote forms	Intracellular amastigote forms	[c]Macrophage toxicity IC_{50}	Promastigote forms	Axenic amastigote forms	Intracellular amastigote forms
Glucantime	25.6±1.6	31.1±3.0	30.4±6.1	15.20±1.3	0.6	0.5	0.5
1	30.5±1.6	43.8±7.7	68.10±6.2	306.9±16.7	10 (17)	7 (14)	5 (10)
2	10.8±0.4	12.3±2.3	26.2±0.9	467.8±20.5	43 (72)	38 (76)	18 (36)
3	22.4±2.6	47.5±6.4	20.9±2.1	98.9±8.9	4 (7)	2 (4)	5 (10)
4	27.2±1.1	32.4±7.1	37.9±0.1	289.7±11.4	11 (18)	9 (18)	8 (16)
5	21.3±0.9	20.7±3.7	23.3±0.5	269.8±13.7	13 (22)	13 (26)	12 (24)
6	12.9±1.3	21.9±4.4	19.9±1.4	299.9±17.9	23 (38)	14 (28)	15 (30)

Results are means of three separate determinations.
[a]IC_{50} was calculated by linear regression analysis from the K_c values at concentrations employed (1, 10, 25, 50 and 100 μM).
[b]Selectivity index (SI) is IC_{50} macrophage toxicity/IC_{50} activity in extracellular or intracellular forms of the parasite. The figure shown in parentheses is the fold increase in the SI of the compound compared with that of the reference drug.
[c]Against J774.2 macrophages after 72 h of culture.

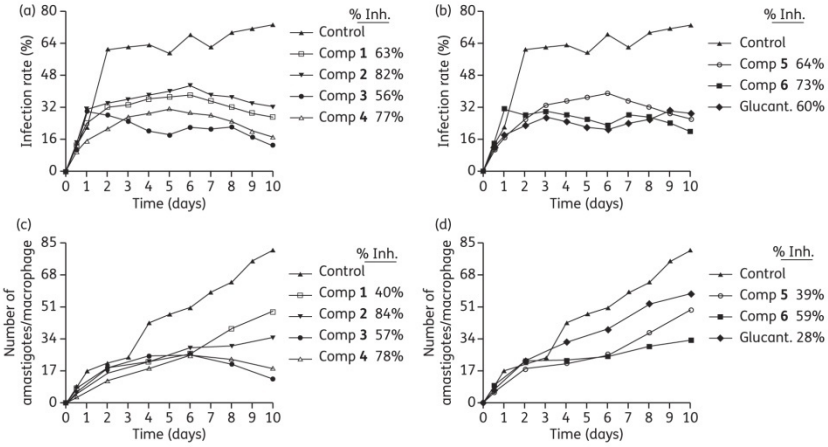

Figure 5. Effects of the imidazole-based (**1**–**4**) and pyrazole-based (**5**–**6**) benzo[*g*]phthalazine derivatives on the infection and growth rates of *Leishmania* spp. (a, b) Rate of infection of *L. infantum*. (c, d) Mean number of amastigotes per infected J774 A.2 macrophage for *L. infantum* (at IC_{25}). Values are means of three separate experiments. Glucant., Glucantime (meglumine antimoniate).

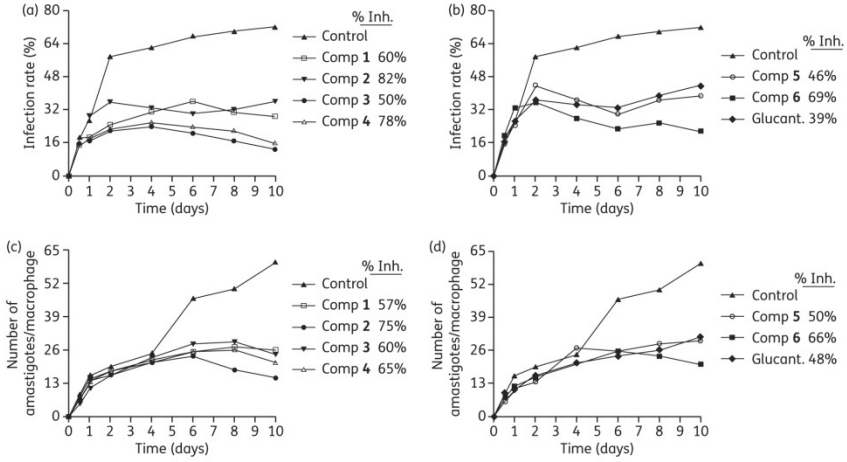

Figure 6. Effects of the imidazole-based (**1**–**4**) and pyrazole-based (**5**–**6**) benzo[*g*]phthalazine derivatives on the infection and growth rates of *Leishmania* spp. (a, b) Rate of infection of *L. braziliensis*. (c, d) Mean number of amastigotes per infected J774 A.2 macrophage for *L. braziliensis* (at IC_{25}). Values are means of three separate experiments. Glucant., Glucantime (meglumine antimoniate).

cases small, strongly electron-dense vesicles. In turn, all six compounds tested led to major morphological changes. The most frequent structural modifications consisted of intense vacuolization (Figure 7, see **1**, **2**, **3**, **4** and **5**), very significant size reduction, distortions in the appearance of the parasite, which in many cases adopted a star-shaped form (see **2**, **5** and **6**;

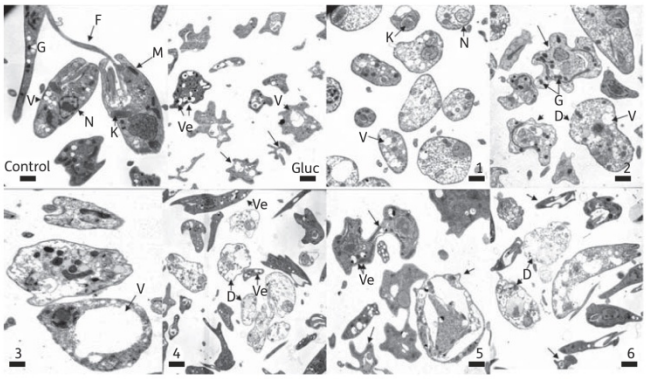

Figure 7. Ultrastructural alterations observed by TEM in *L. infantum* treated with meglumine antimoniate and compounds **1**–**6**. Control, control parasite (bar 1 μm); Gluc, modifications after treatment with meglumine antimoniate (Glucantime) (bar 2.33 μm). **1**, **2**, **3** (bar 1 μm), **4** (bar 2.33 μm), **5** (bar 1 μm) and **6** (bar 1.59 μm), promastigotes treated with the respective compounds. N, nucleus; M, mitochondrion; V, vacuole; F, flagellum; G, glycosome; K, kinetoplast; D, dead parasites; Ve, electron-dense vesicles. Single arrows indicate distorted promastigotes.

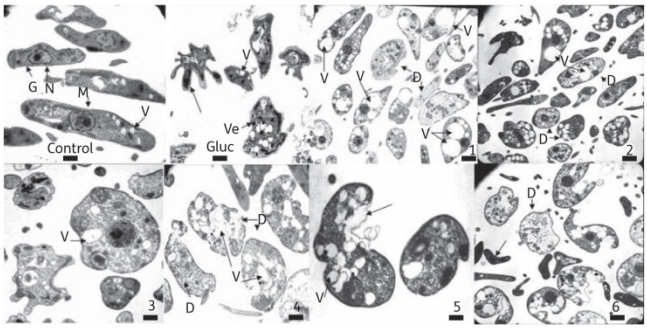

Figure 8. Ultrastructural alterations observed by TEM in *L. braziliensis* treated with meglumine antimoniate and compounds **1**–**6**. Control, control parasite (bar 1 μm); Gluc, modifications after treatment with meglumine antimoniate (Glucantime) (bar 1.59 μm). **1** and **2** (bar 2.33 μm), **3**, **4** and **5** (bar 1 μm) and **6** (bar 1.59 μm), promastigotes treated with the respective compounds. N, nucleus; M, mitochondrion; V, vacuole; F, flagellum; G, glycosome; K, kinetoplast; D, dead parasites; Ve, electron-dense vesicles. Single arrows indicate distorted promastigotes.

marked with an arrow), and breaking of the cytoplasmic membrane (**6**). The cytoplasm was poorly electron dense, and usually had a granular appearance (see **1**), whereas kinetoplasts and nucleus showed the same flabby aspect as the cytoplasm itself. As a confirmation of the infection rate data commented on above, the monosubstituted derivatives **2**, **4** and **6** exhibited the highest numbers of dead and shape-distorted parasites. Thus, although some of the parasites treated with **2** exhibited a normal morphology, many promastigotes were found to be dead and many others were vacuolated, poorly electron dense or star-shaped, and a high number of glycosomes was observed. Many dead and vacuolated promastigotes were also found after treatment with **4**. However, the most harmful compound seemed to be the pyrazole-based monosubstituted derivative **6**, since it caused the death of most of the parasites, and the remaining parasites appeared to be distorted and of a smaller size than normal.

In a similar way, Figure 8 compares the alterations produced by meglumine antimoniate and compounds **1**–**6** in *L. braziliensis* promastigotes. The results obtained are very closely related to those commented on above for *L. infantum*. The presence of meglumine antimoniate led to a high percentage of parasites with many electron-dense vesicles that may well have been parasite excretion vesicles. Treatment with any of the six

Table 3. Variation in the height of the peaks corresponding to catabolites excreted by *L. infantum* and *L. braziliensis* promastigote forms in the presence of imidazole-based (**1**–**4**) and pyrazole-based (**5**–**6**) benzo[*g*]phthalazine derivatives with respect to the control test

	L. infantum				*L. braziliensis*				
Compound	Ac	Suc	Lac	Et	Ac	Suc	Lac	Et	Pyr
1	−20%	−19%	+70%	↑	+9%	=	=	=	−58%
2	−11%	−46%	−50%	ND	=	−100%	−14%	=	−62%
3	=	−11%	−12%	ND	=	−12%	=	=	−14%
4	+9%	+11%	=	↑	−60%	−22%	−11%	=	−44%
5	+22%	+63%	−14%	ND	−42%	−52%	−64%	ND	−100%
6	+32%	+85%	+30%	ND	−64%	−8%	−27%	+100%	−100%

Ac, acetate; Suc, succinate; Lac, L-lactate; Et, ethanol; Pyr, pyruvate. (−) peak inhibition; (+) peak increasing; (=) no difference detected; (ND) peak undetected; (↑) peak undetected in control.

compounds led to a remarkable number of dead parasites, and many others were swollen or presented an unrecognizable and flabby cytoplasm, full of vacuoles and exhibiting a broken cytoplasmic membrane. As observed for *L. infantum*, and again in accordance with the measured infection rates, the monosubstituted derivatives **2**, **4** and **6** seemed to be more harmful than their disubstituted analogues, with a higher amount of dead and shape-distorted parasites.

Metabolite excretion

It is well known that trypanosomatids are unable to degrade glucose completely to CO_2 under aerobic conditions. As a consequence, they excrete into the medium a considerable part of the hexose skeleton as partially oxidized fragments in the form of fermented metabolites, although their nature and percentage depend on the pathway used for glucose metabolism by each of the species considered.[27,28] The final products of glucose catabolism in *Leishmania* are usually CO_2, succinate, acetate, L-lactate, pyruvate, L-alanine and ethanol.[29] Succinate is especially relevant since its main role is in maintaining the glycosomal redox balance, allowing the reoxidation of NADH produced in the glycolytic pathway. Succinic fermentation has the advantage of requiring only half of the phosphoenolpyruvate produced to maintain the NAD^+/NADH balance, and the remaining pyruvate is converted inside the mitochondrion and the cytosol into acetate, L-lactate, L-alanine or ethanol according to the degradation pathway followed by each species.[30]

In order to obtain information concerning the effects of the tested compounds on glucose metabolism in the parasites, we recorded the ^{1}H-NMR spectra of promastigotes from *L. infantum* and *L. braziliensis* after treatment with compounds **1**–**6**, and the final excretion products were identified qualitatively and quantitatively. The results were compared with those found for promastigotes maintained in a cell-free medium (control) for 4 days after inoculation with the parasite. The characteristic presence of acetate, L-lactate and succinate was confirmed in control experiments performed in both species. However, noteworthy differences between them were the extensive presence of pyruvate and also the appearance of ethanol among the catabolites excreted by *L. braziliensis*, whereas these two products were not detected in *L. infantum* promastigotes. After treatment of the parasites with compounds **1**–**6**, the excretion of the catabolites was clearly altered at the concentrations employed. The ^{1}H-NMR obtained in all the tests performed are shown in Figures S1 (*L. infantum*) and S2 (*L. braziliensis*) (available as Supplementary data at *JAC* Online). Table 3 displays the modifications observed in the height of the spectrum peaks corresponding to the most representative final excretion products. From a careful examination of the data it would appear that there were marked differences in the catabolic pathway depending on the structure of the side chains linked to the benzo[*g*]phthalazine moiety. The pair of compounds **1**/**2**, with imidazole rings linked through the C-4 carbon atom to a flexible aliphatic chain, gave rise to a decrease in the excretion of acetate and succinate, whereas the pair **5**/**6**, containing pyrazole rings bonded directly to the benzo[*g*]phthalazine system through an exocyclic nitrogen, caused a sharp increase in the production of both catabolites. The variations originated by the pair **3**/**4** (with imidazole rings linked to the flexible aliphatic chain through the heterocyclic N-1 nitrogen and consequently lacking the NH group present in **1**/**2**) were clearly less significant than those found for the other compounds tested. All these data could be interpreted on the basis of a change in glucose catabolism according to the structure of the respective side chains. The severe damage caused in parasite organelles such as glycosomes or mitochondria by these compounds should be related to the alterations observed in the final products of catabolism. Another point that should be taken into account is the fact that the pairs **1**/**2** and **5**/**6** contain five-membered heterocyclic rings with an acidic NH group, which is not present in **3**/**4**. Therefore, differences in basicity could be responsible for the smaller variations found in the final catabolism products of **3**/**4** with respect to the rest of the compounds, since it is known that modifications in pH may affect metabolism in *Leishmania* species.[31]

The behaviour of the *L. braziliensis* parasites in the presence of **1**–**6** was much more uniform than that described above for *L. infantum*. Variations in the final catabolism products did not seem to be dependent on the structural aspects of the compounds assayed. A decrease in the formation of acetate, succinate, L-lactate and especially pyruvate was observed for most of the test compounds. In fact, the presence of pyruvate was not detected at all in the pair **5**/**6**. Taking into account the high grade of vacuolization and cell death found in *L. braziliensis* when performing the ultrastructural study commented on above, it is likely that such considerable cell and organelle

degeneration could be the cause of the inhibition found in the production of the typical excretion products of this species.

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Transparency declarations

None to declare.

Supplementary data

Figures S1 and S2 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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Supplementary data

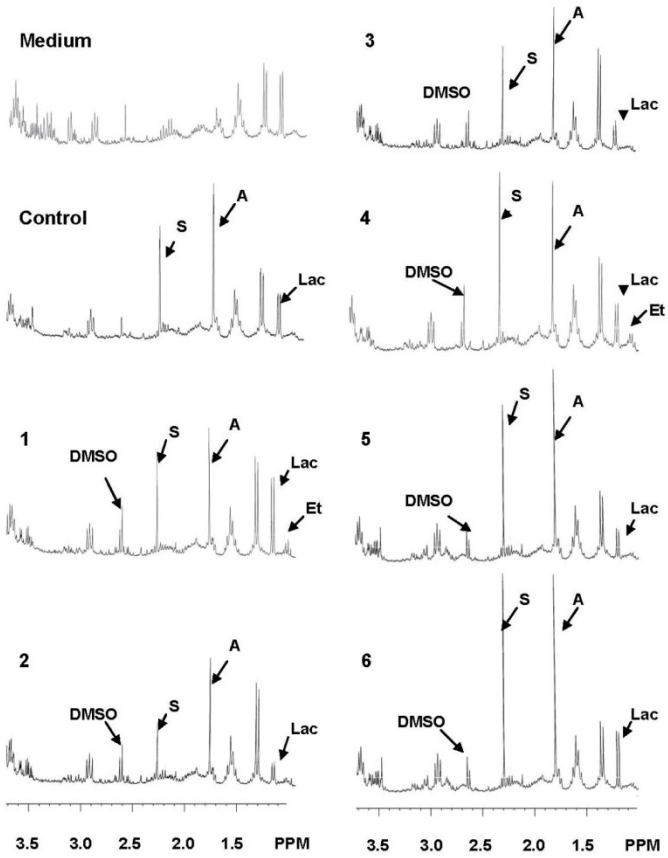

Figure 1S. ^{1}H-NMR spectra showing the medium without parasites and the metabolites excreted by promastigote forms of *L. infantum* untreated (**Control**) and treated with compounds **1**-**6** at IC_{25} dosage. L-lac, L-lactate; Ac, acetate; S, succinate; Et, ethanol.

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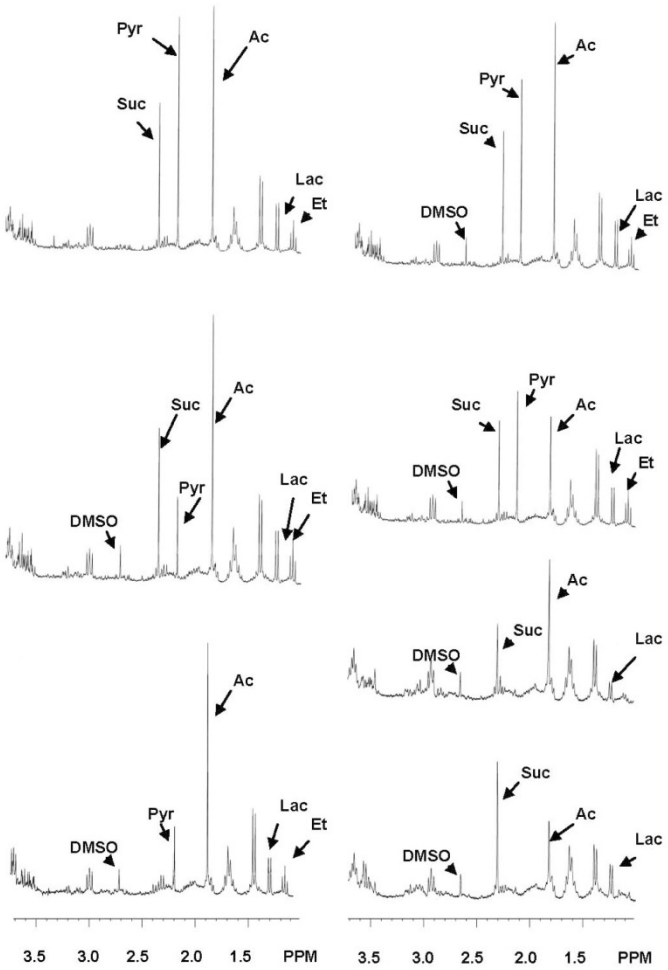

Figure S2. ^{1}H-NMR spectra showing the metabolites excreted by promastigote forms of *L. braziliensis* untreated (**Control**) and treated with compounds **1**-**6** at IC_{25} dosage. L-lac, L-lactate; Ac, acetate; S, succinate; Et, ethanol; Pyr, pyruvate.

Biological activity of three novel complexes with the ligand 5-methyl-1,2,4-triazolo[1,5-a]pyrimidin-7(4H)- one against *Leishmania* spp. *J Antimicrob Chemother*. 2011; 66: 813-9

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Biological activity of three novel complexes with the ligand 5-methyl-1,2,4-triazolo[1,5-*a*]pyrimidin-7(4*H*)-one against *Leishmania* spp.

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Objectives: We report on new 1,2,4-triazolo[1,5-*a*]pyrimidine complexes that have been developed and examined for both antiproliferative *in vitro* activity against *Leishmania infantum* and *Leishmania braziliensis*, and report their possible mechanism of action on *L. infantum* and *L. braziliensis*.

Results: Antileishmanial effects are described for newly synthesized Cu(II) and Co(II) complexes containing 5-methyl-1,2,4-triazolo[1,5-*a*]pyrimidin-7(4*H*)-one (HmtpO) as a ligand. These complexes display a wide structural diversity: (i) mononuclear unit, $[Cu(HmtpO)_2(H_2O)_3](ClO_4)_2{\cdot}H_2O$ (**1**); (ii) two-dimensional framework, $\{[Cu(HmtpO)_2(H_2O)_2](ClO_4)_2{\cdot}2HmtpO\}_n$ (**2**); and (iii) chains, $\{[Co(HmtpO)(H_2O)_3](ClO_4)_2{\cdot}2H_2O\}_n$ (**3**). Compounds **1** and **2** appeared to be the most active against *L. infantum* (IC_{50} 20.0 and 24.4 μM, respectively), and compounds **1** and **3** the most active against *L. braziliensis* (IC_{50} 22.1 and 23.5 μM, respectively), with IC_{50}s similar to those of the reference drug, glucantime (18.0 μM for *L. infantum* and 25.6 μM for *L. braziliensis*). These compounds were not toxic towards J774.2 macrophages. IC_{25} decreased infection capacity and severely reduced the multiplication of intracellular amastigotes, following the trend **1**>**3**>**2** for *L. infantum* and **3**>**1**>**2** for *L. braziliensis*. These complexes had an effect on the energy metabolism of the parasites at the level of the NAD^+/NADH balance and the organelle membranes, causing their degradation and cell death.

Conclusions: Cellular proliferation, metabolic and ultrastructural studies showed that the compounds **2**>**1**>**3** were highly active against *L. infantum* and *L. braziliensis*.

Keywords: *Leishmania infantum*, *Leishmania braziliensis*, triazolopyrimidines, leishmaniasis

Introduction

Leishmaniasis is one of the illnesses called a 'neglected disease', common in tropical and subtropical regions, involving up to 22 countries in the New World and 66 in the Old World.[1] The aetiological agents are different species of protozoa of the genus *Leishmania*, which are transmitted by Diptera of the genera *Phlebotomus* in the Old World and *Lutzomyia* in the New World. These are cosmopolitan or endemic diseases that present serious public health problems and thus are considered by the WHO as one of the seven priority diseases that affect all continents.

More reliable and effective therapies are needed against leishmaniasis. A large number of compounds bearing nitrogen-containing fused heterocyclic skeletons, such as 4-anilinoquinazolines, pyrazolopyrimidines, triazolopyrimidines, pyrrolopyrimidines, pyrazolopyridazines and imidazopyrazines, and natural products from plants (terpenoids, flavonoids, etc.) have been identified, many of which display excellent anti-cancerous, antimicrobial and antiprotozoal activity.[2–6]

Recently, we have shown that it is possible for new coordination compounds with unusual structures and noteworthy physical properties to be synthesized by combining transition metal ions with [1,2,4-]triazolo[1,5-*a*]pyrimidines and pseudohalide auxiliary spacers. These compounds have been the subject of chemical and biological studies due to their promising pharmacology, including antipyretic, analgesic and anti-inflammatory properties, as well as their herbicidal, fungicidal and antileishmanial potential.[7,8] With regard to their antiparasite properties, these bicyclic molecules and some of their metal complexes have proved to be especially active against diverse species such as *Trypanosoma cruzi* and *Leishmania* spp. This makes such compounds potential chemotherapeutic agents for combating these parasites.

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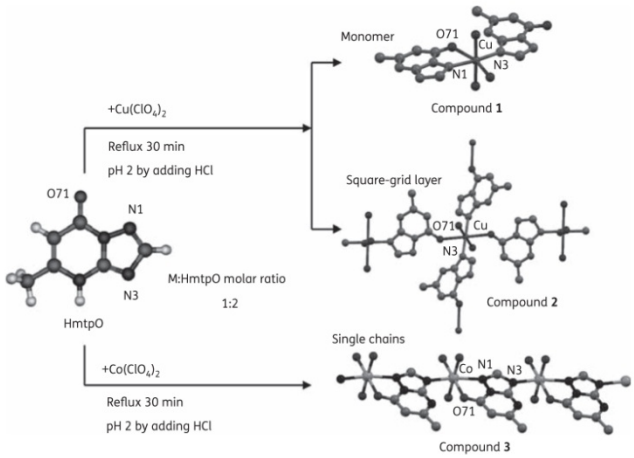

Figure 1. Main structural features of the HmtpO-containing metal compounds assayed.

HmtpO {5-methyl-1,2,4-triazolo[1,5-*a*]pyrimidin-7(4*H*)-one} is a triazolopyrimidine derivative considered to be an analogue of the naturally occurring nucleobase hypoxanthine, which is ideally suited for the study of metal–metal interactions due to its multiple donor positions. In this respect, the synthesis of the anti-tumour drug *cis*-$[PtCl_2(HmtpO)_2]$ has been investigated by our group and its anti-cancer activity has been tested against the human cancer-cell lines MCF-7 breast carcinoma and A121 ovarian carcinoma.[9] Preliminary research on new anti-tumour Pt(IV) HmtpO complexes has just been published.[10] Recently, the first organometallic Pd(II) and Pt(II) complexes with the HmtpO ligand and its anion $mtpO^-$ have been synthesized, showing different coordination modes of this ligand.[11] Moreover, it has been demonstrated that such species interact with DNA. Considering the number of publications regarding HmtpO, it is clear that this ligand has great value due to its structural and biological properties. For this reason novel coordination compounds containing HmtpO were synthesized in order to study their structural and biological properties.

In consideration of the above-mentioned findings, and as a continuation of our efforts[12] to identify new candidates for designing potent, selective and less toxic anti-trypanosomatids, we report here on some new 1,2,4-triazolo[1,5-*a*]pyrimidine complexes that have been developed and examined for antiproliferative *in vitro* activity against *Leishmania infantum and Leishmania braziliensis* (promastigote and amastigote forms). Non-specific mammalian cytotoxicity of the most active compounds was evaluated *in vitro*, and less toxic derivatives were submitted to a more thorough study of their possible mechanism of action. Furthermore, an NMR spectroscopy study (^{1}H-NMR) was run in order to gain details regarding its nature and the percentage of metabolites excreted with inhibitory effects on the glycolytic pathway, since that represents the primary source of energy for the parasite. Finally, the effects of compounds on the ultrastructure of *Leishmania* spp. were considered on the basis of transmission electron microscopy (TEM) studies.

Materials and methods

Chemistry

The compounds assayed are two copper(II) complexes (**1** and **2**) and one cobalt(II) complex (**3**) bearing the neutral form of 5-methyl-1,2,4-triazolo[1,5-*a*]pyrimidin-7(4*H*)-one (HmtpO) and perchlorate as counteranion. The three compounds show different topology and dimensionality ranging from no dimensional (monomers) to two dimensional (layers). This structural diversity is mainly due to the coordination mode of the triazolopyrimidine ligand. Compound **1**, $[Cu(HmtpO)_2(H_2O)_3](ClO_4)_2{\cdot}H_2O$, is a monomeric complex in which HmtpO shows both N3 monodentate and N1,O71 bidentate modes; $\{[Cu(HmtpO)_2(H_2O)_2](ClO_4)_2{\cdot}2HmtpO\}_n$ (**2**) is a two-dimensional framework with the ligand showing an N3,O71 bidentate bridging mode. Finally, the structure of $\{[Co(HmtpO)(H_2O)_3](ClO_4)_2{\cdot}2H_2O\}_n$ (**3**) consists of one-dimensional chains in which HmtpO displays an N1,N3,O71 tridentate bridging mode. It should be noted that these coordination modes of the HmtpO ligand are unique in the case of compounds **2** and **3** (Figure 1).

For the synthesis of the three metal complexes, all analytical reagents were purchased from commercial sources and used without further purification. The procedures that specify the preparation of each of them are indicated below.

*Preparation of $[Cu(HmtpO)_2(H_2O)_3](ClO_4)_2{\cdot}H_2O$ (**1**) and $\{[Cu(HmtpO)_2(H_2O)_2](ClO_4)_2{\cdot}2HmtpO\}_n$ (**2**)*

An aqueous solution of $Cu(ClO_4)_2{\cdot}6H_2O$ (2 mmol, 0.7410 g in 20 mL) was added to another solution containing HmtpO (4 mmol, 0.6006 g in 20 mL) and the mixture was refluxed for 30 min. The pH value of the mixture was decreased from 2.4 to 2 by adding 0.5 N HCl dropwise.

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The resulting clear blue solution was left at room temperature. After 1 week, a mixture of green prismatic and blue cubic crystals corresponding to compounds **1** and **2**, respectively, was isolated from the solution. Crystals of both compounds were separated manually. Several days before these crystals appeared, fractions of monohydrated HmtpO were removed from the solution. Anal. Calcd for $C_{12}H_{20}Cl_2CuN_8O_{14}$ (**1**): C, 22.70; H, 3.18; and N, 17.65. Found: C, 22.28; H, 3.67; and N, 17.39. Anal. Calcd for $C_{24}H_{28}Cl_2CuN_{16}O_{14}$ (**2**): C, 32.06; H, 3.14; and N, 24.92. Found: C, 31.83; H, 3.30; and N, 24.47.

*Preparation of {[Co(HmtpO)(H$_2$O)$_3$](ClO$_4$)$_2$·2H$_2$O}$_n$ (**3**)*

Aqueous solutions of $Co(ClO_4)_2$·6H_2O (0.5 mmol, 0.1830 g in 10 mL) and HmtpO (1 mmol, 0.1501 g in 10 mL) were mixed and refluxed for 30 min. The pH value of the mixture was decreased from 2.92 to 2 by adding 0.5 N HCl dropwise. The resulting light-pink solution was left at room temperature and after 1 week orange needle-shaped crystals of compound **3** were isolated. Fractions of monohydrated HmtpO were removed from the solution before the isolation of compound **3** crystals. Anal. Calcd for $C_6H_{16}Cl_2CoN_4O_{14}$ (**3**): C, 14.47; H, 3.24; and N, 11.25. Found: C, 14.60; H, 3.96; and N, 11.37.

Parasite strain, culture

L. infantum (MCAN/ES/2001/UCM-10) and *L. braziliensis* (MHOM/BR/1975/M2904) were cultivated *in vitro* in medium trypanosomes liquid (MTL) medium with 10% inactive fetal bovine serum and were kept in an air atmosphere at 28°C, in Roux flasks (Corning, USA) with a surface area of 75 cm^2, according to the methodology described by González *et al.*[13]

In vitro activity assay

The compounds obtained were dissolved in DMSO (Panreac, Barcelona, Spain) at a concentration of 0.1% and were afterwards assayed as non-toxic and without inhibitory effects on parasite growth, according to González *et al.*[13] The compounds were dissolved in the culture medium, at dosages of 100, 50, 25, 10 and l μM. The effects of each compound against promastigote forms were tested every 24 h for 3 days using a Neubauer haemocytometric chamber. The antileishmanial effect is expressed as the IC_{50}, i.e. the concentration required to give 50% inhibition, calculated by linear regression analysis from the K_c values of the concentrations employed.

Cell culture and cytotoxicity tests

J774.2 macrophages (ECACC number 91051511) were originally obtained from a tumour in a female BALB/c rat in 1968. The cytotoxicity test for macrophages was performed according to the methodology of González *et al.*[13] After 72 h of treatment, cell viability was determined by flow cytometry. Thus, 100 μL/well of propidium iodide solution (100 μg/mL) was added and incubated for 10 min at 28°C in darkness. Afterwards, 100 μL/well of fluorescein diacetate (100 ng/mL) was added and incubated under the same aforementioned conditions. Finally, the cells were recovered by centrifugation at 400 **g** for l0 min and the precipitate washed with PBS. Flow cytometric analysis was performed with a FACS Vantage™ flow cytometer (Becton Dickinson). The percentage viability was calculated in comparison with the control culture. The IC_{50} was calculated using linear regression analysis from the K_c values of the concentrations employed.

Amastigote assay

J774.2 macrophages were grown in minimum essential medium (MEM) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Cells were seeded at a density of 1×10^5 cells/well in 24-well microplates (Nunc) with rounded coverslips on the bottom and cultured for 2 days. Afterwards the cells were infected *in vitro* with promastigote forms of *L. infantum* and *L. braziliensis*, at a ratio of 10:1. The drugs (at IC_{25} concentrations) were added immediately after infection and were incubated for 12 h at 37°C in 5% CO_2. The non-phagocytosed parasites and drugs were removed by washing, and then the infected cultures were grown within 10 days in fresh medium. Fresh culture medium was added every 48 h. Drug activity was determined on the basis of both the percentage of infected cells and the number of amastigotes per infected cell in treated and untreated cultures in methanol-fixed and Giemsa-stained preparations. The percentage of infected cells and the mean number of amastigotes per infected cell were determined by analysing >200 host cells distributed in randomly chosen microscopic fields. Values are the means of four separate determinations.

Metabolite excretion

Cultures of *L. infantum* and *L. braziliensis* promastigotes (initial concentration 5×10^5 cells/mL) received IC_{25} concentrations of the compounds (except for control cultures). After incubation for 72 h at 28°C, the cells were centrifuged at 400 **g** for l0 min. Then supernatants were collected to determine the excreted metabolites using ^{1}H-NMR, and, eventually, chemical displacements were expressed in parts per million (ppm), using sodium 2,2-dimethyl-2-silapentane-5-sulphonate as the reference signal. The chemical displacements used to identify the respective metabolites were consistent with those described by Fernández-Becerra *et al.*[14]

Ultrastructural alterations

The parasites were cultured at a density of 5×10^6 cells/mL in their corresponding medium, each of which contained drugs at the IC_{25} concentration. After 72 h, those cultures were centrifuged at 400 **g** for 10 min, and the pellets produced were washed in PBS and then mixed with 2% (v/v) *p*-formaldehyde–glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4) for 2 h at 4°C. After that, the pellets were prepared for TEM employing the technique of Gonzalez *et al.*[13]

Results and discussion

Previous studies indicated that newly synthesized 1,2,4-triazolo[1,5-*a*]pyrimidine derivatives are prospective chemotherapeutic drugs in the treatment of diseases caused by members of the Trypanosomatidae.[15] We evaluate the toxic activity of triazolo-pyrimidine compounds and a few of their Cu(II) and Co(II) complexes against two species of *Leishmania* (*L. infantum* and *L. braziliensis*).

In vitro antileishmanial evaluation

In the initial step, the inhibitory effects of the three new complexes with 5-methyl-1,2,4-triazolo[1,5-*a*]pyrimidin-7(4*H*)-one were measured at different times, following established procedures (see the Materials and methods section), at concentrations ranging from 1 to 100 μM.[15] The IC_{50} values registered after 72 h of exposure are shown in Table 1, including glucantime as the reference drug. The antileishmanial activity of the three triazole-pyrimidine derivatives appeared to be similar (IC_{50}s for *L. infantum* were 20.0, 24.4 and 29.0 μM for compounds **1**, **2** and **3**, respectively, and IC_{50}s for *L. braziliensis* were 22.1, 31.5 and 23.5 μM for compounds **1**, **2** and **3**, respectively) to the one found for glucantime (18.0 μM in the case of *L. infantum* and

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Table 1. *In vitro* activity of reference drugs and metal compounds **1**, **2** and **3** against promastigote forms of *Leishmania* spp.

	IC_{50} (μM)			SI[b]	
Compound	*L. infantum*	*L. braziliensis*	Toxicity towards J774.2 macrophages, IC_{50} (μM)[a]	*L. infantum*	*L. braziliensis*
Glucantime	18.0	25.6	15.2	1	1
1	20.0	22.1	723.8	36	33
2	24.4	31.5	945.5	39	30
3	29.0	23.5	843.3	29	36

Results are averages of three separate determinations.
[a]Towards J774.2 macrophages after 72 h of culture. IC_{50}=the concentration required to give 50% inhibition, calculated by linear regression analysis from the K_c values at concentrations employed (1, 10, 25, 50 and 100 μM).
[b]Selectivity index=IC_{50} macrophages/IC_{50} parasite.

Table 2. Effects of compounds **1**, **2** and **3** (at IC_{25} concentrations) on the infection rate of J774.2 macrophages and on the average number of amastigotes per infected macrophage during 10 days of culture

	Macrophages (%)[a]						IP/C[b]					
Compound	12 h	48 h	96 h	144 h	192 h	240 h	12 h	48 h	96 h	144 h	192 h	240 h
L. infantum												
control	35.9	48.4	50.5	54.1	58.2	61.2	6.2±0.3	15.8±0.5	25.2±0.9	34.6±2.4	40.6±1.1	86.2±1.7
glucantime	28.5	40.5	37.3	33.7	30.4	27.1	6.9±0.4	13.9±0.6	18.0±1.4	18.0±0.9	21.6±1.7	33.0±1.2
1	32.4	38.1	28.0	29.8	24.3	20.3	6.0±0.2	11.6±0.2	21.4±1.5	19.8±2.0	22.2±1.6	15.2±3.0
2	34.6	38.1	27.1	23.7	19.5	13.4	5.2±0.1	12.6±0.2	11.3±0.7	16.9±1.1	13.3±1.0	7.8±0.6
3	28.6	40.0	37.3	33.8	30.1	27.3	6.1±0.5	13.2±0.6	17.0±0.5	19.3±0.8	14.0±0.5	12.0±1.0
L. braziliensis												
control	19.1	57.8	62.0	67.6	70.5	72.0	7.2±0.7	20.4±1.0	39.2±3.2	43.4±4.2	53.7±5.9	67.3±4.1
glucantime	17.2	34.1	41.3	48.6	42.4	46.0	6.0±0.3	9.9±0.5	21.0±1.1	22.0±2.2	29.0±1.4	35.0±3.7
1	20.6	42.3	36.5	29.3	24.0	18.0	6.5±0.3	10.6±0.7	17.8±2.2	21.5±1.5	20.3±1.0	18.7±1.3
2	18.0	25.0	34.2	24.0	19.7	10.0	4.6±0.6	11.6±1.0	16.7±1.3	12.6±0.8	10.3±0.8	8.0±0.5
3	16.5	36.0	33.0	24.2	17.2	15.0	6.5±0.0	11.9±0.6	17.4±0.6	17.0±1.3	15.0±1.1	13.2±0.9

IP, intracellular parasite (amastigotes); C, cellule (macrophages).
[a]Percentage macrophage parasitism. Values are means of three separate determinations.
[b]Number of amastigotes per macrophage infected. Values are means±SD of three separate determinations.

25.6 μM for *L. braziliensis*). Cytotoxicity towards mammalian cells, using J774.2 macrophages as the cell model, showed that the three derivatives are much less toxic than glucantime (Table 1). On the other hand, the selectivity index calculated for these derivatives was ~30-fold or more higher than that for glucantime.

Cell assay

Most studies on the *in vitro* biological activity of new compounds against *Leishmania* spp. are performed on promastigote forms because it is much easier to work with these forms *in vitro*. However, in this study, we have also included the effects of these compounds on the forms that develop in the host (amastigotes). This study is of great importance for those areas where the final aim is to determine the effects in the definitive host.[15]

An experimental model designed in our laboratory was used to predict the effect of the complexes on the capacity for infection and growth of the intracellular forms of *L. infantum* and *L. braziliensis*.[13] Adherent J774.2 macrophages (1×10^5 macrophages) were incubated for 2 days and then infected with 1×10^6 promastigote forms of *L. infantum* and *L. braziliensis* for 12 h (control experiment, Table 2). Afterwards, the non-phagocytosed parasites were removed and the culture was kept in fresh medium for 10 days. The parasites invaded the cells and then converted into amastigotes within 1 day after infection. On the 10th day, the rate of host-cell infection reached the maximum. When the drugs were added at their respective IC_{25} concentration to macrophages infected with *Leishmania* spp. promastigote forms in exponential growth phase, the infection rate decreased significantly after 12 h with respect to the control measurement, following the trend **1**>**3**>**2** for *L. infantum* and **3**>**1**>**2** for *L. braziliensis*, with percentages of infestation-inhibition capacity of 84%, 79% and 67%, respectively, in the case of *L. infantum* and 86%, 79% and 75%, respectively, in the case of *L. braziliensis*. These values are remarkably higher than those for inhibition by glucantime (56% and 36% for *L. infantum* and *L. braziliensis*, respectively). The three complexes inhibited *Leishmania* spp. amastigote replication in macrophage cells *in vitro*, following a similar

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behaviour to the inhibition of the infection rate and again being more effective than the reference drug (Table 2). It is difficult to establish a direct relationship between drug action and the extracellular promastigote and the intracellular amastigote forms; in spite of this, compound **3** was effective against both forms.

Studies on the mechanism of action

In order to gain information concerning the possible mechanism of action of compounds **1**, **2** and **3** on the parasite, the following experiments were performed.

Metabolite excretion effect

As far as is known to date, none of the trypanosomatids studied is capable of completely degrading glucose to CO_2 under aerobic conditions, so excreting a great part of the carbon skeleton into the medium as fermented metabolites, which can differ according to the employed species.[16] *Leishmania* spp. have a high rate of glucose consumption, thereby acidifying the culture medium due to incomplete oxidation to acids. ^{1}H-NMR spectra enable us to determine the fermented metabolites excreted by the parasites during their *in vitro* culture. One of the major metabolites excreted by *Leishmania* spp. is succinate, as it is with *T. cruzi*,[17] the main role of which is probably to maintain the glycosomal redox balance by providing two glycosomal oxidoreductase enzymes. These enzymes allow reoxidation of NADH that is produced by glyceraldehyde-3-phosphate dehydrogenase in the glycolytic pathway. Succinic fermentation offers one significant advantage, since it requires only half of the produced phosphoenolpyruvate (PEP) to maintain the NAD^+/NADH balance. The remaining PEP is converted into acetate, depending on the species being considered. Figure 2(a) presents the spectrum given by cell-free medium 4 days after inoculation with *L. infantum*. Additional peaks, corresponding to the major metabolites produced and excreted during growth, were detected when this spectrum was compared with the one made in fresh medium (spectra not shown). *L. infantum* excretes succinate and acetate as the majority of the metabolites. These data agree well with those of other authors.[17] We have identified and assessed one inhibitory effect caused by the compounds using ^{1}H-NMR spectra, which demonstrated that only compound **2** significantly altered the metabolites excreted by *L. infantum*. When the promastigote forms of *L. infantum* were treated with compound **2** at IC_{25} doses, the excretion of catabolites (succinate and acetate) was clearly disturbed (Figure 2b) and a new peak, identified as pyruvate, appeared. These results mean that compound **2** inhibits glycosomal enzymes, causing pyruvate to be excreted as a final metabolite. The other two compounds, **1** and **3**, also slightly inhibited the metabolites excreted (spectra not shown). In the case of *L. braziliensis*, the compounds behaved similarly, but again compound **2** appeared to be the most inhibitory (spectra not shown).

Ultrastructural alterations

By TEM, it was found that morphological alterations were substantial in *Leishmania* spp. promastigotes treated with compounds **1**, **2** and **3**, compared with the control sample (Figure 3).

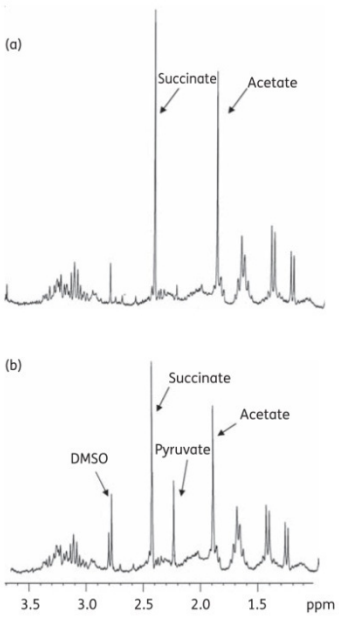

Figure 2. ^{1}H-NMR spectra of promastigote forms of *L. infantum*. (a) Control (untreated). (b) Treated with compound **2** (at a concentration of IC_{25}).

All the products used induced alterations in the *Leishmania* spp. promastigotes, but the most effective one against both of the parasite species was compound **2**. In the case of *L. braziliensis*, compound **2** provoked more alterations compared with *L. infantum*, as can be assessed in Figure 3(f). Many of the parasites appeared dead and others adopted distorted shapes, while in others a uniformly electrodense cytoplasm was formed, in which no cytoplasmic organelles were visible. The vacuolization of the parasites was pronounced and many of these vacuoles contained strongly electrodense inclusions. In the case of *L. infantum*, the effects provoked by this compound were similar (Figure 3c) to cell destruction, which was evident from the presence of a great quantity of cell remains in the supernatant. Likewise, parasites had strongly electrodense cytoplasm with intense vacuolization, with both empty vacuoles and membranes, and reservosomes, which appeared in greater numbers than in non-treated promastigotes (Figure 3a). Against *L. braziliensis*, compound **1** was again very effective, as some parasites appeared dead and others completely altered (Figure 3e), replete with reservosomes and enormous vacuoles. Some promastigotes appeared to be distorted and strongly electrodense, and showed condensed kinetoplast and very swollen mitochondria. In contrast, compound **3** was effective against *L. infantum* (Figure 3d), whose alterations were similar to those already described, with unrecognizable parasites,

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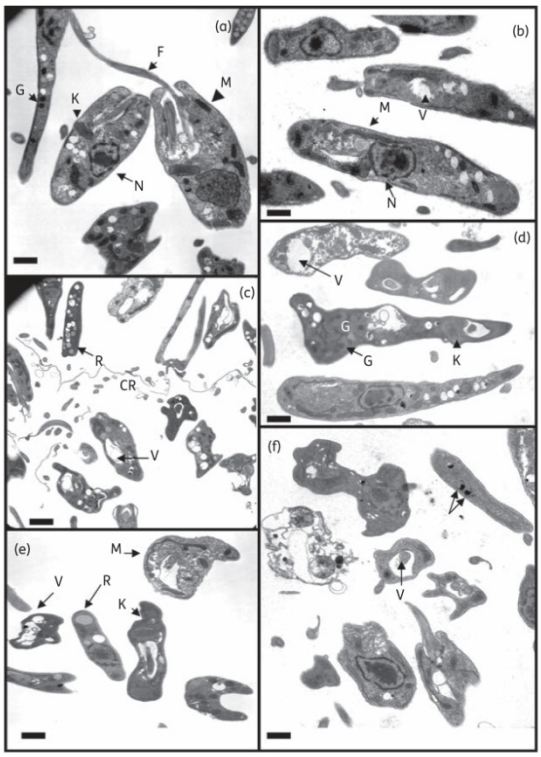

Figure 3. Ultrastructural alterations by TEM in *L. infantum* and *L. braziliensis* treated with compounds **1**, **2** and **3** (at IC_{25} concentrations). (a) Control parasite of *L. infantum* showing organelles with their characteristic aspect, such as nucleus (N), kinetoplast (K), flagellum (F), glycosomes (G) and mitochondrion (M). Bar = 1.00 μm. (b) Control parasite of *L. braziliensis* with structures such as nucleus (N), vacuoles (V) and mitochondrion (M). Bar = 1.00 μm. (c) *L. infantum* treated with compound **2**, showing cellular rest (CR), intense vacuolization (V) and reservosomes (R). Bar = 1.59 μm. (d) *L. infantum* treated with compound **3**, showing electrodense cytoplasm, vacuoles (V), glycosomes (G) and kinetoplast (K). Bar = 1.00 μm. (e) *L. braziliensis* treated with compound **1**, showing intense vacuolization (V), giant reservosomes (R) and kinetoplast (K) and swelling mitochondrion (M). Bar = 1.00 μm. (f) Promastigotes of *L. braziliensis* treated with compound **2**, with vacuoles (V) and electrodense organelles (arrows). Bar = 1.00 μm.

filled with vacuoles, which distorted their morphology, as well as a great quantity of reservosomes that occupied practically the entire cytoplasm. In these parasites the kinetoplast and the mitochondria also appeared swollen, resulting in a strongly electrodense cytoplasm. Dead parasites were also visible.

Conclusions

Our biological tests have shown that the three complexes tested were active *in vitro* against both extracellular and intracellular forms of *L. infantum* and *L. braziliensis* (in the order **2** > **1** > **3**). These compounds are not toxic towards the host cells and are effective at concentrations lower than the reference drug, glucantime. In summary, the *in vitro* growth rate of *Leishmania* spp. was reduced, its capacity to infect cells was negatively affected and multiplication of the amastigote forms was substantially lowered. Moreover, there was a wide range of ultrastructural alterations of *Leishmania* spp. promastigote forms.

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Transparency declarations

None to declare.

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In vitro and *in vivo* antiparasital activity against *Trypanosoma cruzi* of three novel 5-methyl-1,2,4-triazolo[1,5-*a*]pyrimidin-7(4*H*)-one-based complexes

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ABSTRACT

Conventional reactions of the versatile multidentate ligand 5-methyl-1,2,4-triazolo[1,5-*a*] pyrimidin-7(4*H*)-one (HmtpO) with metallic(II) perchlorate salts lead to three novel multidimensional complexes $[Cu(HmtpO)_2(H_2O)_3](ClO_4)_2\cdot H_2O$ (**1**), $\{[Cu(HmtpO)_2(H_2O)_2](ClO_4)_2\cdot 2HmtpO\}_n$ (**2**) and $\{[Co(HmtpO)(H_2O)_3](ClO_4)_2\cdot 2H_2O\}_n$ (**3**). We have tested the antiparasital activity *in vitro* and *in vivo* of the three new complexes against *Trypanosoma cruzi* showing very promising results and overcoming clearly the reference drug commonly used for the Chagas disease treatment, benznidazole.

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1. Introduction

Substituted-pyrimidinic ligands have been demonstrated to be excellent and versatile building blocks with charge and multi-connectivity ability to produce, under conventional and hydrothermal conditions, multidimensional coordination polymers with interesting properties [1–5]. Recently, we have shown that it is possible to synthesise new coordination compounds, with fascinating structures and interesting physical properties, by combining transition metal ions with the 1,2,4-triazolo[1,5-*a*] pyrimidine ligand (tp) and several pseudohalide auxiliary spacers [6]. Triazolopyrimidines are versatile ligands as they have several nitrogen atoms with accessible lone pairs to bind Lewis acids like metal ions [7]. It should also be highlighted that triazolopyrimidine derivatives have been the subject of chemical and biological studies due to their interesting pharmacology including antipyretic, analgesic, antiinflammatory, potential herbicidal, fungicidal, antitumoral and leishmanicidal properties [8–16]. With regard to their antiparasital properties, these bicyclic molecules and some of their metal complexes have shown to be especially active against diverse species like *Leishmania* spp. and *Trypanosoma cruzi* [17–22]. Chagas disease, caused by the protozoan *T. cruzi*, is a neglected disease that affects millions of people in areas of endemicity in Latin America, where approximately 100 million people are at risk [23]. This fact renders this kind of compounds as potential chemotherapeutic agents to combat this disease.

HmtpO molecule (5-methyl-1,2,4-triazolo[1,5-*a*]pyrimidin-7(4*H*)-one) (Scheme 1) is an interesting triazolopyrimidine derivative considered as an analogue of the natural occurring nucleobase hypoxanthine. The synthesis of *cis*-$[PtCl_2(HmtpO)_2]$ has been reported by our group [24], and its anticancer activity has been tested against the human cancer cell lines MCF-7 breast carcinoma and A121 ovarian carcinoma. A preliminary communication on new antitumor Pt(IV) HmtpO complexes has just been reported [25]. Recently, the first organometallic Pd(II) and Pt(II) complexes with the HmtpO ligand and its anion $mtpO^-$ have been prepared, showing different coordination modes of this ligand [26]. Moreover, it has been demonstrated that such species interact with DNA. In view of these publications regarding to HmtpO ligand, it is clear that this ligand possesses a great interest due to its structural and biological properties. For this reason, we decided to synthesise novel coordination compounds containing HmtpO to study their biological properties.

In this paper, we report the antiparasital properties of the following three new metal complexes: (a) the mononuclear unit $[Cu(HmtpO)_2(H_2O)_3](ClO_4)_2\cdot H_2O$ (**1**), (b) the bidimensional framework $\{[Cu(HmtpO)_2(H_2O)_2](ClO_4)_2\cdot 2HmtpO\}_n$ (**2**) and (c) the monodimensional polymer $\{[Co(HmtpO)(H_2O)_3](ClO_4)_2\cdot 2H_2O\}_n$ (**3**). We have studied the antiproliferative *in vitro* and *in vivo* activity of these complexes against *T. cruzi* (epimastigote, amastigote and trypomastigote forms). Moreover, unspecific mammal cytotoxicity was evaluated *in vitro*, and then submitted for *in vivo* experiments to perform a more profound study. Furthermore, we have also included a ^1H NMR study to obtain information about the nature and the percentage of the excretion of

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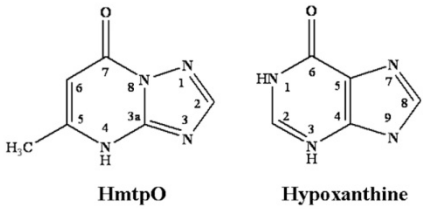


Scheme 1. Structures of the HmtpO ligand (left) and the hypoxanthine nucleobase (right) with their respective ring-numbering (IUPAC for HmtpO and biochemical for hypoxanthine).

metabolites in order to get some clues about the inhibitory effect of the assayed compounds over the glycolitic pathway, since it represents the primary source of energy for the parasite. Finally, the effect of the compounds on the ultrastructure of *T. cruzi* is considered on the basis of transmission electronic microscopy studies (TEM).

2. Experimental

2.1. General

All analytical reagents were purchased from commercial sources and used without further purification.

Preparation of $[Cu(HmtpO)_2(H_2O)_3](ClO_4)_2\cdot H_2O$ (**1**), $\{[Cu(HmtpO)_2(H_2O)_2](ClO_4)_2\cdot 2HmtpO\}_n$ (**2**) and $\{[Co(HmtpO)(H_2O)_3](ClO_4)_2\cdot 2H_2O\}_n$ (**3**).

Detailed synthesis and structural characterization of **1–3** have been recently reported by our group [27]. As it is shown in Scheme 2, the three metal complexes were obtained from refluxing an aqueous solution containing the corresponding metal perchlorate and HmtpO ligand in a M:HmtpO molar ratio of 1:2. The resulting clear solution was slightly acidified until pH 2 by adding diluted HCl, to avoid the potential deprotonation of HmtpO into $mtpO^-$ when metal complex is formed. In all cases, single-crystals were isolated and used for X-ray determination of their crystal structures.

2.2. Biological measurements

2.2.1. Parasite strain, culture

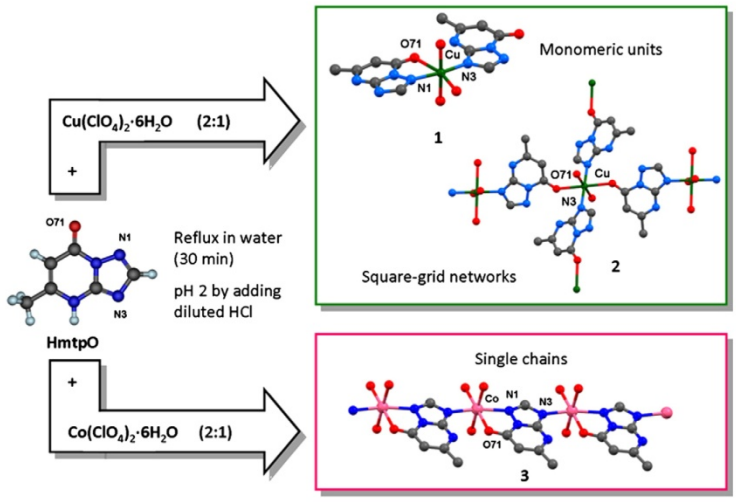
The Maracay strain of *T. cruzi* was isolated at the Institute of Malariology and Environmental Health in Maracay (Venezuela). Epimastigote forms were obtained in biphasic blood-agar NNN medium (Novy–Nicolle–McNeal) supplemented with minimal essential medium (MEM) and 10% inactivated foetal bovine serum and, afterwards, reseeded in a monophasic culture (MTL), following the method of Luque et al. [28].

2.2.2. In vitro activity assay

To obtain the parasite suspension for the assay, the epimastigote culture (in exponential growth phase) was concentrated by centrifugation at 1000 *g* for 10 min and the number of flagellates were counted in a haemocytometric chamber and distributed into aliquots of 2×10^6 parasites/mL. The compounds were dissolved in DMSO and added to culture media so that the final DMSO-concentration was 0.01% in culture, which is non-toxic and without inhibitory effects on the parasite growth. The effect of each compound against epimastigote forms were evaluated at different dosages (100, 50, 25, 10, and 1 μM) after 72 h, using a Neubauer haemocytometric chamber.

2.2.3. Cell culture and cytotoxicity tests

Vero cells (Flow) were grown in MEM (Gibco) supplemented with 10% inactivated foetal calf serum and adjusted to pH 7.2, in a humidified 95% air–5% CO_2 atmosphere at 37 °C for 2 days. For the cytotoxicity test, cells were placed in 25-mL colie-based bottles (Sterling), and centrifuged at 100 *g* for 5 min. The culture medium was removed, and fresh medium was added. Then, the cells were infected *in vitro* with metacyclic forms of *T. cruzi*, to a final concentration of 1×10^5 cells/mL. This cell suspension was distributed in culture trays (with 24 wells) at a rate of 100 μL/well and incubated for 2 days at 37 °C in humid atmosphere enriched with 5% CO_2. The



Scheme 2. Preparative methods of compounds **1–3**.

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medium was removed, and the fresh medium was added together with the product to be studied (at a concentration of 100, 50, 25, 10 and 1 μM). After 72 h of treatment, the cell viability was determined with flow cytometry. Thus, 100 μL/well of propidium iodide (PI) solution (100 μg/mL) was added and incubated for 10 min at 28 °C in darkness. Afterwards, 100 μL/well of fluorescein diacetate (FDA) (100 ng/mL) was added and incubated under the same aforementioned conditions. Finally, the cells were recovered by centrifugation at 400 *g* for 10 min and the precipitate washed with PBS (phosphate-buffered saline). Flow cytometric analysis was performed with a FACS Vantage™ flow cytometer (Becton Dickinson). The number of dead cells was recorded, and the percentage of viability was calculated in comparison to that of the control culture. The IC_{50} was calculated by linear regression analysis from the K_c values at the concentrations employed.

2.2.4. Transformation of epimastigote to metacyclic forms

In order to induce metacyclogenesis, parasites were cultured at 28 °C in modified Grace's medium (Gibco) for 12 days as previously described [28]. Twelve days after cultivation at 28 °C, metacyclic forms were counted in order to infect Vero cells. The proportion of metacyclic forms was around 40% at this stage.

2.2.5. Cell assay

Vero cells were cultured in MEM medium in a humidified 95% air–5% CO_2 atmosphere at 37 °C. Cells were seeded at a density of 1×10^5 cells/well in 24-well microplates (Nunc) with rounded coverslips on the bottom and cultivated for 2 days. Afterwards, the cells were infected *in vitro* with metacyclic forms of *T. cruzi* at a ratio of 10:1. The drugs (at IC_{25}) were added immediately after infection, and were incubated for 12 h at 37 °C in a 5% CO_2. The non-phagocytosed parasites and the drugs were removed by washing, and then the infected cultures were grown for 10 days in fresh medium. Fresh culture medium was added every 48 h.

2.2.6. In vivo trypanocidal activity assay

Groups of five BALB/c female mice (6 to 8 weeks old; 25–30 g) maintained under standard conditions were infected with 10^3 bloodstream *T. cruzi* metacyclic forms by intraperitoneal route. The animals were divided into the following groups: (i) group 1: healthy (not infected and not treated); (ii) group 2: untreated (infected with *T. cruzi* but not treated); (iii) group 3: uninfected (not infected and treated: with 1 mg/kg of body weight/day, for five consecutive days (7 to 12 post-infection) by the intraperitoneal route) [29] and (iv) group 4: treated (infected and treated for five consecutive days (7 to 12 post-infection) with the compounds and benznidazole).

Five days after infection, the presence of circulating parasites was confirmed by the microhematocrit method.

2.2.7. Metabolite excretion

Cultures of *T. cruzi* epimastigotes (initial concentration 5×10^5 cells/mL) received IC_{25} of the compounds (except for control cultures). After incubation for 72 h at 28 °C, the cells were centrifuged at 400 *g* for l0 min. The supernatants were collected to determine excreted metabolites by ^{1}H NMR as previously described by Fernández-Becerra et al. [30]. The chemical displacements were expressed in ppm, using sodium 2,2-dimethyl-2-silapentane-5-sulfonate as reference signal. The chemical displacements used to identify the respective metabolites were consistent with those described by Boutaleb-Charki et al. [21]. The ^{1}H NMR spectra were obtained with a Varian Inova 300 MHz spectrometer at 298 K and using D_2O as solvent. The reference standard was TMS and the water signal was removed by presaturation.

2.2.8. Ultrastructural alterations

The parasites were cultured at a density of 5×10^6 cells/mL in their corresponding medium, each of which containing the drugs at the IC_{25} concentration. After 72 h, the cultures were centrifuged at 400 *g* for 10 min, and the pellets washed in PBS and then fixed with 2% (v/v) *p*-formaldehyde-glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4) for 2 h at 4 °C. Pellets were prepared for transmission-electron microscopy following the technique of Luque et al. [17].

3. Results and discussion

3.1. Structures of the assayed compounds

The three assayed HmtpO-containing metal complexes show a great variety in their structural topologies and dimensionality ranging from 0D (monomers) to 2D (layers). This structural diversity is mainly due to the coordination mode of the triazolopyrimidine ligand. Compound **1**, $[Cu(HmtpO)_2(H_2O)_3](ClO_4)_2\cdot H_2O$, is a mononuclear complex in which two HmtpO show N3-monodentate and N1,O71-bidentate modes. The bidimensional polymer **2** is a sandwich-type compound which consists of alternating cationic square-grid layers $[Cu(HmtpO)_2(H_2O)_2]^{2+}$, perchlorate anions and purely organic layers formed by non-coordinated HmtpO molecules (Fig. 1). In the metal-organic sheets, each metal atom is planarly surrounded by four HmtpO molecules coordinated through N3-O71 in bidentate-bridging mode. Finally, structure of $\{[Co(HmtpO)(H_2O)_3](ClO_4)_2\cdot 2H_2O\}_n$ (**3**) consists of 1D cationic chains in which HmtpO molecules displays a N1,N3,O71-tridentate-bridging coordination mode and are arranged coplanarly with each other [27]. Excepting N3-monodentate mode, the other three coordination modes are showed by the first time for this triazolopyrimidine derivative. In the three complexes, hydrogen bonds play an essential role in their respective crystal packing, leading to three-dimensional supramolecular networks. More structural details have been recently reported [27].

3.2. In vitro antitrypanosomatid activity

The inhibitory effect on the *in vitro* growth of *T. cruzi* epimastigote forms of the three novel complexes with 5-methyl-1,2,4-triazolo[1,5-*a*]pyrimidin-7(4*H*)-one and the isolated HmtpO ligand was measured at different times, following established procedures (see Experimental section). The capacity of such compounds to inhibit the growth of the parasites was evaluated at 1 to 100 μM, and the IC_{50} was determined for the most active compounds, where benznidazole was used as a reference drug. The results, including toxicity values against Vero cells are displayed in Table 1. After 72 h of exposure, the three complexes showed similar or slightly higher IC_{50} values than benznidazol (15.8 μM) against epimastigote forms of *T. cruzi* (27.3, 24.4 and 31.2 μM for **1**, **2** and **3** complexes, respectively). Cytotoxicity of these compounds against mammalian cells, was also evaluated *in vitro* at 1 to 100 μM, using Vero cells as the cellular model (see Table 1). BNZ was

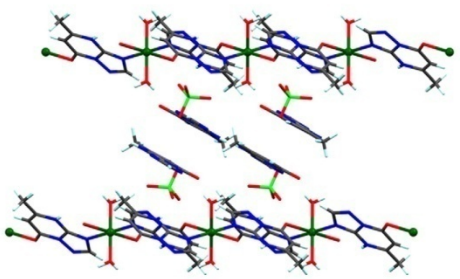

Fig. 1. Sandwich-type crystal structure of compound **2**.

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Table 1
In vitro activity of reference drug, HmtpO ligand and its metal complexes **1**, **2** and **3** on epimastigote forms of *Trypanosoma cruzi*.

Compound	IC_{50} (μM) *T. cruzi*	Toxicity on Vero cells IC_{50} (μM)[a]	SI[b]
Benznidazole	15.8	13.6	1
HmtpO	49.0	97.8	2
1	27.3	579.3	21
2	244	295.3	12
3	31.2	316.7	10

[a] On Vero cells after 72 h of culture. IC_{50} = the concentration required to give 50% inhibition, calculated by linear regression analysis from the K_c values at concentrations employed (1, 10, 25, 50 and 100 μM).
[b] Selectivity index = IC_{50} Vero cells/IC_{50} parasite. Note: Average of three separate determinations.

included, again, as a reference drug. Compound **1** showed the best selectivity indexes (SI = IC_{50} Vero cells/IC_{50} epimastigote of *T. cruzi*), being 21 times less toxic than BNZ. Besides, the other two complexes (**2** and **3**) are also much less toxic than the reference drug (BNZ) with SI values of 12 and 10, respectively. Moreover, all metal compounds show higher selectivity indexes than the isolated HmtpO ligand, revealing clearly the critical role that metal ions play in the antiparasital activity.

3.3. Cell assay

Most of the studies on biological activity *in vitro* of new compounds against *T. cruzi* are usually carried out on epimastigote forms because it is much easier working with these forms *in vitro*. However, in this study, we have also included the effect of these compounds on the forms that are developed in the host (amastigotes and trypomastigotes). This study is of great importance as the final aim is to determine the effects in the definitive host [31].

An experimental model designed in our laboratory has been used to predict the effect of those complexes over the capacity of infection and growth of the intracellular forms and over the transformation to bloodstream forms of *T. cruzi*. Vero cells adherent (10^5 Vero cells) were incubated for 2 days and then were infected with 10^6 metacyclic forms of *T. cruzi*, for 12 h (control experiment, Fig. 2A). Afterwards, the non-phagocytosed parasites were removed and the culture kept in fresh medium for 10 days. The parasites invaded the cells and underwent morphological conversion to amastigotes within 1 day after infection. On day 10, the rate of host-cell infection reaches the maximum. When the drugs were added at their respective IC_{25} concentration to infected Vero cells with *T. cruzi* metacyclic forms in exponential growth phase, the infection rate significantly decreased after 12 h with respect to the control following the trend: 1>3>2, with percentages of inhibition of the capacity of infestation of 75, 70 and 65%, respectively. These values are remarkably higher than the inhibition of the BNZ (56%). In the control experiments, the average number of amastigotes per infected cell increased to 24 amastigotes/cell on day 6, and grew to 41 amastigotes/cell on day 10 (see Fig. 2B). When a cell is completely invaded of amastigote forms, the parasites break the cell and once they are outside, trypomastigote forms can infect new cells in which they multiply, increasing the number of amastigotes per cell again. The three complexes inhibited *T. cruzi* amastigote replication in Vero cells *in vitro*, following a similar relative behaviour for the inhibition of the infection rate and being newly more efficient than the reference drug. The broken Vero cells imply the releasing and subsequent transformation of amastigotes into trypomastigotes (Fig. 2C). The three tested compounds show a significant decrease of trypomastigotes, being higher than the reference drug: 64.5%, 69.6% and 75.5% for compounds **1**, **2** and **3**, respectively, while BNZ produces 52.4%.

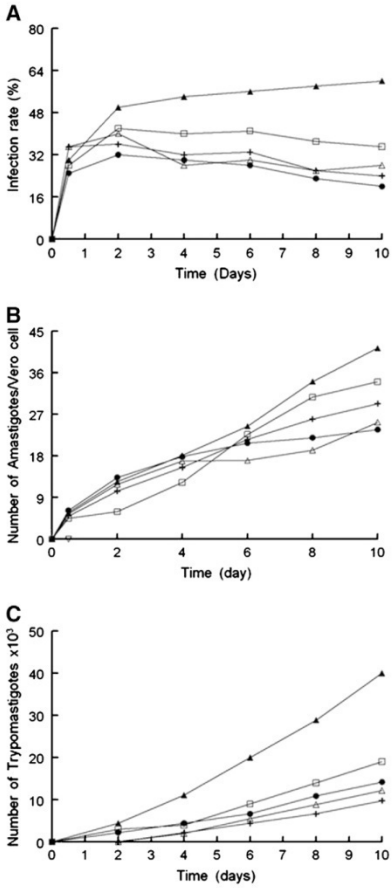

Fig. 2. Effect of the activity of complexes **1**, **2**, **3** on the infection rate and *T. cruzi* growth. (A) Rate of infection, (B) mean number of amastigotes per infected Vero cell, (C) number of trypomastigotes in the culture medium. Control (▲); BNZ (□); Compound **1** (●); Compound **2** (Δ); Compound **3** (+) (at IC_{25}). The values are taken from the average of three independent measurements.

3.4. In vivo anti-Trypanosoma cruzi evaluation

Due to the trypanocidal effect *in vitro* of these three triazolopyrimidine metal complexes, we performed *in vivo* studies to evaluate their activity against *T. cruzi* infection in mice. Previous results had shown that the intravenous doping route resulted in high mortality rates [32]; for this reason, in this study we opted for the intraperitoneal route, using concentrations of 5 mg/kg, which did not result in animal mortality (data not shown). Female Swiss mice were inoculated intraperitoneally with 3000 blood trypomastigotes. The treatment began 7 days after infection, through i.p. route supplying 1 mg/kg per day of each compound for 5 days. The administration was done using a

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saline solution. A group treated in the same manner with vehicle (control) was included. The level of parasitemia was determined every three days (Fig. 3) and the mortality was observed daily. None of the animals treated with the drugs and the control died during the treatment, while in the groups of animals treated with benznidazol the survival fraction was 80%. The three compounds were able to decrease the trypomastigote forms by the day that the experiment reaches the maximum parasitic charge (days 22–24 post-infection) comparing to parasites charged with positive control. In the groups of animals treated with the 3 drugs (in every group only a drug was tested) a significant reduction was observed of the parasitemia by the day 40 post-infection, as we can deduce from Fig. 3 and the percentages of reduction are much higher for the complexes (which vary in a range between 26 and 61%) than for the BNZ (14%). The levels of parasitemia observed for the assayed compounds were in agreement with their behaviour *in vitro* showing, in general, *in vivo* activities in the order: 2>1>3. No signs of toxicity were observed during the animals' treatment with the drugs. These compounds did not entirely reduce all of the circulating bloodstream trypomastigote forms; however, a clear relation between the number of parasites remaining and the chronic stage of the disease and its protection against heart rate alterations characteristic of *T. cruzi* infection could not be found [33].

3.5. Mechanism of action studies

Finally, and in order to confirm or exclude some possible mechanisms of action, studies of the effect on the energy metabolism and alterations at level of the parasite structure were performed.

3.5.1. Metabolite excretion effect

As far as it is known to the date, none of the studied trypanosomatids is capable of completely degrading glucose to CO_2 under aerobic conditions, excreting into the medium a great part of their carbon skeleton in form of fermented metabolites, whose nature differs depending on the parasite specie considered [34]. *T. cruzi*, as well as other trypanosomatids, consumes glucose at a high rate, thereby acidifying the culture medium due to the incomplete oxidation to acids. ^{1}H NMR spectra enable us to determine the fermented metabolites excreted by the trypanosomatids during their *in vitro* culture. Fig. 4A presents the spectrum given by cell-free medium 4 days after inoculation with the *T. cruzi* strain. Additional peaks, corresponding to the major metabolites produced and excreted during growth, were detected when this spectrum (Fig. 4A) was compared with the one made with fresh medium (spectra not shown). *T. cruzi* excretes acetate and succinate as majority metabolites and, in a lower proportion, L-alanine. This data agree well with other authors [35]. We carried out the identification and evaluation of the inhibiting effect caused by the compounds by using ^{1}H NMR, whose spectra showed that only compound **2** leads to an alteration on the metabolites excreted by *T. cruzi*. When epimastigote forms of *T. cruzi* were treated with compound **2** at IC_{25} doses, the excretion of some catabolites (mainly acetate and succinate) is clearly disturbed (Fig. 4B) and a new peak, which was identified as glycerol, appears. These results mean that complex **2** inhibits glycosomal enzymes, which lead to pyruvate formation, forming glycerol instead.

The BZN and compounds **1** and **3** do not produce any significant alteration in the energetic metabolism of the parasites.

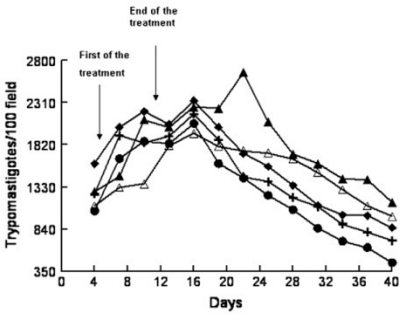

Fig. 3. Parasitemia in the murine modelo of acute Chagas disease: Control (–▲–) and dose receiving 5 mg/kg of: BNZ (♦); Compound **1**(●); Compound **2** (△); Compound **3** (+).

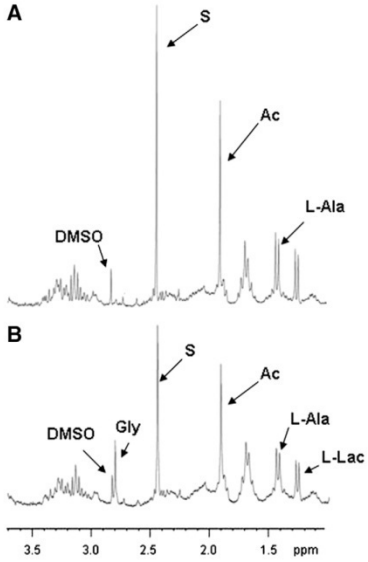

Fig. 4. ^{1}H-NMR spectra of epimastigote forms of *T. cruzi* treated against complexes **1**, **2** and **3** (at a concentration of IC_{25}). (A) Control (untreated), (B) Compound **2**. Ala, L-alanine; Lac, L-lactate; Ac, acetate; S, succinate; Gly, glycerol and DMSO, dimethylsulfoxide.

3.5.2. Ultrastructural alterations

$[Cu(HmtpO)_2(H_2O)_3](ClO_4)_2\cdot H_2O$ (**1**) complex is the most effective compound causing the deepest alterations on the parasite epimastigote cells making them completely unrecognisable, with electrodense aspect and no structural differentiation (Fig. 5). Compound **2** ($\{[Cu(HmtpO)_2(H_2O)_2](ClO_4)_2\cdot 2HmtpO\}_n$) essentially causes a reduction in the size of parasites and the formation of numerous vacuoles, while compound **3** ($\{[Co(HmtpO)(H_2O)_3](ClO_4)_2\cdot 2H_2O\}_n$) causes the lysis of the majority of epimatigote cells, leaving a few of them with a thin and elongated aspect and very electrodense.

Therefore, it is clear that these metal complexes are very effective against *T. cruzi* epimastigotes, specially, complexes **1** and **3**.

4. Conclusions

We have presented the biological properties of three novel complexes of Cu(II) and Co(II) ions, containing 5-methyl-1,2,4-triazolo[1,5-*a*]pyrimidin-7(4*H*)-one (HmtpO), which show interesting

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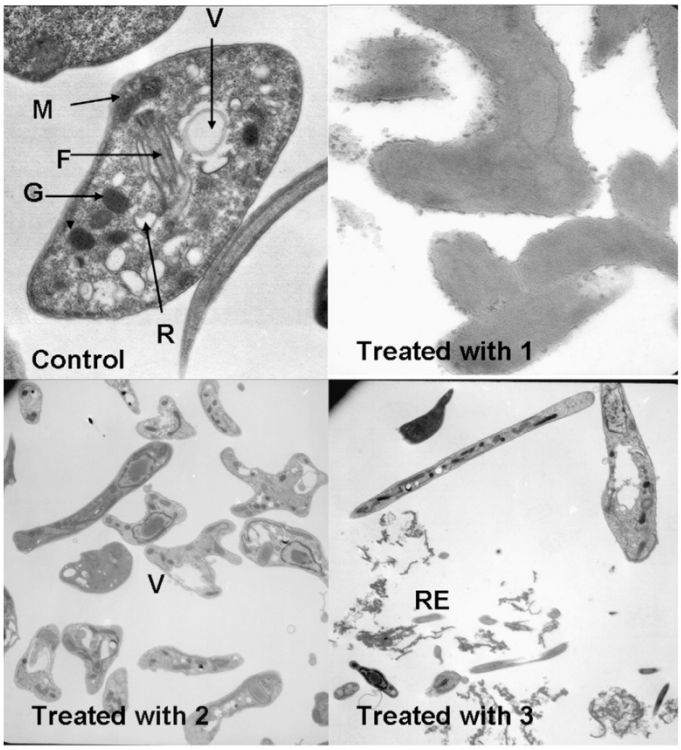

Fig. 5. Ultrastructural alterations in MET of *T. cruzi* treated with triazolopyrimidines. A. In the picture of epimastigotes control, their characteristic structures such as mitochondria (M), flagellum (F), glycosomes (G) and reservosomes (R) are clearly showed. Epimastigotes of *T. cruzi* treated with **1** are apparently unrecognisable. In the picture of parasites treated with **2**, the large number of vacuoles (V) can be observed. In the picture of parasites treated with **3**, cell debris can be seen (RE) and parasites become shortened and electrondenses (→) or elongated.

structural features and a wide structural diversity. Our biological tests showed that the three complexes were very active *in vitro* against both extra and intracellular forms of *T. cruzi* (in the order 1>2≥3). These compounds are effective at concentrations similar to those of the commonly used drug (benznidazole) but are much less toxic for the host cells than benznidazole. Consequently, these compounds significantly reduced *in vitro T. cruzi* growing rate as well as the capacity of the parasite to infect the host cells; therefore, the multiplication of the amastigotes and subsequent transformation to trypomastigotes was greatly lowered. It should be also noted that the antiparasital activity of metal compounds is also much higher than that of isolated HmtpO ligand, which is indicative of the critical role of metal ions in this activity. Moreover, a wide range of ultrastructural alterations in epimastigote forms of *T. cruzi* treated with these complexes were found. *In vivo* study results were consistent with those observed *in vitro*. First of all, no signs of toxicity, during the treatment of mice with complexes **1**, **2** and **3**, were observed. The parasitic charge was significantly decreased in comparison with the reference drug. As a conclusion, a new family of antiparasitic agents has been developed displaying promising *in vivo* activity and deserving a further study.

Acknowledgements

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In Vitro and in Vivo Trypanocidal Activity of Flavonoids from *Delphinium staphisagria* against Chagas Disease

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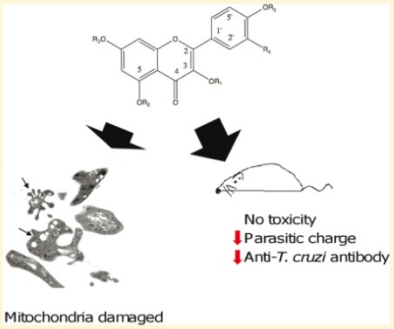
Supporting Information

ABSTRACT: The in vitro and in vivo trypanocidal activities of nine flavonoids (**1**−**9**) isolated from the aerial parts of *Delphinium staphisagria* have been studied in both the acute and chronic phases of Chagas disease. The antiproliferative activity of these substances against *Trypanosoma cruzi* (epimastigote, amastigote, and trypomastigote forms) in some cases exhibited more potent antitrypanosomatid activity and lower toxicity than the reference drug, benznidazole. Studies in vitro using ultrastructural analysis together with metabolism−excretion studies were also performed in order to identify the possible action mechanism of the compounds tested. Alterations mainly at the level of the mitochondria may explain metabolic changes in succinate and acetate production, perhaps due to the disturbance of the enzymes involved in sugar metabolism within the mitochondrion. In vivo studies provided results consistent with those observed in vitro. No signs of toxicity were detected in mice treated with the flavonoids tested, and the parasitic charge was significantly lower than in the control assay with benznidazole. The effects of these compounds were also demonstrated with the change in the anti-*T. cruzi* antibody levels during the chronic stage.



American trypanosomiasis, also known as Chagas disease, is one of the most devastating diseases from parasites of the family Trypanosomatidae. It is caused by the kinetoplastid protozoan *Trypanosoma cruzi*, which is transmitted by an insect vector depositing feces on the skin surface and subsequently biting. Other routes of contamination are infected blood transfusions, organ transplants, and from mother to child during pregnancy or breastfeeding.[1] Chagas disease arises in the form of an acute infection, during which most patients are not aware that they are infected, with further development to chronic and systemic stages, which affect severely the heart, esophagus, and colon. This disease is endemic throughout Latin America and is classified by the World Health Organization as the third most widely spread tropical disease after malaria and schistosomiasis.[2] It is estimated that about 100 million people are at risk of infection and from 15 to 20 million are infected, with some 50 000 persons dying yearly from this disease.

Drugs currently used to treat Chagas disease are the nitro-. heterocyclic compounds nifurtimox (Lampit) and benznidazole (Rochagan, Radanil), a nitroimidazole derivative, for which the anti-*T. cruzi* activity was discovered empirically about 30 years ago. However, neither of these drugs can be regarded as ideal due to (i) their low efficacy in the chronic stage of the illness; (ii) regional variations of effectiveness due to drug-induced resistance; (iii) a high percentage of treatment being discontinued due to side effects: (iv) the long treatment period (30−60 days) required; and (v) the necessity for close medical supervision. Certain drugs used for other diseases, such as allopurinol and itraconazole, have not yet been evaluated for appropriate clinical traits.[3] The goals for specific treatment of Chagas disease should (i) address the removal of the parasite from the infected person in order to reduce the probability of cardiological and digestive pathologies; (ii) stop the transmission chain of *T. cruzi*; (iii) decrease the number of newborns contracting the protozoa; and (iv) increase the number of people able to act as organ and blood donors.[4] Therefore, all these desired properties make the development of new drugs for the treatment of Chagas disease a much needed and challenging research goal.[3,5,6]

One way to discover new drugs is to investigate natural products from plants used medicinally.[7] Folk medicine is very often a valid

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Table 1. In Vitro Activity of Flavonoids Evaluated against the Epimastigote Form of *Trypanosoma cruzi*

compound	*T. cruzi* IC_{50} (μM)	toxicity in Vero cells (IC_{50}, μM)[a]	SI[b]
benznidazole	15.8	13.6	0.9
1	15.7	174.3	11.1
2	6.5	294.1	45.2
3	0.8	164.0	205.0
4	20.5	>1000	48.8
5	21.7	>1000	46.1
6	17.2	>1000	58.1
7	30.3	>1000	33.0
8	27.3	>1000	36.6
9	27.0	362.3	30.0

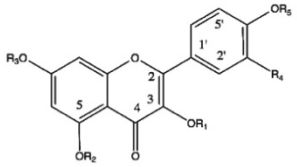
[a] Determined after 72 h of culturing. IC_{50} = the concentration required to give 50% inhibition, calculated by linear regression analysis from the K_c values at concentrations employed (1, 10, 25, 50, and 100 μM). [b] Selectivity index = IC_{50} Vero cells/IC_{50} epimastigote, based on an average of three separate determinations.

source for researchers looking for bioactive substances that are potentially useful against many diseases, as demonstrated by the search for new medicinal agents for treating trypanosomiasis, leishmaniasis, and other diseases.[7] Many potentially trypanocidal and leishmanicidal substances have been isolated from a variety of plants.[8−14] Several trypanocidal flavonoids are strong candidates for use in combination therapy against infections. It has been reported that some substances from plants have antitrypanosomal properties, but unfortunately their side effects are still unknown.[14,15]

Active principles from plants as well as their synthetic and semisynthetic analogues have served as one of the main ways to obtain new chemotherapeutic compounds.[14,16,17] The search for natural products with antitrypanosomal activity has given fresh impetus to the development of new synthetic compounds.[18] Flavonoids have been found in abundance in the fruits, vegetables, and sap of plants and have been demonstrated to have anticarcinogenic, antimicrobial, and antiparasitic activity.[19,20] In a previous study, it was determined that certain flavonoid derivatives exerted significant effects on the in vitro growth of *Leishmania* spp. and *T. cruzi*.[21]

Most studies directed toward the detection of secondary plant metabolites with trypanocidal activity have used the extracellular forms (epimastigote for *T. cruzi*) for easier maintenance under in vitro conditions. However, since the extracellular forms are not the developed form of the parasite in vertebrate hosts, preliminary evaluations need to be complemented using intracellular forms (amastigotes in host cells), and for *T. cruzi* using trypomastigote forms. At the same time, an assessment of the possible cytotoxicity of the metabolite should be made using nonparasitized host cells, in order to establish whether the in vitro activity of the metabolite is due to its general cytotoxic activity or if it is selectively active against the *Trypanosoma* parasite.[22]

In the present work, the inhibitory effects of nine flavonoids (**1**−**9**) from aerial parts of *Delphinium staphisagria* L. (Ranunculaceae) were investigated in relation to the extracellular and intracellular stages of *T. cruzi*. In addition, the cytotoxic effects of these compounds against a host cell line were assessed. A detailed examination of their in vivo antiparasitic activity and toxicity was made, in both the acute and chronic phases. The ^{1}H NMR spectroscopic analysis concerning the nature and percentage of the excretion metabolites was carried out in order to gain information concerning the inhibitory effect of the listed compounds on the parasite metabolism, since the glycolytic pathway represents the prime source of energy for the parasite. Finally, the effect of flavonoids on the ultrastructure of *T. cruzi* was studied on the basis of transmission electronic microscopy experiments.



1 astragalin (R_1 = Glc, R_2 = R_3 = R_4 = R_5 = H)
2 2″-acetylastragalin (R_1 = Glc 2″-Ac, R_2 = R_3 = R_4 = R_5 = H)
3 astragalin heptaacetate (R_1 = Glc-Ac, R_2 = R_3 = R_5 = Ac, R_4 = H)
4 paeonoside (R_1 = R_3 = Glc, R_2 = R_4 = R_5 = H)
5 2″-acetylpaeonoside (R_1 = Glc 2″-Ac, R_3 = Glc, R_2 = R_4 = R_5 = H)
6 paeonoside decaacetate (R_1 = R_3 = Glc-Ac, R_2 = R_5 = Ac, R_4 = H)
7 petiolaroside (R_1 = Glc, R_3 = Rha, R_2 = R_5 = H, R_4 = OH)
8 2″-acetylpetiolaroside (R_1 = Glc 2″-Ac, R_3 = Rha, R_2 = R_5 = H, R_4 = OH)
9 petiolaroside decaacetate (R_1 = Glc-Ac, R_3 = Rha-Ac, R_2 = R_5 = Ac, R_4 = OAc)
(Glc = β-glucopyranosyl; Rha = α- rhamnopyranosyl)

RESULTS AND DISCUSSION

Nine flavonoids (**1**−**9**) derived from the aerial parts of *D. staphisagria* were isolated and identified, as described earlier.[23]

The inhibitory effects on the in vitro growth of *T. cruzi* epimastigote forms of flavonoids **1**−**9** were measured at different times, following established procedures (see Experimental Section). The ability of these compounds to inhibit the growth of the epimastigote forms was evaluated at 1 to 100 μM, and the IC_{50} values were determined for the most active compounds, with benznidazole used as a positive control. The results, including cytotoxicity values against Vero cells, are presented in Table1. After 72 h of exposure, three of the flavonoid compounds, the glycoside astragalin (**1**) and two acetylated derivatives (**2** and **3**), showed significantly lower IC_{50} when the values were compared to benznidazole. The acetylated derivatives were the most effective (**2** and **3**, with IC_{50} values of 6.5 and 0.8 μM, respectively), while **1** showed an IC_{50} value similar to that of benznidazole (15.7 and 15.8 μM, respectively). Of the six remaining compounds, paenoside (**4**), petiolaroside (**7**), and their acetylated compounds (**5**, **6**, **8**, and **9**) showed IC_{50} values close to or slightly greater than those of benznidazole.

The cytotoxicity of these compounds against mammalian cells was also evaluated in vitro at 1 to 100 μM, using Vero cells[14] (see Table 1). Benznidazole was again included as the reference drug. The heptaacetylated compound of astragalin (**3**) showed the best selectivity index (SI = IC_{50} Vero cells/IC_{50} epimastigote). In general, the rest of the eight compounds consistently presented SI values higher (6.7- to 52-fold) than those of benznidazole.

However, also included in this study were the effects of these compounds on the parasitic forms that develop in the host (amastigote and trypomastigote forms), the study of which is of great importance, given that the final aim is to determine the effects in the definitive host.[22] An experimental model designed in our laboratory has been used to predict the effect of those flavonoids that showed promising inhibition in vitro and a higher SI over the growth of extracellular forms, on the capacity of infection and growth of the intracellular forms and subsequent

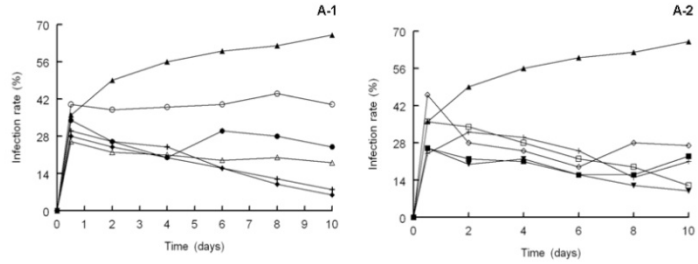


Figure 1. Effect of flavonoids **1**−**9** on the infection rate and *T. cruzi* growth. Rate of infection. **A-1**: (-▲-), control; (-Δ-), benznidazole; (-●-), **1**; (-+-), **2**;(-♦-), **3**; (-Δ-), **4**. **A-2**: (-▲-), control; (-▼-), **5**; (-□-), **6**; (- ◇-), **7**; (- ■ -), **8**; (-+-), **9**. Measured at IC_{25} concentration. Values are means of three separate experiments.

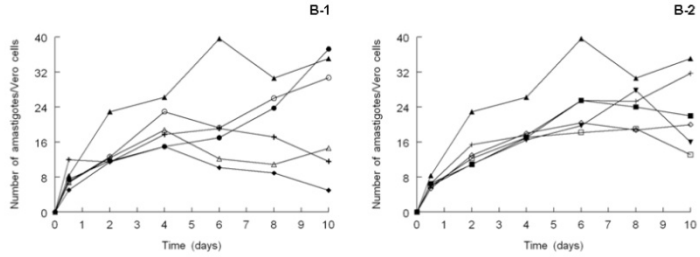


Figure 2. Effect of flavonoids **1**−**9** on the mean number of amastigotes per infected Vero cell. **B-1**: (-▲-), control; (-Δ-), benznidazole; (-●-), **1**; (-+-), **2**; (-♦-), **3**; (-Δ-), **4**. **B-2**: (-▲-), control; (-▼-), **5**; (-□-), **6**; (-◇-), **7**; (-■-), **8**; (-+-), **9**. Measured at IC_{25} concentration. Values are means of three separate experiments.

transformation to bloodstream forms. Adherent Vero cells (1×10^5) were incubated for two days and then were infected with 1×10^3 metacyclic forms for 12 h (control experiment, Figure 1).

Then, the nonphagocytosed parasites were removed and the culture was kept in fresh medium for 10 days. The parasites invaded the cells and underwent morphological conversion to amastigotes within one day after infection. On day 10, the rate of host-cell infection reached its maximum. When the complexes (IC_{25} concentration) were added simultaneously to Vero cells with *T. cruzi* metacyclic forms that had been in the exponential growth phase for 12 h, the infection rate significantly decreased with respect to the control in the order **3** > **2** > **5** > **6** > **4** > **7** > **8** > **1** > **9**, with percentages of infection inhibition within a range of 91, 88, 85, 82, 73, 68, 65, 64, and 59%, respectively (Figure 1, A1 and A2). These values are considerably higher than the inhibition achieved by benznidazole (39%). In the control experiment, the average number of amastigotes per infected cell increased to 39.6 amastigotes/cell on day 6, decreasing to 30.6 amastigotes/cell on day 8, and increasing again to 35 amastigotes/cell on day 10 (see Figure 2, B1and B2); these fluctuations, in the number of amastigote forms per cell, are due to the parasite life cycle. When a cell is completely invaded by amastigote forms, the parasites break the cell, and the breakage of the Vero cells involves the release of amastigotes and their subsequent transformation into trypomastigotes. Afterward, the trypomastigote form can infect new cells, in which they multiply, increasing the number of amastigotes per cell. Flavonoids **1**−**9** inhibited *T. cruzi* amastigote replication in Vero cells in vitro, in a similar manner to how they inhibited the infection rate and again more efficiently than the reference drug, in the order **3** > **2** > **6** > **4** > **7** > **5** > **8** > benznidazole. Compounds **9** and **1** exhibited an inhibition percentage lower than that of benznidazole. In the control experiment, the number of trypomastigotes in culture medium was 4.4×10^3 on day 10 postinfection (Figure 3, C1 and C2). All the compounds tested showed a highly significant decline in the number of trypomastigotes in media (95, 85, 78, 77, 72, 68, 65, 58, and 44% for **3**, **6**, **2**, **5**, **7**, **4**, **1**, **8**, and **9**, respectively), also higher than for the reference drug (benznidazole caused a 37% decrease).

Due to the trypanocidal effect in vitro of flavonoids **1**−**9**, in vivo studies were performed to evaluate their activity against *T. cruzi* infection in mice. Previous results have shown that the intravenous doping route resulted in high mortality rates;[24] therefore, in this study the intraperitoneal route was chosen, using a concentration of 5 mg/kg, which did not result in any animal mortality (data not shown). Female Swiss mice were inoculated intraperitoneally with 1×10^5 blood trypomastigotes, and treatment began seven days postinfection, with an ip route of 1 mg/kg per day of each test compound for five days. Administration was conducted using saline solution. A control group

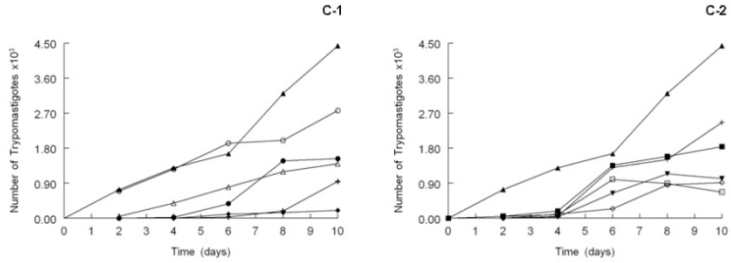


Figure 3. Effect of flavonoids **1**−**9** on the number of trypomastigotes in the culture medium. C-1: (-▲-), control; (-○-), benznidazole; (-●-), **1**; (-+-), **2**; (-♦-), **3**; (-□-), **4**. C-2: (-▲-), control; (-▼-), **5**; (-□-), **6**; (-◇-), **7**; (-■-), **8**; (-+-), **9**. Measured at IC_{25} concentration. Values are means of three separate experiments.

was treated in the same way with the vehicle. The level of parasitemia was determined every two days (Figure 4), with mortality observed daily, and serological tests were performed 40 and 90 days postinfection (Table 2). None of the animals treated with the test compounds or the control died during the treatment, while in the groups treated with benznidazole the survival cohort was 80%. Flavonoids **1**−**9** decreased the trypomastigote forms on the day the experiment reached the maximum parasitic charge (day 22 postinfection), in comparison to the positive control. In the groups of animals treated with the test compounds (only one was tested in each group), a significant decrease in parasitemia was registered on day 40 postinfection with most of the substances tested.

The percentage decrease, with respect to the control, ranged from 59% to 23% for the flavonoids and 16% for benznidazole. In fact, the parasitemia levels observed for the compounds assayed agreed with their in vitro behavior, with the in vivo activity varying, in general, in the order **3** > **6** > **2** > **5** > **4** > **7**. The two acetylated derivatives of petiolaroside (**8** and **9**) exhibited an antiparasite potency greater than the untreated control group.

None of the animals treated with the flavonoids tested in this study or benznidazole, used as control in the antibody studies, showed negative anti-*T. cruzi* serology. However, all compounds lowered antibody levels between days 40 and 90 (Table 2), showing improved results compared with benznidazole in this assay. The differences in the level of anti-*T. cruzi* antibodies were consistent with the parasitemia findings. Although the test flavonoids did not completely eliminate the trypomastigote forms circulating in the bloodstream, no clear relationship was found between the number of remaining parasites and the chronic stage of the disease. Thus, there was no evidence of protection against heart-rate alterations characteristic of *T. cruzi* infection.

To date, none of the trypanosomatids studied is capable of completely degrading glucose to CO_2 under aerobic conditions, and they excrete metabolites into the medium that depend on the species considered.[25,26] *T. cruzi* consumes glucose at a high rate, thereby acidifying the culture medium due to incomplete oxidation to acids. ^{1}H NMR spectroscopy has indicated the nature of the fermented metabolites excreted by this trypanosomatid during in vitro culture. One of the major metabolites excreted by *T. cruzi* is succinate, the probable main role of which is to maintain the glycosomal redox balance by providing two glycosomal oxidoreductase enzymes that allow reoxidation of

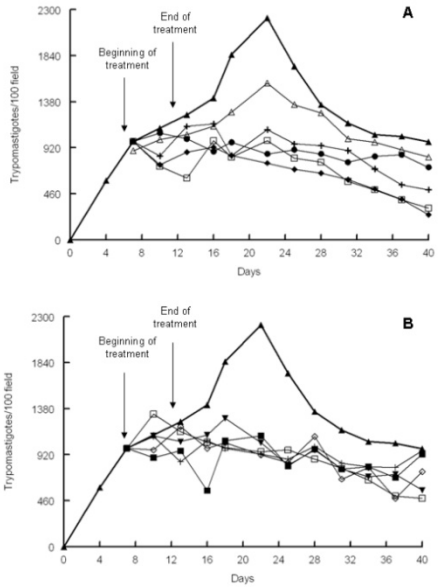


Figure 4. Parasitemia in the murine model of acute Chagas disease by flavonoids **1**−**9**: (-▲-), control and dose receiving 5 mg/kg of C-1: (-Δ-), benznidazole; (-●-), **1**; (-+-), **2**;(-♦-), **3**; (-Δ-), **4**. C-2: (-▲-), control; (-▼-), **5**; (-□-), **6**; (-◇-), **7**; (-■-), **8**; (-+-), **9**.

NADH, produced by glyceraldehyde-3-phosphate dehydrogenase, in the glycolytic pathway. Succinic fermentation offers a significant advantage of requiring only half of the phosphoenolpyruvate (PEP) produced to maintain the NAD^+/NADH balance. The remaining PEP is converted into acetate, L-lactate, L-alanine, and/or ethanol, depending on the species considered.[25]

To gain information concerning the effect of the flavonoids **1**−**9** on metabolite excretion, ^{1}H NMR spectra were measured

Table 2. Differences in the Level of anti-*T. cruzi* Antibodies between Days 40 and 90 Postinfection for Compounds 1−9 and Benznidazole, Expressed in Absorbance Units

compound[b]	ΔA[a]
control (untreated)	0.186
benznidazole	0.116
1	−0.035
2	0.056
3	−0.246
4	0.062
5	−0.104
6	−0.074
7	−0.291
8	0.330
9	0.327

[a] ΔA = Absorbance at 490 nm, day 90 pi − Absorbance at 490 nm, day 40 pi. [b] Dose 1 mg/kg/day, intraperitoneal route administered for five days (see Experimental Section).

from the trypanosomatids treated with these compounds and were compared with those from cell-free medium (control) four days after inoculation with the *T. cruzi* strain. In the control experiment, *T. cruzi* excreted acetate and succinate as the major metabolites and, in a lower percentage, L-alanine (Figure S1A, Supporting Information). These data agreed with those reported previously.[27] When trypanosomatids were treated with the test flavonoids, the excretion of some of these catabolites was slightly altered at the doses employed. Percentages of inhibition in the height of the peaks corresponding to the significant catabolites are shown in Table S1, Supporting Information. It was observed that the excretion of succinate and also of acetate was inhibited by acetylated flavonoid compounds **3**, **5**, **6**, **8**, and **9** (for these compounds only the spectrum shown corresponds to **2**, Figure S1C, Supporting Information). The inhibition of acetate and succinate excretion could explain the observed increase in L-lactate and ethanol production, considering that these compounds operate at the electronic chain level preventing reducing-power recharge, or they may act on the mitochondria and, consequently, oxidative phosphorylation. The acetylated flavonoid **3**, in addition to inhibiting the production of succinate and acetate, also inhibited the excretion of alanine (Figure S1D, Supporting Information). The three nonacetylated flavonoids **1**, **4**, and **7** behaved similarly, clearly inhibiting acetate and augmenting the production of lactate, alanine, and ethanol (Figure S1B shows only the spectrum of the derivative **1**). It should be noted that we found no significant alteration of the energetic metabolism in the presence of benznidazole (spectrum not shown).

The transmission electron microscope evaluation of flavonoids **1**−**9** against *T. cruzi* epimastigotes showed notable ultrastructural alterations, as reflected in Figure 5 (panels 2−6) with respect to the control (Figure 5, panel 1). All compounds induced ultrastructural changes in the parasites, with the most common being greater vacuolization and the shrinkage of the protozoa in the presence of more electrodense structures. The most effective compounds were **1**, **2**, **4**, **6**, and **9**, resulting in a distinct deformation of the parasites, which appeared to have burst and had an undulating cytoplasm membrane, as if extending pseudopodia. For compound **9** (Figure 5, panel 2) most of the epimastigotes had large, empty vacuoles, while the others had a

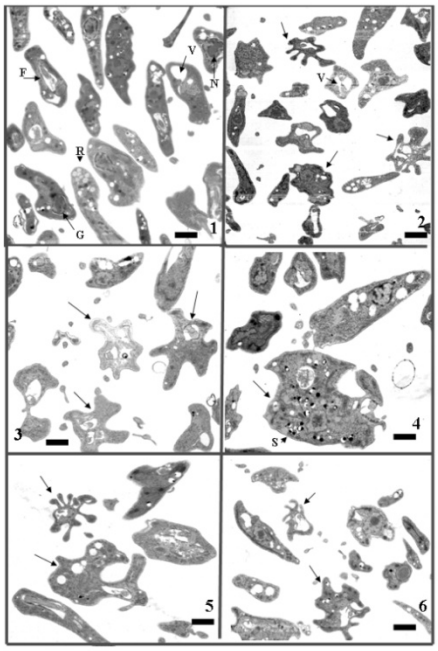


Figure 5. Ultrastructural alterations by TEM in epimastigotes of *Trypanosoma cruzi* treated with flavonoids **1**−**9**. (1) Control parasite of *T. cruzi* showing organelles with their characteristic aspect, such as nucleus (N), reservosomes (R), mitochondrion (M), glycosomes (G), vacuoles (V), and flagellum (F) (bar: 0.583 μm). (2) Epimastigotes of *T. cruzi* treated with **9** with star-shaped (arrow) and strongly vacuolated (V) (bar: 1.59 μm). (3) Epimastigotes of *T. cruzi* treated with **1** with star-shaped and waving cytoplasmatic membrane (arrow) (bar: 1.59 μm). (4) Epimastigotes of *T. cruzi* treated with **4** with strongly electrodense structures (S) and some very dilated (arrow) (bar: 1.59 μm). (5) Parasites treated with **6** with waving shape (arrow) (bar: 1.00 μm). (6) *T. cruzi* treated with **2** with start-shaped (arrow), electrodense structures (S), and dilated mitochondria (M) (bar: 1.00 μm).

strongly electrodense cytoplasm. On the other hand, with compound **1** (Figure 5, panel 3) the effect was reversed, since many of the protozoa showed cytoplasm with low electrodensity and had a dead appearance. Compound **2** (Figure 5, panel 6) also dilated the mitochondria and induced the production of certain small structures that were strongly electrodense, perhaps the products of refuse. These appeared in small quantities, in contrast to the data obtained for flavonoid **4** (Figure 5, panel 4), in which these structures appeared in high abundance, with some parasites appearing dilated and having sizes larger than normal.

EXPERIMENTAL SECTION

Parasite Strain and Culturing. The Maracay strain of *T. cruzi* was isolated at the Institute of Malariology and Environmental Health,

Maracay, Venezuela. Epimastigote forms were obtained in biphasic blood-agar NNN medium (Novy−Nicolle−McNeal), supplemented with minimal essential medium (MEM) and 20% inactivated fetal bovine serum, and reseeded afterward in a monophasic culture (MTL), following the method of Luque et al.[28]

Plant Material. Aerial parts of *Delphinium staphisagria* were collected and processed as described previously.[23] Nine flavonoids (**1**−**9**) were isolated, derivatized, and identified.[23] These substances were dissolved in dimethyl sulfoxide (Panreac, Barcelona, Spain) at a concentration of 0.1%. The effects of each compound against epimastigote forms, as well as the concentrations, were evaluated at 24, 48, and 72 h using a Neubauer hemocytometric chamber, and the trypanocidal effects were expressed as IC_{50} values (the concentration required to give 50% inhibition as calculated by linear regression analysis from the K_c values at the concentrations employed).

In Vitro Trypanocidal Activity Assay. To obtain the parasite suspension for the trypanocidal assay, the epimastigote culture (in the exponential growth phase) was concentrated by centrifugation at 1000*g* for 10 min, and the number of flagellates counted in an hemocytometric chamber and distributed into aliquots of 2×10^6 parasites/mL. Compounds were dissolved in DMSO at a concentration of 0.01%, after being evaluated as nontoxic and without inhibitory effects on parasite growth. The compounds were dissolved in the culture medium, and the dosages used were 100, 50, 25, 10, and 1 μM.

Cell Culture and Cytotoxicity Tests. Vero cells (Flow) were grown in MEM (Gibco), supplemented with 10% inactivated fetal calf serum, and adjusted to pH 7.2, in a humidified 95% air−5% CO_2 atmosphere at 37 °C for two days. For the cytotoxicity testing, cells were placed in 25 mL Colie-based bottles (Sterling) and centrifuged at 100*g* for 5 min. The culture medium was removed, and fresh medium was added to a final concentration of 1×10^5 cells/mL. This cell suspension was distributed in a culture tray (with 24 wells) at a rate of 100 μL/well and incubated for two days at 37 °C in a humidified atmosphere enriched with 5% CO_2. The medium was removed, and fresh medium was added together with each test compound (at concentrations of 100, 50, 25, 10, and 1 μM). After 72 h of treatment, cell viability was determined by flow cytometry. Thus, 100 μL/well of propidium iodide (PI) solution (100 μg/mL) was added and incubated for 10 min at 28 °C in the dark. Afterward, 100 μL/well of fluorescein diacetate (100 μg/mL) was added and incubated under the same conditions as above. Finally, the cells were recovered by centrifugation at 400*g* for 10 min, and the precipitate was washed with PBS. Flow cytometric analysis was performed on a FACS Vantage flow cytometer (Becton Dickinson). The live cells with their plasma membrane intact were associated with a green fluorescence, due to the effect of sterases on fluorescein diacetate. On the other hand, cells that had lost membrane integrity or were dead allowed the penetration of PI by passive diffusion, which specifically bound to their DNA with fluorescence in the range of 580 nm. Percentages of viability were calculated in comparison to that of the control culture, and IC_{50} values were calculated by linear regression analysis from the K_c values at the concentrations employed.

Transformation of Epimastigote to Metacyclic Forms. Metacyclogenesis was induced by culturing the parasites at 28 °C in modified Grace's medium (Gibco) for 12 days, as described previously.[29] Twelve days after cultivation at 28 °C, metacyclic forms were counted in order to infect Vero cells. The proportion of metacyclic forms was around 40% at this stage.

Cell Selectivity Assay. Vero cells were cultured in MEM medium in a humidified 95% air−5% CO_2 atmosphere at 37 °C. Cells were seeded at a density of 1×10^5 cells/well in 24-well microplates (Nunc) with rounded coverslips on the bottom and then cultivated for two days. Afterward, the cells were infected in vitro with metacyclic forms of *T. cruzi*, at a ratio of 10:1. The test compounds (IC_{25} concentrations) were added immediately after infection and incubated for 12 h at 37 °C in a 5% CO_2 atmosphere. The nonphagocytosed parasites and the test compounds were removed by washing, and the infected cultures were grown for 10 days in fresh medium. Fresh culture medium was added every 48 h.

The activity of each test compound was determined from the percentage of infected cells and the number of amastigotes per cell infected in treated and untreated cultures in methanol-fixed and Giemsa-stained preparations. The percentage of infected cells and the mean number of amastigotes per infected cell were determined by analyzing more than 100 host cells distributed in randomly chosen microscopic fields. Values are the means of four separate determinations. The number of trypomastigotes in the medium was determined as described previously.[29]

In Vivo Trypanocidal Activity Assay. Groups of five BALB/c female mice (6 to 8 weeks old; 25−30 g) maintained under standard conditions were infected with 1×10^3 bloodstream *T. cruzi* metacyclic forms by the intraperitoneal route. The animals were divided into the following groups: (i) group 1: uninfected (not infected and not treated); (ii) group 2: untreated (infected with *T. cruzi* but not treated); (iii) group 3: uninfected (not infected and treated with 1 mg/kg body weight/day, for five consecutive days [7 to 12 days postinfection] by the intraperitoneal route);[23] and (iv) group 4: treated (infected and treated for five consecutive days [7 to 12 days postinfection] with the test compounds and benznidazole). This animal experiment was performed with the approval of an ethical committee of the University of Granada.

Five days after infection, the presence of circulating parasites was confirmed by the microhematocrit method. A blood sample (5 μL) drawn from the tail of each treated mouse was taken and diluted 1:15 (50 μL of citrate buffer and 20 μL of lysis buffer at pH 7.2), and this vehicle was also employed as a negative control. The parasites were counted in a Neubauer chamber. The number of deaths was recorded every two days.

One group of four animals treated with each compound and benznidazole was included for serological studies. Treatments were started seven days after animal infection. Compounds were administered in a similar way to that explained above and at the same concentrations.

Circulating anti-*T. cruzi* antibodies, at days 40 and 90 postinfection, were evaluated quantitatively by an enzyme-linked immunoassay. The sera, diluted to 1:100, were reacted with an antigen composed of an excreted Fe-SODe of *T. cruzi* epimastigotes. The results are expressed as the ratio of the absorbance of each serum sample at 490 nm to the cutoff value. The cutoff for each reaction was the mean of the values determined for the negative controls plus three times the standard deviation.

Metabolite Excretion. Cultures of *T. cruzi* epimastigotes (initial concentration 5×10^5cells/mL) received the IC_{25} dosage of the test compounds (except for control cultures). After incubation for 72 h at 28 °C, the cells were centrifuged at 400*g* for 10 min. The supernatants were collected to determine the excreted metabolites by ^{1}H NMR spectroscopy as previously described by Fernández-Becerra et al.[30] Chemical shifts were expressed in parts per million (ppm), using sodium 2,2-dimethyl-2-silapentane-5-sulfonate as the reference signal, and were used to identify the respective metabolites, with the results proving to be consistent with those described previously.[30]

Ultrastructural Alterations. The parasites, at a density of 5×10^6 cells/mL, were cultured in their corresponding medium, containing the test compounds at their IC_{25} concentration. After 72 h, the cultures were centrifuged at 400*g* for 10 min, and the pellets washed in PBS and then fixed with 2% (v/v) *p*-formaldehyde-glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4) for 2 h at 4 °C. The pellets were prepared for transmission electron microscopy following the technique of Luque et al.[28]

■ ASSOCIATED CONTENT

Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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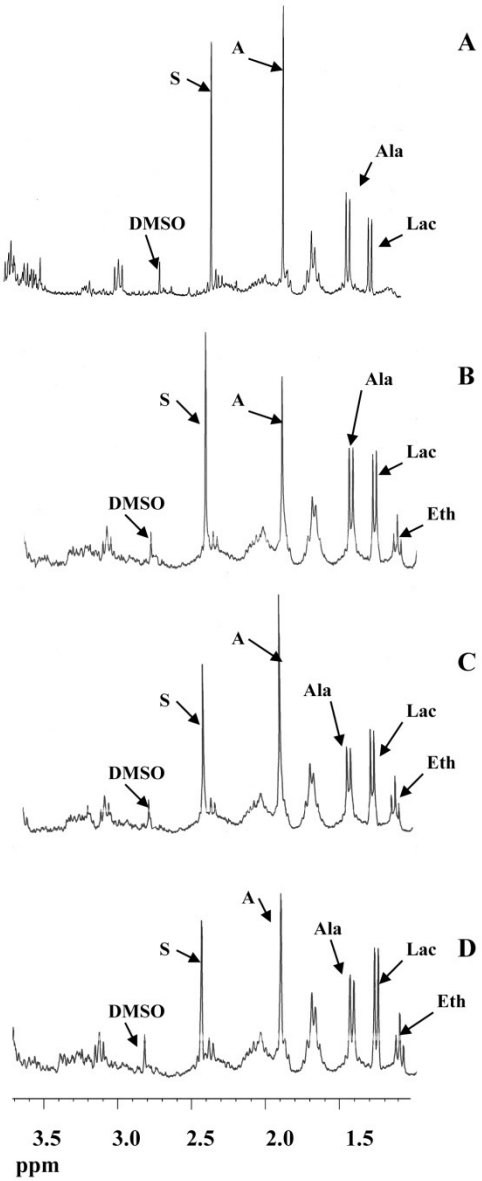

Figure S1. ^{1}H-NMR spectra epimastigote forms of *T. cruzi* treated against flavonoid derivative (at a concentration of IC_{25}): (**A**) Control (untreated); (**B**) **1**; (**C**) **3** and (**D**) **5**. Lac, L-lactate; A, acetate; S, succinate; Ala, L-alanine; Eth, ethanol and DMSO, dimethylsulfoxide.

Table S1. Variation percentages in the height of the peaks corresponding to catabolites excreted by *T. cruzi* epimastigotes in the presence of compounds **1**-**9** with respect to the control test.

Comp.	Lac	Ala	A	S	Eth
1	+ 53 %	+ 18%	-30.3%	=	+100%
2	+ 47 %	=	- 27 %	-26.5%	+100%
3	+ 33 %	- 20 %	-19 %	-35%	+100%
4	+ 94 %	+ 22 %	-12.3%	=	+100%
5	+ 63 %	=	- 37 %	- 39 %	+100%
6	+ 47 %	=	- 33 %	-39.6%	+100%
7	+ 86 %	+ 20 %	-12.5%	=	+100%
8	+ 57 %	=	-14.0%	-24%	+100%
9	+ 19 %	=	-32.1%	-29.9%	+100%

Lac: L-lactate; Ala: L-alanine; A: acetate; S: succinate; Eth: ethanol. (-): peak inhibition; (+): peak increasing.

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In Vitro and *In Vivo* Studies of the Trypanocidal Activity of Four Terpenoid Derivatives against *Trypanosoma cruzi*

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Abstract. Four terpenoid derivatives were examined for their activity against *Trypanosoma cruzi.* Our results show that two compounds were very active *in vitro* against both extra- and intracellular forms. These compounds, non-toxic for the host cells, are more effective than the reference drug benznidazole. The capacity to infect cells was negatively affected and the number of amastigotes and trypomastigotes was reduced. A wide range of ultrastructural alterations was found in the epimastigote forms treated with these compounds. Some metabolic changes occurred presumably at the level of succinate and acetate production, perhaps caused by the disturbance of the enzymes involved in sugar metabolism inside the mitochondria. *In vivo* results were consistent with those observed *in vitro*. The parasitic load was significantly lower than in the control assay with benznidazole. The effects of these products showed the reduction of the anti-*T. cruzi* antibodies level during the chronic stage.

INTRODUCTION

Tropical and subtropical diseases caused by protozoal parasites remain a major public health problem in many of the less developed countries of the world, because of the lack of effective drugs or increasing resistance against the few affordable drugs available.[1] American trypanosomiasis, also known as Chagas disease, is one of the most devastating parasitic diseases. It is caused by the kinetoplastid protozoan *Trypanosoma cruzi*, which is vectorially transmitted by a hemiptera depositing feces on the skin surface, containing metacyclic forms, after the blood meal. Other pathways of infection include contaminated blood transfusions, organ transplants, oral contamination caused by food/beverage,[2,3] and even transmission from mother to child during pregnancy or breastfeeding.[4]

Chagas disease manifests itself in the form of an acute infection, during which most patients do not know that they are infected. Actually, the acute phase can be asymptomatic but also the Romana's sign can be present in around 15% of the cases. Only a 30–40% of acute individuals undergo further development and it becomes chronic and systemic, severely affecting the heart, esophagus, and colon.[5] Endemic throughout Latin America, it is the third most widespread tropical disease after malaria and schistosomiasis, according to the World Health Organization (WHO).[5]

An estimated 10 million people are infected worldwide, mostly in Latin America where Chagas disease is endemic. More than 25 million people are at risk of the disease. It is estimated that in 2008 Chagas disease killed more than 10,000 people[6]; unfortunately, current treatment of this disease is very limited, and no successful vaccine has been developed.[7]

Available drugs are mainly nitroheterocyclic compounds such as nitrofuranenifurtimox or the nitroimidazole derivative benznidazole (BZN), but they have proved effective only against the acute phase, exhibiting very limited efficacy in the chronic stage.[8] Furthermore, they are quite toxic, causing severe side effects such as pancreatitis and cardiac toxicity.[2] The search for more effective drugs focuses mainly on their potential action over essential and exclusive components of the trypanosomatids.

The use of natural products as new chemotherapeutic agents has been investigated for some time. Among these, terpenoids, an important group of secondary metabolites, must be emphasized, mainly because of their structural diversity and natural abundance. During the last few years trypanocidal activities for some triterpenes[9] and diterpenes, with *ent*-kaurene,[10] pimarane,[11] and spongiane skeleton,[12] have been reported. Recently, the trypanocidal activity of several oxygenated abietane diterpenoids has been described.[13] Diterpene resin acids are important defense compounds from conifers against potential herbivores and pathogens.[14] The biological activity of natural abietane acids has been reviewed.[15] In recent years, interest in these types of terpenoids has increased as a result of the isolation of compounds, mainly phenols and related derivatives, showing remarkable biological activities.[16–18] Other significant oxidized abietane diterpenes have shown strong inhibition of various human tumors and oncogen-transformed cells.[19] The widespread use of these agents has not yet been established, however, and chemotherapeutic armament against kinetoplastic parasites remains limited. New drug options are clearly needed to fight these pathogens.

Recently, our group synthesized abietane phenols 1–4[20,21] (Figure 1), and their antiproliferative *in vitro* and *in vivo* F1 activities against *T. cruzi* (epimastigote, amastigote, and trypomastigote forms) have been investigated in this work. Unspecific mammal cytotoxicity of the most active compounds was evaluated *in vitro*, and less toxic derivatives have been submitted to *in vivo* experimentation in a more thorough study. Furthermore, we also included a nuclear magnetic resonance (1H NMR) study concerning the nature and percentage of the excretion metabolites to gain information concerning the inhibitory effect of our compounds over the glycolytic pathway, because it represents the prime source of energy for the parasite. Finally, the effect of compounds on the ultrastructure of *T. cruzi* is considered the basis of transmission electronic microscopy (TEM) experiments.

MATERIALS AND METHODS

Chemical compounds. Compound 1, the methyl ester of 12-hydroxydehydroabietic acid, recently described as a new

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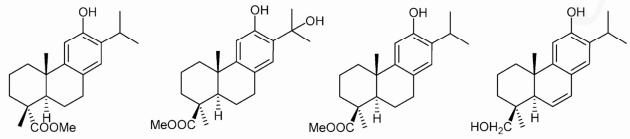

1 2 3 4

FIGURE 1. Terpenoid derivatives structure.

natural product,[22] has been synthesized from commercial abietic acid.[20]

Compounds 2–4 were prepared from *trans*-communic acid,[21] a labdane diterpene very abundant in some species of *Juniperus* and *Cupressus*. Compound 3 is the methyl ester of lambertic acid, isolated from *Podocarpus lambertius* and compound 4, 6,7-dehydroabieta-8,11,13-trien-12,19-diol, named sugikurojin A, is a new diterpene recently isolated from *Cryptomeria japonica*. Compound 2, methyl 12,15-dihydroxyabieta-8,11,13-trien-19-oate, has not yet been found in nature.

Parasite strain, culture. *Trypanosoma cruzi* SN3 strain of (IRHOD/CO/2008/SN3) was isolated from domestic *Rhodnius prolixus* and the biological origin is Guajira (Colombia).[23]

Epimastigote forms were obtained in biphasic blood-agar AU1 NNN medium (Novy-Nicolle-McNeal) supplemented with minimal essential medium and 20% inactivated fetal bovine serum and afterwards reseeded in a monophasic culture (MTL), following the method of Luque and others.[24]

Cell culture and cytotoxicity tests. Vero cells (Flow) were AU2 grown in RPMI (Gibco) supplemented with l0% inactivated fetal bovine serum and adjusted to pH 7.2, in a humidified 95% air-5% CO_2 atmosphere at 37°C for 2 days. For the cytotoxicity test, cells were placed in 30 mL sterile polystyrene container (Deltalab, Barcelona, Spain), and centrifuged at 100 *g* for 5 min. The culture medium was removed, and fresh medium was added to a final concentration of 1×10^5 cells/mL. This cell suspension was distributed in a culture tray (with 24 wells) at a rate of 100 μL/well and incubated for 2 days at 37°C in humid atmosphere enriched with 5% CO_2.

The medium was removed, and the fresh medium was added together with the product to be studied (at concentrations of 100, 50, 25, l0, and 1 μM). After 72 h of treatment, the cell viability was determined by flow cytometry. Thus, 100 μL/well of propidium iodide (PI) solution (100 μg/mL) was added and incubated for 10 min at 28°C in darkness. Afterward, 100 μL/well of fluorescein diacetate (FDA) (100 ng/mL) was added and incubated under the same conditions as above. Finally, the cells were recovered by centrifugation at 400 *g* for l0 min and the precipitate washed with phosphate buffer solution (PBS). Flow cytometric analysis was performed on a AU3 FACS Vantageflow cytometer (Becton Dickinson). The live cells with their plasma membrane intact were associated with the green fluorescence, because of the effect of sterases on FDA. On the other hand, cells that had lost the membrane integrity and were dead allowed the penetration of the IP by passive diffusion and specifically bound to their DNA and then, fluoresce in the range of 580 nm. The percentage of viability was calculated in comparison to that of the control culture (infected but untreated cultures), and the IC_{50} (the concentration required to give 50% of inhibition) was calculated by linear-regression analysis from the Kc values at the concentrations used.

***In vitro* trypanocidal activity assay.** *Epimastigote assay.* The parasite suspension was obtained for the trypanocidal assay by concentrating the epimastigote culture in the exponential growth phase by centrifugation at 1,000 *g* for 10 min, whereupon the number of flagellates were counted in a hemocytometric chamber and distributed into aliquots of 5×10^5 parasites/mL. The compounds were dissolved in dimethyl sulfoxide at a concentration of 0.01%, after being assayed as non-toxic and without inhibitory effects on the parasite growth. The compounds were dissolved in the culture medium, and the dosages used were 100, 50, 25, 10, and 1 μM. After 72 h of incubation, the effect of each compound was evaluated by light microscopy, through the quantification of viable parasite using a Neubauer chamber.

Metacyclic trypomastigotes assay. Metacyclogenesis was induced by culturing parasites at 28°C in modified Grace's medium (Gibco) for 12 days as described previously.[25] Twelve days after cultivation at 28°C, metacyclic forms were counted to infect Vero cells. The proportion of metacyclic forms was around 40% at this stage.

After the metacyclic forms were obtained, Vero cells were cultured in RPMI medium in a humidified 95% air-5% CO_2 atmosphere at 37°C. Cells were seeded at a density of 1×10^4 cells/well in 24-well microplates (Nunc) with rounded AU4 coverslips on the bottom and cultivated for 2 days. Afterward, the cells were infected *in vitro* with metacyclic forms of *T. cruzi* at a ratio of 10:1. The compounds (IC_{25} concentrations, concentration required to give 25% of inhibition) were added immediately after infection and were incubated for 12 h at 37°C in a 5% CO_2. The non-phagocytosed parasites and the drugs were removed by washing, and the infected cultures were then grown for 10 days in fresh medium without drugs. Fresh culture medium was added every 48 h.

The activity of the compounds was determined from the percentage of infected cells and the number of amastigotes founded per cells infected in treated and untreated cultures in methanol-fixed and Giemsa-stained preparations. The percentage of infected cells and the mean number of amastigotes per infected cell were determined by analyzing more than 100 host cells distributed in randomly chosen microscopic fields. Values are the means of three separate determinations. The number of trypomastigotes in the medium was determined as described previously.[24]

Axenic amastigotes assay. Axenic amastigote forms of *T. cruzi* were cultured following the methodology described

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previously by Moreno.[26] Thus, epimastigotes transformed into amastigotes after 3 days of culture in M199 medium (Invitrogen, Leiden, The Netherlands) supplemented with 10% heat-inactivated FCS, 1 g/L β-alanine, 100 mg/L L- asparagine, 200 mg/L sucrose, 50 mg/L sodium pyruvate, 320 mg/L malic acid, 40 mg/L fumaric acid, 70 mg/L succinic acid, 200 mg/L α-ketoglutaric acid, 300 mg/L citric acid, 1.1 g/L sodium bicarbonate, 5 g/L MES, 0.4 mg/L hemin, and 10 mg/L gentamicin pH 5.4 at 37°C. The effect of each compound against 1×10^6 axenic amastigote forms/mL, was tested at 48 h using a Neubauer hemocytometric chamber. The inhibitory effect is expressed as IC_{50} values.

***In vivo* trypanocidal activity assay.** This experiment was performed with the permission of the ethical committee of the University of Granada, Spain. Groups of three BALB/c female mice (6 to 8 weeks of age; 25 g) maintained under standard conditions were infected with 1×10^5 *T. cruzi* metacyclic forms by the intraperitoneal route. The animals were divided into the following groups: i) group 1: uninfected (not infected and not treated); ii) group 2: untreated (infected with *T. cruzi* but not treated); iii) group 3: uninfected (not infected and treated: with 1 mg/kg of body weight/day, for five consecutive days (7–11 post-infection) by the intraperitoneal route[27]; and iv) group 4: treated (infected and treated with 1 mg/kg of body weight/day for five consecutive days (7 to 11 post-infection) by the intraperitoneal route with the compounds and BZN).

Treatments were started 7 days after infection with the parasites. Compounds were administered in a similar way to that explained previously and at the same concentrations.

A blood sample (5 μL) drawn from the mandibular vein of each treated mouse was taken and diluted 1:15 (50 μL of citrate buffer and 20 μL of lysis buffer at pH 7.2). The parasites were counted by fields with the immersion objective. The number of bloodstream *T. cruzi* metacyclic forms were recorded every 2 days from 7 to 40 days post-infection. The number of metacyclic forms was expressed per 100 microscopic fields.

Circulating anti-*T. cruzi* antibodies were quantitatively evaluated at Days 40 and 120 post-infection by the use of an enzyme-linked immunoassay. The blood, diluted to 1:50 in PBS, was reacted with an antigen constituted by a soluble Fe-SODe from *T. cruzi* epimastigotes. The results are expressed as the ratio of the absorbance (*Abs*) of each serum sample at 490 nm to the cutoff value. The cutoff for each reaction was the mean of the values determined for the negative controls plus three times the standard deviation.[28]

Metabolite excretion. Cultures of *T. cruzi* epimastigotes (initial concentration 5×10^5 cells/mL) received IC_{25} of the compounds (except for control cultures). After incubation for 96 h at 28°C, the cells were centrifuged at 400 *g* for l0 min. The supernatants were collected to determine excreted metabolites by 1H-NMR spectroscopy as previously described.[29] The chemical displacements were expressed in parts per million (ppm), using sodium 2,2 dimethyl-2-silapentane-5-sulfonate as the reference signal. The chemical displacements used to identify the respective metabolites were consistent with that described.[29]

Ultrastructural alterations. The parasites, at a density of 5×10^5 cells/mL, were cultured in their corresponding medium, containing the drugs at the IC_{25} concentration. After 96 h, the cultures were centrifuged at 400 *g* for 10 min, and the pellets washed in PBS and then fixed with 2% (v/v) *p*-formaldehyde-glutaraldehyde in 0.05M cacodylate buffer (pH 7.4) for 5 h at 4°C. Pellets were prepared for TEM (Zeiss model) following the technique of Luque.[24]

RESULTS

***In vitro* anti-*T. cruzi* and cytotoxicity evaluation**. The inhibitory effect of the new terpenoid Compounds 1–4 was measured at concentrations ranging from 1 to 100 μM on the *in vitro* growth of. *T. cruzi* epimastigotes.[30] The IC_{50} values registered after 72 h of exposure are shown in Table 1, including BZN as the reference drug. The trypanocidal activity of the derivatives was slightly higher (Compounds 1 and 2, IC_{50} 6.10 and 7.98 μM, respectively) or even slightly less (Compounds 3 and 4, IC_{50} 50.03 and 69.01 μM) with respect to that found for BZN (15.89 μM).

The cytotoxicity evaluation against mammalian cells by using Vero cells as the mammal-cell model (Table 1) showed that the four derivatives were much less toxic than BZN. On the other hand, the selectivity index calculated for the derivatives was about 20-fold (Compound 1) and 24-fold (Compound 2) higher than that of BZN. The SI values found for Compounds 3 and 4 were significantly lower than those found for Analogues 1 and 2.

Axenic amastigotes obtained following the technique described in the Materials and Methods section, were assayed to determine the IC_{50} against the four terpenoids, using BZN as the reference drug (Table 1). Compounds 1 and 2 proved the most effective with IC_{50} of 6.03 and 6.81 μM, respectively. When the SI was determined for Compound 2, it was 32-fold better than the reference drug, whereas Compound 1 was 23-fold better that the BZN again.

TABLE 1

In vitro activity, toxicity, and selectivity index found for the terpenoids derivatives on epimastigote and axenic-amastigote forms of *Trypanosoma cruzi**

Compound	Activity IC_{50} (mM)†		Toxicity on Vero cell IC_{50} (mM)‡	SI§	
	Epimastigote forms	Axenic amastigote forms		Epimastigote forms	Axenic amastigote forms
Benznidazole	15.89 ± 1.1	18.92 ± 1.1	13.60 ± 0.9	0.8	0.7
Compound 1	6.10 ± 0.2	6.03 ± 0.3	98.95 ± 6.2	16.2 (20)	16.4 (23)
Compound 2	7.98 ± 0.8	6.81 ± 0.1	154.66 ± 17.3	19.4 (24)	22.7 (32)
Compound 3	50.03 ± 6.6	30.44 ± 2.3	117.67 ± 21.8	2.4 (3)	3.9 (6)
Compound 4	69.01 ± 7.7	38.72 ± 3.6	163.14 ± 14.5	2.4 (3)	4.2 (6)

* Results are averages of three separate determinations.
† IC_{50} = the concentration required to give 50% inhibition, calculated by linear regression analysis from the Kc values at concentrations used (1, 10, 25, 50, and 100 μM).
‡ On Vero cells after 72 h of culture. IC_{50} = the concentration required to give 50% inhibition, calculated by linear regression analysis from the Kc values at concentrations used (1, 10, 25, 50 and 100 μM).
§ Selectivity index = IC_{50} Vero cells/IC_{50} epimastigote and axenic amastigote forms of the parasite. In brackets: number of times that compound SI exceeds the reference drug SI.

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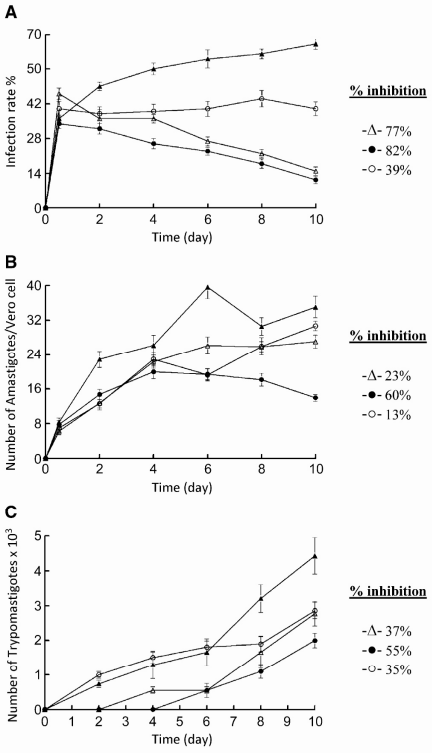

FIGURE 2. Effect of activity of terpenoid compounds on the infection rate and *T. cruzi* growth. (**A**) Rate of infection. (**B**) Mean number of amastigotes per infected Vero cell. (**C**) Number of trypomastigotes in the culture medium. Control (-▲-); benznidazole (BZN) (-○-); Compound **1** (-△-) and Compound **2** (-●-) (at IC_{25} conc.). The values are means of three separate experiments.

Compounds 3 and 4 did not reach values of SI $\geq$ 20-fold and therefore these compounds were not included in the subsequent studies.[31]

Because the metacyclic trypomastigote forms are responsible for the infection of the mammalian host, Compounds 1 and 2 were assayed against the metacyclic forms to evaluate the infection rate. When 1×10^4 Vero cells were incubated for 2 days and then infected with 1×10^5 metacyclic forms, obtained in the way described in the Materials and Methods section (control experiment; Figure 2A), the parasites invaded the cells and underwent the morphological transformation to amastigotes within 1 day after infection, following the parasite's normal life cycle. On Day 10, the rate of host-cell infection peaked. When Compounds 1 or 2 were added to the infected Vero cells with *T. cruzi* metacyclic forms (IC_{25} concentration), the infection rate significantly decreased with respect to the control, reaching 77% (Compound 1) and 82% (Compound 2) on Day 10 of the experiment. The infection-rate decline found using the compounds tested was substantially more pronounced than that measured for BZN (39%). Furthermore, another indication of the effectiveness of the infection-rate decrease was the average number of amastigotes per infected cell (Figure 2B) increased to 39.6 amastigotes/cell in the control experiment on Day 6, decreased to a value of 30.6 on Day 8, and increased again to 35.0 on Day 10. The break-up of Vero cells implies the transformation of amastigotes into trypomastigotes and release to spread the infection. Therefore, the variation of the trypomastigote number in the culture medium was also measured (Figure 2C) as a third way to test whether the infection rate was initially affected as a consequence of the exposure to the compounds. The control experiment afforded a trypomastigote number of 4.4×10^3 on Day 10, and reductions of 37% and 55% are found for Compounds 1 and 2, respectively. The reduction was higher for Compound 2 than that found for BZN (35%).

***In vivo* anti-*T. cruzi* evaluation.** The good results obtained *in vitro* with Compound 1 and 2 prompted us to study their *in vivo* activity in mice. Their impact on the two significant stages of Chagas disease was evaluated: the acute phase, considered until 60 days post-infection, and the chronic phase, from 60 days post-infection. It has been published that the intravenous doping route results in high mortality rates,[27] and therefore we used the intraperitoneal route, using a concentration of 5 mg/kg,[32] which did not result in any animal mortality. Female Swiss mice were inoculated intraperitoneally with 1×10^5 metacyclic trypomastigotes, and treatment began 7 days post-infection with the intraperitoneally route of 1 mg/kg/day of each compound for 5 days. Administration was performed using a saline solution. A group treated in the same manner with the vehicle (control) was included. During the study of the acute-phase activity, the level of parasitemia was determined every 2 days (Figure 3).

None of the animals treated with Compounds 1 and 2 died during the treatment, whereas the surviving percentage of the mice treated with BZN was 80%.

The data represented in Figure 3 show that the two compounds tested diminished the trypomastigote number on the day of maximum parasitic load, which was Day 25 post-infection, with respect to the control with untreated mice. On Day 40 post-infection a reduction of the parasitemia was found for the two compounds. From these data, the following order for *in vivo* activity could be established: Compound 1 $\approx$ Compound 2 > BZN.

Concerning the activity in the chronic phase, serological tests were performed 40 and 120 days post-infection (Table 2). Compounds 1 and 2 decreased antibody levels between Days 40 and 120, showing higher performance than did BNZ in this assay.

Studies on the action mechanism. To gain information on the possible mechanism of action of Compounds 1 and 2 on the parasite, we performed the following experiments:

Metabolite-excretion effect. For information concerning the effect of Compounds 1 and 2 on metabolite excretion, the 1H-NMR spectra were registered. As a control, a culture of parasites untreated was used (Figure 4A). In this control experiment, *T. cruzi* excreted acetate and succinate as the major metabolites and, in a lower percentage, L-alanine. These data agree with those reported previously.[33] When the

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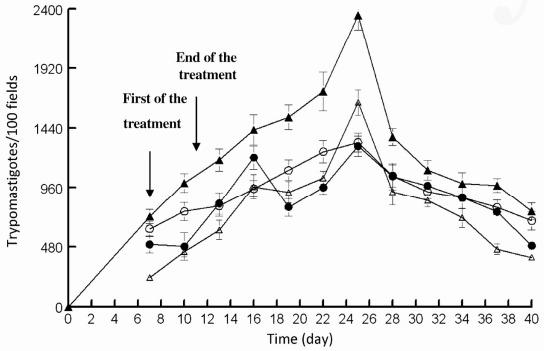

FIGURE 3. Parasitemia in the murine model of acute Chagas disease. Control (-▲-) and dose receiving 5 mg/kg of: Benznidazole (BZN) (-○-); Compound 1 (-△-) and Compound 2 (-●-).The values are means of three separate experiments.

TABLE 2

Differences in the level of anti-*Trypanosoma cruzi* antibodies between Days 40 and 120 post-infection for Compounds **1** and **2** and benznidazole (BZN), expressed in absorbance units (abs)

Compounds*	A†
Control (untreated)	0,206 ± 0.07
BZN	0,116 ± 0.03
Compound 1	−0.237 ± 0.01
Compound 2	−0.005 ± 0.00

*1 mg/kg/day, intraperitoneal route administered during 5 days (see Material and Methods).
†ΔA = absorbance at 490 nm, Day 120 p.i. absorbance at 490 nm, day 40 p.i.

trypanosomatids were treated with Compound 2, the excretion of some of these catabolites (mainly acetate) was clearly disturbed (Figure 4B) at the dosages assayed (IC_{25}). Similarly, succinate, L-alanine and ethanol levels rose in comparison with the control experiment.

Compound 1 provoked similar effects, although at a lesser magnitude (spectra not shown). The BZN did not trigger any alteration in the energy metabolism of the parasites (spectra not shown).

Ultrastructural alterations. The study of the ultrastructural alterations caused by Compounds 1 and 2 in the *T. cruzi* epimastigotes reflected notable changes in parasites, as reflected in (Figure 5B, C, and D), with respect to the control cultures (Figure 5A). Both compounds induced ultrastructural alterations. The most evident changes were the destruction of the treated parasites. Furthermore, there was evident intense vacuolization in a high number of parasites. Compound 2 triggered the dilatation of the kinetoplast of some parasites (Figure 5D).

DISCUSSION

In most studies on activity assays of new compounds against parasites, forms that develop in invertebrate host are used because they are easier to handle *in vitro*, but a preliminary test using extracellular epimastigote forms should always be complemented by a subsequent evaluation using

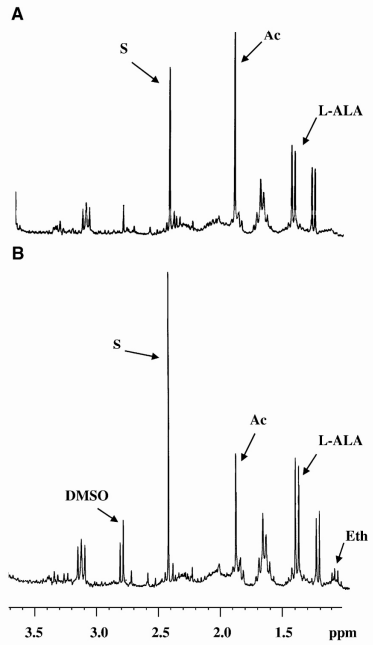

FIGURE 4. Nuclear magnetic resonance (1H-NMR) spectra epimastigote forms of *Trypanosoma cruzi* treated against terpenoids compounds (at a concentration of IC_{25}): (**A**) Control (untreated) and (**B**) Compound 2. Ala = L-alanine; Ac = acetate; S = succinate; Eth = ethanol, and DMSO = Dimethyl sulfoxide.

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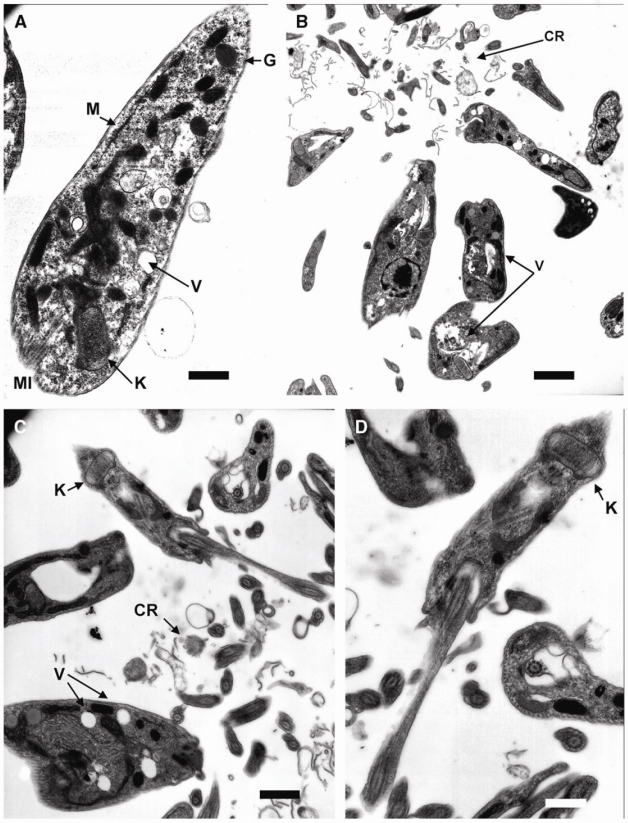

FIGURE 5. Ultrastructural alterations by transmission electron microscopy (TEM) in *Trypanosoma cruzi* treated with terpenoids compounds. (**A**) Control parasite of *T. cruzi* with structures as vacuoles (V) and mitochondrion (M), kinetoplast (K), glycosomes (G), and microtubules (MI) (Bar: 583 μm). (**B**) Epimastigotes of *T. cruzi* treated with Compound **1** with cellular debris from dead parasites (CR) and vacuoles (V) (Bar: 1.59 μm). (**C** and **D**) Epimastigotes of *T. cruzi* treated with Compound 2 with cellular rest (CR), vacuoles (V), and swelling kinetoplast (K) (Bar: 1.00 μm and Bar: 583 μm, respectively).

intracellular forms (amastigotes in vertebrate host cells) for a better understanding of the activity results found. For this reason, we studied the activity of axenic amastigote forms.

The data on the axenic amastigotes match those found for the extracellular forms, where Compounds 1 and 2 were the most effective.

The trypanocidal effect of terpenoid compounds against epimastigote and amastigote forms, has been previously shown. Cassane diterpenes isolated from leaves of *Myrospermum frutescens*, have anti-*T. cruzi* activity against these forms.[34]

According to the activity criteria for the development of new drugs,[31] only the compounds that SI $\geq$ 20-fold were included in the following studies.

The assays showed that Compounds 1 and 2 were effective enough to undergo the next stage. To study the drug's effect on the infective parasite forms, we performed the metacyclic assay. The IC_{25} of each product was used as the test dosage. This concentration was chosen for being harmful but not totally lethal,[35] revealing that Compound 2 was the most effective.

Diterpenes isolated from *Aristolochia cymbifera* have been shown to be selective against trypomastigotes.[36]

The *in vivo* assays showed that the differences in the level of anti-*T. cruzi* antibodies are consistent with the parasitemia findings.

As far as is known, no trypanosomatid studied to date is capable of completely degrading glucose to CO_2 under

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aerobic conditions, and thus these parasites excrete into the medium a great part of their carbon skeleton as fermented metabolites, which differ depending on the species considered.[37,38] *Trypanosoma cruzi* consumes glucose at a high rate, thereby acidifying the culture medium caused by incomplete oxidation to acids. The ^{1}H-NMR spectra enables us to determine the fermented metabolites excreted by the trypanosomatid during *in vitro* culture. One of the major metabolites excreted by *T. cruzi* is succinate, the main role of which is probably to maintain the glycosomal redox balance, by providing two glycosomal oxidoreductase enzymes that allow reoxidation of NADH, produced by glyceraldehydes-3-phosphate dehydrogenase in the glycolytic pathway. Succinic fermentation offers the significant advantage of requiring only half of the phosphoenolpyruvate produced to maintain the NAD+/NADH balance. The remaining phosphoenolpyruvate is converted into acetate, L-lactate, L-alanine, and/or ethanol, depending on the species considered. The role of the acetate is probably to maintain the glycosomal redox balance. In the case of Compound 2 the inhibition of acetate excretion explains the observed increase in succinate, L-alanine, and ethanol production, probably because these compounds act over any level in the energy metabolism, still unknown.

In conclusion, our results show that four new terpenoid derivative compounds were active *in vitro* against both extra- and intracellular forms of *T. cruzi* (in the order 2 > 1 > 3 > 4 > BZN). These compounds are not toxic for the host cells and are effective at concentrations lower than the reference drug used in this study. The *in vitro* growth rate of *T. cruzi* was reduced, its capacity to infect cells was negatively affected, and the multiplication of the amastigotes and subsequent transformation into trypomastigotes was greatly lowered. Moreover, a wide range of ultrastructural alterations in epimastigote forms of *T. cruzi* treated with these new terpenoid compounds were found. These alterations mainly at the mitochondria level could explain the metabolic changes in the productions of succinate and acetate, which may be caused by the disturbance of the enzymes involved in sugar metabolism within the mitochondria. The *in vivo* studies revealed results that were consistent with those observed *in vitro*. On the one hand, during the treatment of mice with the compounds (Compounds 1 and 2), no signs of toxicity were observed. On the other hand, the parasitic load was significantly decreased in comparison with the reference drug. The effects of these products were also demonstrated with the anti-*T. cruzi* antibody level modification during the chronic stage. So far nothing is known about the structure-activity relationship for these substances, and the results described here do not allow it. Additional research is needed for the activity of other AU10 structurally related molecules. These results support further research of terpenoid compounds as potential agents against Chagas disease. The synthesis of new derivatives and preclinical studies, such as doses, schedule, strains, and toxicological studies, is currently in progress.

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Article Type: Regular Paper

Keywords: Nickel complexes; H-bonds; Triazolopyrimidines; Leishmania infantum; Leishmania braziliensis; Biological activity.

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Abstract: Studies on the anti-proliferative activity in vitro of seven ternary nickel (II) complexes with a triazolopyrimidine derivative and different aliphatic or aromatic amines as auxiliary ligands against promastigote and amastigote forms of Leishmania infantum and Leishmania braziliensis have been carried out. These compounds are not toxic for the host cells and two of them are effective at lower concentrations than the reference drug used in the present study (Glucantime). In general, the in vitro growth rate of Leishmania spp. was reduced, its capacity to infect cells was negatively affected and the multiplication of the amastigotes decreased. Ultrastructural analysis and metabolism excretion studies were executed in order to propose a possible mechanism for the action of the assayed compounds. Our results show that the potential mechanism is at the level of organelles membranes, either by direct action on the microtubules or by their disorganization, leading to vacuolization, degradation and ultimately cell death.

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In vitro anti-leishmania evaluation of nickel complexes with a triazolopyrimidine derivative against *Leishmania infantum* and *Leishmania braziliensis*

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ABSTRACT

Studies on the anti-proliferative activity *in vitro* of seven ternary nickel (II) complexes with a triazolopyrimidine derivative and different aliphatic or aromatic amines as auxiliary ligands against promastigote and amastigote forms of *Leishmania infantum* and *Leishmania braziliensis* have been carried out. These compounds are not toxic for the host cells and two of them are effective at lower concentrations than the reference drug used in the present study (Glucantime). In general, the *in vitro* growth rate of *Leishmania* spp. was reduced, its capacity to infect cells was negatively affected and the multiplication of the amastigotes decreased. Ultrastructural analysis and metabolism excretion studies were executed in order to propose a possible mechanism for the action of the assayed compounds. Our results show that the potential mechanism is at the level of organelles membranes, either by direct action on the microtubules or by their disorganization, leading to vacuolization, degradation and ultimately cell death.

Keywords: nickel complexes, H-bonds, triazolopyrimidines, *Leishmania infantum*, *Leishmania braziliensis*, and biological activity.

1. Introduction

Leishmaniasis is one of the so-called neglected diseases, considered by WHO as one of the seven primary sicknesses that affect to all continents and usually located in tropical and subtropical regions, including 22 countries in the New World and 66 in the Old World [1]. The etiologic agents are different species of protozoa of the genus *Leishmania*, transmitted by dipteral insects that belong to the genera *Phlebotomus* in the Old World and *Lutzomyia* in the New World.

Pentavalent antimonial compounds, sodium stibogluconate (Pentostam) and meglumine antimoniate (Glucantime), have been recommended as the first line drugs in the treatment of leishmaniasis for over 50 years. However, these compounds, that selectively inhibit leishmania enzymes required for glycolytic and fatty acid oxidation [2], cause several toxic side effects including anorexia, vomiting, peripheral polyneuropathy, and allergic dermopathy [3-4] and drug resistance [5]. Thus, there is an urgent need for new and more efficient therapies to fight leishmaniasis and minimize its impact in society.

Previous studies have indicated that newly synthesised triazolopyrimidine metal complexes are promising chemotherapeutic drugs in the treatment of diseases caused by members of Trypanosomatidae [6-10]. Following this research line, we have started to explore the coordination possibilities and biological applications of another triazolopyrimidine family: 1,2,3-triazolo[4,5-*d*]pyrimidines which possess three contiguous N atoms in the imidazole ring and may also be regarded as purine mimetics, replacing the external C atom of the imidazole ring with a N atom (Scheme 1). Previously reported studies describe some of their potential biological applications [11-14].

In view of the above findings and, as a continuation of our effort to identify new candidates that are more potent, selective and less toxic antitrypanosomals, we report in this article the anti-proliferative *in vitro* activity of seven nickel complexes bearing the anionic form of 4,6-dimethyl-1,2,3-triazolo[4,5-*d*]pyrimidine-5,7-dione against promastigote and

amastigote forms of *L. infantum* and *L. braziliensis*. Furthermore, unspecific mammal cytotoxicity was evaluated *in vitro*, and the non-toxic derivatives were thoroughly studied in order to elucidate their possible mechanisms of action. For this purpose, we have included ^{1}H-NMR studies about the nature and percentage of the excreted metabolites, in order to gather information about the inhibitory effect of our compounds over the glycolytic pathway which is the primary energy source for the parasite, and we have analysed the effect of these compounds on the ultrastructure of *Leishmania* spp. by transmission electronic microscopy studies (TEM).

2. Materials and methods

2.1. Synthesis of the compounds

The assayed compounds are seven nickel(II) complexes bearing the anionic form of 4,6-dimethyl-1,2,3-triazolo[4,5-*d*]pyrimidin-5,7-dione (dmax) and different aliphatic amines [ethylenediamine (en), 1,3-diaminopropane (dap), bis(3-aminopropyl)-amine (bapa)] or aromatic amines [1,10-phenanthroline (phen), 2,2´-bipyridyl (bpy), 2,2´-dipyridylamine (dpyamin)] as auxiliary ligands. The synthesis of the ligand 4,6-dimethyl-1,2,3-triazolo[4,5-*d*]pyrimidine-5,7-dione (**Hdmax**) and its nickel salt (**Nidmax**) were carried out according to previously published procedures [15,16]. Other reagents were obtained from commercial sources and used without further purification.

Detailed synthesis and structural characterization of $[Ni(dmax)_2(en)_2]\cdot 3H_2O$ (**Nidmax-en**), $Ni(dmax)_2(dap)_2(H_2O)_{1.5}$ (**Nidmax-dap**) and $Ni(dmax)_2(bapa)H_2O$ (**Nidmax-bapa**) [17] and $[Ni(dmax)_2(phen)_2]$ (**Nidmax-phen**), $[Ni(phen)_3](dmax)_2\cdot 7H_2O$ (**Nidmax-3phen**), $[Ni(dmax)_2(bpy)_2](H_2O)_{0.5}$ (**Nidmax-bpy**) and $Ni(dmax)_2(dpyamin)_2(H_2O)$ (**Nidmax-dpyamin**) have been recently reported by our group [14]. As it is shown in Scheme 2, the three metal complexes including aliphatic amines in their structures were obtained by mixing three aqueous solutions containing $Ni(NO_3)_2\cdot 6H_2O$, the triazolopyrimidine ligand and the

corresponding aliphatic amine in 1:2:2 molar ratio. On the other hand, those including aromatic amines were obtained by mixing two organic solutions containing $Ni(H_2O)_6(dmax)_2$ (**Nidmax**) and the corresponding aromatic amine in 1:2 (**Nidmax-phen** and **Nidmax-bpy**) or 1:3 (**Nidmax-3phen** and **Nidmax-dpyamin**) molar ratio.

2.2. Parasite Strain, Culture

L. infantum (MCAN/ES/2001/UCM-10) and *L. braziliensis* (MHOM/BR /1975/M2904) were cultured *in vitro* in medium trypanosomes liquid (MTL) plus 10% inactivated foetal bovine serum (IFBS) and were kept in an air atmosphere at 28 °C in Roux flasks (Corning, USA) with a surface area of 75 cm^2, according to the methodology described by González *et al.* [18].

2.3. Extracellular forms. Promastigotes assay

The synthesized compounds (Scheme 2) were dissolved in the culture medium with a final DMSO concentration not exceeding 0.1%, this percentage of DMSO was previously assayed as nontoxic and without inhibitory effects on the parasite growth according to Magán *et al*. [7]. The dosages of the compounds used in this study were 100, 50, 25, 10 and l μM. The effect of each compound against promastigote forms, at different concentrations, was evaluated at 72h using a Neubauer hemocytometric chamber. The leishmanicidal effect on promastigotes is expressed as IC_{50} values, i.e. the concentration required to give 50% inhibition, calculated by linear-regression analysis from the *Kc* values at the concentrations employed.

2.4. Cell Culture and Cytotoxicity Tests

J774.2 macrophages (ECACC number 91051511) were originally obtained from a tumour in a female BALB/c rat in 1968. The cytotoxicity tests on macrophages were performed

according to a previously published methodology [18]. After 72 h of treatment, the cell viability was determined by flow cytometry. Thus, 100 μL/well of propidium iodide solution (100 μg/mL) was added and incubated for 10 min at 28 ºC in darkness. Afterwards, 100 μL/well of fluorescein diacetate (100 ng/mL) was added and incubated under the same aforementioned conditions. Finally, the cells were recovered by centrifugation at 400 *g* for 10 min and the precipitate was washed with PBS. Flow cytometric analyses were performed with a FACS Vantage™ flow cytometer (Becton Dickinson). The percentage viability was calculated in comparison to the control culture. The IC_{50} was calculated using linear-regression analysis from the *Kc* values at the employed concentrations.

2.5. Intracellular forms: Amastigote-macrophage assay

Two experimental procedures were designed for this study. In all cases, J774.2 macrophages were grown in minimum essential medium (MEM) plus Glutamine (2 mM) and 20% IFBS in a humidified atmosphere of 95% air and 5% CO_2 at 37ºC.

2.5.1. Experimental model no. 1 ([Mϕ + Leishmania spp.] + drug)

Cells were seeded at a density of 1×10^4 cells/well in 24-well microplates (Nunc) with rounded coverslips on the bottom and cultured for 48 hours. Afterwards, adherent macrophage cells were infected with promastigotes in stationary growth phase at a 10:1 ratio and kept for 24 h at 37 ºC in 5% CO_2. Non phagocytosed parasites were removed by washing, and afterwards the infected cultures were incubated with the compounds (1, 10, 25, 50 and 100 µM concentrations) and cultured for 72 h. The activity of the compounds, expressed as amastigote IC_{50} values, was determined from the percentage of the amastigote number reduction in treated and untreated cultures in methanol-fixed and Giemsa-stained preparations. Values are the average of four separate determinations [18].

2.5.2. Experimental model no. 2 (Mϕ + Leishmania spp. + drug)

Cells were seeded at a density of 1×10^4 cells/well in 24-well microplates (Nunc) with rounded coverslips on the bottom and cultured for 48 hours. Afterwards, the cells were infected *in vitro* with promastigote forms of *L. infantum* and *L. brazilensis* at a ratio of 10:1. The drugs (IC_{25} concentrations) were added at the same time and the trays were incubated for 12 h at 37 °C in 5% CO_2. The non-phagocytosed parasites and drugs were removed by washing, and then the infected cultures were grown within 10 days in fresh medium. Fresh culture medium was added every 48 h. The drug activity was determined on the basis of both the percentage of infected cells and the number of amastigotes per infected cell in treated and untreated cultures in methanol-fixed and Giemsa-stained preparations. The percentage of infected cells and the mean number of amastigotes per infected cell were determined by analysing more than 200 host cells distributed in randomly chosen microscopic fields. Values are the average of four separate determinations.

2.6. Metabolite Excretion

Cultures of *L. infantum* and *L. braziliensis* promastigote forms (initial concentration 5×10^5 cells/mL) were incubated with the assayed compounds at IC_{25} concentrations (except for control cultures) for 96 h at 28 °C. Afterwards, the cells were centrifuged at 400 *g* for 10 min. The supernatants were collected to determine the excreted metabolites using ^{1}H-NMR and chemical shifts were expressed in parts per million (ppm), using sodium 2,2´-dimethyl-2-silapentane-5-sulfonate as the reference signal. The chemical shifts of the signals used to identify the respective metabolites were consistent with those described by Fernández-Becerra *et al*. [19].

2.7. Ultrastructural alterations

The parasites were cultured at a density of 5×10^5 cells/mL in their corresponding medium, each of which contained drugs at the IC_{25} concentration. After 96 h, those cultures were centrifuged at 400 *g* for 10 min, and the pellets were washed in PBS and then mixed with 2% (v/v) *p*-formaldehyde-glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4) for 4 h at 4 ºC. After that, the pellets were prepared for TEM following a previously described procedure [19].

3. Results and Discussion

3.1. Structures of the assayed compounds

For **Nidmax-en**, **Nidmax-phen**, **Nidmax-3phen** and **Nidmax-bpy**, suitable single-crystals were isolated and used for the X-ray determination of their crystal structures. Except for **Nidmax-3phen**, where only the phenanthroline ligands are directly bonded to the nickel ion and the two triazolopyrimidine anions (dmax) act as non-coordinated counteranions, both ligands (dmax and the amine) are directly bonded to the metal centre, with dmax linked through the less-hindered triazole nitrogen atom (N2) and the auxiliary ligand, as usual, through their two nitrogen atoms forming five-membered chelate rings (Scheme 2).

As it has been previously reported for **Nidmax-en** [17], the presence of potential acceptor (in triazolopyrimidine ligand) and donor sites (in auxiliary ligand and interstitial water molecules) generates a hydrogen-bonded network that increases the dimensionality form 0D (monomers) to 2D (layers).

Now, a more thorough and detailed analysis of our previously published crystal structure of **Nidmax-3phen** [14] reveals that the presence of a large amount of interstitial water molecules and the well-know capacity of dmax to establish H-bonds promotes the formation of a very extensive H-bond network. Table 1 lists hydrogen bonds parameters for **Nidmax-3phen**. The X-ray diffraction measurements were taken at room temperature, for this reason

O7W and O8W have partial occupancy and some of the hydrogen atoms have not been accurately located in the ΔF maps. Nevertheless, we can describe the presence of a water motif that is repeated throughout the crystal. Considering only the hydrogen bonds between water molecules, they are grouped in centrosymmetric, finite and well-defined clusters containing 16 molecules each, and built by one chair conformation central R_6 ring, with two R_5 rings, one at each side, and two side chains C_2. These finite water clusters establish new H-bonds with the triazolopyrimidine derivatives (dmax) generating a two-dimensional wavy superstructure (Fig. 1). It is worthy to note that the only "glue" that builds these supramolecular structures is the hydrogen bond, because there are not pi-pi interactions between the dmax counteranions that may strengthen this 2D structure.

Finally, more unspecific interactions between aromatic hydrogens of phen entities and water molecules and/or some of the acceptor groups of the triazolopyrimidine ligand generate the 3D packing of the solid, in which we can find channels (along the a crystallographic axis) where the interstitial water molecules are hosted (Fig. 2).

3.2. Unspecific Cytotoxicity

The inhibitory effect of the seven ternary nickel complexes, the free triazolopyrimidine ligand and its nickel salt on the *in vitro* growth of *L. infantum* and *L. braziliensis* promastigote and amastigote forms, has been determined. IC_{50} values obtained after 72 h of exposure are shown in Table 2 and Table 3, including glucantime as reference drug.

On promastigote forms, the IC_{50} of two of the assayed compounds (**Nidmax-bpy** and **Nidmax-dpyamin)** were slightly lower or similar to those found for glucantime for both species of *Leishmania* (IC_{50} values are 18.0 and 25.6 μM for glucantime, 16.3 and 14.9 μM for **Nidmax-bpy** and 20.0 and 20.5 μM for **Nidmax-dpyamin** on *L. infantum* and *L. braziliensis* respectively), while the rest of the compounds also presented a significant inhibitory effect on *Leishmania* spp. growth, although with IC_{50} values higher than the values of the reference drug. On the other hand, five (*L. infantum*) and three (*L. braziliensis*) of the

ternary nickel complexes, besides Hdmax, displayed IC_{50} values lower than glucantime on the *in vitro* growth of the amastigote forms.

Cytotoxicity evaluation against mammalian cells, using macrophages J774.2 as cell model (Table 2 and Table 3), showed that the nickel complexes containing both the triazolopyrimidine derivative and the amine as auxiliary ligand are much less toxic than glucantime or even the free triazolopyrimidine (**Hdmax**) and its nickel salt (**Nidmax**) for both species of *Leishmania*. Regarding the co-ligands employed in this study (see supplementary information), five of them (en, dap, bapa, bpy and dpyamin) are non-active against promastigote and amastigote forms of both species of *Leishmania* and very toxic on the host cells. On the other hand, phenanthroline shows a great antileismania activity but due to its high cytotoxicity on macrophages it cannot be considered as a real candidate for subsequent studies.

The selectivity index (SI) is an indicator of a compound's activity, with higher values meaning greater selectivity of the drug. All these values have been referred to glucantime (see Table 2 and Table 3, values in parentheses). On promastigote forms of both species of *Leishmania*, the higher SI values were found for **Nidmax-bpy** and **Nidmax-dpyamin** (17 and 26 (Table 2) in *L. infantum* and 24 and 33 (Table 3) in *L. braziliensis*). Moreover, **Nidmax-bapa** and **Nidmax-3phen** (both species) and **Nidmax-en** and **Nidmax-phen** (*L. braziliensis*) have also significantly high SI values (12-7 fold higher than glucantime).

On amastigote forms, the best selectivity indexes ($\geq$ 20) were obtained for **Nidmax-dpyamin** (both species) and **Nidmax-phen** and **Nidmax-bpy** against *L. infantum*, followed by **Nidmax-dap** and **Nidmax-bapa** (*L. infantum*) with SI = 14 (Table 2) and by **Nidmax-en**, **Nidmax-bapa**, **Nidmax-bpy** and **Nidmax-3phen** (*L. braziliensis*) with SI $\geq$ 11 (Table 3). According to these results, the compounds selected to carry on the subsequent studies were those with a SI $\geq$ 10 in amastigote forms.

3.3. Cell Assay

In most studies on the activity of new compounds against parasites, forms developed in vectors are used (promastigotes in the case of *Leishmania* spp.), for the ease of working with these forms *in vitro*. However, in this study, we have included the effect of these compounds on the forms that are developed in the host (amastigotes) as the final goal is to determine the effects in the definitive host [18]. For this task, as well as for the studies on the mechanism of action, we have selected the compounds that had the greatest inhibitory effect on the *in vitro* growth of the parasites and that at the same time had less toxic effect on macrophage cells (see criteria described above), using the IC_{25} of each compound as the tested dosage.

Adherent macrophage J.774.2 cells (1×10^4 macrophage cells) were cultured for 2 days, afterwards fresh medium was added with 1×10^5 metacyclic promastigote forms of *L. infantum* or *L. braziliensis* and the selected drugs at a IC_{25} concentration. After 12 hours (when the parasites have invaded the cells and become morphologically into amastigotes), the non-phagocytised parasites were removed and the culture was kept in fresh medium for 10 days.

In all the cases, the infection rate significantly decreased after 240 h (10 days) with respect to the control (Table 4) following the trend: **Nidmax-bpy > Nidmax-dpyamin > Nidmax-bapa > Nidmax-3phen** for *L. infantum* with 84, 75, 66 and 63% of infestation-inhibition capacity respectively and **Nidmax-bpy > Nidmax-dpyamin > Nidmax-bapa > Nidmax-3phen > Nidmax-phen** for *L. braziliensis* with 80, 75, 70, 67 and 61% of infestation-inhibition capacity respectively. All these values are remarkably higher than the inhibition of glucantime (56 and 48% for *L. infantum* and *L. braziliensis*, respectively). In the same way, all the complexes (except **Nidmax-phen** on *L. braziliensis*) inhibited *in vitro* *Leishmania* spp. amastigote replication in macrophage cells, following a similar trend to the above mentioned for the inhibition of the infection rate and being more effective than the reference drug after 10 days (Table 4).

3.4. Mechanism of action studies

Finally, in order to confirm or exclude some possible mechanisms of action, studies of the effect on the energy metabolism and on alterations of the parasite structure were preformed.

3.4.1. Metabolite Excretion Effect

As far as it is known to date, none of the studied trypanosomatids can completely degrade glucose to CO_2 under aerobic conditions, excreting into the medium a great part of their carbon skeleton as fermented metabolites, whose nature differs depending on the considered species [20]. *Leishmanias* spp., as well as other trypanosomatids, consumes glucose at a high rate, thereby acidifying the culture medium due to incomplete oxidation to acids. ^{1}H-NMR spectra enabled us to determine the fermented metabolites excreted by the leishmanias during their *in vitro* culture [20,21].

Fig. 3A and 3B show the ^{1}H-NMR spectra signals given by cell-free medium 4 days after inoculation with *L. infantum* and *L. braziliensis* respectively. Additional peaks, corresponding to the major metabolites produced and excreted during growth, were detected when these spectra were compared to that obtained with just with fresh medium (spectrum not shown). *L. infantum* and *L. braziliensis* excrete acetate and succinate as major metabolites and, in the case of *L. braziliensis* , a small amount of ethanol also appears. These data agree well with those reported by other authors [20,21]. The analysis of the ^{1}H-NMR spectra showed that only **Nidmax-bapa** significantly altered the metabolites excreted by *L. infantum* and *L. braziliensis* at IC_{25} doses (Fig. 3C and 3D respectively). The excretion of acetate is clearly decreased (45% and 26% for *L. infantum* and *L. braziliensis*, respectively), while production of succinate increases (45% and 110% for *L. infantum* and *L. braziliensis*, respectively) compared with the control (Fig. 3A and 3B respectively). The role of acetate is probably to maintain the glycosomal redox balance. The inhibition of acetate excretion explains the observed increase in succinate and L-lactate production, probably due to the action of this

compound at the pyruvate dehydrogenase complex level or to disturbance of the mitochondria and, consequently, of the oxidative phosphorylation. The rest of tested compounds and glucantime did not show any remarkable effect over the excretion of metabolites (spectra not shown). Thus, we cannot establish any clear relation between the observed antileishmania activities of these nickel complexes and the alterations of the parasite's glycolytic pathway.

3.4.2. Ultrastructural Alteration Effect

In contrast to the results found on the study of the metabolism of the parasite, significant morphological alterations have been found by electron microscopy (TEM) in *L. infantum* and *L. braziliensis* promastigotes treated with the most active nickel compounds. In general, an intense induction of vacuolization must be highlighted, although there are others specific disruptions produced depending on the parasite and the tested drug.

In Fig. 4.1, the ultrastructure of *L. infantum* without treatment (control) is shown, with the typical components of these cells, nucleus (N), mitochondria (M), vacuoles (V), flagellum (F) and microtubules (MI). Fig. 4.2-4.5 display the promastigotes of *L. infantum* treated with the different nickel complexes. It can be noted that the cells are really disrupted and electrodense (arrow) and there are lots of cellular remains (CR) for **Nidmax-3phen**, while there are many vacuoles (V) and distorted shapes for **Nidmax-bapa**. When *L. infantum* is treated with **Nidmax-bpy**, an increase in the number of vacuoles (V) and a clear disruption of the cytoskeleton (arrow) are observed, while **Nidmax-dpyamin** brings on substantial increase in the number of vacuoles (V) as well as fragmentation of the mitochondria (M). Fig. 4.6 shows *L. infantum* treated with glucantime, where a high amount of cellular remains (CR), cellular distortion (arrow), vacuoles (V) and vacuoles with electrodense material (E) were found.

The morphologic alterations induced on the promastigotes of *L. braziliensis* were very similar to those produced on *L. infantum* (Fig. 5). Non-treated promastigote forms have a characteristic morphology with the classical components mitochondria (M), vacuoles (V),

glycosomes (G) and flagellum (F). The treatment with **Nidmax-bpy** (Figure 5.2) shows many cellular remains (CR) and disrupted and vacuolated (arrow) parasites while an intense vacuolization (V) was noticed when the parasites were incubated with **Nidmax-bapa** (Fig. 5.5). A notable agglutinating effect was observed for **Nidmax-dpyamin** (Fig. 5.3), that also can produce a massive destruction of parasites generating many cellular remains (CR) (Fig. 5.4). Finally, Fig. 5.6 shows *L. braziliensis* treated with glucantime, where very electrodense shapes (arrow) and distortion (D) were observed.

4. Concluding remarks

We have presented the biological properties of seven novel ternary nickel complexes containing the anionic form of 4,6-dimethyl-1,2,3-triazolo[4,5-*d*]pyrimidin-5,7-dione (dmax). From a structural point of view, the tested compounds are molecular entities that, in some cases, build 2D networks through well defined H-bonds. Our biological studies showed that five of the tested nickel complexes were very active *in vitro* against both extra and intracellular forms (in the order **Nidmax-bpy** > **Nidmax-dpyamin** > **Nidmax-bapa** > **Nidmax-3phen** for *L. infantum* and **Nidmax-bpy** > **Nidmax-dpyamin** > **Nidmax-bapa** > **Nidmax-3phen** > **Nidmax-phen** for *L. braziliensis*) and they are not toxic in the host cells. Consequently, the tested compounds significantly reduced *in vitro* Leishmanias ssp. growing rate as well as the capacity of the parasite to infect cells and the multiplication of the amastigotes.

Moreover, a wide range of ultrastructural alterations in promatigote forms of *L. infantum* and *L. braziliensis* treated with these triazolopyrimidine complexes were found, suggesting that one of the possible mechanisms of action is at the level of the membranes of organelles, either by direct action on the microtubules or by their disorganization, all of this leading to a vacuolization, degradation, and ultimately cell death.

Abbreviations

Hdmax	4,6-dimethyl-1,2,3-triazolo[4,5-*d*]pyrimidin-5,7-dione
en	ethylenediamine
dap	1,3-diaminopropane
bapa	bis(3-aminopropyl)-amine
phen	1,10-phenanthroline
bpy	2,2´-bipyridyl
dpyamin	2,2´-dipyridylamine
SI	selectivity index

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Table 1. Hydrogen-bond geometry (A° , º) for **Nidmax-3phen**.

D-H...A	d(D-H)	d(H...A)	d(D...A)	<(DHA)
O(1W)-H(11W) ··· O(5A) #1	0.840(10)	2.041(12)	2.873(3)	171(3)
O(1W)-H(12W) ··· N(2B)	0.833(10)	2.015(11)	2.841(3)	172(3)
O(2W)-H(21W) ··· N(2A)	0.843(10)	1.981(11)	2.820(3)	174(4)
O(2W)-H(22W) ··· O(1W)	0.832(10)	1.957(11)	2.784(3)	173(4)
O(3W)-H(31W) ··· O(6W)	0.845(10)	1.99(3)	2.745(5)	148(5)
O(3W)-H(32W) ··· O(2W)	0.838(10)	1.933(16)	2.754(4)	166(5)
O(4W) ··· N(3B)	—	—	2.820(4)	—
O(4W) ··· O(7A)#2	—	—	2.886(5)	—
O(5W) ··· N(1A)	—	—	2.828(4)	—
O(5W) ··· O(3W)	—	—	2.830(5)	—
O(6W) ··· O(7W)	—	—	2.773(7)	—
O(7W) ··· O(4W)#3	—	—	2.811(6)	—
O(7W) ··· O(8W)	—	—	2.619(8)	—
O(8W) ··· O(5W)	—	—	2.698(7)	—
O(8W) ··· O(4W)#4	—	—	2.828(6)	—

Symmetry transformations used to generate equivalent atoms
#1 -x,-y,-z+1 #2 x+1,y,z #3 -x+1,-y+1,-z+2 #4 x-1,y,z

Table 2. *In vitro* activity, toxicity, and selectivity index found for the reference drug and the 4,6-dimethyl-1,2,3-triazolo[4,5-d]pyrimidin-5,7-dione derivatives on promastigote and amastigote forms of *L. infantum*.

Compounds	[IC_{50} μM]		Toxicity [IC_{50} μM] on J774.2 ϕ[a]	SI[b]	
	Promatigote	Amastigote		Promatigote	Amastigote
Glucatim®	18.0	33.9	15.2	0.8	0.5
Hdmax	24.1	24.1	26.5	1.1 (1)	1.1 (2)
Nidmax	42.7	65.0	71.2	1.7 (2)	1.1 (2)
Nidmax-en	29.6	30.2	112.4	3.8 (5)	3.7 (5)
Nidmax-dap	73.0	27.4	185.9	2.5 (3)	6.8 (14)
Nidmax-bapa	40.1	35.5	248.0	6.2 (8)	7.0 (14)
Nidmax-bpy	16.3	11.9	216.5	13.3(17)	18.2 (36)
Nidmax-phen	46.7	8.0	201.2	4.3 (5)	25.2 (50)
Nidmax-3phen	30.8	53.4	173.2	5.6 (7)	3.2 (6)
Nidmax-dpyamin	20.0	32.7	410.6	20.5 (26)	12.6 (25)

IC_{50} = the concentration required to give 50% inhibition, calculated by linear regression analysis from the *K*c values at concentrations employed (1, 10, 25, 50 and 100 μM).

[a] On J774.2 Macrophages cells after 72 h of culture.

[b] Selectivity index = IC_{50} Macrophages/IC_{50} promastigote and amastigote forms.

Note: Average of three separate determinations.

In brackets: number of times that compound SI exceeds the reference drug SI.

Table 3. *In vitro* activity, toxicity, and selectivity index found for the reference drug and the 4,6-dimethyl-1,2,3-triazolo[4,5-d]pyrimidin-5,7-dione derivatives on promastigote and amastigote forms of *L. braziliensis*.

Compounds	[IC_{50} µM]		Toxicity [IC_{50} µM] on J774.2 ϕ[a]	SI[b]	
	Promatigote	Amastigote		Promatigote	Amastigote
Glucatim®	25.6	46.1	15.2	0.6	0.3
Hdmax	31.4	38.8	26.5	0.8 (1)	0.7 (2)
Nidmax	38.0	48.4	71.2	1.9 (3)	1.5 (5)
Nidmax-en	25.4	31.1	112.4	4.4 (7)	3.6 (12)
Nidmax-dap	65.6	76.8	185.9	2.8 (5)	2.4 (8)
Nidmax-bapa	36.1	69.0	248.0	6.9 (12)	3.6 (12)
Nidmax-bpy	14.9	64.2	216.5	14.5 (24)	3.4 (11)
Nidmax-phen	38.5	81.7	201.2	5.2 (9)	2.7 (9)
Nidmax-3phen	32.0	37.8	173.2	5.4 (9)	4.6 (15)
Nidmax-dpyamin	20.5	25.8	410.6	20.0 (33)	15.9 (53)

IC_{50} = the concentration required to give 50% inhibition, calculated by linear regression analysis from the *K*c values at concentrations employed (1, 10, 25, 50 and 100 µM).

[a] On J774.2 Macrophages cells after 72 h of culture.

[b] Selectivity index = IC_{50} Macrophages/IC_{50} promastigote and amastigote forms.

Note: Average of three separate determinations.

In brackets: number of times that compound SI exceeds the reference drug SI.

Table 4. Effects of the selected nickel complexes (IC_{25} dosage) on the infection rate of J774.A macrophages and on the average number of amastigotes per infected macrophage during 10 days of culture.

Leishmania infantum

Compound	(%) Mϕs[a]							Compound	IP/C[b]						
	12 h	24 h	48 h	96 h	144 h	192 h	240 h		12 h	24 h	48 h	96 h	144 h	192 h	240 h
Control	20	52	50	46	48	53	64	Control	3±0.2	9±1.0	29±0.7	43±2.2	36±0.9	54±4.5	69±6.1
Glucantime	16	37	38	26	24	30	28	Glucantime	4±0.3	7±0.5	28±0.9	22±3.3	21±2.2	30±4.3	56±6.3
Nidmax-bapa	13	36	39	28	26	24	22	Nidmax-bapa	7±0.6	9±0.4	30±0.6	39±3.9	17±2.0	23±2.9	44±7.2
Nidmax-bpy	16	56	42	30	22	14	10	Nidmax-bpy	2±0.1	16±0.6	16±0.5	20±1.7	25±3.8	22±1.7	20±3.4
Nidmax-3phen	12	40	32	28	22	27	24	Nidmax-3phen	6±0.2	7±0.2	14±0.6	51±3.7	17±1.3	27±2.3	38±1.0
Nidmax-dpyamin	17	48	44	28	18	20	16	Nidmax-dpyamin	8±0.2	11±0.3	14±0.8	21±2.0	30±1.9	19±2.6	24±2.3

Leishmania brazilensis

Compound	(%) Mϕs[a]							Compound	IP/C[b]						
	12 h	24 h	48 h	96 h	144 h	192 h	240 h		12 h	24 h	48 h	96 h	144 h	192 h	240 h
Control	14	36	56	58	48	50	56	Control	5±0.0	9±0.4	21±1.5	43±5.2	40±8.1	58±8.2	80±10.1
Glucantime	12	30	37	36	26	27	29	Glucantime	3±0.0	8±0.4	12±0.9	18±0.6	29±0.8	29±5.3	45±7.3
Nidmax-bapa	7	34	33	28	24	22	17	Nidmax-bapa	4±0.1	7±0.3	14±1.1	21±0.5	59±3.4	58±6.6	42±3.2
Nidmax-bpy	8	40	28	24	20	16	11	Nidmax-bpy	3±0.0	7±0.7	11±0.3	16±0.8	20±2.2	28±3.2	21±2.0
Nidmax-phen	10	36	37	32	28	25	22	Nidmax-phen	5±0.4	6±0.3	20±0.4	24±1.3	31±3.0	45±1.7	63±5.5
Nidmax-3phen	10	40	34	33	24	20	19	Nidmax-3phen	6±0.7	7±0.2	13±0.1	19±0.9	23±4.1	29±3.6	29±3.8
Nidmax-dpyamin	9	56	50	38	23	16	14	Nidmax-dpyamin	4±0,0	8±0.7	20±1.2	17±0.8	23±0.8	24±0.9	17±3.1

[a] Percentage macrophage parasitism. Values are mean deviation average of four separate determinations.

[b] Number of amastigotes per macrophage infected. Values are mean ± Standard deviation of four separate determinations

Figure legends

Scheme 1. Purine and 1,2,3-triazolo[4,5-*d*]pyrimidine skeletons displaying the biochemical and IUPAC numbering schemes.

Scheme 2. Chemical composition of the nickel complexes under study, including the skeletons of the triazolopyrimidine derivative and the amines used as auxiliary ligands.

Figure 1. Detail of the water cluster and 2D structure generated by the interaction of these clusters and the triazolopyrimidine ligand in **Nidmax-3phen**. Hydrogen bonds between interstitial water molecules (in blue) and between these water molecules and the dmax counteranions (in green).

Figure 2. 3D-packing in **Nidmax-3phen** (*bc* plane). Generation of channels where the interstitial water molecules are hosted (not represented on the picture).

Figure 3. ^{1}H-NMR spectra showing metabolites excrete by promastigote forms of *L. infantum* untreated (**A**), *L. braziliensis* untreated (**B**), *L. infantum* treated with **Nidmax-bapa** at IC_{25} dosage (**C**) and *L. braziliensis* treated with **Nidmax-bapa** at IC_{25} dosage (**D**). L-lac, L-lactate; Ac, acetate; S, succinate; EtOH, ethanol; DMSO, dimethyl sulfoxide.

Figure 4. Ultrastructural alterations by TEM in promastigote forms of *L.infantum* treated with nickel complexes (IC_{25} dose). Control of *L.infantum* showing organelles with their characteristic aspect, Bar= 1.00 μm **(1)**, *L.infantum* treated with **Nidmax-3phen**, Bar= 2.33 μm **(2)**, **Nidmax-bapa**, Bar= 2.33 μm **(3)**, **Nidmax-bpy**, Bar= 0.583 μm **(4)**, **Nidmax-**

dpyamin, Bar= 1.00 μm **(5)** and glucantime, Bar= 1.59 μm **(6)**. Nucleus (N), mitochondria (M), vacuoles (V), flagellum (F) microtubules (MI) and cellular rest (CR).

Figure 5. Ultrastructural alterations by TEM in promastigote forms of *L.braziliensis* treated with nickel complexes (IC_{25} dose). Control of *L. braziliensis* showing organelles with their characteristic aspect , Bar= 1.59 μm **(1)**. *L. braziliensis* treated with **Nidmax-bpy**, Bar= 1.59 μm **(2)**, **Nidmax-dpyamin**, Bar= 2.33 μm **(3)**, **Nidmax-dpyamin**, Bar= 1.59 μm **(4)**, **Nidmax-bapa**, Bar= 1.59 μm **(5)** and Glucantime, Bar= 1.00 μm **(6)**. Nucleus (N), glicosomes (G), mitochondrion (M), vacuoles (V), flagellum (F) microtubules (MI) and cellular rest (CR).

Scheme1
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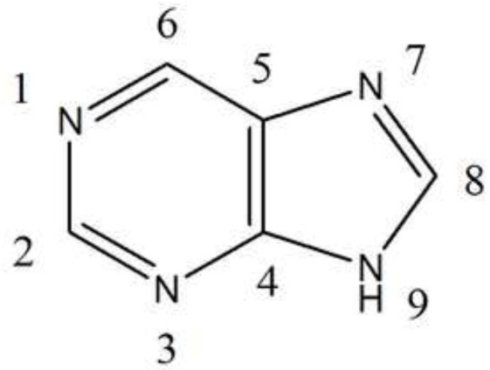
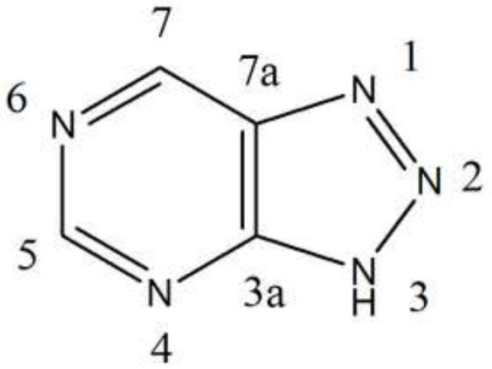


Purines



1,2,3-triazolo-[4,5-*d*]pyrimidines
(8-azapurines)

Scheme2
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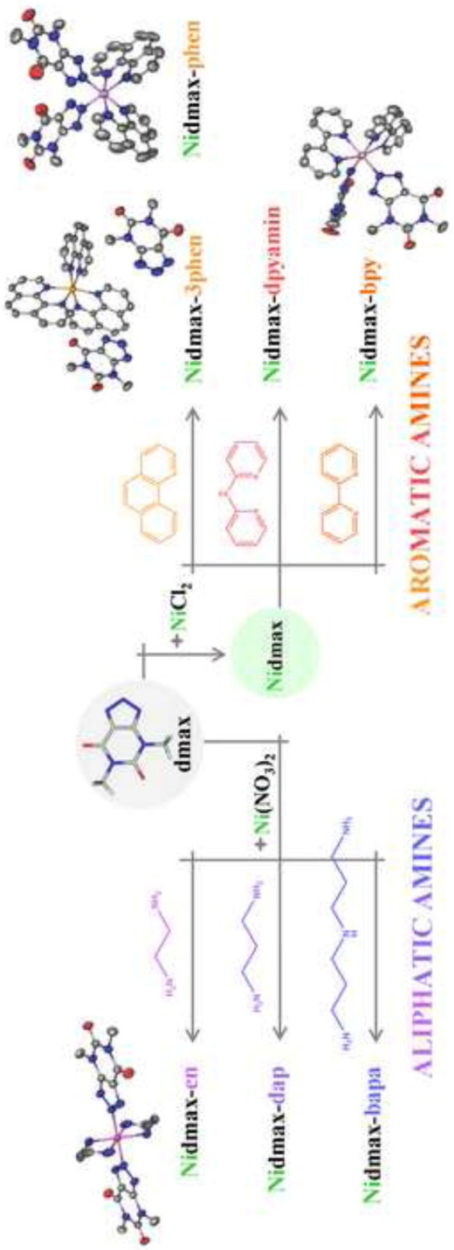

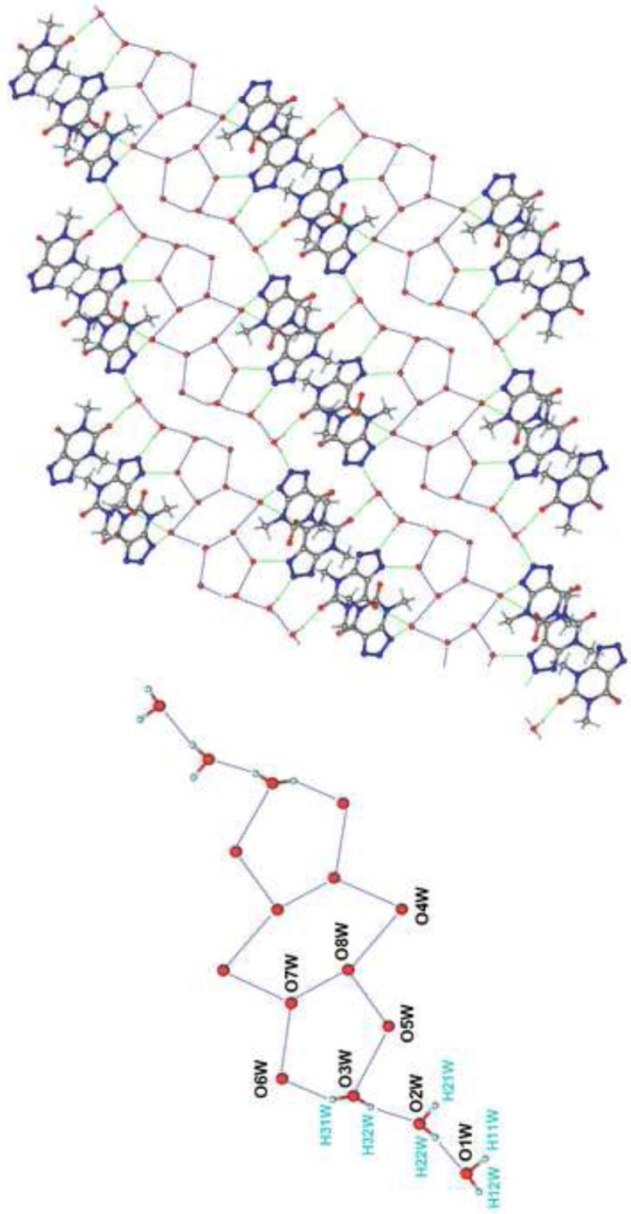

Figure1
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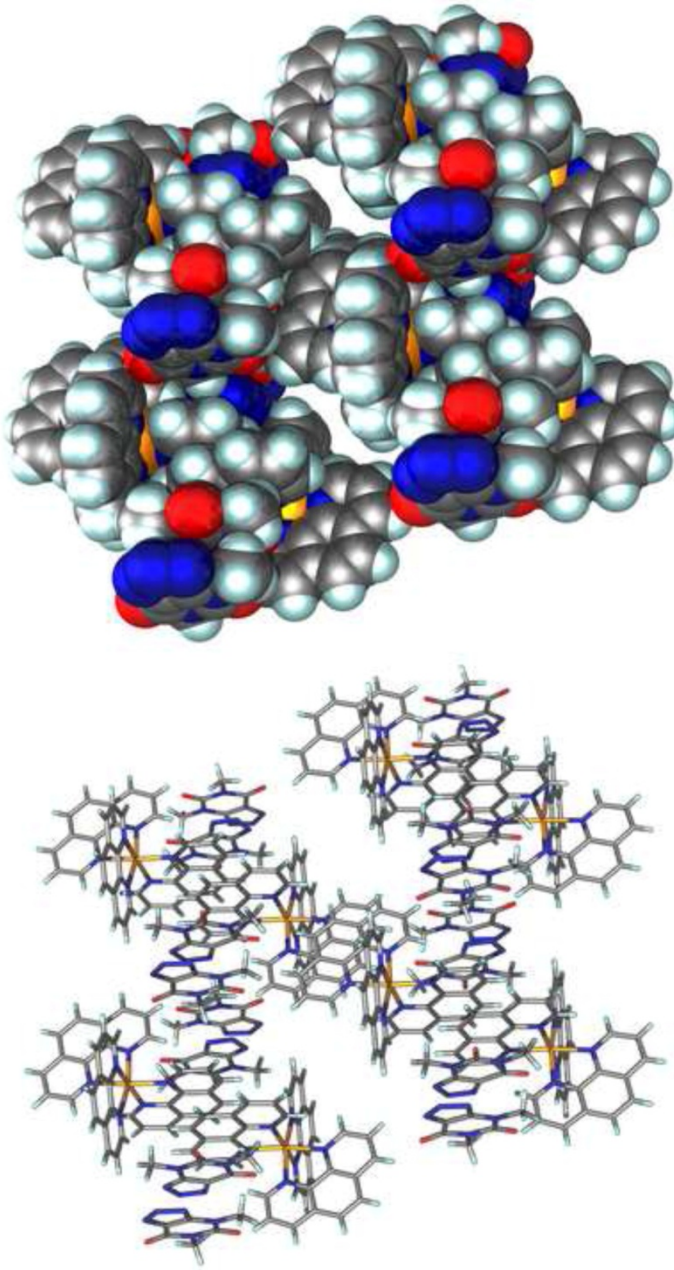

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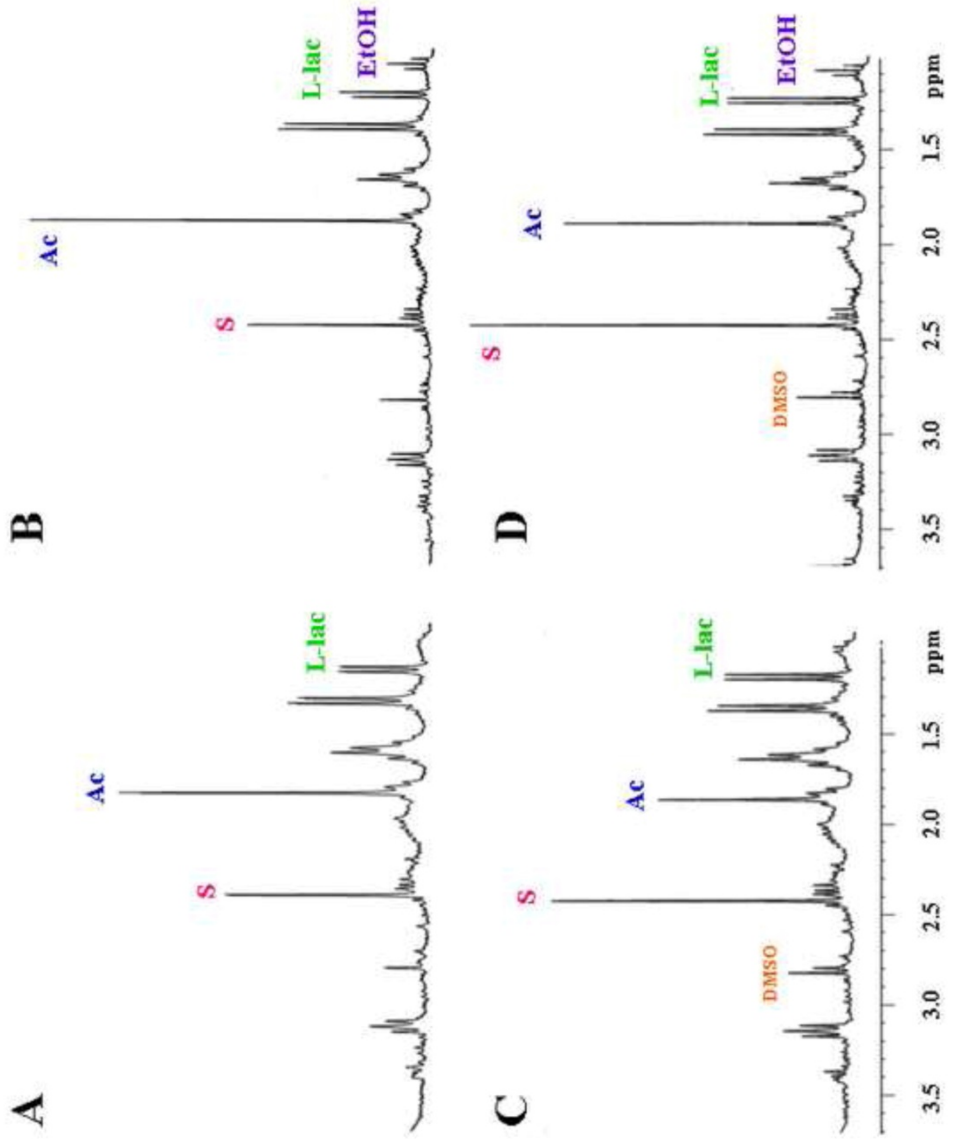

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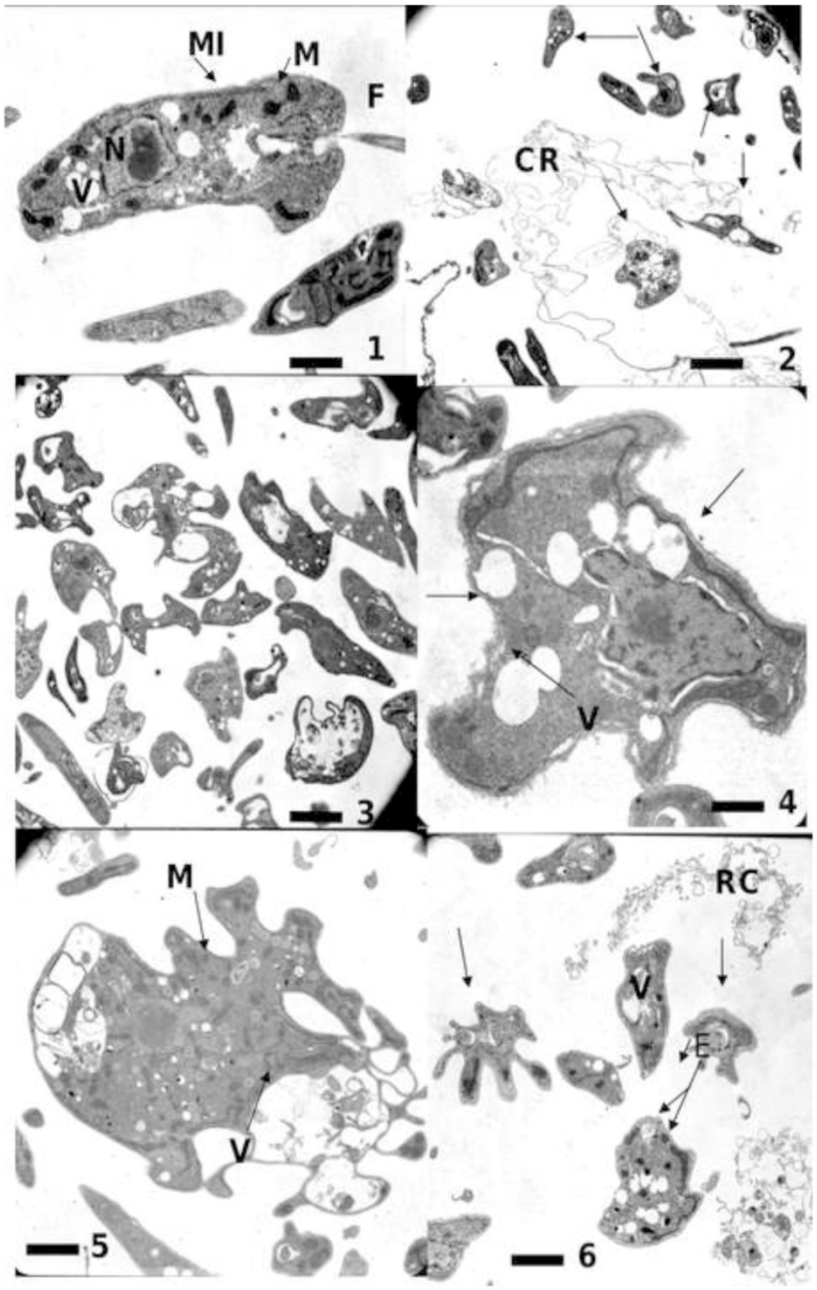

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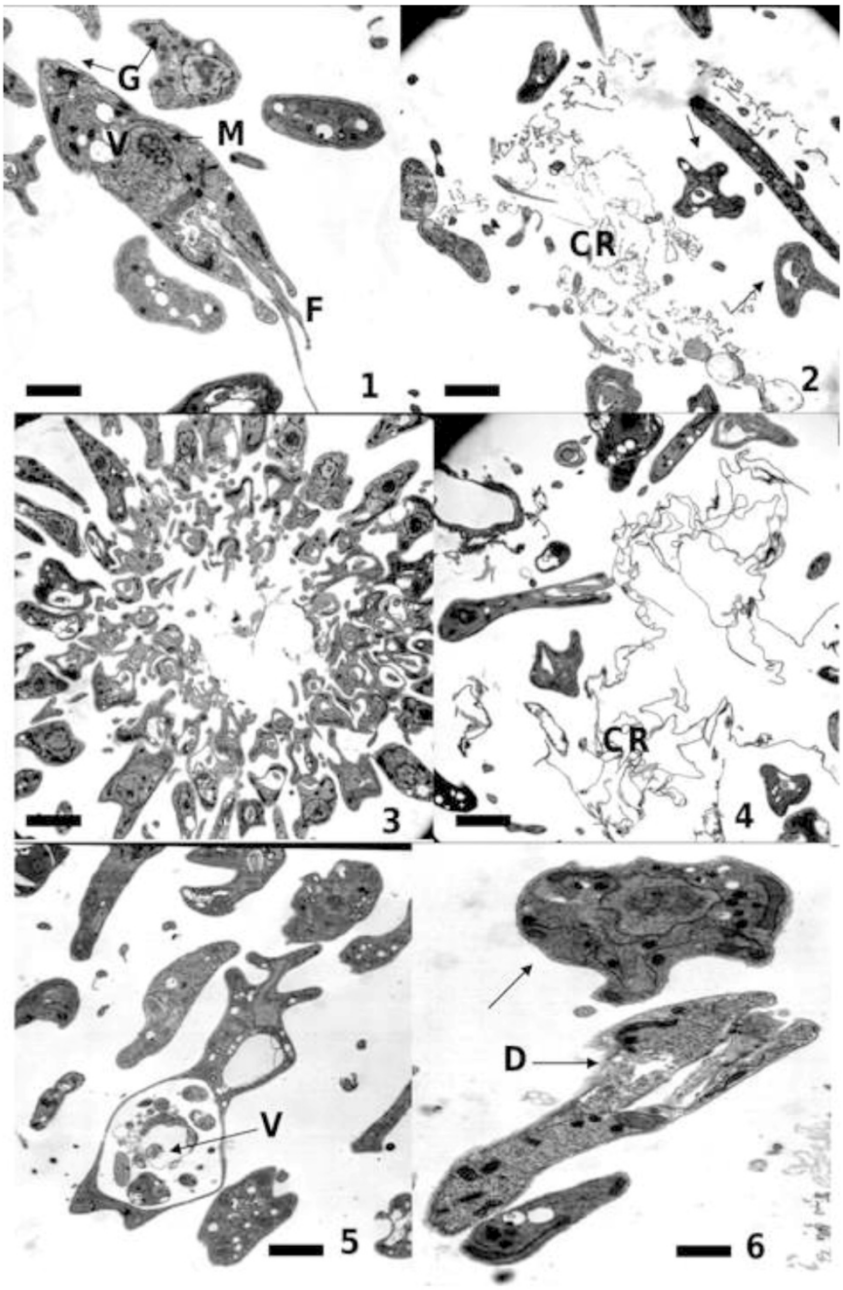

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SUPPLEMENTARY INFORMATION

In vitro anti-leishmania evaluation of nickel complexes with a triazolopyrimidine derivative against *Leishmania infantum* and *Leishmania braziliensis*

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Table S1. *In vitro* activity, toxicity, and selectivity index found for the reference drug and the co-ligands on promastigote and amastigote forms of *L. infantum*.

Compounds	[IC_{50} µM]		Toxicity [IC_{50} µM] on J774.2 ϕ[a]	SI[b]	
	Promatigote	Amastigote		Promatigote	Amastigote
Glucatim®	18.0	33.9	15.2	0.8	0.5
Ethylenediamine (en)	223.9	64.3	2.3	0.0 (0)	0.0 (0)
1,3-diaminopropane (dap)	135.4	107.1	17.6	0.1 (0)	0.2 (0)
Bis(3aminopropyl)amine (bapa)	92.1	66.4	2.6	0.0 (0)	0.0 (0)
1,10-phenanthroline (phen)	12.3	72.7	2.3	0.2 (0)	0.0 (0)
2,2'-bipyridyl (bpy)	174.7	110.9	2.5	0.0 (0)	0.0 (0)
2,2'-dipyridylamine (dpyamin)	88.0	252.8	2.9	0.0 (0)	0.0 (0)

IC_{50} = the concentration required to give 50% inhibition, calculated by linear regression analysis from the *K*c values at concentrations employed (1, 10, 25, 50 and 100 µM).

[a] On J774.2 Macrophages cells after 72 h of culture.

[b] Selectivity index = IC_{50} Macrophages/IC_{50} promastigote and amastigote forms.

Note: Average of three separate determinations.

In brackets: number of times that compound SI exceeds the reference drug SI.

Table S2. *In vitro* activity, toxicity, and selectivity index found for the reference drug and the co-ligands on promastigote and amastigote forms of *L. braziliensis*.

Compounds	[IC_{50} µM]		Toxicity [IC_{50} µM] on J774.2 ϕ[a]	SI[b]	
	Promatigote	Amastigote		Promatigote	Amastigote
Glucatim®	25.6	46.1	15.2	0.6	0.3
Ethylenediamine (en)	158.0	161.4	2.3	0.0 (0)	0.0 (0)
1,3-diaminopropane (dap)	201.6	142.9	17.6	0.1 (0)	0.1 (0)
Bis(3aminopropyl)amine (bapa)	120.1	54.5	2.6	0.0 (0)	0.0 (0)
1,10-phenanthroline (phen)	1.1	64.2	2.3	2.1 (0)	0.0 (0)
2,2'-bipyridyl (bpy)	191.3	102.0	2.5	0.0 (0)	0.0 (0)
2,2'-dipyridylamine (dpyamin)	154.1	72.7	2.9	0.0 (0)	0.0 (0)

IC_{50} = the concentration required to give 50% inhibition, calculated by linear regression analysis from the *K*c values at concentrations employed (1, 10, 25, 50 and 100 µM).

[a] On J774.2 Macrophages cells after 72 h of culture.

[b] Selectivity index = IC_{50} Macrophages/IC_{50} promastigote and amastigote forms.

Note: Average of three separate determinations.

In brackets: number of times that compound SI exceeds the reference drug SI.

In Vitro evaluation of new terpenoid derivatives against *Leishmania infantum* and *Leishmania braziliensis*. Artículo sometido en la revista *Mem Inst Oswaldo Cruz*. 2011

Runnig title: **Anti-leishmania terpenoid derivatives**

In Vitro evaluation of new terpenoid derivatives against *Leishmania infantum* and *Leishmania braziliensis*

Inmaculada Ramírez-Macías[a], Clotilde Marín[a], Rachid Chahboun[b], Francisco Olmo[a], Ibtisam Messouri[b], Oscar Huertas[a], María Jose Rosales[a], Ramón Sánchez-Gutierrez[c], Enrique Alvarez-Manzaneda[b,] Manuel Sánchez-Moreno[a*].

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Summary
The activity of five (**1-5**) Abietane phenol derivatives has been studied against *L. infantum* and *L. braziliensis,* using promastigotes, axenic and intracellular amastigotes. Infectivity and cytotoxicity tests were carried on J774.2 macrophage cells using Glucantime as the reference drug. The mechanisms of action were analysed performing metabolite excretion and TEM ultrastructural alteration studies. Compounds 1-5 were more active and less toxic than Glucantime. Infection rates and amastigotes mean numbers data showed that **2**, **4** and **5** were the most effective in both *L. infantum* and *L. braziliensis.* The ultrastructural alterations observed in treated promastigote forms confirmed the greater cell damage caused by the most active compound **4**. The modifications observed by ^{1}H NMR in the nature and amounts of catabolites excreted by the parasites after treatment was demostrated only for compound **5**. All the compounds assayed were active *in vitro* against the two *Leishmania* species and were less toxic against mammalian cells than the reference drug.

Keywords: Abietane phenols compounds, *Leishmania infantum*, *Leishmania braziliensis*, biology evaluation activity, promastigote, amastigote forms.

Financial support: Proyecto de Excelencia de la Junta de Andalucia Project (P07-FQM-03101) and MEC (Spain) (CGL2008-03687-E/BOS).

INTRODUCTION

Leishmaniasis, which is widely considered to be a “neglected disease”, is common in tropical and subtropical regions, including 22 countries in the New World and 66 in the Old World (http://www.who.int/leishmaniasis/). The etiological agents for this disease are different species of protozoa from the genus *Leishmania*, which are transmitted by flies (Diptera) from the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World. These are cosmopolitan or endemic diseases that present serious public-health problems and are thus considered by the WHO to be one of the seven priority diseases that affect all continents.

Pentavalent antimonial compounds, such as sodium stiboglucanate (Pentostam) and meglumine antimoniate (Glucantime), have been recommended as first-line drugs for the treatment of leishmaniasis for 50 years. Their most frequent side-effects of these compounds include anorexia, vomiting, peripheral polyneuropathy,

and allergic dermopathy, probably a result of oxidative or reductive damage to the host's tissue, and are thus inextricably linked to their antiparasitic activity (Momeni et al. 2002; Natera et al. 2007; Palumbo 2009). These effects therefore make the development of new drugs for the treatment of leishmaniasis a highly relevant and challenging research goal (Docampo & Moreno 1986; Cerecetto & Gonzalez 2002; Palumbo 2009).

One method for discovering new drugs is to investigate natural products obtained from plants with medicinal uses (Braña et al. 2005). Indeed, folk medicine is very often a valid source for researchers looking for bioactive substances that could potentially be useful against many diseases, as demonstrated by the search for new medicinal agents for treating trypanosomiasis, leishmaniasis, and other diseases (Braña et al. 2005). Indeed, a broad range of plant families and species contain active trypanocidal and leishmanicidal substances (Muhammad et al. 2002, Cui et al. 2003, González et al. 2005, Takahashi et al 2004, Tasdemir et al. 2006, Corrêa et al. 2011). Diterpene resin acids, for example, which are found in conifers, are known to be potent defence compounds against herbivores and pathogens (Martin et al. 2002). Likewise, the reported biological activity of natural abietane acids includes antimicrobial, antiulcer, cardiovascular activities, antiallergic, filmogenic, surfactant, and anti-feedant properties (San Feliciano et al. 1993). The interest in this type of terpenoid has increased in recent years as a result of the isolation of compounds, mainly phenols and related derivatives, with remarkable biological activities (Marrero et al. 2002; Tan et al. 2002). Other significant oxidized abietane diterpenes have been shown to strongly inhibit various human tumours and oncogene-transformed cells (Son et al. 2005).

Our group has recently synthesised abietane phenols **1–5** (Alvarez-Manzaneda et al. 2007a, b and c), and in order to determine the benefits of these compounds we studied their antiproliferative activity against extra- and intracellular forms of two species of *Leishmania*, namely *L. infantum* and *L. braziliensis*, *in vitro*. The possible cytotoxicity of these compounds was also assessed using non-parasitized host cells in order to establish whether the *in* v*itro* activity of the metabolite is due to its general cytotoxic activity or whether it is selectively active against the *Leishmania* parasites (Luque et al. 2000). Furthermore, we also performed a nuclear magnetic resonance spectroscopy (^{1}H NMR) study of the nature and percentage of the excretion metabolites in order to gain information concerning the potential inhibitory effect of the compounds on the glycolytic pathway, which is the prime source of energy for the parasite. Finally, the effect of these compounds on the ultrastructure of *Leishmania* spp. was monitored by transmission electron microscopy (TEM).

MATERIALS AND METHODS

Chemical Compounds **-** Compound **1**, the methyl ester of 12-hydroxydehydroabietic acid, recently described as a new natural product (Kinouchi et al. 2000) has been synthesised from commercial abietic acid (Alvarez-Manzaneda et al. 2007a) (**Scheme 1**). Compounds **2-4** were prepared from *trans*-communic acid (Alvarez-Manzaneda et al., 2007b), a labdane diterpene very abundant in some species of *Juniperus* and *Cupressus*. Compound **3** is the methyl ester of lambertic acid, isolated from *Podocarpus lambertius* and compound **4**, 6,7-dehydroabieta-8,11,13-trien-12,19-diol, named sugikurojin A, is a new diterpene recently isolated from *Cryptomeria japonica*. Compound **2**, methyl 12,15-dihydroxyabieta-8,11,13-trien-19-oate, has not yet been found in nature. Compound **5**, abieta -8,11,13-trien-14-ol, prepared from Abietic acid (Alvarez-Manzaneda et al. 2007c) is a immediate precursor for the synthesis of the antileishmanial agent 12-deoxy royleanone.

Parasite strain, culture **-** *L. infantum* (MCAN/ES/2001/UCM-10) and *L. braziliensis* (MHOM/BR/1975/M2904) were cultivated in vitro in medium trypanosomes liquid (MTL) with 10% inactive fetal bovine serum and were kept in an air atmosphere at 28 ºC, in Roux flasks (Corning, USA) with a surface area of 75 cm2, according to the methodology described by González and coworkers (2005).

Cell culture and cytotoxicity tests **-** J774.2 macrophages (ECACC number 91051511) were originally obtained from a tumour in a female BALB/c rat in 1968. The cytotoxicity test for macrophages was performed according to the methodology reported by González and coworkers (2005). After 72 hours of treatment, cell viability was determined by flow cytometry. Thus, 100 µl/well of propidium iodide solution (100 mg/ml) was added and incubated for 10 min at 28 ºC in darkness. Afterwards, 100 µl /well of fluorescein diacetate (100 ng/ml) was added and incubated under the same conditions. Finally, the cells were recovered by centrifugation at 400 g for l0 min and the precipitate washed with phosphate buffered saline (PBS). Flow cytometric analysis was performed with a FACS VantageTM flow cytometer (Becton Dickinson). The percentage viability was calculated in comparison with the control culture. The IC_{50} was calculated using linear regression analysis from the Kc values of the concentrations employed.

In vitro activity assay**:**

Promastigote forms – Promastigote forms were collected in the exponential growth phase and distributed in cultures trays (with 24 wells) to a final concentration of $5x10^4$ parasites/well.

Compounds were dissolved in medium trypanosomes liquid (MTL) and dimethyl sulfoxide (DMSO) (Panreac, Barcelona, Spain) at a concentration of 0.01%, after being assayed as non-toxic and without inhibitory effects on the parasite growth. Compounds were tested at final concentration of 1 to 100 μM. The effects of each compound against promastigote forms were tested at 72 hours using a Neubauer haemocytometric chamber. The antileishmanial effect is expressed as the IC_{50}, i.e. the concentration required to give 50% inhibition, calculated by linear regression analysis from the Kc values of the concentrations employed.

Amastigote assay - J774.2 macrophages were grown in minimum essential medium (MEM) plus Glutamine (2 mM) and supplemented with 20% inactive fetal bovine serum and were kept in a humidified atmosphere of 95% air and 5% CO_2 at 37 ºC.

Cells were seeded at a density of 1×10^4 cells/well in 24-well microplates (Nunc) with rounded coverslips on the bottom and cultured for 2 days. Afterwards the cells were infected in vitro with promastigote forms of *L. infantum* and *L. braziliensis*, at a ratio of 10:1 during 24 hours. The non-phagocytosed parasites, were removed by washing, and then the drugs (at 1, 10, 25, 50 and 100 μM) were added. Macrophages with the drugs were incubated for 72 hours at 37 ºC in 5% CO_2.

Drug activity was determined on the basis of number of amastigotes in treated and untreated cultures in methanol-fixed and Giemsa-stained preparations. The number of amastigotes was determined by analyzing 200 host cells distributed in randomly chosen microscopic fields. The antileishmanial effect is expressed as the IC_{50}.

Axenic amastigote assay - Axenic amastigotes forms of *L. braziliensis* and *L. infantum* were cultured following the methodology described previously by Moreno and coworkers (2011). Thus, promastigote transformation to amastigotes was obtained after three days of culture in M199 medium (Invitrogen, Leiden, The Netherlands) supplemented with 10% heat-inactivated FCS, 1 g/l β-alanine, 100 mg/l L- asparagine, 200 mg/l sacarose, 50 mg/l sodium pyruvate, 320 mg/l malic acid, 40 mg/l fumaric acid, 70 mg/l succinic acid, 200 mg/l α-ketoglutaric acid, 300 mg/l citric acid, 1.1 g/l sodium bicarbonate, 5 g/l MES, 0.4 mg/l hemin, 10 mg/l gentamicine pH 5.4 at 37 ºC. The effect of each compound against axenic

amastigotes forms was tested at 48 hours using a Neubauer haemocytometric chamber. The antileishmanial effect is expressed as the IC_{50}.

Infection assay **-** J774.2 macrophage cells were grown under the same conditions expressed in amastigote assay during two days. Afterwards, the cells were infected *in vitro* with promastigote forms of *L. infantum* and *L. braziliensis*, at a ratio of 10:1. The drugs (IC_{25} concentrations) were added at the same time and were incubated for 12 hours at 37 °C in 5% CO_2. The non phagocytosed parasites and the drugs were removed by washing, and then the infected cultures were grown for 10 days in fresh medium. Fresh culture medium was added every 48 h. The drug activity was determined from the percentage of infected cells and the number of amastigotes per infected cell (in treated and untreated cultures) in methanol-fixed and Giemsa-stained preparations. The percentage of infected cells and the mean number of amastigotes per infected cell were determined by analyzing 200 host cells distributed in randomly chosen microscopic fields.

Metabolite excretion **-** Cultures of *L. infantum* and *L. braziliensis* promastigotes (initial concentration 5×10^5 cells/ml) received IC_{25} concentrations of the compounds (except for control cultures). After incubation for 96 hours at 28 ºC, the cells were centrifuged at 400 g for l0 min. The supernatants were collected to determine the excreted metabolites using ^{1}H-NMR, and, eventually, chemical displacements were expressed in parts per million (ppm), using sodium 2,2-dimethyl-2-silapentane-5-sulphonate as the reference signal. The chemical displacements used to identify the respective metabolites were consistent with those described by Fernández-Becerra and coworkers (1997).

Ultrastructural alterations **-** The parasites were cultured at a density of 5×10^5 cells/ml in MTL medium, and the cultures contained drugs at the IC_{25} concentration. After 96 hours, those cultures were centrifuged at 400 g for 10 min, and the pellets produced were washed in PBS and then mixed with 2% (v/v) p-formaldehyde–glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4) for 4 hours at 4 ºC. After that, the pellets were prepared for TEM employing the technique of Gonzalez and coworkers (2005)

RESULTS

In vitro antileishmania activity. The IC_{50} values registered 72 h post-exposure of promastigote forms, axenic amastigote forms, and intracellular amastigote forms of *L. infantum* and *L. braziliensis* to compounds **1**–**5** were determined and are shown in Table 1. The values for the reference drug Glucantime are also included in all cases for comparison.

The results displayed in **Table 1** for activity against *L. infantum* show that the leishmanicidal activity is similar to, or in most cases higher than, that found for Glucantime for both extra- and intracellular forms. The toxicity data are more revealing, with all five compounds tested proving to be far less toxic to macrophages than the reference drug. Thus, compounds **5** and **4** were 31- and 25-fold less toxic than Glucantime, and even the most toxic amongst them, namely derivative **1**, is 12-fold more benign. The best values for the selectivity index (SI) were again found for compounds **5**, **4** and **3**, with a values exceeding those of the reference drugs by 36-, 36-, and 36-fold in the case of **5**, 34-, 40- and 34-fold for **4**, and 21-, 28- and 26-fold for **3**.

Very similar conclusions can be drawn from the results for *L. braziliensis* shown in **Table 1**. Thus, compounds **5** and **4** again gave the best SI values in the three assays performed, with values exceeding those of the reference drugs by 37-, 52-, and 37-fold in the case of **5** and by 32-, 24-, and 22-fold for **4**. Compound **2** also presented very good SI values, which in this case were even slightly higher than those for compound **4**.

Different authors have pointed out that a compound should have an **SI** value 20-fold higher than that for the reference in order to be considered to have leishmanicidal properties (Nwaka & Hudson 2006), a requisite that is satisfied by compounds **2**–**5** in the case of *L. infantum*, and *L. braziliensis*. Compound **1** was not included in further studies due to its low Si values.

The following step in the activity study involved determining the effect of these compounds on intracellular replication of the amastigote forms. Thus, macrophage cells were grown and infected with promastigotes in the stationary phase. The parasites invaded the cells and converted to amastigotes within one day post-infection, and the rate of host-cell infection reached its maximum on day 10 (control experiment). The IC_{25} of each product was used as the test dosage for these assays, with Glucantime as the reference drug. As shown in **Figure 1A**, addition of compounds **2**–**5** to macrophage cells infected with *L. infantum* promastigotes decreased the infection rate significantly with respect to the control for all compounds tested, with all of them proving to be more effective than Glucantime. Compounds **5** and **2** (infectivity reduction of 93% and 85%, respectively) were the most effective. Determination of the average number of amastigotes per infected

macrophage cell (**Figure 1C**) led to similar conclusions, with all four compounds proving to be much more effective than Glucantime (only 59% decrease).

The same experiment was performed with *L. braziliensis*, and the results concerning infection rates and amastigote numbers are presented in **Figures 1B** and **1D**, respectively. In both cases, all four compounds (**2**–**5**) were more effective than Glucantime, with compounds **5** and **2** again proving to be the most active.

Studies on the mechanism of action. We performed the following experiments to gain information concerning the possible mechanism of action of abietane phenols **2-5** on the parasite.

Metabolite excretion effect. *Leishmania* species have a high rate of glucose consumption, which results in acidification of the culture medium due to incomplete oxidation to acids. ^{1}H NMR spectroscopy enables us to identify the fermented metabolites excreted by the parasites during their *in vitro* culture. **Figure 2A** presents the spectrum of cell-free medium four days after inoculation with *L. infantum*. Additional peaks corresponding to the major metabolites produced and excreted during growth were detected when this spectrum was compared with that for fresh medium (spectra not shown). *L. infantum* excretes succinate and acetate as majority metabolites. We have identified and assessed one inhibiting effect caused by these compounds from the ^{1}H NMR spectra, which demonstrated that only compound **5** significantly altered the metabolites excreted by *L. infantum*. When the promastigote forms of *L. infantum* were treated with compound **5** at IC_{25} doses, the excretion of catabolites (succinate and acetate) was clearly altered (**Figure 2B**), with a new peak, which was subsequently identified as pyruvate, appearing. All compounds behaved similarly towards *L. braziliensis*, with compound **5** again appearing to have the largest inhibitory effect (spectra not shown).

Ultrastructural alterations. Electron microscopy (TEM) showed that morphological alterations in *Leishmania* spp. promastigotes after treatment with the newly synthesised abietane phenols **2–5** were substantial when compared with the control sample (**Figure 3**). All the compounds tested induced alterations in *L. infantum* promastigotes but not in *L. braziliensis*, with only compounds **5** and **4** proving to be effective against the latter. The ultrastructural alterations induced by these compounds in the two *Leishmania* species can be seen in **Figure 3** (**1-4**), together with the respective controls.

The most effective product against *L. infantum* was compound **4** (see panel **3**), which induced a marked formation of vacuoles, some of which were completely empty, with others containing cellular debris. These vacuoles occupied the entire

cytoplasm in some cases and the parasites appeared swollen and deformed, with swollen kinetoplasts and mitochondria. Some parasites were filled with lipid vacuoles. In addition, circular structures similar to glycosomes but strongly electrodense, which may be metabolic products of the parasites, appeared; these structures were not found in the untreated parasites. Likewise, numerous promastigotes were found to be strongly electrodense, thus meaning that the cytoplasmic organelles could not be distinguished. Although the remaining compounds were less effective against *L. infantum*, they nevertheless all induced the formation of a large number of empty vacuoles or cellular remains resulting from destruction of the parasites (data not shown).

Compound **4** was also the most effective against *L. braziliensis*, as reflected in panel **4** of **Figure 3**. In this case a high percentage of parasites appeared deformed, with vacuoles, both empty and filled with membranous structures, occupying almost the entire cytoplasm. Many of these parasites were strongly electrodense and appeared dead, whereas others contained a cytoplasm filled with lipid vacuoles.

DISCUSSION

Natural products from plants are frequently studied by researchers looking for bioactive substances that could be useful against many diseases (Braña et al. 2005). The isolation and structural elucidation of sulfated glucoside and *seco*-A-ring oleanane-type triterpenoids has been described recently and the anti-leishmanial activity of these compounds has been examined. The results showed that only the acylated *seco*-A ring oleanane derivatives are active, with the tricarboxylic acids being inactive (Macahig et al. 2011).

PX-6518 (1,10 13,28-epoxy-oleanane triterpene saponins), possesses potent in vivo activity against cutaneous leishmaniasis species (Inocêncio da Luz et al 2011) and visceral species (Maes et al 2004).

A previous study has indicated that the newly synthesised abietane phenols **1–5** are prospective chemotherapeutic drugs in the treatment of diseases caused by *Trypanosoma cruzi* (unpublished data). Most studies on the *in vitro* biological activity of new compounds against *Leishmania* spp. are performed on promastigote forms as it is much easier to work with these forms *in vitro*. However, since extracellular forms are not the developed forms of the parasite in vertebrate hosts, studies undertaken with extracellular forms are merely indicative of the potential leishmanicidal activity of the compounds tested. Consequently, a preliminary test using extracellular promastigote forms should always be complemented by a

subsequent evaluation using intracellular forms (amastigotes in vertebrate host cells) in order to gain a better understanding of the activity results (González et al. 2005). For this reason, we studied the activity on both intra- and extracellular forms.

The inhibitory effect of abietane compounds on promastigote forms has been studied previously, with totarol, ferruginol and 7β-hydroxyabieta-8,13-diene-11,12-dione showing good antileishmanial activity (IC_{50} values of 3.5–4.6 μg/mL vs. 1.3 μg/mL for pentamidine) against *L. donovani* promastigotes (Samoylenko1 et al. 2008). Abietane derivatives also showed appreciable *in vitro* antileishmanial activity against intracellular amastigote forms of *L. donovani* and *L. major* in other studies (Tan et al. 2002).

Our results indicated that the leishmanicidal activity of these compounds against both extra- and intracellular forms of *L. infantum* and *L. braziliensis* is similar to that found for Glucantime, although they are less toxic than the reference drug. Furthermore, the infection rate decreased significantly. To the best of our knowledge, none of the trypanosomatids studied to date is capable of completely degrading glucose to CO_2 under aerobic conditions, thus meaning that they excrete a large proportion of their carbon skeleton into the medium as fermented metabolites, which can differ according to the species (Bringaud et al. 2006).

As is also the case with *T. cruzi* (Ginger 2005), one of the major metabolites excreted by *Leishmania* spp. is succinate, the main role of which is probably to maintain the glycosomal redox balance by providing two glycosomal oxidoreductase enzymes. These enzymes allow the reoxidation of NADH, which is produced by glyceraldehyde-3-phosphate dehydrogenase in the glycolytic pathway. Succinic fermentation offers one significant advantage, namely that it requires only half of the phosphoenolpyruvate (PEP) produced to maintain the NAD^+/NADH balance. The remaining PEP is converted into acetate, depending on the species.

The metabolites excreted when the parasites were cultured ion the absence of **2**–**5** agree well with the findings of other authors (Ginger 2005). Thus, the new peak that appears when the promastigote forms were treated with compound **5** at IC_{25} doses, which was subsequently identified as pyruvate, means that complex **5** inhibits glycosomal enzymes, thus causing pyruvate to be excreted as a final metabolite.

The sequiterpene polygodial isolated from stem barks of *Drimys brasiliensis* affected the *Leishmania* mitochondria (Corrêa et al 2011)

The ultrastructural alterations induced by these terpenoid-based products, especially compound **4**, on extracellular forms of *L. infantum* and *L. braziliensis* can explain the changes observed in the metabolic studies, mainly as regards their effect on the cytoplasm, kinetoplasts and mitochondria.

It can therefore be concluded from these *in vitro* results that terpenoid compounds are potentially promising agents against *Leishmania* infections and that further *in vivo* studies are warranted in order to develop an efficient treatment.

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Legends for figures

Scheme 1. Chemical structures of the terpenoid derivatives.

Figure 1. Effect of terpenoid derivatives on J774.2 macrophages infected with *L. infantum* (A and C) and *L. braziliensis* (B and D): rate of infection (A and B) and mean number of amastigotes per infected cell (C and D). Control (-▲-); Gluc (- -); compound **2** (-●-); compound **3** (-+-); compound **4** (-♦-) and compound **5** (-□-) (at IC_{25} conc.). Each value is the mean of three separate experiments. ▽

Figure 2. ^{1}H-NMR spectra of promastigote forms of *L. infantum:* (A) Control and (B) Treated with Compound **5** at the concentration of IC_{25} (8 μM). ala, L-alanine: doublet, 1,50 ppm, 7,25 Hz; ac, acetate: singlet, 1,94 ppm; suc, succinate: singlet, 2,43 ppm; pyr, pyruvate: singlet, 2,26 ppm; lac, lactate: doublet, 1,35 ppm, 6,84 Hz, and DMSO, Dimethyl sulfoxide.

Figure 3. TEM images of the ultrastructural alterations induced in *L. infantum* and *L. braziliensis* upon treatment with terpenoid derivatives. Panel **1**. Control parasite of *L. infantum* showing organelles with their characteristic aspect, namely a nucleus (N), kinetoplast (K), lipid vacuoles (LV), mitochondrion (M), glycosomes (G), flagellum (F) (Bar: 7,000x). Panel **2**. Control parasite of *L. braziliensis* containing structures such as a nucleus (N), lipid vacuoles (LV), vacuoles (V), glycosomes (G), kinetoplast (K), flagellum (F) and mitochondrion (M) (Bar: 4,400x). Panel **3**. *L. infantum* treated with compound **4** shows intense vacuolisation (V), lipid vacuoles (LV), and abundant electrodense structures (arrow) (Bar: 7,000x). Panel **4**. *L. braziliensis* treated with compound **4**; some parasites are more electrodense, deformed and with intense vacuolisation (V) and lipid vacuoles (LV) (Bar: 4,400x).

Table 1. *In vitro* activity, toxicity, and selectivity index found for the reference drugs and the terpenoides derivatives on promastigote, axenic amastigote and amastigote forms of *Leishmania spp*.

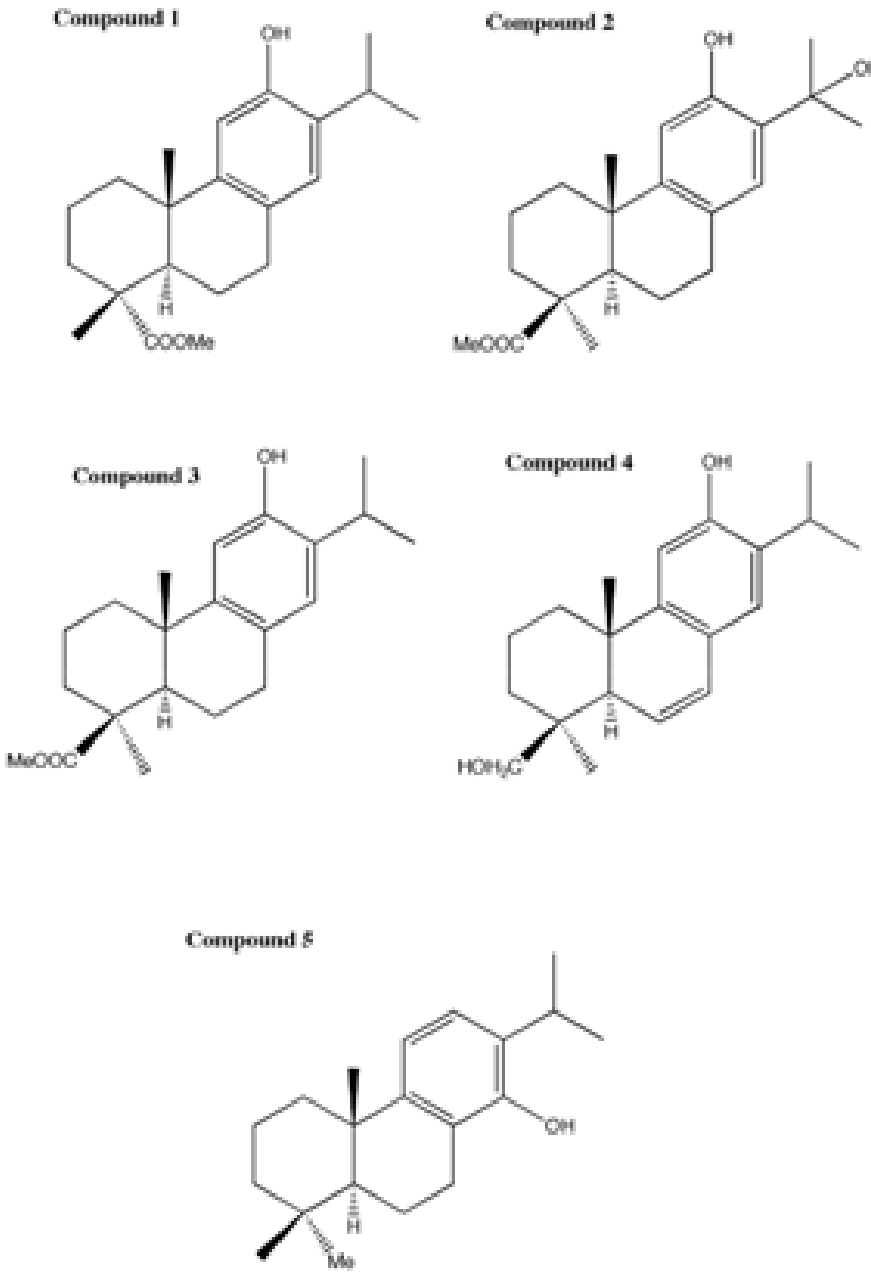
Leishmania infantum

Compds	[IC_{50} µM]			Toxicity [IC_{50} µM] on J774.2 ϕ[a]	SI[b]		
	Promatigote	Axenic Amastigote	Intracells Amastigote		Promatigote	Axenic Amastigote	Intracells Amastigote
Glucatime®	18.0 ± 1.1	30.0 ± 3.4	31.1 ± 5.7	15.2 ± 1.0	0.8	0.5	0.5
Comp 1	17.8 ± 3.3	27.5 ± 3.1	26.3 ± 3.9	189.6 ± 10.8	11 (14)	7 (14)	7 (14)
Comp 2	18.5 ± 2.1	18.9 ± 2.1	25.6 ± 3.0	257.6 ± 16.1	14 (17)	13 (26)	10 (20)
Comp 3	17,7 ± 0.4	20.9 ± 0.8	22.3 ± 2.6	300.0 ± 15.3	17 (21)	14 (28)	13 (25)
Comp 4	14.0 ± 1.5	19.2 ± 1.3	22.3 ± 1.8	379.0 ± 9.9	27 (34)	20 (40)	17 (34)
Comp 5	16.1 ± 2.4	26.3 ± 2.7	26.6 ± 5.3	467.4 ± 22.2	29 (36)	18 (36)	18 (35)

Leishmania braziliensis

Compds	[IC_{50} µM]			Toxicity [IC_{50} µM] on J774.2 [a]	SI [b]		
	Promastigote	Axenic Amastigote	Intracells Amastigote		Promastigote	Axenic Amastigote	Intracells Amastigote
Glucatime®	25.6 ± 1.6	29.6 ± 2.3	28.3 ± 4.3	15.2 ± 1.0	0.6	0.5	0.6
Comp 1	24.9 ± 5.7	40.1 ± 2.7	35.0 ± 5.5	189.6 ± 10.8	8 (13)	5 (10)	5 (8)
Comp 2	15.0 ± 1.5	12.7 ± 1.4	18.0 ± 2.8	257.6 ± 16.1	20 (33)	16 (32)	14 (25)
Comp 3	19.5 ± 0.5	30.9 ± 2.8	26.5 ± 2.9	300.0 ± 15.3	15 (25)	10 (20)	11 (19)
Comp 4	19.6 ± 1.1	31.6 ± 1.7	29.0 ± 1.0	379.0 ± 9.9	19 (32)	12 (24)	13 (22)
Comp 5	20.7 ± 5.2	18.0 ± 0.8	21.2 ± 2.2	467.4 ± 22.2	22 (37)	26 (52)	22 (37)

[a] On J774.2 Macrophages cells after 72 h of culture. IC_{50} = the concentration required to give 50% inhibition, calculated by linear regression analysis from the Kc values at concentrations employed (1, 10, 25, 50 and 100 µM).
[b] Selectivity index = IC_{50} Vero cells/IC_{50} promastigote, axenic amastigote, intracells amastigote forms. In brackets: number of times that compound SI exceeds the reference drug SI.
Note: Average of three separate determinations.


Compound 1
OH
H
COOMe
Compound 2
OH
OH
H
MeOOC
Compound 3
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H
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Compound 4
OH
H
HOH2C
Compound 5
OH
H
Me

Scheme 1

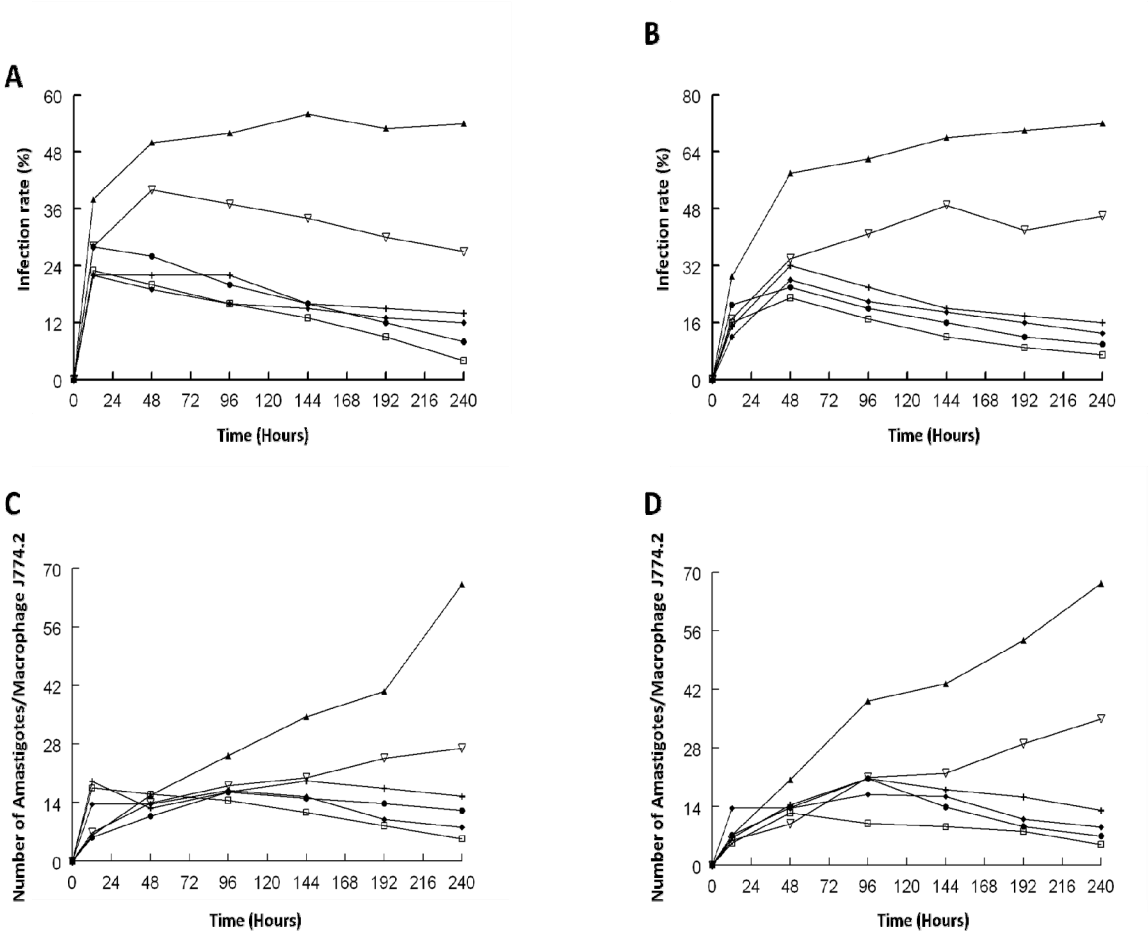
A
Infection rate (%)
60
48
36
24
12
0
0 24 48 72 96 120 144 168 192 216 240
Time (Hours)
B
Infection rate (%)
80
64
48
32
16
0
0 24 48 72 96 120 144 168 192 216 240
Time (Hours)
C
Number of Amastigotes/Macrophage J774.2
70
56
42
28
14
0
0 24 48 72 96 120 144 168 192 216 240
Time (Hours)
D
Number of Amastigotes/Macrophage J774.2
70
56
42
28
14
0
0 24 48 72 96 120 144 168 192 216 240
Time (Hours)

Figure. 1

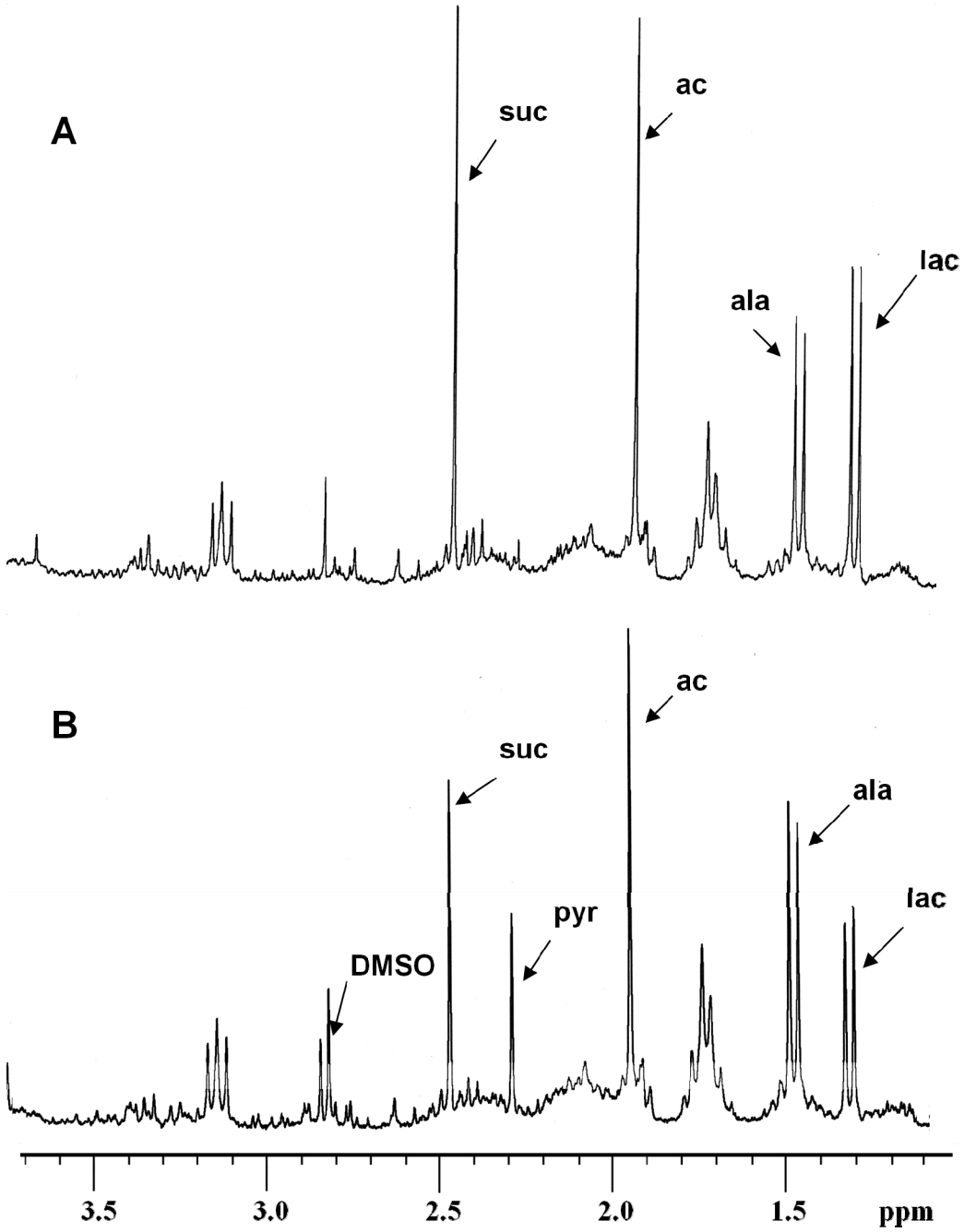

Figure. 2

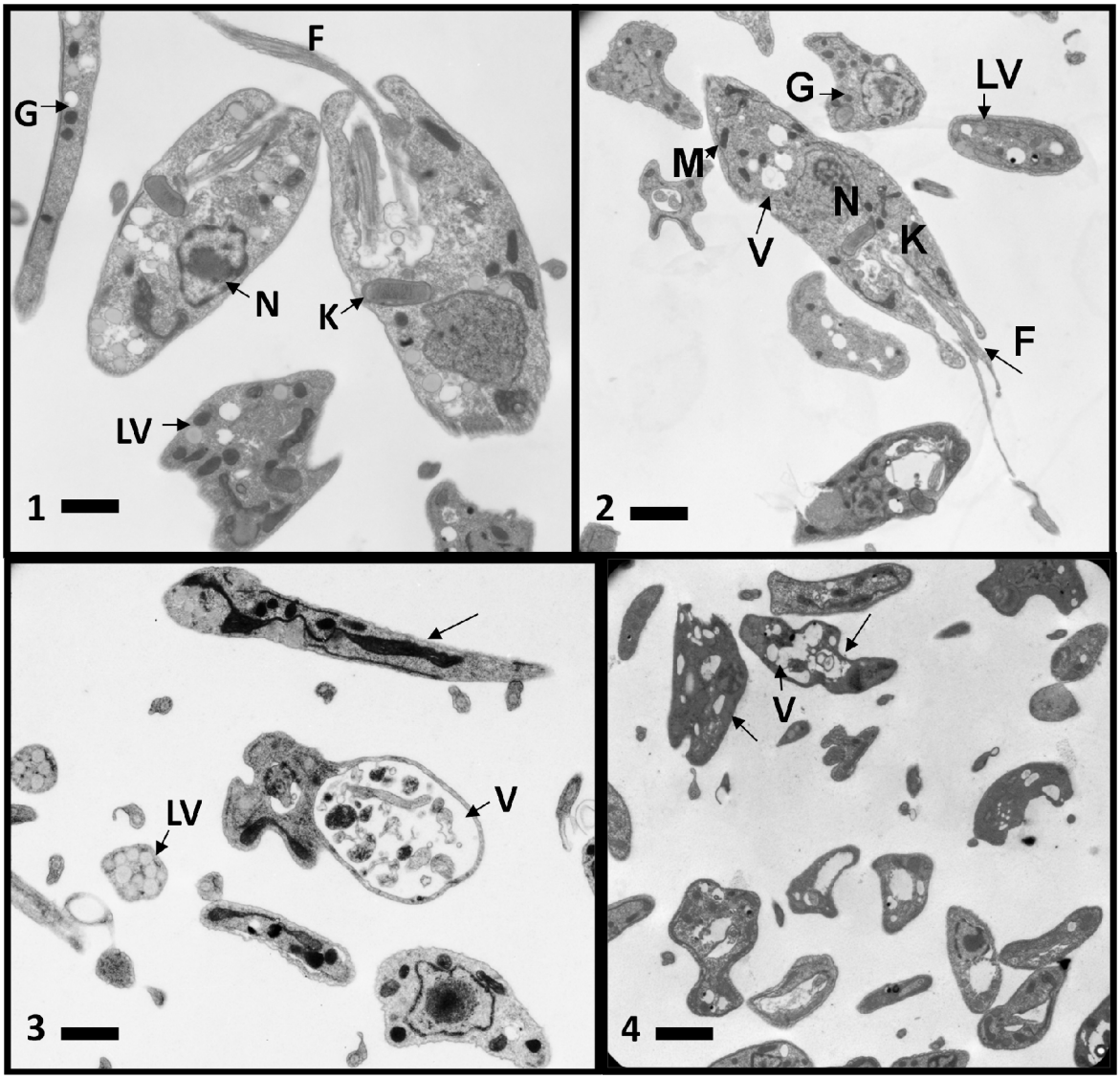

Figure. 3

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Taiwaniaquinoid and abietane quinone derivatives with trypanocidal activity against *T. cruzi* and *Leishmania spp.* Artículo sometido en la revista *Parasitol Int.* 2011

Taiwaniaquinoid and abietane quinone derivatives with trypanocidal activity against *T. cruzi* and *Leishmania* spp..

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ABSTRACT

The *in vitro* leishmanicidal (*Leishmania infantum* and *L. braziliensis*) and trypanocidal (*Trypanosoma cruzi*) activities of different compounds were evaluated. These compounds, of vegetal origin but synthesised in our laboratory, included five taiwaniaquinoid derivatives (**S-567**; **S-569**; **S-589; S-602** and **A-246)** and one abietane quinone (**P-1**). The *in vitro* activity of the compounds on extracellular and intracellular forms of the two *Leishmania* species and *T. cruzi* was assayed. Infectivity and cytotoxicity tests for the *Leishmania* species were conducted on J774.2 macrophage cells using Glucantime as the reference drug. From all the compounds assayed, the derivatives **P-1** > **S-567** were more active and less toxic than Glucantime. Infection rates and amastigote means indicated that these two compounds were the most active in both *Leishmania* species. In the case of *T. cruzi*, the best derivatives were **P-1** and **S-567**, at the same levels as for the *Leishmania* species. These compounds exhibited the most potent anti-proliferative activity against the extracellular vector form (the epimastigote), the extracellular host form (the trypomastigote), and the intracellular host form (the amastigote), with lower toxicity than that of the reference drug Benznidazole. Metabolite excretion studies showed that alterations mainly at the level of the mitochondria may explain observed metabolic changes in succinate and acetate production, perhaps due to the disturbance of enzymes involved in sugar metabolism within the mitochondrion. The *in vivo* studies for *T. cruzi* provided results consistent with those found *in vitro*. No signs of toxicity were detected in mice treated with the compounds tested, and the parasitic charge was slightly lower than in the control assay with Benznidazole. The effects of these two compounds were also demonstrated with the change in the anti-*T. cruzi* antibody levels during the chronic stage.

Keywords: antiproliferative activity, taiwaniaquinoid derivatives, abietane quinone derivatives, *Leismania infantum*, *Leishmania braziliensis*, *Trypanosoma cruzi*.

1. Introduction

The protozoan diseases leishmaniasis and Chagas disease are responsible for substantial global morbidity, mortality, and economic adversity in tropical and subtropical regions [1]. Leishmaniasis refers to a group of diseases caused by kinetoplastid protozoan parasites of the genus *Leishmania*. Their impact on public health is evident in the rapid expansion of endemic zones over recent years. It is now endemic in 98 countries, mainly in the New World, but also in Europe and Asia. More than 350 million people are at risk, and two million new cases arise every year, with an annual mortality rate higher than 60,000, a number surpassed only by malaria among parasitic diseases [2].

Leishmaniasis appears as three major clinical forms in humans: a) visceral, the most severe and life-threatening, b) cutaneous, causing nodules and ulcers that may persist for years, and c) mucocutaneous, causing permanent lesions in the mouth, nose, or the genital mucosa [3].

The forms of Leishmaniasis are caused by multiple phylogenetically distinct species. Among them, *L. infantum* is considered the main aetiological agent of visceral leishmaniasis in southern Europe. With dogs as the reservoir, it affects mainly children from one to four years old, although, since the emergence of HIV infection and increased use of immunosuppression for transplants and chemotherapy, nearly half of the new cases are now adults [4,5]. Another significant species, derived from the subgenus *Viannia*, is *L. braziliensis*, which occurs mainly in Andean countries and the Amazon basin and causes cutaneous and mucocutaneous leishmaniasis [6].These two species were selected as representative targets for the biological assays performed in the present work.

There is an urgent need for new active drugs against the different forms of these parasitic diseases, especially against visceral leishmaniasis. The drugs currently used are largely ineffective or cause a multitude of severe toxic side effects and the parasites are rapidly developing strong drug resistance [9]. To date, no effective vaccination is available, and diagnostic tools are not specific due to deficient vector-control measures [7,8].

For more than 50 years, heavy-metal derivatives, mainly pentavalent antimonials, have been used as standard drugs to treat leishmaniasis. Two of the most representative are Pentostam (sodium stibogluconate) and Glucantime (meglumine antimoniate). Antimonials cause many toxic effects, including dizziness, nausea, vomiting, diarrhoea, skin eruptions, cardiac arrhythmia, hypotension, hepatitis, or pancreatitis. Furthermore, drug administration is difficult, and low doses favour resistance in the parasite while higher doses are dangerously toxic [10,11].

Other types of drugs have recently been tested. Pentamidines are effective only against cutaneous leishmaniasis and although they are better tolerated, they cause diabetes mellitus at high doses. Fluconazole, amphotericin B and miltefosine are not useful against visceral leishmaniasis and despite their lower toxicity, they have limited effectiveness against other forms of the disease [10,11]. In most countries, existing strategies for the control and treatment of leishmaniasis are either failing or under serious threat. Environmental changes, drug resistance, and immunosuppression contribute to disease outbreak and transmission. However, despite the side-effects of available drugs, chemotherapy, combined with vector control, remains the most important measure to control trypanosomatid diseases in the absence of safe and efficient vaccines [12].

Trypanosoma cruzi causes Chagas disease which currently affects 12 to 15 million people [13,14]. Some patients develop chronic conditions that lead to cardiac, gastrointestinal, or neurological damage. Chemotherapy together with vector control remains the most important elements in the control of trypanosomatid disease, as there are currently no vaccines to prevent either *Leishmania* or *Trypanosoma* infection [15].

As with leishmaniasis, the arsenal of available drugs is limited, and all current treatments suffer from significant drawbacks including parenteral administration, length of treatment, toxicity, and/or cost, which limit their use in areas where the disease is endemic.

The treatment of Chagas disease relies largely on two drugs, Nifurtimox and Benznidazole, which are capable of curing at least 50% of acute-stage infections. These drugs require long (60 days) courses of treatment, and both have serious and frequent side-effects. Benznidazole has proved to be effective in early-stage chronic disease and an extensive clinical trial is in progress. New and safer drugs for treating Chagas disease, especially for the chronic phase, are urgently required [12].

In summary, despite some progress in treatment options, no ideal drugs are available against trypanosomatids that fulfil the major requirements of safety, high efficacy, ease of administration, low cost, and high patient compliance. Novel approaches towards improving and protecting current therapies, as well as towards the identification and optimisation of drug leads, are required to effectively combat and eventually eliminate these deadly diseases.

One way to discover new drugs is to investigate natural products from plants used medicinally. Folk medicine is often a valid indicator of sources for researchers looking for bioactive substances that are potentially useful against many diseases. This has already been demonstrated by the searches for new medicinal agents for treating a range

of diseases including trypanosomiasis and leishmaniasis [16]. Many potentially trypanocidal and leishmanicidal substances have been isolated from a variety of plants [17-20]. Substances from plants with anti-trypanosomal properties have been reported, but their side-effects are still unknown [20,21]. Active principles from plants and their synthetic and semi-synthetic analogues have served as one of the main routes towards developing new chemotherapeutic compounds [20,22]. The trypanocidal activity of several oxygenated abietane diterpenoids has been reported [23]. Such natural products with anti-trypanosomal activity have given fresh impetus to the development of new synthetic compounds as effective anti-trypanosomal drugs.

We synthesised a number of compounds including taiwaniaquinoid derivatives (**S-567**; **S-569**; **S-589** and **S-602)** and an abietane quinone (P-**1)**. Here we present their anti-proliferative activities *in vitro* against extracellular and intracellular forms of *T. cruzi* and two species of *Leishmania* (*L. infantum* and *L. braziliensis*). Their toxicity against macrophage J774.2 and Vero cells was determined and compared with values found for the reference drugs Benznidazole (BZN) and Glucantime. In the case of *T. cruzi*, trypanosomicidal activity on BALB/c female mice was measured *in vivo* both in the acute and chronic phases. To confirm the type of damage caused on the parasite cells, the effect of the compounds on the ultrastructure of the two *Leishmania* species and *Trypanosoma cruzi* was studied by transmission electronic microscopy (TEM). As it represents the prime source of energy for the parasite, the inhibitory effect of our compounds over the glycolytic pathway was assessed using (^{1}HNMR) analysis of the nature and percentage of the excretion metabolites.

2. Materials and methods

2.1. Chemistry

The compounds **S-567, S-569, S-589,** and **S-602 (Figure 1),** are enantiomerically pure and have a taiwaniaquinoid structure. Compound **S-569**, known by the name of (-)-taiwaniaquinone G, has been isolated from *Taiwania cryptomerioides*, a conifer found in Taiwan [24]. The four taiwainaquinone compounds were synthesised from homofarnesyl acetate and (+)-sclareolide in our laboratory [25,26] (Fig. 1). The cytotoxic activity for some members of this family of compounds has been described [27,28].

Compound **A-246** is a racemic mixture of taiwaniaquinoid, which is found in nature as (-)-enantiomer and was isolated from *Salvia dichroantha*, known as (-)-dichroanone. The racemic synthesis of compound **A-246** was also performed in our

laboratory, using ß-cyclocitral and sesamol as starting products, and its synthesis having been described elsewhere [29]. The enantioselective synthesis of (-)-dichroanone from (+)-sclareolide and homofarnesyl acetate has also been described [26] (Figure 1).

Compound **P-1**, enantiomerically pure (Figure 1), is a quinone with an abietane skeleton not found until now in nature. The synthesis of this compound starting from dehydroabietic acid has been described [30]. Quinone **1** has been synthesised in our laboratory starting from abietic acid [31]. Structurally related compounds have been described, such as 12-desoxyroyleanone, which have anti-leishmania activity [32,33].

2.2 Parasite strain, culture

Promastigote forms of *L. infantum* (MCAN/ES/2001/UCM-10) and *L. braziliensis* (MHOM/BR/1975/M2904) and epimastigote forms of *T. cruzi* SN3 strain of (IRHOD/CO/2008/SN3) isolated from domestic *Rhodnius prolixus* and the biological origin is Guajira (Colombia) [34] were cultivated in vitro in medium trypanosomes liquid (MTL) with 10% inactive fetal bovine serum and were kept in an air atmosphere at 28 ºC, in Roux flasks (Corning, USA) with a surface area of 75 cm2, according to the methodology described by [20].

2.3. Cell culture and cytotoxicity tests

J774.2 macrophages (ECACC number 91051511) originally obtained from a tumour in a female BALB/c rat in 1968 and Vero cells (Flow) were grown in minimum essential medium (MEM) plus Glutamine (2 mM) and supplemented with 20% inactivated foetal bovine serum and RPMI (Gibco), supplemented with 10% inactivated foetal bovine serum, in a humidified 95% air, 5% CO_2 atmosphere at 37 ºC respectively for two days. The cytotoxicity test for macrophages and Vero cells, was performed according to the methodology of [20]. After 72 hours of treatment, cell viability was determined by flow cytometry. Thus, 100 µl/well of propidium iodide solution (100 mg/ml) was added and incubated for 10 min at 28 ºC in darkness. Afterwards, 100 µl /well of fluorescein diacetate (100 ng/ml) was added and incubated under the same conditions. Finally, the cells were recovered by centrifugation at 400 g for l0 min and the precipitate washed with phosphate

buffered saline (PBS). Flow cytometric analysis was performed with a FACS VantageTM flow cytometer (Becton Dickinson). The percentage viability was calculated in comparison with the control culture. The IC_{50} was calculated using linear regression analysis from the Kc values of the concentrations employed.

2.4. In vitro activity assay:

2.4.1. Promastigote and epimastigote forms (extracellular forms)

The compounds obtained and the reference drugs (Glucantime and Benznidazole), were dissolved in DMSO (Panreac, Barcelona, Spain) at a concentration of 0.01% and were afterwards assayed as nontoxic and without inhibitory effects on parasite growth, according to [20]. The compounds were dissolved in the culture medium, at dosages of 100, 50, 25, 10 and 1 µM. The effects of each compound against promastigote of *L. infantum* and *L. braziliensis*) and epimastigote of *T. cruzi* forms were tested at 72 hours using a Neubauer haemocytometric chamber. The antitrypanosomatids effect is expressed as the IC_{50}, i.e. the concentration required to give 50% inhibition, calculated by linear regression analysis from the Kc values of the concentrations employed.

2.4.2. Amastigote assay (Intracellular forms)

J774.2 macrophages and Vero cells were grown in minimum essential medium (MEM) plus Glutamine (2 mM) and supplemented with 20% inactive fetal bovine serum and RPMI (Gibco), supplemented with 10% inactivated foetal bovine serum respectively and were kept in a humidified atmosphere of 95% air and 5% CO_2 at 37 ºC. Cells were seeded at a density of 1×10^4 cells/well in 24-well microplates (Nunc) with rounded coverslips on the bottom and cultured for 2 days. Afterwards the cells were infected in vitro with promastigote forms of *L. infantum* and *L. braziliensis* (in the stationary phase) and metacyclic forms of *T. cruzi* (obtained as described by [35], at a ratio of 10:1 during 24 hours. The non-phagocytosed parasites, were removed by washing, and then the drugs (at 1, 10, 25, 50 and 100 µM) were added. Macrophages and Vero cells with the drugs were incubated for 72 hours at 37 ºC in 5% CO_2. Drug activity was determined on the basis of number of amastigotes in treated and untreated cultures in methanol-fixed and Giemsa-stained preparations.

The number of amastigotes was determined by analyzing 200 host cells distributed in randomly chosen microscopic fields. The antitrypanosomats effect is expressed as the IC_{50}.

2.5. Infection assay

J774.2 macrophage and Vero cells were grown under the same conditions expressed in amastigote assay during two days. Afterwards, the cells were infected *in* v*itro* with promastigote forms of *L. infantum* and *L. braziliensis* and metacyclic forms of *T. cruzi*, at a ratio of 10:1. The drugs (IC_{25} concentrations) were added immediately after infection and were incubated for 12 hours at 37 °C in 5% CO_2. The non phagocytosed parasites and the drugs were removed by washing, and then the infected cultures were grown for 10 days in fresh medium. Fresh culture medium was added every 48 h. The drug activity was determined from the percentage of infected cells and the number of amastigotes per infected cell and the case of *T. cruzi*, the number of trypomastigotes in the medium was also determined [36] (in treated and untreated cultures) in methanol-fixed and Giemsa-stained preparations. The percentage of infected cells and the mean number of amastigotes per infected cell were determined by analyzing 200 host cells distributed in randomly chosen microscopic fields.

2.6. Metabolite excretion

Cultures of *L. infantum* and *L. braziliensis* promastigotes and *T. cruzi* epimastigote forms (initial concentration 5×10^5 cells/ml) received IC_{25} concentrations of the compounds (except for control cultures). After incubation for 96 hours at 28 ºC, the cells were centrifuged at 400 g for l0 min. The supernatants were collected to determine the excreted metabolites using ^{1}H-NMR, and, eventually, chemical displacements were expressed in parts per million (ppm), using sodium 2,2-dimethyl-2-silapentane-5-sulphonate as the reference signal. The chemical displacements used to identify the respective metabolites were consistent with those described by [37].

2.7. Ultrastructural alterations

The parasites were cultured at a density of 5×10^5 cells/ml in MTL medium, and the cultures contained drugs at the IC_{25} concentration. After 96 hours, those cultures were centrifuged at 400 g for 10 min, and the pellets produced were washed in PBS and then mixed with 2% (v/v) p-formaldehyde–glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4) for 4 hours at 4 ºC. After that, the pellets were prepared for TEM employing the technique of [20].

2.8. In Vivo Trypanocidal Activity Assay

Groups of three BALB/c female mice (6 to 8 weeks old; 20-25g) maintained under standard conditions were infected with $1x10^5$ *T. cruzi* metacyclic forms by the intraperitoneal route. The animals were divided into the following groups: (I) group 1: uninfected (not infected and not treated); (II) group 2: untreated (infected with *T. cruzi* but not treated); (III) group 3: uninfected (not infected and treated with 1 mg/kg body weight/day, for five consecutive days [7 to 12 days postinfection] by the intraperitoneal route) [38] and (IV) group 4: treated (infected and treated for five consecutive days [7 to 12 days postinfection] with the test compounds and benznidazole). This animal experiment was performed with the approval of an ethical committee of the University of Granada.

Treatments were started seven days after animal infection. Compounds were administered in a similar way to that explained above and at the same concentrations. A blood sample (5 µl) drawn from the mandibular vein of each treated mouse was taken and diluted 1:15 (50 µl of citrate buffer: citric acid 0.1 M, sodium citrate 0.1 M and 20 µL of lysis buffer at pH7.2: Tris-Cl 2M, $MgCl_2$), The parasites were counted by fields with the inmersion objetive. The number of bloodstream *T. cruzi* forms were recorded every three days from 7 to 40 days postinfection. The number of trypomastigote forms was expressed per 100 microscopic fields. Circulating anti-T. cruzi antibodies, at days 40 and 90 postinfection, were evaluated quantitatively by an enzyme-linked immunoassay. The blood, diluted to 1:50 in PBS, were reacted with an antigen composed of an excreted Fe-SODe of *T. cruzi* epimastigotes [39]. The results were expressed as the ratio of the absorbance of each sample at 490 nm to the cutoff value. The cutoff for each reaction was the mean of the values determined for the negative controls plus three times the standard deviation.

3. Results and discussion

As prospective chemotherapeutic drugs in the treatment of diseases caused by members of Trypanosomatidae [32], the taiwaniaquinoid (**S-567, S-569, S-589, S-602, A-246**) and abietane derivatives (**P-1**) were assessed for their toxic activity against two species of *Leishmania* (*L. infantum* and *L. braziliensis*) and a strain of *T. cruzi*.

Most *in vitro* studies of the biological activity of new compounds against trypanosomatid species have been performed on extracellular forms due to their ease to manipulate. However, since extracellular forms develop in the vector, the evaluations made with them are only indicative of the potential trypanocidal and leishmanicidal activity of the compounds in the vertebrate host. Consequently, a preliminary test using extracellular promastigote and epimastigote forms needs to be complemented with a subsequent evaluation using intracellular forms (amastigotes) for a more accurate assessment of the activity [20]. Intracellular assays were performed by infecting macrophage cells with promastigotes of *L. infantum* and *L. braziliensis*, and Vero cells, with metacyclic forms of *T. cruzi*. These infective forms invade the mammalian cells and are transformed into amastigotes within one day following infection. The inhibition is expressed in terms of the IC_{50} values after 72 h of exposure to the compounds. Results of inhibition by the compounds assayed in promastigote forms of *L. infantum* and *L. braziliensis* and epimastigote forms of *T. cruzi*, and in intracellular amastigote forms of the three trypanosomatids, are shown in Table 1 and Table 2 respectively. Values for the reference drugs Glucantime and Benznidazole are also included in all cases for comparison.

Toxicity values against mammalian macrophage J774.2 and Vero cells after 72 h of exposure to the compounds were also determined, and the selectivity indexes (**SI**, IC_{50} macrophage and/or Vero cell toxicity/IC_{50} activity of extracellular and intracellular forms of both parasites) are shown in the last three columns of Tables 1 and 2. The number of times that the compound **SI** exceeds the Glucantime or the Benznidazole **SI** appears in brackets. This value is highly representative of the potential *in vitro* efficacy of the compounds tested compared to the reference drugs.
The best activity against *L. infantum* and the best leishmanicidal activity on both extra- and intracellular forms were shown by taiwaniaquinone (**S-567**) and the abietane quinone (**P-1**, see the results displayed in the first column of Tables 1 and 2). All other compounds tested exhibited lower activity than that of Glucantime in the two parasitic forms assayed. More noteworthy are the toxicity data, as all six compounds tested were found to be much less toxic to macrophages than the reference drug. Compound **P-1** was 53-fold less toxic than Glucantime. Toxicity values substantially influenced the highly informative selectivity index (SI) data.

The highest SI values were again registered for the compounds **P-1** and **S-567**, with **P-1**exceeding those of the reference drugs by 61-fold (extracellular form) and 81-fold (intracellular form), and **S-567** by 24-fold (extracellular form) and 31-fold (intracellular form)**.**

Similar conclusions can be drawn from the results for *L. braziliensis* (see the second column of **the** Tables 1 and 2). The compounds **P-1** and **S-567** again showed the highest **SI** values in the two assays performed, which exceeded those of the reference drugs by 75- and 65-fold in the case of **P-1**, and by 25- and 40-fold for **S-567** (for intracellular and extracellular forms respectively).

The IC_{50} values of the compounds against the epimastigote forms of *T. cruzi*, along with the results of the reference drug Benznidazole, are displayed in the third column of Table 1. Toxicity values against mammalian Vero cells after 72 h of culture are also shown and the **SI** values are in the last column of Table 1. It can be seen that the taiwaniaquinone (**S-567**) and the abietane quinone derivative (**P-1**) are once again the most active compounds, not only against the extracellular forms but also against the intracellular forms (Table 2, third column). Moreover, they were far more active than Benznidazole against both the extracellular and intracellular forms of the parasite.

The two compounds (**S-567** and **P-1**) were also substantially less toxic to Vero cells than the reference drug (IC_{50} 394.7 and 781.4 □M, respectively, against 13.6 □M for Benznidazole). **SI** values indicate that these compounds were again the most promising, with SI values 36- and 41-fold higher than those of Benznidazole in the case of **S-567**, and 69- and 75- times higher in **P-1**, for intracellular and extracellular forms respectively.

Various authors have highlighted that **SI** values should be higher than 20-fold the **SI** of the reference [40] for a compound to be considered to have trypanocidal properties. This requisite is satisfied by compounds **S-567** and **P-1** against all the parasites studied. These compounds were therefore selected for subsequent investigations.

The activity study was extended by determining the effects of the compounds on the infection rate and the intracellular replication of the amastigote forms. Macrophage J774.2 and Vero cells were grown and infected with promastigotes in the stationary phase of *L. infantum* and *L. braziliensis* and metacyclic forms of *T. cruzi*. The control experiment showed that the parasites invaded the cells and underwent morphological conversion to amastigotes within one day after infection and on day 10, the rate of host-cell infection reached its maximum. For the assays, the IC_{25} of each product was used as the test dosage, with Glucantime and Benznidazole as the reference drugs.

As shown in Fig. 2, when the taiwaniaquinone (**S-567**) and abietane (**P-1**) compounds were added to macrophages infected with *L. infantum* promastigotes, the infection rate significantly decreased relative to the control in the two compounds

tested. The infection rate by *L. infantum* declined significantly relative to the control 72 h after treatment (Fig. 2 A-1), following the trend **P-1 > S-567**, declining by 73% and 68% respectively. These values are substantially higher than the inhibition by Glucantime (57%). Similarly, the two compounds inhibited *L. infantum* amastigote replication in macrophages *in vitro* in a similar way to the above-mentioned inhibition of the infection rate. The compounds were again more effective than the reference drug 10 days following treatment (Fig. 2 A-2).

The same experiment was performed with *L. braziliensis*, and the results of infection rates (B-1) and amastigote numbers (B-2) are shown in Fig. 2. In both cases, the two compounds were more effective than Glucantime. The data on infectivity rates calculated from Fig. 2 B-1 showed the efficacy to be **P-1** (80%)> **S-567** (72%) > Glucantime (36%), and the decrease in amastigote number was **P-1** (77%) > **S-567** (70%) > Glucantime (42%).

The results corresponding to *T. cruzi* propagation in Vero cells for compounds **S-567** and **P-1** are shown in Fig. 3. Using the same method used for the *Leishmania* species, the IC_{25} of each product was used as the test dosage and Benznidazole was used as the reference drug. When Vero cells were incubated for two days and then infected with metacyclic forms, the parasites invaded the cells and underwent morphological conversion to amastigotes within one day after infection. On day 10, the rate of host-cell infection reached its maximum. When compound **S-567** or **P-1** (IC_{25} concentration) was added to the infected Vero cells with *T. cruzi* metacyclic forms, the infection rate significantly decreased relative to the control, reaching 62% (**S-567**) and 85% (**P-1**) on day 10 of the experiment (Fig. 3 A). This decrease in infection rate shown by these compounds was substantially higher than that shown by Benznidazole (9%). In the control experiment, the average number of amastigotes per infected cell increased to 39.6 amastigotes/cell on day 6, decreasing to a value of 30.6 on day 8 and increasing again to 35.0 on day 10 (Fig. 3 B). Such fluctuations in the number of amastigotes may be due to their leaving the cell and invading it again followed by new cycles of replication resulting in variation in the number ofamastigotes per cell over time.

Both the taiwaniaquinoid and abietane compounds inhibited *T. cruzi* amastigote replication in Vero cells. The addition of a concentration equivalent to the IC_{25} of these compounds substantially reduced the amastigote number per infected cell by day 10, reaching a 26% reduction for **S-567** and 46% reduction for **P-1** compared to the control experiment. These results are striking when compared with those for Benznidazole, which effected only a 14% reduction in the amastigote number. After intracellular amastigote replication, amastigotes are transformed into trypomastigotes which burst out of the cell to be released into the circulation. The circulating parasites can then invade new cells to initiate new cycles of replication and are available to infect vectors that feed on the host [41]. We therefore measured

the variation in the trypomastigote number in the culture medium (Fig. 3 C). The control experiment had a trypomastigote number of 4.4 x10^3 on day 10, and reductions of 32% and 60% were found for compounds **S-567** and **P-1**, respectively. These reductions were more significant than for Benznidazole (17%).

When the results above were compared for both *Leishmania* species and *T. cruzi*, the abietane derivative (**P-1**) demonstrated clearly to be the most active and was much less toxic against the host than either Glucantime or Benznidazole. On the other hand, while the taiwaniaquinoid derivative was active against both *Leishmania* species and *T. cruzi*, it did not reach the levels of effectiveness of **P-1**.

The inhibitory effect of abietane compounds on promastigote forms of *Leishmania* species and *T. cruzi* has been studied previously, with totarol, ferruginol and 7β-hydroxyabieta-8,13-diene-11,12-dione showing good anti-leishmanial activity against *L. donovani* promastigotes [42]. Abietane derivatives also showed appreciable *in vitro* anti-leishmanial activity against intracellular amastigote forms of *L. donovani* and *L. major* in other studies [32]. Furthermore, cytotoxic effects against trypomastigote and epimastigote forms of *T. cruzi*, and against fibroblastic Vero cells, were shown by abietanes 1, 1A and 3-5, and ferruginol [23].

It is well established that trypanosomatids are unable to completely degrade glucose to CO_2 under aerobic conditions. Consequently, they excrete a considerable part of the hexose skeleton as partially oxidised fragments in the form of fermented metabolites into the medium. The nature and percentage of the partially oxidised fragments depend on the pathway used for glucose metabolism by each of the species considered [43,44]. The final products of glucose catabolism in *Leishmania* are usually CO_2, succinate, acetate, **L**-lactate, pyruvate, **L**-alanine, and ethanol [45]. *T. cruzi* also consumes glucose at a high rate, thereby acidifying the culture medium due to incomplete oxidation to acids [46]. Among them, succinate is especially relevant. Its main role is to maintain the glycosomal redox balance, which allows the reoxidation of NADH produced in the glycolytic pathway. Succinic fermentation has the advantage of requiring only half of the phosphoenolpyruvate produced to maintain the NAD+/NADH balance, while the remaining pyruvate is converted inside the mitochondrion and the cytosol into acetate, **L**-lactate, **L**-alanine or ethanol, according to the degradation pathway followed by each species [47].

To gain information concerning the effect of the experimental compounds on glucose metabolism in the parasites, the final excretion products were qualitatively and quantitatively identified. These data were obtained by recording the ^{1}H NMR spectra of promastigotes from *L. infantum* and *L. braziliensis*, and epimastigotes from *T. cruzi*, after treatment with the compounds studied. The results were compared with a control of promastigotes and epimastigotes maintained in a cell-free medium for four days after inoculation with the parasite. The characteristic

presence of acetate, L-lactate, and succinate was confirmed in the control experiments performed on all three species. However, noteworthy differences between them were the extensive presence of pyruvate and the appearance of ethanol among the catabolites excreted by *L. braziliensis* (Fig. 5A). These catabolites were not detected in *T. cruzi* epimastigotes (Fig. 6A) and *L. infantum* promastigotes (Fig. 4A).

When the parasites were treated with compound **P-1**, similar activity patterns appeared. For *T. cruzi* (Fig. 6B) and *L. braziliensis* (Fig. 5B) the excretion of acetate increased, while for *L. infantum* (Fig. 4B) values remained as for the control. The excretion of one of the most significant metabolites, succinate, was also common among the three species, with excretion into the medium lower compared to the control. On the other hand, the end metabolites of anaerobic oxidation, such as L-lactate and ethanol, were slightly higher in the culture medium, suggesting that despite the availability of glucose in the medium, compound **P-1** interfered with one or more steps of the TCA cycle. This might prevent the parasite from using this fundamental pathway forcing it to resort to means of compensation for energy production such as those used in absence of glucose. Another mechanism of action for this compound that may be postulated is the inhibition of transaminases, as pyruvate cannot be transformed into alanine in an amount sufficient for it to appear in the culture medium [48]. The other compound tested, **S-567**, showed a performance profile similar to that of the above compound, except that acetate excretion by both *T. cruzi* (Fig. 6C) and by *L. infantum* (Fig. 4C) decreased significantly, whereas no variations were found in the case of *L. braziliensis* (Fig. 5C). The cause of this metabolic change appears to be an action of the compounds on the enzymes acetate: succinate CoA transferase and succinyl-CoA synthetase, which are directly involved in mitochondrial production of acetate from acetyl-CoA [49]. Future studies are needed to elucidate the exact mechanisms of action of these compounds over relevant metabolic components.

Recent studies showed that doxorubicin and mitomycin C, two quinone derivatives, disturb redox homeostasis and increase ROS concentration by acting as inhibitors and as subversive substrates of TryR [50].

Ultrastructural alterations of *L. infantum*, *L. braziliensis* and *T. cruzi* caused by the compounds **S-567** and **P-1** were investigated. The two compounds were similarly effective and caused the same ultra-structural alterations. Product **S-567** induced an unusual effect in the parasites in the appearance of strongly electrodense, circular organelles (see the image corresponding to *L. infantum* treated with this compound in Fig. 7-2), which did not appear in any case in the non-treated trypanosomatids (Fig.7-1). It is not certain whether these are products of the metabolism of these parasites. A different effect of compound **P-1** (Fig. 7-3) on *L. infantum* was observed, with less alteration of the parasites. The parasites appeared almost normal

with the exception of an enormous quantity of lipid and swelling vacuoles visible in most of the promastigotes. In the case of *T. cruzi*, the effects of the two compounds were milder, manifested mainly as agglutination and vacuolization, but compound **S-567** was very effective, inducing the death of a high percentage of the parasites (Fig. 7-5), which shows dead parasites and others heavily vacuolated with enormous reservosomes and small, electrodense granules. These features also appear in the promastigotes of *L. infantum* treated with this compound. The parasites treated with **P-1** showed multiple alterations under TEM examination (Fig. 7-6). There was a notable quantity of glycosomes in most of the epimastigotes, many of which were highly vacuolated (V) with empty vacuoles, membranes or with electrodense vesicles (EV). The presence of large lipid vacuoles (arrow) is also notable as they were not present in control cultures. Many of the parasites had a star-like or festoon shape, in contrast to the shape of normal epimastigotes. The effect of these two compounds on *L. braziliensis* was very similar to the effect on *L. infantum* (data not shown).

The positive results of the *in vitro* tests using taiwaniaquinoid and abietane phenol derivatives prompted us to study their *in vivo* activity in mice. We evaluated their impact on the two significant stages of Chagas disease, the acute phase, regarded as the first 40 days post-infection, and the chronic phase, from 80 days post-infection. It was recently reported that the intravenous doping route results in high mortality rates [51]. We therefore opted for the intra-peritoneal (ip) route of doses of 5 mg/kg, which did not result in any animal mortality. Female Swiss mice were inoculated intra-peritoneally with $1x10^5$ metacyclic trypomastigotes, and treatment began seven days post-infection by the ip route with 1 mg/kg/day of each compound for five days. Administration was performed with a saline solution. A control group treated in the same manner with the vehicle alone was included. During the study of the acute phase activity, the level of parasitemia was determined every three days (Fig 8). None of the animals treated with either the control or the two compounds died during the treatment, whereas the survival of the mice treated with BZN was 80%. A comparison of the data represented in Fig. 8 showed that relative to control mice, the two compounds tested were able to diminish the trypomastigote number on the day of maximum parasitic burden (day 22-23 post-infection). On day 40 post-infection, a reduction of the parasitemia was found for the taiwaniaquinoid and abietane phenol derivatives relative to BZN. From these data, the following order for *in vivo* activity was established as **S-567≈P-1>BZN**. The activity in the chronic phase was assayed by serological tests performed at 40 and 90 days post- infection (Table 3). None of the animals treated with the **S-567** compound or with the control drug BZN showed negative anti-*T. cruzi* serology, while the **P-1** compound resulted in the greatest fall in antibody levels between days 40 and 90, showing higher performance than BZN in this assay. Differences in the levels of anti-*T. cruzi* antibodies agreed with the parasitemia findings.

4. Conclusions

In conclusion, **S-567** and **P-1** show activity against *T. cruzi, L. infantum* and *L. braziliensis*. These results support further investigation of taiwaniaquinoid and abietane quinone derivatives as potential agents against trypanosomatids.

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Figure captions

Fig. 1. Structures of the taiwaniaquinoid and abietane quinone derivatives

Fig. 2. Effect of activity of taiwaniaquinoid and abietane quinone derivatives on the infection rate and growth of *L. infantum* (**A**) and *L. braziliensis* (**B**). (**A-1** and **B-1**) Rate of infection, (**A-2** and **B- 2**) Mean number of amastigotes per infected macrophage J774.2 cell. Control (-▲-); Gluc (-●-); S-567 (-Δ-) and P-1 (-+-), (at IC_{25} conc.). The values are means of three separate experiments.

Fig. 3. Effect of activity of taiwaniaquinoid and abietane quinone derivatives on the infection rate and *T. cruzi* growth. **(C-1)** Rate of infection, **(C-2)** mean number of amastigotes per infected Vero cell, **(C-3)** number of trypomastigotes in the culture medium. Control (-▲-); BZN (-●-); **S-567** (- Δ -) and P-1(-+-), (at IC_{25} conc.). The values are means of three separate experiments.

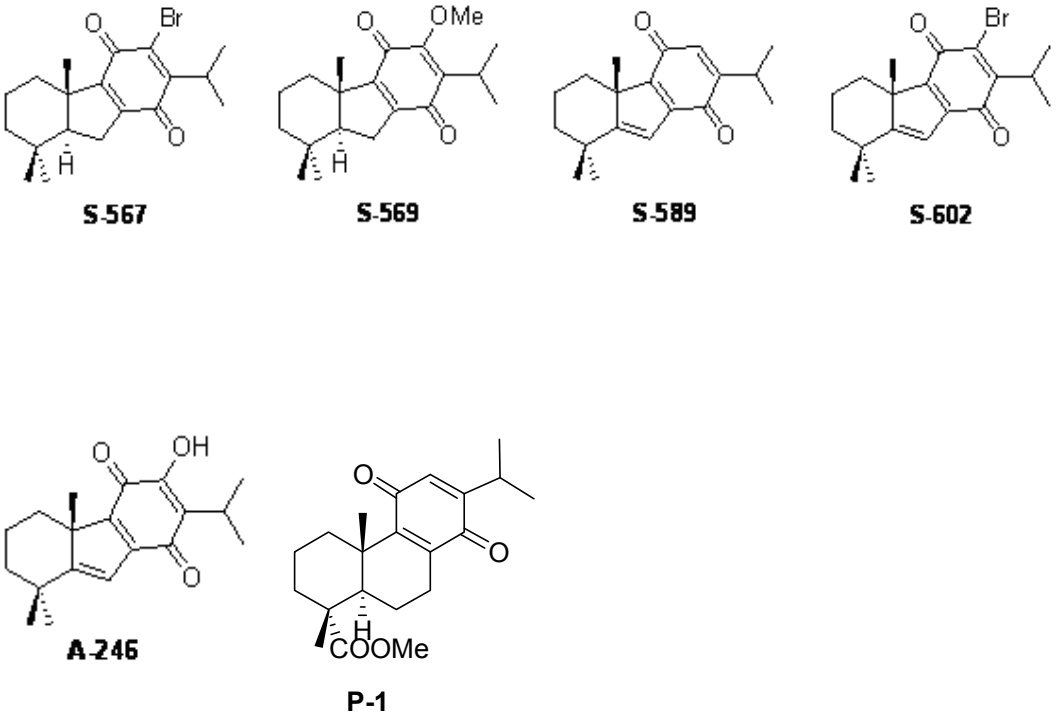
Fig. 4. ^{1}H-NMR spectra of promastigote forms of *L. infantum* treated with taiwaniaquinoid and abietane quinone derivatives (at concentrations at IC_{25}): (A) Control of *L. infantum* (untreated); (B) *L. infantum* treated with **P-1**, and (C) *L. infantum* treated with **S-567**. Lac, D-lactate; A, acetate; S, succinate; Pyr, pyruvate; Eth, ethanol; DMSO, dimethyl sulfoxide.

Fig. 5. ^{1}H-NMR spectra promastigote forms of *L. braziliensis* treated with taiwaniaquinoid and abietane quinone derivatives (at concentrations at IC_{25}): (A) Control of *L. braziliensis* (untreated); (B) *L. braziliensis* treated with **P-1**, and (C) *L. braziliensis* treated with **S-567**. Lac, D-lactate; A, acetate; S, succinate; Pyr, pyruvate; Eth, ethanol; DMSO, dimethyl sulfoxide.

Fig. 6. ^{1}H-NMR spectra of epimastigote forms of *T. cruzi* treated with taiwaniaquinoid and abietane quinone derivatives (at concentrations at IC_{25}): (A) Control of *T. cruzi* (untreated); (B) *T. cruzi* treated with **P-1**, and (C) *T. cruzi* treated with **S-567**. Lac, D-lactate; A, acetate; S, succinate; Pyr, pyruvate; Eth, ethanol; DMSO, dimethyl sulfoxide.

Fig. 7. Ultrastructural alterations visualised by TEM in *L. infantum* and *T. cruzi* treated with taiwaniaquinoid and abietane quinone derivatives (at concentrations at IC_{25}). **Fig. 7-1**. Control parasite of *L. infantum* showing organelles with their characteristic appearances, such as the nucleus (N), a kinetoplast (K), a mitochondrion (M), glycosomes (G), flagellum (F), vacuoles (V). **Fig. 7-2**. *L. infantum* treated with **S-567**, with electron-dense organelles (E) and vacuoles (V). **Fig. 7-3**. Promastigotes of *L. infantum* treated with **P-1**, with lipid and swelling vacuoles (V). **Fig. 7-4**. Control parasite of *T. cruzi* showing glycosomes (G), microtubules (MT), a mitochondrion (M) and a kinetoplast (K). **Fig. 7-5**. Epimastigotes of *Trypanosoma cruzi* treated with **S-567** showing many dead parasites (arrow), vacuoles (V) and electron-dense organelles (E). **Fig. 7-6** Epimastigotes of *T. cruzi* treated with P-1. Electrodense vesicles (EV) and vacuoles (V).

Fig. 8. Parasitemia in the murine model of acute Chagas disease: Control (-▲-) and receiving 5 mg/kg doses of BZN (-Δ-); **S-567** (-●-) and **P-1** (-+-).


O
Br
O
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S-567
O
OMe
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H
S-569
O
O
S-589
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Br
O
S-602
O
OH
O
A-246
O
O
H
COOMe
P-1

Fig. 1

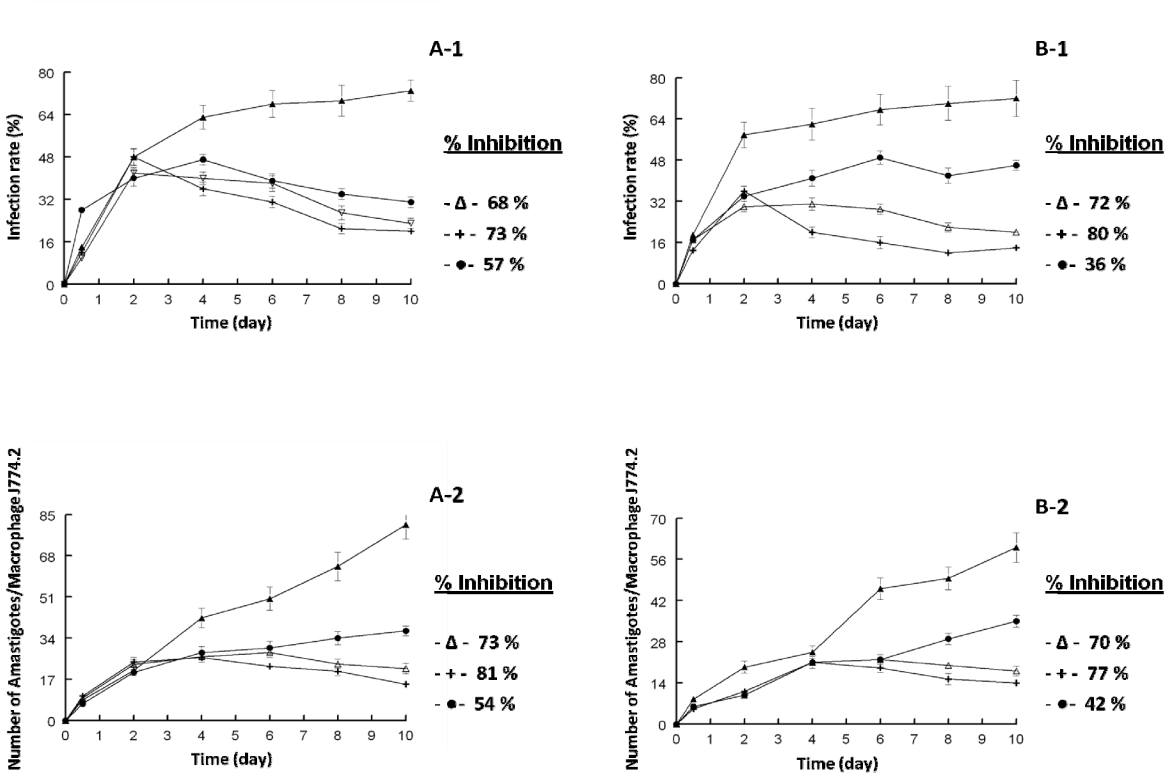

A-1
Infection rate (%)
Time (day)
% Inhibition
- Δ - 68 %
- + - 73 %
- ● - 57 %
B-1
Infection rate (%)
Time (day)
% Inhibition
- Δ - 72 %
- + - 80 %
- ● - 36 %
A-2
Number of Amastigotes/Macrophage J774.2
Time (day)
% Inhibition
- Δ - 73 %
- + - 81 %
- ● - 54 %
B-2
Number of Amastigotes/Macrophage J774.2
Time (day)
% Inhibition
- Δ - 70 %
- + - 77 %
- ● - 42 %

Fig. 2

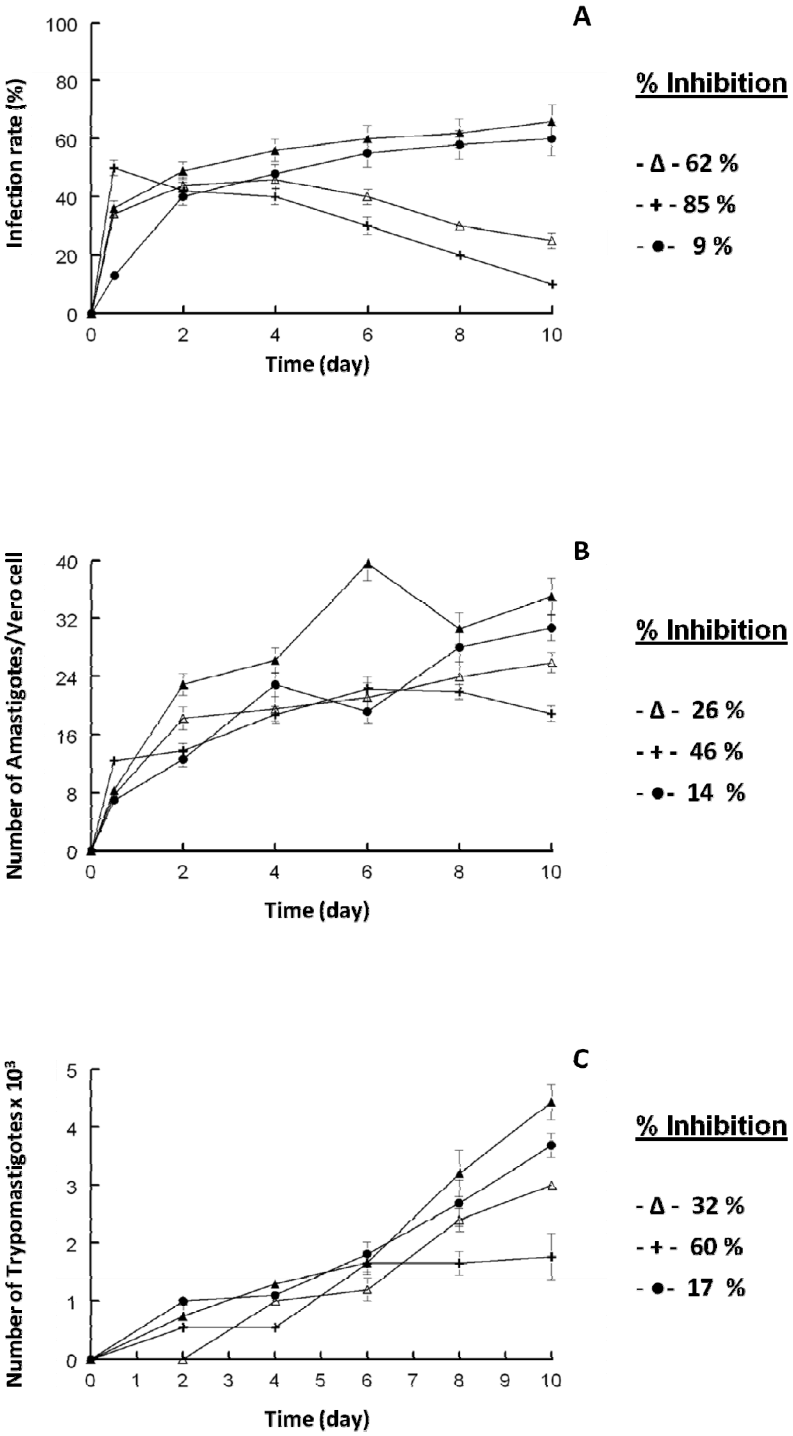
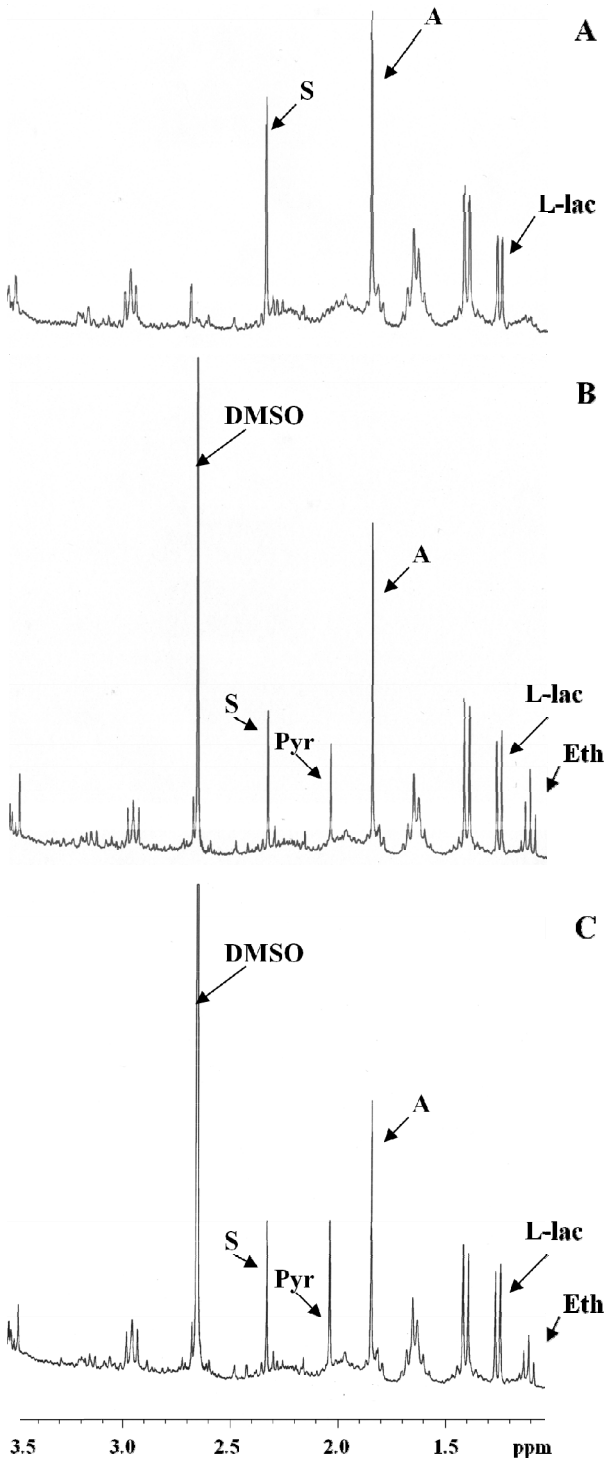

A
Infection rate (%)
Time (day)
% Inhibition
- Δ - 62 %
- + - 85 %
- ● - 9 %
B
Number of Amastigotes/Vero cell
Time (day)
% Inhibition
- Δ - 26 %
- + - 46 %
- ● - 14 %
C
Number of Trypomastigotes x 10³
Time (day)
% Inhibition
- Δ - 32 %
- + - 60 %
- ● - 17 %

Fig. 3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A
L-lac
B
DMSO
A
S
Pyr
L-lac
Eth
C
DMSO
A
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Pyr
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Eth
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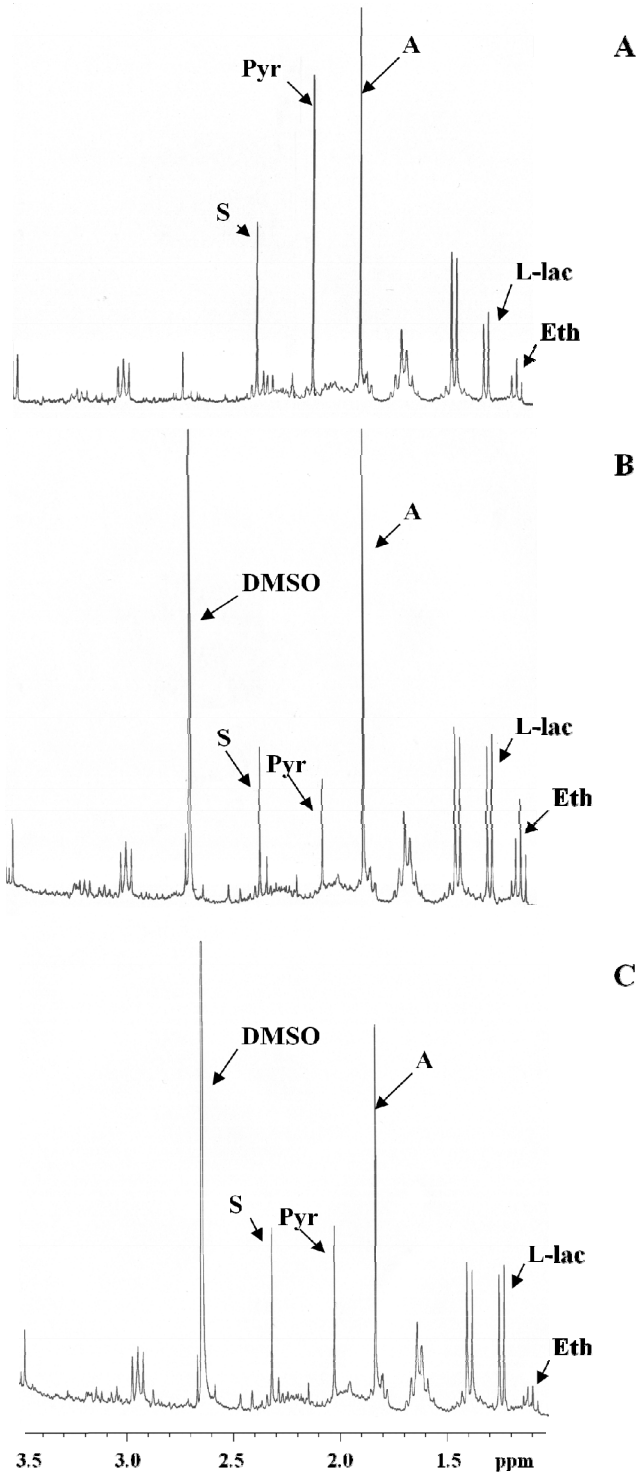
3.0
2.5
2.0
1.5
ppm

Fig.4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Pyr
A
S
L-lac
Eth
B
DMSO
A
S
Pyr
L-lac
Eth
C
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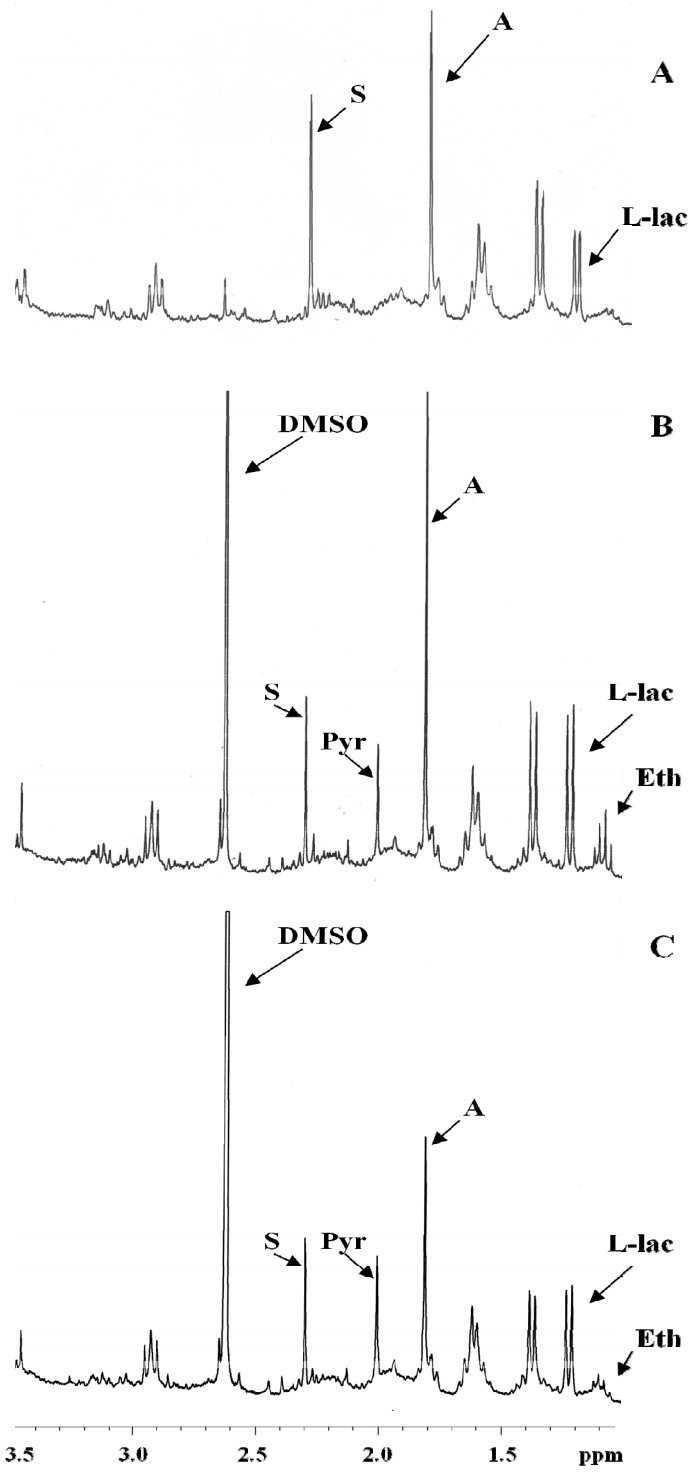
Pyr
L-lac
Eth
3.5
3.0
2.5
2.0
1.5
ppm

Fig. 5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L-lac
DMSO
B
A
S
L-lac
Pyr
Eth
DMSO
C
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S
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3.0
2.5
2.0
1.5
ppm

Fig. 6

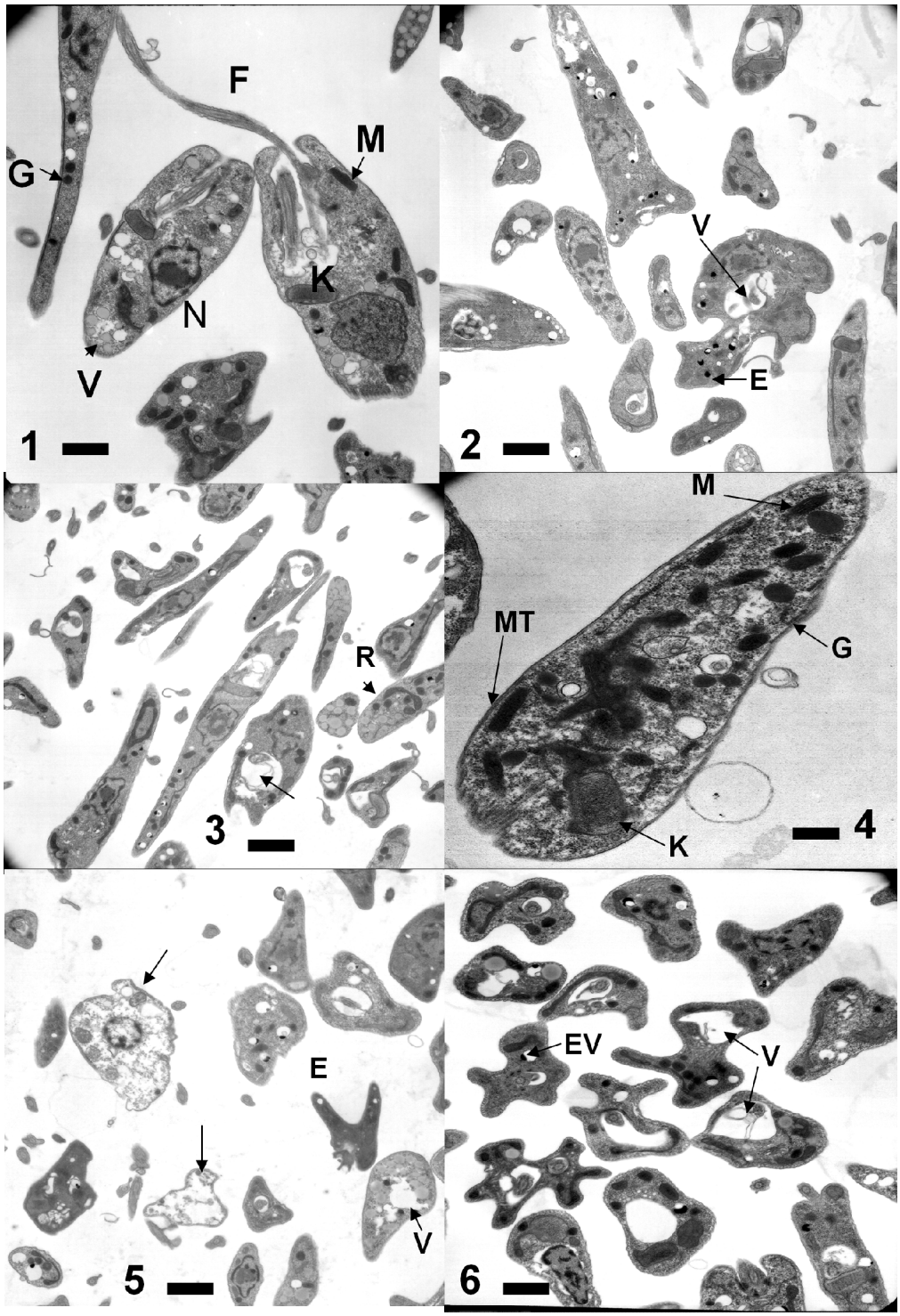

Fig. 7

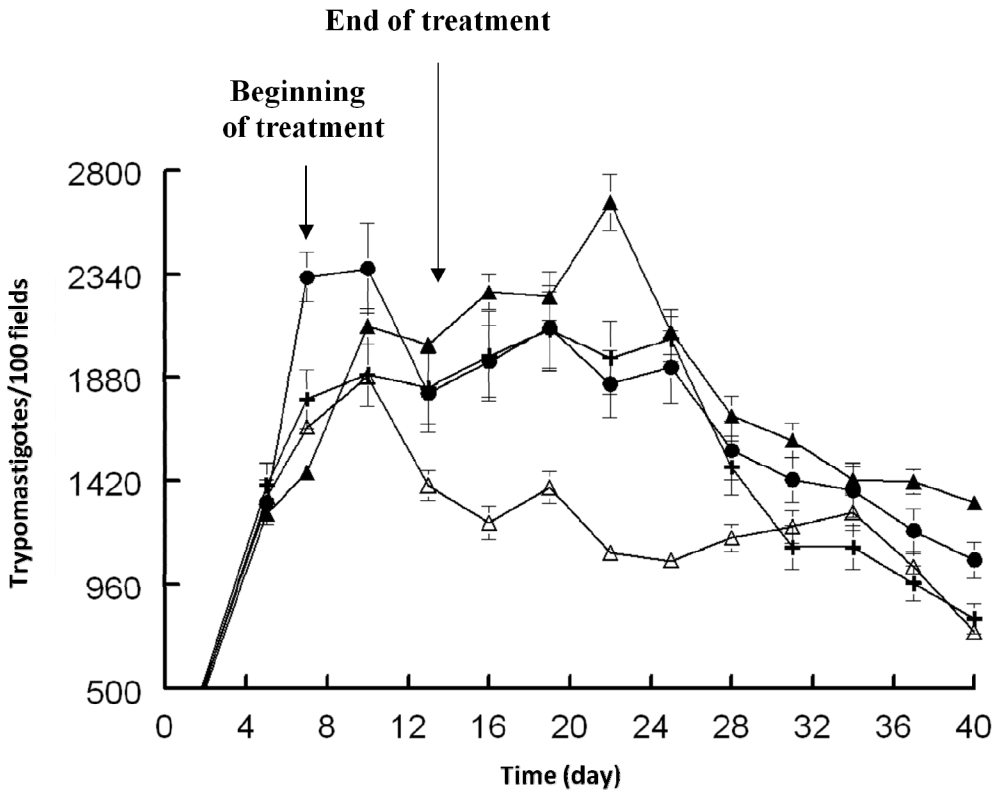
End of treatment
Beginning
of treatment
Trypomastigotes/100 fields
2800
2340
1880
1420
960
500
0
4
8
12
16
20
24
28
32
36
40
Time (day)

Fig. 8

Table 1. In Vitro Activity, Toxicity and Selectivity Index found for the taiwaniaquinoid and abietane quinone derivatives on extracellular forms (promastigotes and epimastigotes) of *Leishmania infantum*, *L. braziliensis* and *Trypanosoma cruzi*, respectively.

Compounds	IC50 (μM) [a]			Toxicity IC50 (μM)[b]		IS[c]		
	L. infantum	*L. braziliensis*	*T. cruzi*	on Macrophages J774.2	On Vero cells	*L. infantum*	*L. braziliensis*	*T. cruzi*
Benznidazole	-	-	15.8 ± 1.1	-	13.6 ± 0.9	-	-	0.8
Glucatim®	18.0 ± 3.1	25.6 ± 1.6	-	15.2 ± 1.0	-	0.8	0.6	-
S-567	13.4 ± 1.1	16.7 ± 1.3	13.7 ±0.7	253.6 ± 11.2	394.7 ± 6.9	18.9 (24)	15.2 (25)	28.8 (36)
S-569	18.3 ± 2.3	37.9 ± 4.0	41.7 ± 3.9	90.0 ± 8.7	43.6 ± 6.4	4.9 (6)	2.4 (4)	1.0 (1)
S-589	24.1 ± 1.9	26.9 ± 2.1	38.8 ± 4.4	116.5 ± 14.8	22.2 ± 0.4	4.8 (6)	4.3 (7)	0.6 (1)
S-602	19.7 ± 1.7	27.6 ± 2.8	34.2 ± 2.5	55.5 ± 6.7	18.3 ± 5.3	2.8 (4)	2.0 (3)	0.5 (1)
A-246	126.3 ± 11.4	55.4 ± 4.4	49.9 ± 6.1	187.5 ± 19.4	99.5 ± 11.3	1.5 (2)	3.4 (6)	2.0 (3)
P-1	16.3 ± 1.8	17.8 ± 2.1	14.2 ± 1.1	800.3 ± 83.4	781.4 ± 71.2	49.1 (61)	45.0 (75)	55.0 (69)

Results are averages of three separate determinations.

[a] IC_{50} = the concentration required to give 50% inhibition, calculated by linear regression analysis from the Ec values at concentrations employed (1, 10, 25, 50 and 100 μM).

[b] Towards macrophages J774.2 and Cell Vero after 72 h of culture.

[c] Selectivity index = IC_{50} macrophage J774.2 and Cell Vero/IC_{50} extracellular form of parasite. In brackets: number of times that compound SI exceeds the reference drug SI.

Table 2. In Vitro Activity, Toxicity and Selectivity Index found for the taiwaniaquinoid and abietane quinone derivatives on intracellular forms (amastigotes) of *Leishmania infatum*, *L. braziliensis* and *Trypanosoma cruzi*, respectively.

Compounds	IC_{50} (μM)[a]			Toxicity IC_{50} (μM)[b]		SI[c]		
	L. infantum	*L. braziliensis*	*T. cruzi*	on Macrophage J774.2	on Vero cells	*L. infantum*	*L. braziliensis*	*T. cruzi*
Benznidazole	-	-	23.3± 4.6	-	13.6± 0.9	-	-	0.6
Glucatim®	31.1± 5.7	28.3± 4.3	-	15.2± 1.0	-	0.5	0.5	-
S-567	16.3± 2.4	12.8± 1.6	15.9± 1.3	253.6± 11.2	394.7± 6.9	15.6 (31)	19.8 (40)	24.8 (41)
S-569	35.5± 5.4	37.1± 4.2	31.9± 6.1	90.0± 8.7	43.6± 6.4	2.5 (5)	2.4 (5)	1.4 (2)
S-589	36.3± 3.8	32.8± 5.2	25.5± 2.2	116.5± 14.8	22.2± 0.4	3.2 (6)	3.6 (7)	0.9 (2)
S-602	53.3±10.5	30.1± 4.6	35.6± 3.7	55.5± 6.7	18.3± 5.3	1.0 (2)	1.8 (4)	0.5 (1)
A-246	44.4± 7.3	55.0± 8.9	23.6± 1.6	187.5± 19.4	99.5± 11.3	4.2 (8)	3.4 (7)	4.2 (7)
P-1	19.8± 2.4	24.5± 7.2	17.3± 1.6	800.3± 83.4	781.4± 71.2	40.4 (81)	32.7 (65)	45.2 (75)

Results are averages of three separate determinations.

[a] IC_{50} = the concentration required to give 50% inhibition, calculated by linear regression analysis from the Kc values at concentrations employed (1, 10, 25, 50 and 100 μM).

[b] Towards macrophage J774.2 and Cell Vero after 72 h of culture.

[c] Selectivity index = IC_{50} macrophage J774.2 and Cell Vero/IC_{50} intracellular form of parasite. In brackets: number of times that compound SI exceeds the reference drug SI.

Table 3.- Differences in the level of anti-*T. cruzi* antibodies between days 40th and 90th post-infection for compounds taiwaniaquinoid and abietane quinone derivatives and BZN, expressed in absorbance units (abs).

Compounds[b]	ΔA[a]
Control (untreated)	**0,206**
BZN	**0,116**
S-567	**0,037**
P-1	**- 0,009**

[a]ΔA = Absorbance at 490 nm, day 90 p.i. – Absorbance at 490 nm, day 40 p.i.
[b] 1mg/kg/day, intraperitoneal route administered during 5 days.

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Leishmanicidal activity of nine novel flavonoids from *Delphinium staphisagria*. Artículo sometido en la revista *TheScientificWorldJOURNAL*. 2012

LEISHMANICIDAL ACTIVITY OF NINE NOVEL FLAVONOIDS FROM *DELPHINIUM STAPHISAGRIA*

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ABSTRACT

Objetives: To evaluate the *in vitro* leishmanicidal activity of nine flavonoids derivatives from *Delphinium staphisagria* against *L. infantum* and *L. braziliensis.*

Desing and methods: The *in vitro* activity of compounds **1-9** was assayed on extracellular promastigote and axenic amastigote forms, and on intracellular amastigote forms of the parasites. Infectivity and cytotoxicity tests were carried on J774.2 macrophage cells using Glucantime as the reference drug. The mechanisms of action were analysed performing metabolite excretion and TEM ultrastructural alteration studies.

Results: Nine flavonoids showed leishmanicidal activity against promastigote as well as amastigote forms of *Leishmania infantum* and *L. braziliensis*. These compounds were nontoxic to mammalian cells and were effective at similar concentrations up to or lower than that of the reference drug (Glucantime) . The results showed that 2´´-acetylpetiolaroside (compound **8**) was clearly the most active.

Conclusion: This study has demonstrated that flavonoid derivatives are active against *L. infantum* and *L. braziliensis.*

Keywords: Flavonoids, *Leishmania infantum*, *Leishmania braziliensis*, leishmanicidal activity.

1. Introduction

Leishmaniasis is an infection caused by different species of the protozoan genus *Leishmania*, which are transmitted by diptera of the genera *Phlebotomus* in the Old World and *Lutzomyia* in the New World.

Leishmaniasis represents one of the most significant of a collection of neglected tropical diseases. According to the latest report of the World Health Organization [1], 350 million people in 88 countries are considered at risk of contracting leishmaniasis, and some 2 million new cases occur annually.

Drug treatment for leishmaniasis has been available since the beginning of the 20th Century, [1] but only a few drugs have been developed for use and there are numerous drawbacks to each of the treatments. Two pentavalent antimonials are available (meglumine antimoniate or Glucantime and sodium stibogluconate or Pentostan), but they have common side-effects such as anorexia, vomiting, nausea, abdominal pain, malaise, myalgia. They are available in several formulations, which are administered by intravenous infusion, including liposomal amphotericin B, amphotericin B lipid complex and amphotericin B colloidal dispersion. However, side-effects such as fever, chills, rigor and back pain, and transient nephrotoxicity or thrombocytopenia, occur in some patients. Other antileishmanial medicines are paromomycin (aminosidine) and pentamidine isethionate, which are usually administered intramuscularly. The alkyl phospholipid (hexadecylphosphocholine), commonly known as mitelfosine, induces gastrointestinal side-effects such as anorexia, nausea, vomiting (38%) and diarrhea (20%), and also skin allergies, elevated hepatic transaminase concentrations and, occasionally, renal failure. It is now used in combination with different classes of azole oral antifungal agents including ketoconazole, fluconazole and itraconazole.

In addition to the adverse effects of the drugs, resistance to these treatments is appearing in the parasites. For all these reasons, further novel drug development is necessary to treat these infections.

Considerable attention is currently being paid to phytotherapy in the search for new drugs. One method used to discover new drugs is to investigate natural products from plants used medicinally. [2] The broad range of plant families and species available offer many potentially active leishmanicidal substances. [3,4] Leishmanicidal capacity of alkaloid compounds isolated from medicinal plants has been studied [5] and the anti-leishmania capacity of Aloe Vera exudate against *L. donovani* has been established. [6]

Leishmanicidal properties may reside in phytochemicals such as flavonoids, which are hence strong candidates for use in combination therapy against these infections. Flavonoids are abundant in fruits, vegetables, and saps of plants and are

demonstrated to have anti-carcinogenic, anti-microbial, and anti-parasitic activity. [7,8] Flavonoid analogues derived from *Consolida oliveriana* exerted a significant effect on the *in vitro* growth of species of *Leishmania* spp. [9]

In this work, the inhibitory effects of flavonoids from aerial parts of *Delphinium staphisagria* L. (Ranunculaceae) on the extracellular and intracellular stages of *L. infantum* and *L. braziliensis* were investigated. In addition, the cytotoxic effects of these compounds against a host cell line were assessed. We also used ^{1}H-NMR spectroscopic analysis to determine the nature and percentage of the excretion metabolites, and to elucidate any inhibitory effect that the compounds have on the glycolytic pathway. Finally, the effects of the compounds on the ultrastructure were studied.

2. Material and methods

2.1. Plant Material

Aerial part of the *Delphinium staphisagria* were collected and processed as described previously Díaz *et al*. [10] Nine flavonoids (1-9) were isolated, derivatized, and identified. (Fig. 1). [10]

2.2. Parasite strain, culture

L. infantum (MCAN/ES/2001/UCM-10) and *L. braziliensis* (MHOM/BR/1975/M2904) were cultivated in vitro in medium trypanosomes liquid (MTL) with 10% inactive fetal bovine serum and were kept in an air atmosphere at 28ºC, in Roux flasks (Corning, USA) with a surface area of 75 cm^2, according to the methodology described by González *et al*. [4]

2.3. Cell culture and cytotoxicity tests

J774.2 macrophages (ECACC number 91051511) were originally obtained from a tumour in a female BALB/c rat in 1968. The cytotoxicity test for macrophages was performed according to the methodology of González *et al*. [4] After 72 hours of treatment, cell viability was determined by flow cytometry. Thus, 100 µl/well of propidium iodide solution (100 mg/ml) was added and incubated for 10 min at 28ºC in darkness. Afterwards, 100 µl /well of fluorescein diacetate (100 ng/ml) was added and incubated under the same conditions. Finally, the cells were recovered by centrifugation at 400 g for 10 min and the precipitate washed with phosphate buffered saline (PBS). Flow cytometric analysis was performed with a FACS VantageTM flow cytometer (Becton Dickinson). The percentage viability was

calculated in comparison with the control culture. The IC_{50} was calculated using linear regression analysis from the Kc values of the concentrations employed.

2.4. In vitro activity assay

2.4.1. Promastigote forms assay

The compounds obtained were dissolved in the culture medium, at dosages of 100, 50, 25, 10 and 1 μM. The effects of each compound against promastigote forms were tested at 72 hours using a Neubauer haemocytometric chamber. The antileishmanial effect is expressed as the IC_{50}, i.e. the concentration required to give 50% inhibition, calculated by linear regression analysis from the Kc values of the concentrations employed.

2.4.2. Amastigote forms assay

J774.2 macrophages were grown in minimum essential medium (MEM) plus Glutamine (2 mM) and supplemented with 20% inactive fetal bovine serum and were kept in a humidified atmosphere of 95% air and 5% CO_2 at 37ºC.

Cells were seeded at a density of 1×10^4 cells/well in 24-well microplates (Nunc) with rounded coverslips on the bottom and cultured for 2 days. Afterwards the cells were infected in vitro with promastigote forms of *L. infantum* and *L. braziliensis*, at a ratio of 10:1 during 24 hours. The non-phagocytosed parasites, were removed by washing, and then the drugs (at 1, 10, 25, 50, 100 μM) were added. Macrophages with the drugs were incubated for 72 hours at 37ºC in 5% CO_2.

Drug activity was determined on the basis of number of amastigotes in treated and untreated cultures in methanol-fixed and Giemsa-stained preparations. The number of amastigotes was determined by analyzing 200 host cells distributed in randomly chosen microscopic fields. The antileishmanial effect is expressed as the IC_{50}

Values are the means of three separate determinations.

2.4.3. Axenic amastigote forms assay

Axenic amastigotes forms of *L. infantum* and *L. braziliensis* were cultured following the methodology described previously by Moreno *et al.* [11] Thus, promastigote transformation to amastigotes was obtained after three days of culture in M199 medium (Invitrogen, Leiden, The Netherlands) supplemented with 10% heat-inactivated FCS, 1 g/l β-alanine, 100 mg/l L- asparagine, 200 mg/l sacarose, 50 mg/l

sodium pyruvate, 320 mg/l malic acid, 40 mg/l fumaric acid, 70 mg/l succinic acid, 200 mg/l α-ketoglutaric acid, 300 mg/l citric acid, 1.1 g/l sodium bicarbonate, 5 g/l MES, 0.4 mg/l hemin, 10 mg/l gentamicine pH 5.4 at 37°C. The effect of each compound against Axenic amastigotes forms was tested at 48 hours using a Neubauer haemocytometric chamber. The antileishmanial effect is expressed as the IC_{50}.

2.5. Infection assay

J774.2 macrophage cells were grown under the same conditions expressed in amastigote forms assay during two days. Afterwards, the cells were infected *in vitro* with promastigote forms of *L. infantum* and *L. braziliensis*, at a ratio of 10:1. The drugs (IC_{25} concentrations) were added immediately after infection and were incubated for 12 hours at 37°C in 5% CO_2. The non phagocytosed parasites and the drugs were removed by washing, and then the infected cultures were grown for 10 days in fresh medium. Fresh culture medium was added every 48 h. The drug activity was determined from the percentage of infected cells and the number of amastigotes per infected cell (in treated and untreated cultures) in methanol-fixed and Giemsa-stained preparations. The percentage of infected cells and the mean number of amastigotes per infected cell were determined by analyzing 200 host cells distributed in randomly chosen microscopic fields. Values are the average of three separate determinations.

2.6. Metabolite excretion

Cultures of *L. infantum* and *L. braziliensis* promastigotes (initial concentration

5×10^5 cells/ml) received IC_{25} concentrations of the compounds (except for control cultures). After incubation for 96 hours at 28ºC, the cells were centrifuged at 400 g for l0 min. Then supernatants were collected to determine the excreted metabolites using ^{1}H-NMR, and, eventually, chemical displacements were expressed in parts per million (ppm), using sodium 2,2-dimethyl-2-silapentane-5-sulphonate as the reference signal. The chemical displacements used to identify the respective metabolites were consistent with those described by Fernández-Becerra *et al*. [12]

2.7. Ultrastructural alterations

The parasites were cultured at a density of 5×10^5 cells/ml in MTL medium, and the cultures contained drugs at the IC_{25} concentration.

After 96 hours, those cultures were centrifuged at 400 g for 10 min, and the pellets produced were washed in PBS and then mixed with 2% (v/v) p-formaldehyde–glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4) for 4 hours at 4ºC. After that, the pellets were prepared for TEM employing the technique of González *et al*. [4]

3. Results

3.1. In vitro antileishmanial evaluation

The IC_{50} values obtained on promastigote, axenic amastigote, and intracellular amastigote forms of *L. infantum* and *L. braziliensis* after 72 h of exposure with compounds **1-9** are displayed in the first three columns of Table 1. Values of the reference drug, Glucantime, are also included for all cases for comparison.

The anti-leishmania activity in both extra- and intracellular forms is similar or, in most cases, less than that found for Glucantime, with the compounds **7**, **8** and **9** presenting the lowest IC_{50}.

The macrophage toxicity of these compounds is of particular interest as all compounds tested show significantly less toxicity to macrophages than the reference drug, from 15 to 65 times (Table 1). Thus, compounds **4, 5, 6, 7** and **8** present IC_{50} values greater than 1000 μM, while compounds **1, 2, 3** and **9** give smaller IC_{50} values of 235.9 μM, 267.0 μM, 350.6 μM and 489.8 μM respectively.

Toxicity values substantially influence the more informative selectivity index data (SI, IC_{50} macrophage toxicity/IC_{50} activity of extracellular or intracellular forms of the parasite) which are shown in the last three columns of Table 1. The numbers of times that the compound SI exceeds the Glucantime SI are given in brackets. These values are very illustrative of the *in vitro* potential of the compounds tested with respect to the reference drug. Compounds **7**, **8**, **4**, **5** and **6**, showed the best SI for *L. infantum* (Table 1.A), with indexes exceeding that of the reference drug SI by more than 50 times. Compounds **1**, **2**, **3** and **9** were more effective than Glucantime, however, SI values of 50 times or greater were not reached, which is the criterion for a compound to be included in subsequent studies. [13]

Similar results can be extracted from the *L. braziliensis* data shown in Table 1B. The compounds **7**, **8**, **4** and **6** again gave the best SI results in the three assays performed, with values exceeding those of the reference drugs by 79, 103, and 59 times in the case of compound **7**, by 58, 78 and 100 times for compound **8,** by 59, 84 and 61 times for compound **4**, and by 53, 71 and 66 times for compound **6.** Although compound **5** did not give SI values greater than or equal to 50 times those of the reference drugs it was included in subsequent studies because promastigote and intracellular amastigote SI values were close to 50 times that of the reference drug, while the axenic amastigote SI was two times (Table 1.B).

The effectiveness of the compounds on the infection rate and the intracellular replication of the amastigote forms was determined by the infection assay (Fig. 2).

When selected flavonoid compounds **4-8** and Glucantime were added at their respective IC_{25} concentrations to macrophages infected with *Leishmania spp.* promastigote forms, the infection rate decreased significantly after 12 h with respect to the control measurement. The infection rate decreases followed the trend **8**>**5**>**7**=**4**>**6** for *L. infantum* (Fig. 2A) and **8**>**6**>**5**>**7**>**4** for *L. braziliensis* (Fig. 2B) with percentages of inhibition capacity of 95%, 79%, 77% and 71%, respectively, in the case of *L. infantum* and 88%, 82%, 79%, 69% and 68%, respectively, in the case of *L. braziliensis.* These values are remarkably higher than those for inhibition by Glucantime (63% and 58% for *L. infantum* and *L. braziliensis,* respectively).

All five compounds were more effective than Glucantime (only 12% decrease for *L. infantum* and 33% decrease for *L. braziliensis*) at decreasing the average number of amastigotes per infected macrophage cells, (Fig. 2C and Fig. 2D). Compound **8** was the most effective in *L. infantum* and *L. braziliensis.* The amastigote number decreases measured were as follows: **8** (78%) > **7** (49%) > **5** (45%) > **4** (37%) > **6** (24%) for *L. infantum* and **8** (82%) > **5** (72%) > **6** (69%) > **4** (61%) > **7** (55%) for *L. braziliensis.*

3.2. Metabolites excretion effect

After treatment of the parasites with compounds **4-8** at IC_{25}**,** the excretion of the catabolites was clearly altered. Fig. 3 displays the modifications observed in the height of the spectra peaks corresponding to the most representative final excretion products. From a careful examination of the data it would appear that there are marked differences in the catabolic pathway. In the case of *L. infantum*, compounds **4, 5, 6**, and **8** gave similar spectra, with an increase in the excretion of succinate and acetate, followed by malate, alanine, lactate and ethanol. On the other hand, compound **7** causes an increase in the production of malate, succinate, ethanol and lactate, but a decrease of excreted acetate and alanine levels (Fig. 3A).

In the case of *L. braziliensis,* the increase of excreted succinate was the most significant change when the parasites were treated with the compounds. Compounds **6, 7** and **8** caused decreases in the excretion of acetate (Fig. 3B).

3.3. Ultrastructural alterations

The transmission electron microscope evaluation of flavonoid compounds **4-8** against *Leishmania spp* promastigotes showed notable ultrastructural alterations, as reflected in Fig. 4 and Fig. 5 (panels B, C, D, E) with respect to the control (Fig. 4 and 5, panel A). All compounds produced significant alterations to *L. infantum*, but the compounds most effective were **4** and **8**, as illustrated in Fig. 4 (panel B and E). All of the compounds induced the formation of strongly electrodense inclusions that

appeared inside and outside vacuoles with different sizes, or directly in the cell cytoplasm. The major effect of compound **8** was that many of the parasites adopted distorted shapes with distorted cytoplasm, while no cytoplasmic organelles were visible due to the altered appearance of promastigotes. However, large vacuoles containing cellular debris were visible. With compound **4** (Fig. 4, panel B), many of the parasites appeared dead, while another showed pronounced vacuolization, and many of these vacuoles contained strongly electrodense inclusions. Compound **5** produced similar effects (Fig. 4, panel C). In the same way, compound **6** induced empty vacuoles, lipid vacuoles and a large number of electrodense vesicles (Fig. 4, panel D). By contrast, compound **7** showed no induction of substantial alterations (photograph not shown).

Studies of ultrastructural alterations made on *L. braziliensis* showed that the compounds produced changes similar to those they produced in *L. infantum.* Another effect of the compounds was the expansion of the kinetoplast, which appeared swollen when treated with compound **4** (Fig. 5, panel B). **C**ompound **8** (Fig. 5, panel E) induced changes in the shape of parasites, with them showing completely altered forms. All these changes occurred after treatment with compound **7** (Fig. 5, panel D), while compound **5** produced no significant changes compared to the control (data not shown).

4. Discussion

Many people living in rural areas have no easy access to conventional allopathic treatments, largely due to the limited availability of health services and their low socioeconomic status. Therefore, plants may provide an important and necessary source of therapeutic medicinal compounds. Flavones and their derivatives, flavonoids and flavonols, are among the most attractive plant derivatives that might enrich the current therapy options, due to their extremely large range of biological properties. [14]

In this study, *in vitro* biological activity of nine flavonoid compounds against extracellular and intracellular forms *Leishmania spp.* was investigated. This is important as many studies of the activity of compounds against *Leishmania* spp. are performed on promastigote forms, which are much easier to work *in vitro.* However, since extracellular forms are not the developed forms of the parasite in vertebrate hosts, evaluations made with these forms are merely indicative of the potential leishmanicidal activity of the compounds tested. Consequently, a preliminary test using extracellular promastigote forms should always be complemented by a subsequent evaluation using intracellular forms (amastigotes in vertebrate host cells), so that a better understanding of the activity may be obtained. [4]

The petiolaroside-based derivatives **(7-9)** presented the lowest IC_{50} values against extra and intracellular forms.

Previously, the nine flavonoids investigated here were tested against epimastigote forms of *Trypanosoma cruzi* and the most effective compounds were astragalin (**1**) and two acetylated derivatives (**2** and **3**). These had significantly lower IC_{50} values compared with Benznidazole. [15] By contrast, in this study, we saw that the astragalin and its derivates were the least effective agents against *Leishmania spp*.

Petiolaroside, 2´´ acetylpetiolaroside (**7** and **8** respectively), and paenoside and its derivates (**4, 5** and **6**), showed the best selectivity indexes. These indexes exceeded the reference drug SI by more than 50 times. The astragalin and its derivates (**1, 2** and **3**), and petiolaroside decacetate (**9**), were all more effective than Glucantime.

If we compare the results obtained for the infection rate and the number of amastigotes per infected macrophage cells for both *Leishmania* species, it can be concluded that 2´´-acetylpetiolaroside (**8**) is clearly the most active. The acetylated derivatives 2´´-acetylpetiolaroside (**8**) and 2´´-acetylpaeonoside (**5**) are more active than petiolaroside (**7**) and paeonoside (**4**) respectively.

As far as is known to date, none of the trypanosomatids studied are capable of completely degrading glucose to CO_2 under aerobic conditions. When this occurs, it results in the excretion of a major part of the carbon skeleton into the medium as fermented metabolites, which can differ according to the employed species. [16] ^{1}H-NMR spectra enabled us to determine the fermented metabolites excreted by the parasites during their *in vitro* culture. The final products of glucose catabolism in Leishmania are usually CO_2, succinate, acetate, L-lactate, pyruvate, L-alanine, and ethanol. [17] As with *T. cruzi*, [18] one of the major metabolites excreted by Leishmania spp. is succinate, with the main role likely to be to maintain the glycosomal redox balance by providing two glycosomal oxidoreductase enzymes. These enzymes allow reoxidation of NADH produced by glyceraldehyde-3-phosphate dehydrogenase in the glycolytic pathway. Succinic fermentation offers one significant advantage as it requires only half the produced phosphoenolpyruvate (PEP) to maintain the NAD+/NADH balance. The remaining PEP is converted to acetate, depending on the species under consideration.

The majority of metabolites excreted by *L. infantum* are succinate and acetate. In the case of *L. infantum,* variations in the final catabolism products seem to be dependent on the structural aspects of the compounds assayed. These data agree well with those of other authors. [18]

Analyses of *in vitro* and *in vivo* results of *Trypanosoma cruzi* showed that the best potential flavonoids to treat Chagas disease were the acetylated compounds **2**, **5** and **6**. [15] The present work confirmed this conclusion in the case of visceral

leishmaniasis (*L. infantum*) and mucocutaneous leishmaniasis (*L. braziliensis*). Moreover, these data are consistent with others previously published, which showed that several acetylated flavonoids derived from *Consolida oliweriana* were very active *in vitro* against both extracellular and intracellular forms of *L. (V.) peruviana* and *L. (V.) braziliensis.* [9]

In conclusion, this study has demonstrated that flavonoid derivatives are active against *L. infantum* and *L. braziliensis.* These results support further investigation of flavonoid compounds as potential agents against Leishmaniasis.

5. Acknowledgments

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Figure captions

Figure 1. Flavonoid compounds investigated.

Figure 2. Effects of flavonoids **4, 5, 6, 7** and **8** on the infection and growth rates of *Leishmania spp*. (A), rate of infection of *L. infantum*; (C), mean number of amastigotes per infected J774 A.2 macrophage cells of *L. infantum*; (B), rate of infection of *L. braziliensis*; (D), mean number of amastigotes per infected J774 A.2 macrophage cells of *L. braziliensis*. (-▲-, control; - Δ -, comp. **4**; -▼-, comp. **5**; - □ -, comp. **6**; - ◊ -, comp. **7**; - ▽ -, comp. **8**; - ○ -, Glucantime). At IC_{25} conc., values are means of three separate experiments.

Figure 3. Variation in the height of the peaks corresponding to catabolites excreted by *L. infantum* (A) and *L. braziliensis* (B) promastigote forms in the presence of flavonoid derivatives with respect to the control test.

Figure 4. Ultrastructural alterations by TEM in *L. infantum* treated with the flavonoid derivatives. Panel A: Control parasite of *L. infantum* showing organelles with their characteristic features such as the nucleus (N), kinetoplast (K), reservosomes (R), mitochondrion (M) and vacuoles (V). (Bar: 1µm). Panel B**.** *L. infantum* treated with compound **4.** Electrodense organelles (arrow) (Bar: 1µm). Panel C. *L. infantum* treated with compound **5** with intense vacuolization (V) (Bar: 1.59 µm). Panel D**.** *L. infantum* treated with compound **6**, vacuoles (V), lipid vacuoles (LV), electrodense vesicles (arrow) (Bar: 1.59 µm). Panel E**.** *L. infantum* treated with compound **8** with distorted parasites (arrow) (Bar: 1 µm).

Figure 5. Ultrastructural alterations by TEM in *L. braziliensis* treated with flavonoid derivatives**.** Panel A. Control parasite of *L. braziliensis* with structures such as the nucleus (N), reservosomes (R), vacuoles (V) and mitochondrion (M). (Bar: 1µm). Panel B. *L. braziliensis* treated with compound **4** with vacuoles (V) and swelling kinetoplast (arrow) (Bar: 1µm). Panel C. *L. braziliensis* treated with compound **6** (Bar: 1.59 µm). Panel D. *L. braziliensis* treated with compound **7** with intense vacuolization (V), electrodense organelles (arrow) and swelling kinetoplast (K) (Bar: 2.33µm). Panel E. *L. braziliensis* treated with compound **8** with distorted forms (Bar: 1.59 µm).

Table 1. *In vitro* activity, toxicity and selectivity index found for the astragalin-based (1-3), paeonoside-based (4-6) and petioloroside-based (7-9) derivatives on extracellular and intracellular forms of *Leishmania spp.*

***Leishmania infantum* (1)**

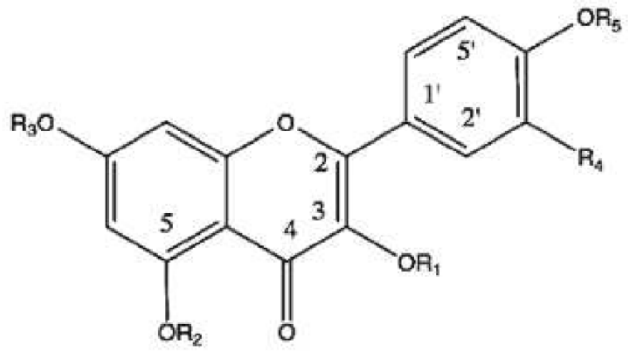
Compounds	Activity IC_{50} (μM)[a]			[b]Macrophage toxicity IC_{50}	SI		
	Promastigote forms	Axenic amastigote forms	Intracellular amastigote forms		Promastigote forms	Axenic amastigote forms	Intracellular amastigote forms
Glucantime®	18.0 ± 3.1	24.2 ± 2.6	30.0 ± 2.7	15.2 ± 1.3	0.8	0.6	0.5
1	35.1± 3.3	29.2 ± 2.8	45.9 ± 2.7	235.9 ± 17.9	6.7 (8)	8.1 (14)	5.1 (10)
2	34.1 ± 1.6	32.7 ± 5.0	42.3 ± 3.9	267.0 ± 11.0	7.8 (10)	8.2 (14)	6.3 (13)
3	40.4 ± 3.4	29.2 ± 3.1	38.1 ± 1.6	350.6 ± 26.8	8.7 (11)	12.0 (20)	9.2 (18)
4	25.1 ± 1.9	26.9 ± 1.4	31.4 ± 2.0	>1000.0 ± 60.0	39.8 (50)	37.2 (62)	31.8 (64)
5	24.6 ± 3.7	29.8 ± 2.2	37.2 ± 3.5	>1000.0 ± 71.4	40.7 (51)	33.6 (56)	26.9 (54)
6	23.1 ± 4.6	30.5 ± 2.3	40.3 ± 2.4	>1000.0 ± 82.0	43.3 (54)	32.8 (55)	24.8 (50)
7	24.5 ± 1.1	19.4 ± 1.4	27.8 ± 1.4	>1000.0 ± 55.6	40.8 (51)	51.5 (86)	36.0 (72)
8	19.1 ± 0.9	18.4 ± 1.3	32.1 ± 2.0	>1000.0 ± 38.9	52.4 (66)	54.3 (91)	31.2 (62)
9	22.8 ± 1.7	21.5 ± 0.9	31.0 ± 1.7	489.8 ± 22.4	21.5 (27)	22.8 (38)	15.8 (32)

Leishmania braziliensis (B)

Compounds	Activity IC_{50} (μM)[a]			[b]Macrophage toxicity IC_{50}	SI[c]		
	Promastigote forms	Axenic amastigote forms	Intracellular amastigote forms		Promastigote forms	Axenic amastigote forms	Intracellular amastigote forms
Glucantime®	25.6 ± 1.6	30.4 ± 6.1	31.1 ± 3.0	15.2 ± 1.3	0.6	0.5	0,5
1	28.1 ± 1.0	22.5 ± 1.7	31.2 ± 1.6	235.9 ± 17.9	8.4 (14)	10.4 (21)	7.3 (15)
2	25.1 ± 2.5	27.9 ± 2.7	30.2 ± 3.8	267.0 ± 11.0	10.6 (18)	9.6 (19)	8.8 (18)
3	28.4 ± 3.0	32.9 ± 3.0	41.9 ± 4.0	350.6 ± 26.8	12.3 (21)	10.7 (21)	8.4 (17)
4	28.1 ± 5.2	23.7 ± 2.1	31.7 ± 2.1	>1000.0 ± 60.0	35.6 (59)	42.2 (84)	30.6 (61)
5	34.1 ± 6.6	19.4 ± 0.9	50.9 ± 6.2	>1000.0 ± 71.4	29.3 (49)	51.5 (100)	20.0 (40)
6	31.3 ± 3.0	28.3 ± 1.6	30.4 ± 2.7	>1000.0 ± 82.0	31.9 (53)	35.7 (71)	32.9 (66)
7	21.1 ± 1.7	19.5 ± 0.8	33.9 ± 3.5	>1000.0 ± 55.6	47.4 (79)	51.3 (100)	29.5 (59)
8	29.0 ± 1.5	25.5 ± 0.7	20.1 ± 1.7	>1000.0 ± 38.9	34.5 (58)	39.2 (78)	49.8 (100)
9	32.3 ± 3.3	24.5 ± 1.3	21.0 ± 1.3	489.8 ± 22.4	15.2 (25)	19.9 (40)	23.3 (47)

Results are averages of three separate determinations.
[a] IC_{50}: the concentration required to give 50% inhibition, calculated by linear regression analysis from the Kc values at concentrations employed (1, 10, 15, 50 and 100 μM).
[b] Against J774.2 macrophages after 72 h of culture.

[c] Selectivity index = IC_{50} macrophages/IC_{50} extracellular and intracellular form of the parasite. In brackets: number of times that compound SI exceeds the reference drug SI.



1 astragalin (R_1 = Glc, R_2 = R_3 = R_4 = R_5 = H)
2 2″-acetylastragalin (R_1 = Glc 2″-Ac, R_2 = R_3 = R_4 = R_5 = H)
3 astragalin heptaacetate (R_1 = Glc-Ac, R_2 = R_3 = R_5 = Ac, R_4 = H)
4 paeonoside (R_1 = R_3 = Glc, R_2 = R_4 = R_5 = H)
5 2″-acetylpaeonoside (R_1 = Glc 2″-Ac, R_3 = Glc, R_2 = R_4 = R_5 = H)
6 paeonoside decaacetate (R_1 = R_3 = Glc-Ac, R_2 = R_5 = Ac, R_4 = H)
7 petiolaroside (R_1 = Glc, R_3 = Rha, R_2 = R_5 = H, R_4 = OH)
8 2″-acetylpetiolaroside (R_1 = Glc 2″-Ac, R_3 = Rha, R_2 = R_5 = H, R_4 = OH)
9 petiolaroside decaacetate (R_1 = Glc-Ac, R_3 = Rha-Ac, R_2 = R_5 = Ac, R_4 = OAc)
(Glc = β-glucopyranosyl; Rha = α- rhamnopyranosyl)

Figure 1.

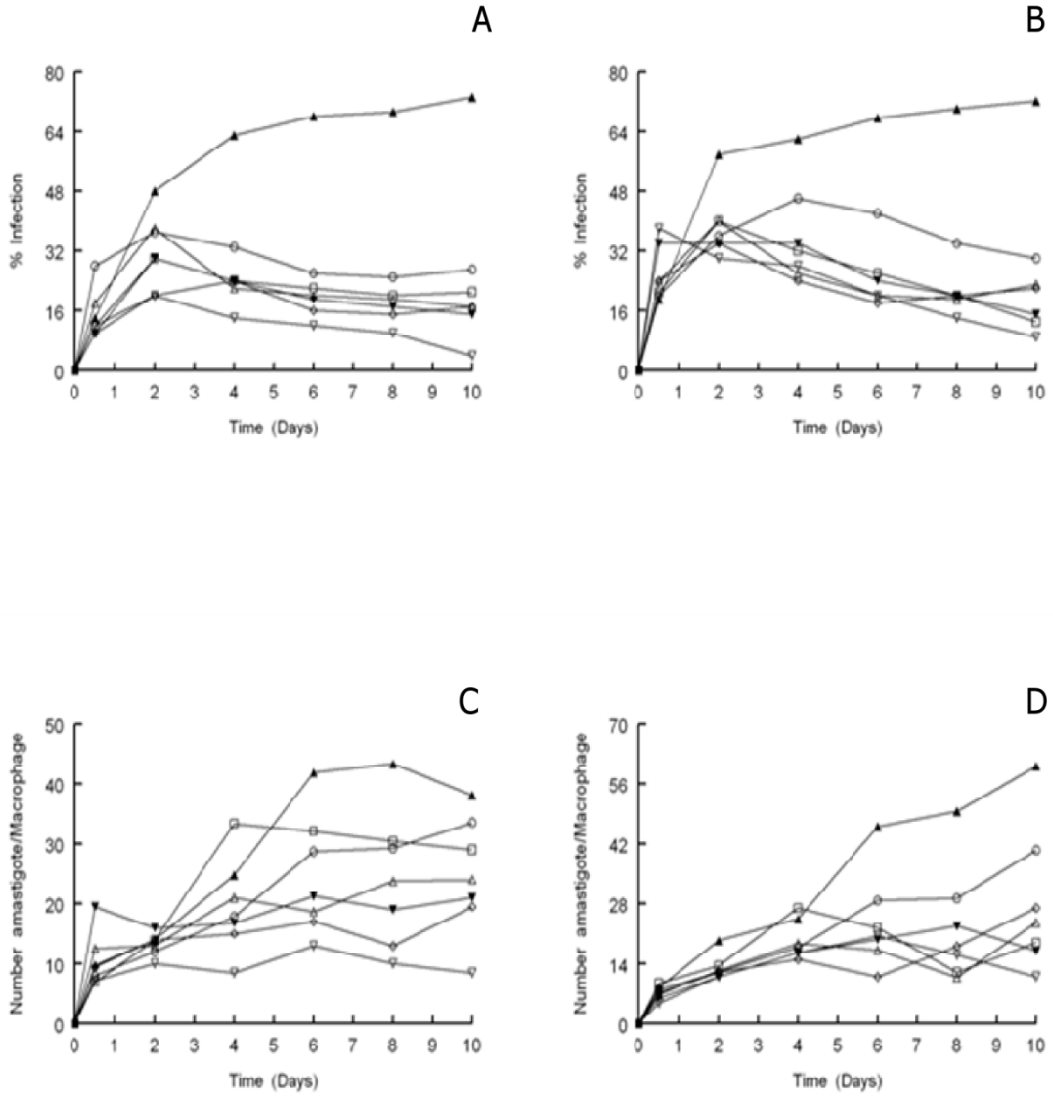


Figure 2.

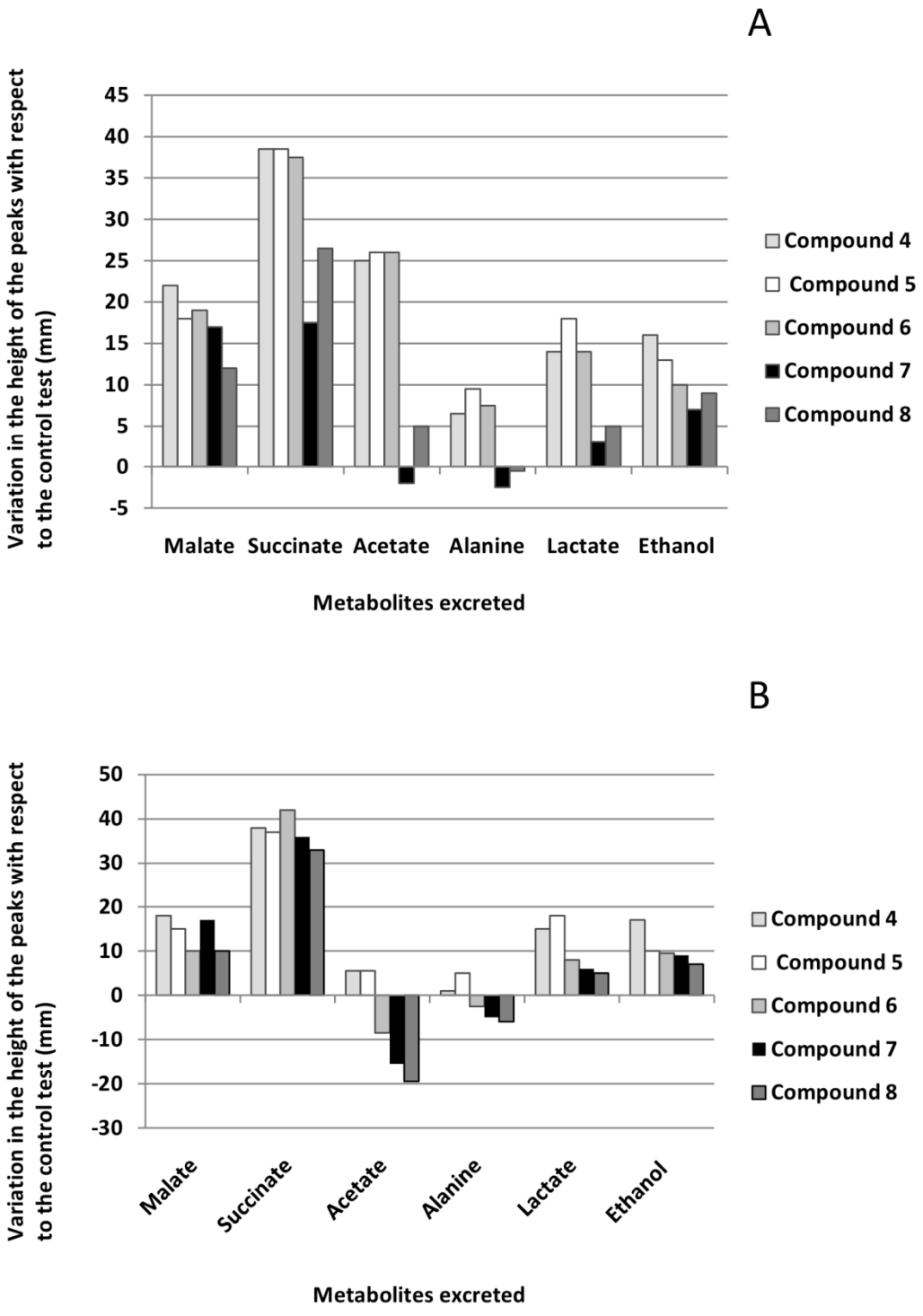

A
Variation in the height of the peaks with respect to the control test (mm)
45
40
35
30
25
20
15
10
5
0
-5
Malate
Succinate
Acetate
Alanine
Lactate
Ethanol
Metabolites excreted
Compound 4
Compound 5
Compound 6
Compound 7
Compound 8
B
Variation in the height of the peaks with respect to the control test (mm)
50
40
30
20
10
0
-10
-20
-30
Malate
Succinate
Acetate
Alanine
Lactate
Ethanol
Metabolites excreted
Compound 4
Compound 5
Compound 6
Compound 7
Compound 8

Figure 3.

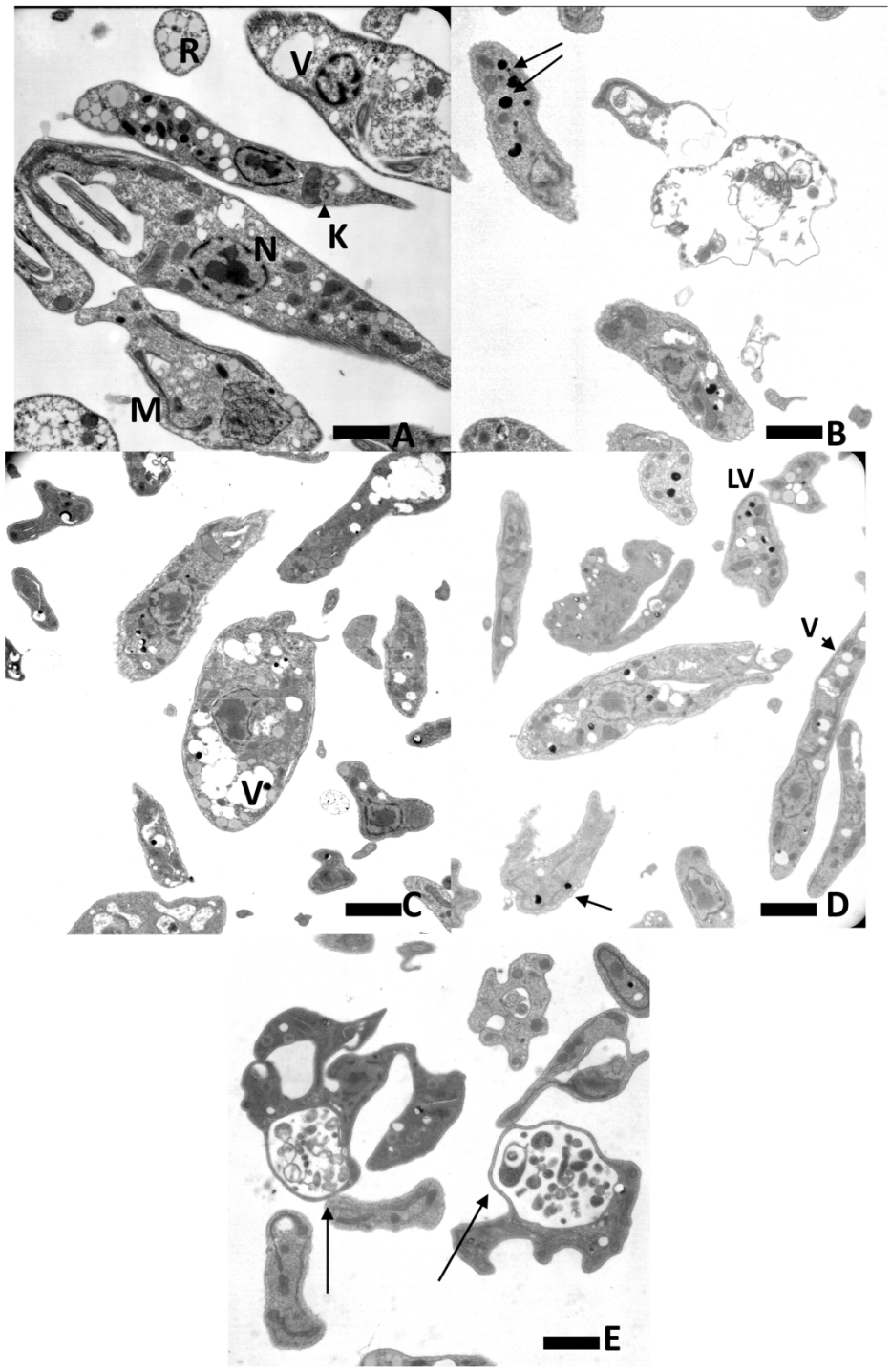


Figure 4.

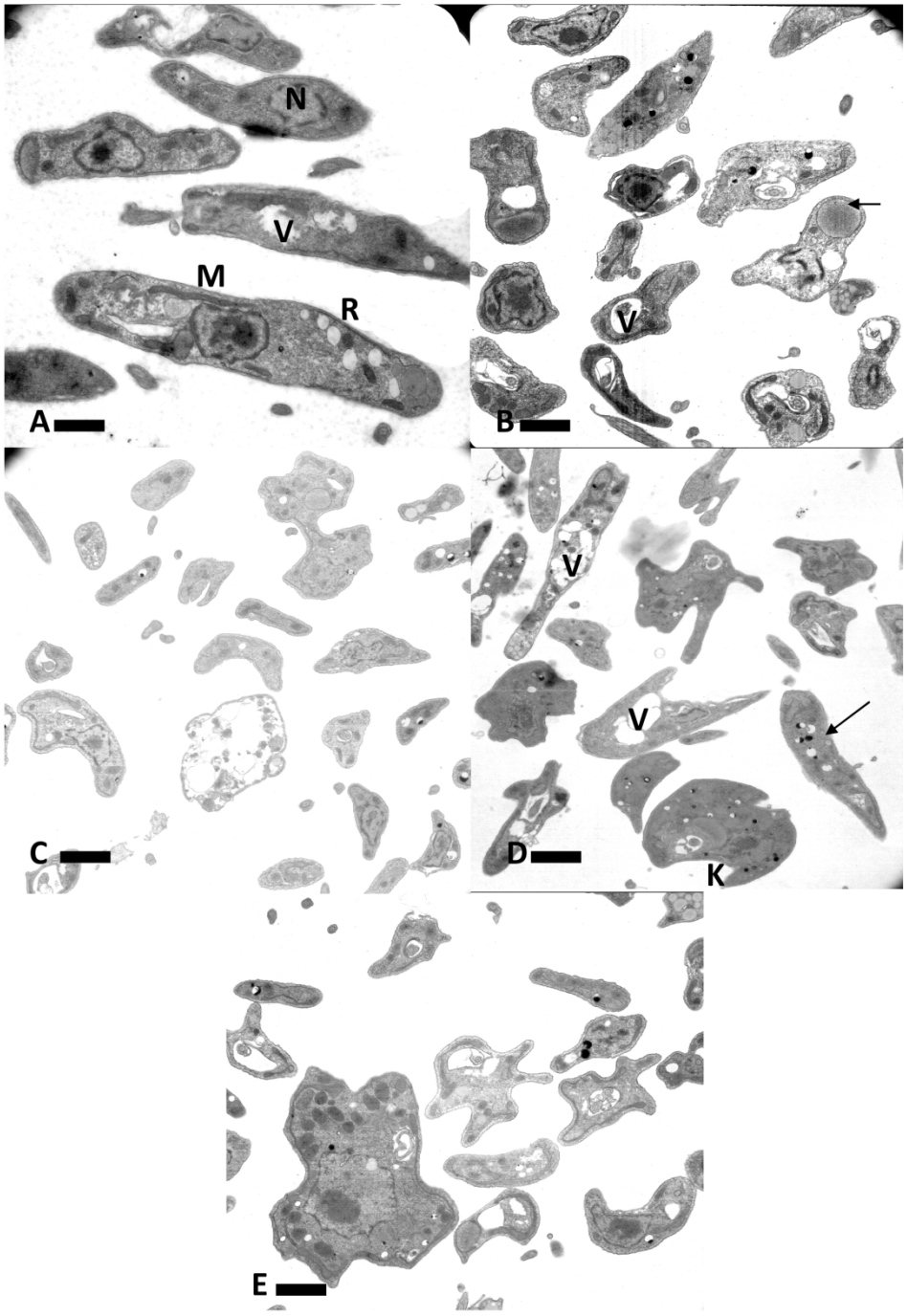


Figure 5.

Estado actual del proceso de publicación:

Dear Dr. Marín,

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5.- DISCUSIÓN.

El acceso a los medicamentos esenciales se ha convertido en un importante motivo de preocupación internacional. Un tercio de la población mundial, la mayoría de ella, residente en países pobres, carece de acceso a estos medicamentos esenciales.

Dentro de estos medicamentos esenciales, se encuentran muchos medicamentos de las enfermedades olvidadas como la enfermedad de Chagas y las leishmaniasis, cuyo acceso es con frecuencia inhibido por varias razones interconectadas, incluyendo los altos precios de los medicamentos, la distribución desigual de los mismos, la falta de centro de salud o del personal necesario para su prescripción y administración y el mal diagnóstico. En los últimos años, la falta de disponibilidad de medicamentos o la falta de acceso a ellos se han considerado como violaciones de los derechos a la salud y a la vida. (Hunt P. TDR/SDR/SEB/ST/07.2)

La necesidad de desarrollar nuevos tratamientos seguros, eficaces y económicos contra la enfermedad de Chagas y las distintas formas de leishmaniasis, constituye la idea fundamental del trabajo presentado en esta tesis.

Por ello, y gracias a colaboraciones con otros grupos: grupo del Prof. Fernando Gómez Contreras de la Universidad Complutense de Madrid y de la Doctora Pilar Navarro (CSIC); grupo del Prof. Juan Manuel Salas Peregrín de la Universidad de Granada; grupo del Prof. Enrique Álvarez de Manzaneda Roldán de la Universidad de Granada; grupo del Prof. Jesús González Díaz de la Universidad

de La Laguna, se han estudiado los efectos de distintos compuestos de nueva síntesis y extraídos de plantas sobre los parásitos causantes de dichas enfermedades y sobre las células de sus hospedadores.

Los compuestos se pueden clasificar en:

- Derivados imidazol y pirazol Benzo[*g*]ftalazina.
- Derivados triazolopirimidinas y complejos metálicos.
- Derivados flavonoides.
- Derivados terpenoides.

5.1. Derivados imidazol y pirazol Benzo[*g*]ftalazina.

(*J Antimicrob Chemother* 2012; 67: 387–397; *J Med Chem* 2011; 54: 970-9.)

La supervivencia de los parásitos protozoarios está estrechamente relacionada con la capacidad de determinadas enzimas para evadir el daño originado por radicales libres tóxicos procedentes de sus hospedadores. Como se ha comentado con anterioridad, la superóxido dismutasa de hierro (Fe-SOD), juega un papel relevante como defensa antioxidante en las especies de la familia de los tripanosomátidos. Por tanto, aquellos compuestos que inhiben la acción protectora

de la Fe-SOD son buenas dianas de actividad antiparasitaria que afecta tanto al crecimiento como a la supervivencia de los parásitos.

En estudios previos, se han encontrado una serie de derivados de benzo[g]ftalazina con capacidad para complejar iones metálicos de transición, que en forma libre se comportan como inhibidores de Fe-SOD. (Rodríguez-Ciria y col. 2007, Sanz y col. 2008).

Por ello, en esta tesis, hemos estudiado el efecto de cuatro derivados imidazol (compuestos **1**, **2**, **3**, **4**) y dos nuevos derivados pirazol benzo[*g*]ftalazina (compuestos **5**, **6**) frente a los parásitos *T. cruzi, L. infantum* y *L. braziliensis.*

El estudio de actividad *in vitro* de los compuestos **1**, **2**, **3** y **4**, frente a las formas epimastigotas del parásito, la capacidad de invasión celular, la posterior multiplicación intracelular y la liberación de formas tripomastigotas al adicionar los productos, el daño producido por los compuestos sobre las células de mamíferos y el marcado efecto inhibidor de la enzima originado por los cuatro derivados imidazol, se ha estudiado anteriormente. (Sanz y col. 2008). Ahora, se ha ampliado su estudio y se han comparado los resultados con los obtenidos por los compuestos **5** y **6.**

La actividad tripanocida de los derivados pirazol, es similar a la presentada por Benznidazol en el caso del compuesto monosustituido (compuesto **6**). El

compuesto disustituido resulta menos activo que la droga de referencia. (compuesto **5**). Los derivados imidazol, presentan mejores resultados.

Estudiando el efecto de los compuestos derivados de pirazol sobre las células de mamífero, se ve que estos productos son muy poco tóxicos para ellas con respecto al Benznidazol, por lo que el Índice de Selectividad (SI) de estos productos supera al del Benznidazol en tres (compuesto **5**) y nueve (compuesto **6**) veces.

Comparando estos resultados con los de los derivados imidazol, se observa que los SI de los derivados pirazol, son significativamente menores que los de los derivados imidazol.

La capacidad de invasión celular, cuando los parásitos y los productos se añaden al mismo tiempo, se ve afectada de forma significativa, llegando a disminuir un 63% (compuesto **5**) y un 68% (compuesto **6**) en el día 10 post-infección con respecto al control. Estos porcentajes de inhibición también exceden a los del Benznidazol, que disminuyen un 39%.

Con respecto al número de formas amastigotas presentes en las células Vero, se observa que los derivados pirazoles inhiben la replicación de la forma intracelular del parásito, siendo la inhibición mayor en el compuesto **6**. La liberación de las

formas tripomastigotas al medio también se reduce con respecto al control y al Benznidazol.

Al comprobar la actividad de los derivados imidazol y pirazol *in vivo*, en modelo murino, sobre la fase aguda y crónica de la enfermedad, se comprueba que durante el conteo de la parasitemia en la fase aguda, los seis compuestos ensayados presentan una disminución del número de formas tripomastigotas libres en el medio, y en el día 40 post-infección, la disminución de la parasitemia en los derivados monoalquiloamino es mayor que la de los derivados disustituidos.

La técnica de la ELISA, permite el estudio de la fase crónica, demostrando que los compuestos monosustituidos **6**, **4** y **2**, disminuyen los niveles de anticuerpos entre los días 40 y 120, mostrando un mayor rendimiento que el Beznidazol. Además, la diferencia en el nivel de anticuerpos anti-*T. cruzi*, concuerda con los resultados del conteo de la parasitemia.

Haciendo un análisis histopatológico del hígado de estos ratones el día noventa después de la infección, se observa que los compuestos monosustituidos presentan menos alteraciones hepáticas que el Benznidazol, lo que indica la menor toxicidad de estos productos. El compuesto **4** es el que presenta menos daños.

Frente a *L. infantum* y *L. braziliensis,* se han realizado ensayos de inhibición de la actividad de las formas promastigotas, de unas formas creadas artificialmente

en el laboratorio a las que llamamos formas axenic-amastigotas, (Moreno y col. 2011) y de las formas amastigotas intracelulares.

El ensayo de inhibición de estas tres formas y el cálculo de su IC_{50} (concentración necesaria para inhibir al 50% el crecimiento del parásito), nos permite tener un estudio más detallado de cómo afectan los productos a cada estadío del parásito presente tanto en el hospedador mamífero como en el vector.

Los ensayos de actividad de los productos frente a las dos especies de *Leishmania,* presentan un comportamiento muy similar en ambas especies. Tampoco se observan grandes diferencias frente las distintas formas del parásito, siendo los compuestos **2** y **6** los que muestran mejor índice de selectividad.

Los ensayos realizados para ver el porcentaje de infección y la replicación intracelular de los amastigotes nos permite ver que todos los compuestos ensayados son más efectivos que el Glucantime. Como ocurre en el estudio de los productos frente a *T. cruzi,* se observa que los derivados monosustituidos son más activos que los derivados disustituidos, y los derivados de imidazol más activos que los derivados pirazol. La toxicidad que presentan sobre los macrófagos es menor que la del Glucantime.

Haciendo mención a la inhibición de la actividad de la enzima Fe-SOD, se observa un significativo descenso de la actividad enzimática cuando los seis compuestos son administrados a las tres especies de parásitos, mostrando los

mejores resultados los compuestos **2** y **4** en *T. cruzi* y los compuestos **5** y **6** frente a *L. infamtum*. En *L. braziliensis,* la inhibición de la actividad fue similar con los seis compuestos.

Estos compuestos, además de producir una fuerte inhibición en la actividad de la Fe-SOD del parásito, no inhiben a la Cu/Zn-SOD humana, por lo que se convierten en unos fuertes candidatos para el tratamiento de la enfermedad de Chagas y la leishmaniasis.

Los tripanosomátidos, no son capaces de degradar la glucosa completamente a CO2 en condiciones aeróbicas, por lo que excretan al medio de cultivo metabolitos fermentativos según las necesidades de las especies consideradas (Ginger 2005, Bringaud y col. 2006).

La Resonancia Magnética Nuclear de Protones (^{1}H-RMN), nos permite determinar los metabolitos fermentativos excretados por los tripanosomátidos durante su cultivo in vitro.

T. cruzi excreta succinato y acetato como metabolitos mayoritarios y L-alanina como metabolito minoritario. En el caso de las formas promastigotas de *L. infantum*, los metabolitos mayoritariamente excretados son acetato, succinato, L-alanina y en un menor proporción D-lactato; datos que están de acuerdo con los obtenidos por otros autores (Turrens 1999, Ginger 2005). En el caso de *L. braziliensis* también se observa la presencia de piruvato y etanol.

Cuando los tripanosomátidos se tartan con los seis compuestos, los catabolitos excretados se alteran. En *T. cruzi,* la excreción de succinato y acetato se inhibe y aumenta la producción de L-alanina con los compuestos **1**-**4**. Por lo que se puede pensar que los derivados imidazol actúan a nivel de la cadena transportadora de electrones. Los derivados de pirazol, exhiben un comportamiento diferente. El succinato es el único metabolitos inhibido por el compuesto **5**, y un nuevo pico, identificado como malato aparece. Esto podría explicarse como una actuación a nivel de la enzima fumarato reductasa. El compuesto **6** puede inhibir la succinato deshidrogenasa.

En *L. infantum,* se puede interpretar que los cambios metabólicos pueden ser debidos a la estructura de las cadenas laterales, ya que los compuestos **1** y **2** disminuyen la excreción de acetato y succinito, los compuestos **5** y **6** producen un aumento de los catabolitos y los compuestos **3** y **4** no presentan una alteración muy marcada. Por otra parte, *L. braziliensis,* sufre cambios similares en presencia de los distintos compuestos.

El marcado efecto tripanocida y leishmanicida, de los derivados imidazol y pirazol Benzo[*g*]ftalazina, produce importantes alteraciones morfológicas tanto en las formas epimastigotas como en las formas promastigotas llegando incluso a producir la lisis del parásito.

5.2. Derivados triazolopirimidinas y complejos metálicos.

(*J Antimicrob Chemother*. 2011; 66: 813-9; *J Inorg Biochem*. 2011; 105: 770-6; **In vitro anti-leishmania evaluation of nickel complexes with a triazolopyrimidine derivative against *Leishmania infantum* and *Leishmania braziliensis.*** Artículo sometido en la revista *J Inorg Biochem.* 2011.)

La fusión de un anillo triazol con un anillo pirimidínico da lugar a la formación de heterociclos nitrogenados llamados triazolopirimidinas.

Estos derivados guardan una gran similitud con las bases nucleicas púricas tanto en su estructura como en su comportamiento. Esto, confiere a los derivados y a sus complejos metálicos una potente actividad biológica, (Holý y col. 1996, El-Koussi y col. 2004, Havlicek y col. 2005, Giorgi y col. 2006, Chen y col. 2009, Guijar y col. 2009, Tang y Shi 2010, Guijar y col. 2011), actuando como antifúngicos, antimaláricos, herbicidas, antivirales, antitumorales… Además, se han realizado diversos estudios anteriores que muestran la actividad que presentan estos compuestos frente a distintos géneros y especies de la familia de los tripanosomátidos. (Luque y col. 2000[a],[b], Magán y col. 2004[a,b], 2005, Boutaleb-Charki y col. 2009, Maldonado y col. 2010).

- **Complejos de cobre y cobalto con triazolopirimidinas** (*J Antimicrob Chemother.* 2011; 66: 813-9; *J Inorg Biochem.* 2011; 105: 770-6)

Se han ensayado tres compuestos, los cuales son complejos que resultan de la interacción de 7-hidroxi-5-metil-1,2,4-triazolo[1,5-*a*]pirimidina (HmtpO) con metales de transición divalentes (cobre y cobalto).

El compuesto **1,** es un compuesto de Cu II monomérico. El compuesto **2**, es un complejo de Cu II, que a diferencia del complejo anterior, la estructura de este compuesto consiste en capas catiónicas formando redes aproximadamente cuadradas. El compuesto **3**, es un complejo de Co II y presenta una estructura polimérica en cadenas.

Al realizar los ensayos de inhibición del crecimiento *in vitro* de las formas epimastigotas de *T. cruzi* y las formas promastigotas de *L. infantum* y *L. braziliensis* se comprueba que con excepción de los compuesto **1** y **3**, frente a *L. braziliensis,* todos los compuestos presentan menor actividad que la droga de referencia, pero la toxicidad que originan en las células de mamíferos es mucho menor que la causada por el Benznidazol y el Glucantime. Estos buenos resultados de toxicidad en las células vero y macrófagos hacen que el SI de estos productos frente a los distintos parásitos sea mucho mayor que los que presentan el Benznidazol y Glucantime. Tanto en *L. infantum* como en *L. braziliensis,* los SI de estos compuestos superan

alrededor de 30 veces a los presentados por el Glucantime y son parecidos en los distintos compuestos. En el caso de *T. cruzi*, el compuesto **1**, presenta un SI mayor a los que presentan los dos compuestos restantes, siendo este 21 veces mayor que el SI del Benznidazol. Los SI de los compuestos **2** y **3** superan al del Benznidazol 12 y 10 veces respectivamente.

Es interesante destacar que los tres complejos metálicos presentaron también unos SI mucho mayores al presentado por el derivado triazolopirimidina (Frente *T. cruzi,* SI presentado por HmtpO: 2; Frente a *L. infantum* y *L. braziliensis,* SI presentado por HmtpO: 1,6 en ambos casos). (Caballero 2010). Estos resultados muestran el importante papel que juegan los iones metálicos en la actividad antiparasitaria.

Con respecto al porcentaje de infección de las células vero con *T. cruzi* cuando los productos se añaden durante 12 horas, se observa que los tres compuestos presentan una inhibición significativa de la infección con respecto al control y mayor a la inhibición presentada por el Benznidazol, y siendo el compuesto **1**, el que presenta mayor inhibición. Este compuesto también es el más eficaz al inhibir la replicación intracelular de las formas amastigotas.

Al igual que con *T. cruzi,* el porcentaje de células infectada disminuye significativamente con los tres compuestos, y los resultados son mejores que los de la droga de referencia, siendo los compuestos **1**, **3** y **2** los más efectivos frente a *L.*

infantum inhibiendo la infección un 84%, 79%, 67% respectivamente, y los compuestos **3**, **1** y **2** los más eficaces frente a *L. braziliensis.*(inhibición del 86%, 79% y 75%).

La replicación intracelular también se ve afectada negativamente con la presencia de estos compuestos en las dos especies de *Leishmania* estudiadas.

Frente a *T. cruzi,* además de llevarse a cabo los experimentos *in vitro,* se han realizado ensayos *in vivo*.

Después de tratar a los ratones durante cinco días con una dosis de 1 mg/Kg de peso por día, se comprueba que la parasitemia decrece, apreciándose esta disminución claramente a partir del día 22-24 postinfección y continuándose hasta el día 40. El compuesto más efectivo, al igual que en los ensayos *in vitro,* se corresponde con el compuesto **1**.

Al estudiar los metabolitos excretados al medio cuando los tres complejos triazolopirimidinas son añadidos, se aprecia que tanto en las dos especies del género *Leishmania* como en *T. cruzi,* el único compuesto que origina cambios en dichos metabolitos es el compuesto **2**. Cuando este compuesto se añade a un cultivo de formas epimastigotas de *T. cruzi,* en los espectros de ^{1}H-RMN se observa una disminución del acetato y succinato, apareciendo glicerol como nuevo metabolito excretado. Esto puede deberse a que el compuesto **2**, inhibe las enzimas glicosomales y esta inhibición conduce a la formación de glicerol.

La excreción de succinato y acetato disminuye también tanto en *L. infantum* como en *L. braziliensis* y aparece un nuevo pico que se corresponde con la excreción de piruvato. Este cambio al igual que en el caso de *T. cruzi* se debe a la inhibición de las enzimas glicosomales.

Analizando las alteraciones ultraestructurales que aparecen en las formas promastigotas de sendas especies de *Leishmania,* se puede apreciar que todos los compuestos alteran a dichos parásitos, pero el compuesto **2** es el que mayores cambios presenta. Estos cambios pueden estar provocados por la inhibición de las enzimas glicosomales como hemos señalado en el apartado de los metabolitos excretados.

Frente a *T. cruzi,* los mayores cambios se observan cuando las formas epimastigotas se tratan con el compuesto **1**.

Por tanto se puede concluir que el compuesto **1** es el que mejores resultados presenta frente a *T. cruzi* y el compuesto **2** frente a las dos especies de *Leishmania.*

- **Complejos de níquel con triazolopirimidinas.** (**In vitro anti-leishmania evaluation of nickel complexes with a triazolopyrimidine derivative against *Leishmania infantum* and *Leishmania braziliensis.*** Artículo sometido en la revista *J Inorg Biochem.* 2011.)

Como se ha expuesto en el apartado anterior, la actividad antiprotozoaria de las 1,2,4-triazolo[1,5-*a*]pirimidina se ha estudiado con anterioridad. (Luque y col. 2000[a],[b], Magán y col. 2004[a,b], 2005, Boutaleb-Charki y col. 2009).

Siguiendo con la búsquedad de compuestos efectivos frente a estos protozoos kinetoplástidos, se ha empezado a estudiar otra familia de triazolopirimidinas: 1, 2, 3-triazolo[4, 5-*d*]pirimidina.

En este trabajo, hemos realizado la evaluación biológica de siete nuevos complejos de niquel II, con la forma aniónica del 4, 6-dimetil-1, 2, 3-triazol[4, 5-*d*]pirimidina-5, 7-diona (dmax) y diferentes aminas alifáticas como la etilendiamina (en), 1, 3-diaminopropano (dap), bis 3-aminopropil-amina (bapa) o aminas aromáticas como la 1, 10-fenantrolina (phen), 2, 2'-bipiridilo (bpy) y 2, 2'-dipiridilamina (dpyamin) como ligandos auxiliares, frente a dos especies distintas del género *Leishmania: L. infantum* y *L. braziliensis.* Además de estudiar la evaluación biológica de estos siete complejos, también se ensaya el efecto del 4, 6-dimetil-1, 2, 3-triazol[4, 5-*d*]pirimidina-5, 7-diona (Hdmax) y de su sal de niquel (Nidmax) sobre dichas especies de parásitos.

Previamente, estos compuestos han sido estudiados como una posible alternativa al tratamiento de la enfermedad de Chagas. (Maldonado y col. 2010).

Los ensayos de actividad de los nueve compuestos sobre las formas promastigotas y amastigotas de *L. infantum* y *L. braziliensis*, nos permite apreciar

que la IC_{50} de los productos **Nidmax-bpy** y **Nidmax-dpyamin** son menores o similares a la presentada por el Glucantime en las formas promastigotas de ambas especies. También se observa que frente a las formas amastigotas, cinco de los complejos de níquel mas el compuesto **Hdamx** en el caso de *L. infantum* y tres complejos de níquel y el **Hdamx** en *L. braziliensis,* presentan mejores IC_{50} que la droga de refencia.

En *L. infantum*, se puede ver que los productos **Nidmax-dap** y **Nidmax-phen**, son más activos frente a las formas amastigotas. En el caso de *L. braziliensis,* todos los compuestos ensayados son más activos frente a las formas promastigotas que a las amastigotas.

Al ensayar los productos sobre las células de mamíferos se puede ver que los complejos con níquel, son mucho menos tóxicos para estas que el Glucantime. También se observa que los siete complejos con níquel son menos tóxicos que el derivado triazolopirimidina libre y su sal de níquel.

Para los siguientes experimentos, sólo se han ensayado los productos que presentan un SI≥10. Los compuestos **Nidmax-bpy** y **Nidmax-dpyamin**, son los que mejores SI presentan en ambas especies.

Al estudiar cómo afectan los productos sobre la capacidad de infección, se puede observar que el porcentaje de células infectadas disminuye con respecto al

control y con respecto a la droga de referencia en las dos especies. Siendo los compuestos **Nidmax-bpy** y **Nidmax-dpyamin** los que presentan mejores resultado con respecto a la inhibición que causan estos productos sobre la infección. El número de formas amastigotas que aparece en cada célula, también disminuye.

El estudio de los metabolitos excretados, muestra que sólo el compuesto **Nidmax-bapa** produce cambios en las dos especies, apareciendo una disminución de acetato y un incremento en la producción del succinato.

La inhibición de la excreción de acetato puede explicar el incremento de succinato y L-lactato, probablemente debido a la acción del compuesto **Nidmax-bapa** sobre la piruvato deshidrogenasa.

Haciendo un examen ultraestructural de las formas promastigotas, se aprecia una gran cantidad de alteraciones ultraestructurales en las dos especies de *Leishmania* tratadas con los complejos triazolopirimidinas. De estas alteraciones, se puede decir que tal vez un posible mecanismo de acción se encuentre a nivel de las membranas de los orgánulos.

La importancia de la fitoterapia en la búsqueda de nuevas drogas tripanocidas está en auge, (González y col. 2005, Singh y col. 2004, Urbina 2002) por lo que la utilización de compuestos naturales extraídos de plantas se ha intensificado en los últimos años, y la posibilidad de encontrar una molécula activa

y de baja toxicidad aumenta a medida que se realizan más estudios. Principios activos derivados de plantas así como sus análogos semisintéticos y sintéticos han servido como la principal vía para obtener nuevos compuestos quimioterapéuticos. (Urbina 2002, Newman y col. 2007, Torres y col. 2008)

5.3. Derivados flavonoides.

(*J Nat Prod* 2011; 74: 744-50; **Leishmanicidal activity of nine novel flavonoids from *Delphinium staphisagria*.** Artículo sometido en la revista *TheScientificWorldJOURNAL*. 2012)

Las flavonas y sus derivados flavonoides, son algunos de los derivados de plantas más atractivos para enriquecer las opciones actuales de tratamientos, debido a su amplio rango de propiedades biológicas. (Cazarolli y col. 2008)

Los flavonoides se encuentran con abundancia en frutas, vegetales y jugos de plantas y han demostrado tener actividad anticancerígena, antimicrobiana y antiparasitaria. (Taleb-Contini y col. 2004, Grael y col. 2005).

En publicaciones anteriores se determinó que derivados flavonoides presentaron un efecto inhibidor significativo sobre el crecimiento in vitro de especies de *Leishmania spp* y *Trypanosoma cruzi*. (Marín y col. 2009, Boutaleb-Charki y col. 2011)

Seis de los nueve derivados flavonoides ensayados, se obtuvieron de la planta anual *Delphinium staphisagria*, de la familia Ranunculaceae (compuesto **1**, **2**, **4**, **5**, **7**, **8**). Los tres compuestos restantes, se obtuvieron por acetilación de los anteriores (compuesto **3**, **6**, **9**). (Díaz y col. 2008)

Hay que señalar que los nueve compuestos ensayados, son muy poco tóxicos para las células vero de mamíferos, superando la IC_{50} de los compuestos de 12 a más de 74 veces la IC_{50} del Benznidazol.

Los nueve compuestos presentan un efecto inhibidor *in vitro* de las formas epimastigotas de *T. cruzi*, aunque el astragalín y sus derivados acetilados (compuesto **1**, **2** y **3**) presentan una IC_{50} menor a la obtenida con el Benznidazol.

El resto de compuestos presentan una IC_{50} superior a la de la droga de referencia, pero al poseer unas IC_{50} mucho mayores que el Benznidazol frente a las células de mamífero, sus SI son muy elevados.

Estos compuesto flavonoides también presentan una gran actividad leishmanicida. Los valores de IC_{50} son semejantes o ligeramente superiores a los obtenidos por el Glucantime, pero es interesante destacar el efecto que producen estos compuestos sobre los macrófagos. Al igual que en los ensayos con las células vero, estos productos son muy poco tóxicos.

El petiolaroside, 2´´ acetylpetiolaroside (compuestos **7** y **8** respectivamente), y el paenoside y sus derivados (**4, 5** y **6**), son los compuestos más efectivos frente a las formas intra y extacelulares de *Leishmania* y presentan los mejores índices de selectividad.

En los ensayos de infectividad de *T. cruzi,* se comprueba que de nuevo los derivados del astragalín (compuestos **2** y **3**) son los compuestos que inhiben más la infección de las células, la replicación intracelular de los amastigotes y la cantidad de tripomastigotas liberadas al medio.

Realizando los estudios sobre las dos especies de *Leishmania,* se puede ver que el compuesto 2´´-acetilpetiolaroside (**8**) es claramente el más activo y que los derivados acetilados 2´´-acetylpetiolaroside (**8**) y 2´´-acetylpaeonoside (**5**) son más efectivos que el petiolaroside (**7**) y paeonoside (**4**) respectivamente.

Se ha demostrado que la acetilación de algunos flavonoides incrementa los efectos antiproliferativos de los compuestos frente a líneas celulares HL-60 entre otras. El derivado acetilado del trifolín y el compuesto astragalín heptaacetato, inducen la muerte celular en células humanas de leucemia. (Torres y col. 2008, Burmistrova y col. 2011). Esta gran eficiencia, puede ser debida a que la acetilación facilita la absorción del compuesto. (Manach y col. 2004).

En el caso de *T. cruzi*, los ensayos se completan con estudios *in vivo*. Ningún ratón tratado con los nueve compuestos flavonoides muere a lo largo del

experimento debido a la cantidad de producto administrado. Además haciendo un control de la parasitemia, se puede ver que los animales tratados no presentan el pico máximo de parásitos que aparece en el control sobre el día 22 post-infección. La parasitemia decrece a lo largo de 40 días con respecto al control y el Benznidazol.

En el estudio serológico, ningún animal infectado presenta niveles negativos de anticuerpos anti *T. cruzi,* sin embargo a excepción de los compuestos **8** y **9,** los niveles de anticuerpos disminuyen entre los días 40 y 90 con respecto al control y la droga de referencia.

Como en todos los casos anteriormente citados, los espectros de ^{1}H-RMN nos proporcionan los metabolitos excretados por los tripanosomátidos durante su cultivo.

La excreción de algunos de los catabolitos en *T. cruzi* se altera con la adición de los derivados flavonoides, donde la excreción de succinato y acetato disminuye en los compuestos acetilados. Esta inhibición puede explicar el incremento de L-lactato y etanol considerando que estos compuestos pueden actuar a nivel de la cadena de electrones o a nivel de mitocondria y como consecuencia en la fosforilación oxidativa.

En el caso de *L. infantum,* los compestos **4**, **5**, **6** y **8**, presentan un espectro similar, con un incremento en la excreción del succinato y acetato seguido por el

malato, alanina, lactato y etanol. El compuesto **7** causa un incremento de la producción de malato, succinato, etanol y lactato y produce una disminución de acetato y alanina.

En *L. braziliensis,* el incremento de succinato es el mayor cambio presentado. En los compuestos **6**, **7** y **8** se produce una disminución de la excreción de acetato.

Se observan cambios estructurales en las tres especies de parásitos estudiadas siendo las más frecuentes la presencia de grandes vacuolas, vesículas electrodensas y muerte de los parásitos. Estos cambios se pueden deber a las alteraciones en los metabolitos.

5.4. Derivados terpenoides.

(*Am J Trop Med Hyg.* 2012. doi:10.4269/ajtmh.2012.11-0471; **In Vitro evaluation of new terpenoid derivatives against *Leishmania infantum* and *Leishmania braziliensis.*** Artículo sometido en la revista *Mem Inst Oswaldo Cruz*. 2011; **Taiwaniaquinoid and abietane quinone derivatives with trypanocidal activity against *T. cruzi* and *Leishmania spp***. Artículo sometido en la revista *Parasitol Int.* 2011.)

Los terpenoides de la resina de las coníferas, son compuestos importantes de defensa frente herbívoros y patógenos de plantas. (Martin y col. 2002).

El interés de los terpenoides se ha incrementado, habiéndose aislado nuevos compuestos principalmente fenoles y derivados relacionados que muestran unas actividades biológicas remarcables. (Gao y col. 1997, Marrero y col. 2002, Tan y col, 2002) Además presentan una importante inhibición del crecimiento de células tumorales y células transformadas oncogénicas. (Son y col. 2005)

Hay varios estudios que demuestran la actividad tripanocida de los terpenoides. Así el efecto antitripanosomátido frente a las formas epimastigotas y amastigotas de *T. cruzi* se ha demostrado en compuestos diterpenos casanos, aislados de hojas de *Myrospermum frutescens*. (Mendoza y col. 2003). Otros diterpenos aislados de *Aristolochia cymbifera,* han mostrado selectividad frente a las formas tripomastigotas. (Sartorelli y col. 2010), al igual que los diterpenos pimaranos aislados de *Viguiera arenaria.* (Ambrósio y col. 2008). Además el ferruginol y varios compuestos abietanos aislados de la raíz de *Craniolaria annua* presentan actividad citotóxica frente a las formas epimastigotas y tripomastigotas de *T. cruzi.* (Herrera y col. 2008).

Recientemente, se ha visto que la acetilación de triterpenos tipo oleano produce un efecto leishmanicida. (Macahig et al. 2011).

El efecto antileismania de los abietanos sobre las promastigotas y amastigotas de distintas especies de *Leishmania,* también se ha comprobado. (Tan y col. 2002, Samoylenko y col. 2008)

Se han ensayado once compuestos muy distintos entre ellos.

El compuesto **1**, es el metil éster de ácido 12-hidroxidehidroabietico, un producto natural, (Kinouchi y col. 2000) y se ha sintetizado del ácido abiético comercial. (Alvarez-Manzaneda y col. 2007a)

Los compuestos **2, 3** y **4,** se han preparado del ácido trans-comúnico. (Alvarez-Manzaneda y col. 2007b) El compuesto **2**, no se ha encontrado en la naturaleza. El compuesto **3**, es el metil éster del ácido lambertico, aislado de *Podocarpus lambertius.* El compuesto **4**, es un diterpeno aislado de *Cryptomeria japonica*, llamado sugikurojin A. (Arihara y col. 2004)

El compuesto **5**, se prepara a partir del ácido abiético (Alvarez-Manzaneda et al. 2007c) y es un precursor del agente antileishmania obtenido de la planta *Salvia silícica,* 12-desoxyroyleanona (Tan y col. 2002)

El compuesto **P-1**, es una quinona con esqueleto de abietano, no encontrada hasta ahora en la naturaleza. (Matsushita y col. 2005, Álvarez-Manzaneda y col. 2007)

Al igual que el compuesto **5**, relacionado estructuralmente con 12-desoxyroyleanona.

Los compuestos **S-567**, **S-569**, **S-589** y **S-602**, poseen estructura de taiwaniaquinoide. El compuesto **S-569**, conocido con el nombre de (-)-taiwaniaquinone G, ha sido aislado de *Taiwania cryptomerioides*. (Chang y col. 2005) Los compuestos S-567, S-569, S-589 y S-602 han sido sintetizados a partir de acetato de homofarnesilo y de (+)-sclareolida (Alvarez-Manzaneda y col. 2009a,b)

El compuesto **A-237** es un taiwaniaquinoide. No es un producto natural, y su síntesis aún no ha sido descrita.

Para hacer más sencilla la discusión de todos los resultados obtenidos por los diferentes compuestos estudiados, los voy a dividir en grupos. En primer lugar los compuestos **1-4,** después los compuestos **5** y **P-1** y por último los cinco derivados taiwaniaquinoides.

De los compuestos **1-4,** ensayados frente al agente etiológico de la enfermedad de Chagas en sus formas extracelulares e intracelulares, se comprueba un efecto inhibidor del crecimiento de dichas formas al adicionar los compuestos, siendo los compuestos **1** y **2** incluso más efectivos que la droga de referencia. Si sumamos estos resultados a los presentados por citometría de flujo sobre las células vero, en los que se observa que los compuestos son poco tóxicos para ellas, se obtiene que ambos compuestos superan a la droga de referencia en 20 y 23 veces.

El porcentaje de infección de las células con formas metacíclicas tratadas con los compuestos **1** y **2**, se ve reducido significativamente con respecto al control, alcanzando un porcentaje de inhibición de 77% y 82% respectivamente en el día diez después de la infección. Este porcentaje también es mayor al que presenta el Benznidazol (39%). Estos datos de decrecimiento de la infección se ven corroborados porque el número de amastigotas presentes en las células infectadas disminuye y también las formas tripomastigotas presentes en el medio libre. Las fluctuaciones que presentan las células infectadas en el número de formas amastigotas presentes en su interior a lo largo de los diez días de experimento, se debe a las transformaciones ocurridas en el ciclo del parásito de amastigotas a tripomastigotas y estas vuelven a infectar nuevas células.

Gracias a los buenos resultados presentados en los experimento *in vitro*, se procede a realizar experimentos *in vivo*, donde se verifican los resultados anteriores, ya que la parasitemia disminuye al administrar a los ratones los dos compuestos. Así el máximo pico de parasitemia observado sobre el día 25 se ve disminuido con respecto al control y al día cuarenta, la parasitemia es menor a la presentada por el control y Benznidazol, pero sin llegar a la eliminación total del parásito. Ambos compuestos presentan un efecto muy silimar.

Analizando el efecto de los compuestos **1-4** sobre *L. infantum* y *L. braziliensis,* se observa que los tres derivados del ácido trans-comúnico,

(compuestos **2**-**4**) son los más efectivos frente a las formas amastigotas y promastigotas.

Como los cuatro compuestos son menos tóxicos para los macrófagos que el Glucantime, estos productos presentan SI mayores a los de la droga de referencia. El compuesto **1** no se incluye en los siguientes ensayos puesto que su SI no supera en 20 veces o más al SI del Glucantime. (Nawaka y Hudson 2006).

De los ensayos de infectividad, deducimos que los tres compuestos derivados del ácido trans-comúnico son más eficaces impidiendo la invasión del parásito al interior de la célula de mamífero, presentando mejor resultado el compuesto **2**, que inhibe la infección un 85% respecto al control en *L. infantum.* Frente a *L. braziliensis,* ocurre lo mismo.

El compuesto **5**, presenta una actividad leishmanicida interesante frente a las dos especies del género *Leishmania* estudiadas, mostrando datos mejores a los obtenidos por el compuesto **2.**

El compuesto **P-1**, merece especial atención ya que en los ensayos de citotoxicidad sobre las células de mamíferos, se obtiene una IC_{50} de 800,3 μM en macrófagos y de 781,4 μM en células Vero frente a la IC_{50} de 15,2 μM y 13,6 μM que presentan el Glucantime y Benznidazol. Además de la poca toxicidad que presenta frente a este tipo de células, cabe destacar que la actividad antiparasitaria

que presenta frente a las formas intracelulares y extracelulares, es mayor que la de la droga usada en la actualidad, por lo que el SI de este compuesto es muy elevado frente a los tres parásitos estudiados. En todos los casos supera en más de sesenta veces al de la droga de referencia.

Mirando cómo afecta este compuesto a la infección de las células por las formas infectivas de los parásitos, comprobamos que el porcentaje de células infectadas disminuye en presencia de dicho compuesto. Esto ocurre en *L. infantum, L. braziliensis* con porcetajes de inhibición del 74% y 80% respectivamente y en *T. cruzi,* donde disminuye la infección en un 85%. Todos estos resultados también son significativamente mayores a los presentados por las drogas de referencia donde la inhibición es del 56%, 36% y 9%. Estos datos se ven apoyados porque también se aprecia una disminución en el número de formas amastigotas presentes en el interior celular y en el caso de *T. cruzi,* también ocurre con las formas tripomastigotas liberadas al medio.

El estudio *in vivo* de este producto nos muestra que la parasitemia se reduce a lo largo de cuarenta días pero no logra la eliminación completa del parásito. Lo que si presenta gran interés es que en la prueba serológica los aminales tratados con el compuesto experimentan un descenso en los niveles de anticuerpos entre los días 40 y 90 post-infección.

De los cinco compuestos taiwaniaquinoides, sólo el compuesto **S-567** presenta actividad antiparasitaria frente las formas amastigotas y epimastigotas o promastigotas. Los cuatro restantes presentan unos valores similares a los presentados por el Benznidazol y Glucantime.

El compuesto **S-567**, además de presentar actividad tripanosomátida frente a estas formas, también afectan a las formas infectivas ya que disminuyen la infección de las células Vero o macrófagos.

En los ensayos *in vivo* se obtienen resultados muy similares a los que presenta el compuesto **P-1.**

A través de los espectros de ^{1}H-RMN, se aprecia que cuando un cultivo de formas epimastigotas de *T. cruzi* es tratado con el compuesto **2**, se produce una inhibición de la excreción de acetato. Esta disminución puede explicar el incremento observado en los niveles de succinato, L-alanina y etanol, probablemente debido a que estos compuestos actúan a nivel del metabolismo energético.

Cuando las dos especies de *Leishmania* son tratadas con el compuesto **5**, aparece un nuevo pico correspondiente a la excreción de piruvato. Esto podría significar que dicho compuesto inhibe las enzimas glicosomales originando piruvato como un metabolito final de dichas especies.

Cuando los parásitos se tratan con el compuestos **P-1**, tanto en *T. cruzi* como en *L. braziliensis* la excreción de acetato aumenta, mientras que en *L. infantum* los valores no sufren alteraciones con respecto al control. El succinato disminuye su excreción en las tres especies.

El compuesto **S-567**, muestra un perfil muy similar al del producto anterior en todas las especies estudiadas.

Haciendo una observación al microscopio electrónico de transminsión (MET), se aprecia que todos los compuestos terpenoides producen grandes alteraciones ultraestructurales tanto en las formas epimastigotas como en las promastigotas, presentando grandes orgánulos electrodensos, abundancia de reservosomas en el caso de *T. cruzi*, vacuolas tanto lipídicas como vacias, glicosomas, y perdiendo la conformación estructural normal, adoptando formas estrelladas por ejemplo y en muchos casos produciendo la muerte del parásito.

Todos los resultados expuestos en esta discusión, nos lleva a pensar que nuevas alternativas para el tratamiento de la enfermedad de Chagas y los distintos tipos de leishmaniasis son posibles, sólo hay que seguir investigando y haciendo estudios sobre estos datos.

6.- CONCLUSIONES.

1. Se han estudiado el efecto tripanocida y leishmanicida de una serie de derivados de nueva síntesis (derivados imidazol y pirazol Benzo[*g*]ftalazina, derivados triazolopirimidinas y complejos metálicos) y otros de origen natural (derivados flavonoides y derivados terpenoides.)
2. Se han realizado estudios *in vitro* e *in vivo* frente a *T. cruzi*, e *in vitro* frente a *L. braziliensis* y *L. infantum*
3. Entre los compuestos de nueva síntesis Benzo[*g*]ftalazina, los derivados de imidazol, presentan mejores resultados de actividad tripanocida frente a las formas epimastigotas de *T. cruzi,* que los derivados de pirazol. Frente a las dos especies de *Leishmania,* estos compuestos presentan un comportamiento muy similar en ambas especies, sin observarse grandes diferencias frente las distintas formas del parásito.
4. Los derivados Benzo[*g*]ftalazina, son muy poco tóxicos para las células de mamífero con respecto al Benznidazol y Glucantime utilizados como drogas de referencia.
5. La eficacia tripanocida de los derivados de imidazol, es significativamente mayor que la de los derivados de pirazol.
6. Frente a las dos especies de *Leishmania* los derivados Benzo[*g*]ftalazina monosustituidos presentan una eficacia mayor que los derivados disustituids. Ambos son más eficaces que el Glucantime utilizado como droga de referencia.

7. Estos derivados han demostrado una capacidad inhibitoria de la capacidad de invasión celular y la replicación de las formas amastigotas intracelulares en las tres especies parasitas estudiadas.
8. Los ensayos *in vivo* de los derivados imidazol y pirazol frente a *T. cruzi*, muestran una reducción de la parasitemia durante la fase aguda de la enfermedad, donde en los derivados monosustituidos es mayor que en los derivados disustituidos, como corrobora la detección de anticuerpos mediante la técnica de la ELISA entre los días 40 y 120 post-infección.
9. Los compuestos monosustituidos son menos tóxicos que la droga de refencia tal y como demuestran los estudios histopatológicos realizados en el hígado.
10. Uno de los posibles mecanismos de acción de estos compuestos puede ser sobre la enzima Fe-SOD, que presenta un significativo descenso de la actividad enzimática cuando los compuestos son administrados, sin afectar a la enzima Cu Zn-SOD humana.
11. Al tratar a los tripanosomátidos con los seis compuestos imidazol y pirazol Benzo[*g*]ftalazina, el metabolismo glucolítico se altera, así mismo se producen alteraciones morfológicas a nivel ultraestructural que están en perfecta concordancia con los cambios metabolicos observados.
12. Los resultados de estos estudios convierten a estos productos, especialmente a los derivados monosustituidos, en sólidos candidatos para el tratamiento de la Enfermedad de Chagas y las Leishmaniasis.

13. Otro grupo de compuestos ensayados son los complejos resultantes de la interacción de 7-hidroxi-5-metil-1,2,4-triazolo[1,5-*a*]pirimidina (HmtpO) con metales de transición divalentes (cobre y cobalto), que inhiben el crecimiento *in vitro* de las formas epimastigotas de *T. cruzi* y las formas promastigotas de *L. infantum* y *L. braziliensis,* y un grado de toxicidad sobre células de mamíferos menor que el causado por las drogas de referencia.
14. La capacidad infectiva y la replicación intracelular de los tres parásitos es alterada por el efecto de estos compuestos.
15. Los derivado triazolopirimidinas de cobr*e* frente a *T. cruzi,* producen un descenso de la parasitemia
16. El metabolismo energético se ve afectado por el efecto del compuesto **2**, seguramente por la acción directa de este compuesto sobre las enzimas glicosomales. Estos resultados se ven refrendados por las alteraciones ultraestructurales observadas en los parástios.
17. De los estudios de los complejos de niquel con 1as triazolopirimidinas: 1, 2, 3-triazolo[4, 5-*d*]pirimidina, se aprecia que los productos de los complejos con niquel, son mucho menos tóxicos para las células de mamífero que el Glucantime
18. Algunos de estos complejos (**Nidmax-bpy y Nidmax-dpyamin**) inhiben la capacidad de replicación de las formas intracelulares y el porcentaje de infección.

19. El compuesto **Nidmax-bapa,** altera significativamente al metabolismo energético de los parásitos y estos cambios pueden deberse a la acción directa del compuesto sobre la enzima piruvato deshidrogenasa. Hecho reforzado por cambios ultraestructurales que se observan a nivel de la membrana de los orgánulos.
20. Una parte importante de esta memoria está constituida por los estudios de los compuestos de origen vegetal, entre ellos los compuestos flavonoides obtenidos de la planta *Delphinium staphisagria* y sus derivados acetilados. Todos ellos son muy poco tóxicos para las células de mamíferos.
21. De los resultados obtenidos en los estudios *in vitro,* se observa que los derivados acetilados del astragalín son los más efectivos frente a *T. cruzi,* mientras que para las especies de *Leishmania,* es el compuesto 2´´-acetilpetiolaroside.
22. Estudios *in vivo* muestran una disminución de la parasitemia en la fase aguda de la Enfermedad de Chagas y una disminución de los niveles de anticuerpos anti *T. cruzi,* entre los días 40 y 90 con respecto al control y al fármaco de referencia.
23. Uno de los posibles mecanismos de acción puede ser a nivel de la cadena de electrones o a nivel de la fosforilación oxidativa mitocondrial.

24. Dentro del heterogéneo grupo de compuestos terpenoides estudiados, los compuestos **1** y **2**, presentan una notable actividad *in vitro* e *in vivo* frente a las distintas formas de *T. cruzi*.
25. Los tres derivados del ácido trans-comúnico, son efectivos frente a las formas amastigotas y promastigotas de las dos especies de *Leishmania*.
26. Sólo uno de los compuestos taiwaniaquinoides estudiados, **S-567,** presenta actividad antiparasitaria.
27. El compuesto abietano quinona **P-1**, presenta los mejores resultados obtenidos *in vitro* de los compuestos terpenoides estudiados frente a las tres especies de tripanosomátidos.
28. El compuesto **S-567** y **P-1**, presentan similares resultados en los estudios *in vivo*. La parasitemia se reduce a lo largo de la fase aguda de la infección al igual que los niveles de anticuerpos detectados en la fase crónica.

Conclusión general.

Los resultados expuestos en esta memoria nos llevan a pensar que nuevas alternativas para el tratamiento de la Enfermedad de Chagas y los distintos tipos de Leishmaniasis son posibles.

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