

**Exploring Trisubstituted Adenine Derivatives as Adenosine A1 Receptor
Ligands with Antagonist Activity: Synthesis, Biological Evaluation and
Molecular Modelling**

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Abstract: The therapeutic potential of adenosine receptor (AR) ligands is becoming increasingly important as our understanding of the physio-pathological functions of ARs advances. This study presents the synthesis and biological screening of a novel library of trisubstituted adenine analogues and reports the discovery of derivatives that displaying selective binding affinity towards hA_1AR . Compounds were synthesized using a cyclization approach by combining 4,6-bisalkylamino-5-aminopyrimidines with three different trialkyl/arylorthoesters, thereby generating adenines featuring three different substituents at the C-8 position: H, methyl or phenyl. Most promising derivatives presented a phenyl ring at such position and displayed selective antagonistic activity against hA_1AR . N,9-diisopropyl-8-phenyl-9H-purin-6-amine (**14c**) was identified as the most potent compound with a K_i of 2 nM, motivating the synthesis of new derivatives including **19c**. Docking modelling predicted key interactions between the lead compounds and hA_1AR . Determination of their anti-proliferative activity on six cancer cell lines found **19c** to be the most potent derivative with low micromolar EC_{50} values. Our findings support further exploration around the adenine scaffold for cancer research and AR drug development.

Keywords: Adenine scaffold, docking simulation, cancer cell lines, antiproliferation, orthosteric binding site, selectivity index, pharmacokinetic properties

1. Introduction

One of the most ubiquitous receptors in human cell membranes is the adenosine receptor (AR). It is a purinergic G protein-coupled receptor (GPCR) and is widely distributed in many tissues with adenosine as its endogenous agonist. There are four different types of ARs:

55 A₁, A_{2A}, A_{2B} and A₃, each with a variety of functions. Purines are privileged structures because
56 they are one of the most widely occurring heterocycles in nature.¹ They have a low cost,
57 commercial availability, and a range of well-established synthetic protocols existing in the
58 literature. Additionally, purines have potential pharmacological properties that make them
59 attractive for use in drug discovery. Furthermore, their structure allows for the generation of new
60 purine analogues through a range of different reactions, making them an efficient chemical
61 "navigator" towards novel compounds with desired biological activities.² To date, dozens of
62 purine-based drugs have been approved by regulatory agencies, including fludarabine,
63 tecadenoson, cladribine and regrelor.³ Despite purines being privileged structures and ARs
64 receptors being one of the most targeted biological entities,⁴ to date no drugs have been
65 approved as agonists or antagonists of ARs.

66 Small molecules that interact with AR have been shown to be anti-inflammatory,
67 cytotoxic, and modulators of neurological, cardiac and kidney diseases. In the early 1990s, an
68 adenine derivative, N⁶-endonorboran-2-yl-9-methyladenine (N-0861),⁵ was introduced with great
69 promise as an antagonist of adenosine receptors, but clinical trials did not yield the expected
70 results. Since then other small molecules, in particular, xanthines have been used as hA₁AR
71 antagonists⁶ in clinical trials. For instance, Biogen Inc. brought to clinical trials a xanthine
72 derivative, tonapofylline (BG9928, ClinicalTrials.gov Identifier: NCT00745316, NCT00709865 &
73 NCT00858156) for treating heart failure and renal insufficiency. Unfortunately, Biogen stopped
74 the trials in phase 2b. While there have been great efforts to develop new hA₁AR antagonist
75 drugs, there are just few of them with high affinity for human A₁AR (hA₁AR) and high selectivity
76 index over other hAR types.^{7,8,9}

77 Not only xanthine but also polysubstituted adenines have been shown to target AR.
78 Some of them, as it was the case of SLV320, went into clinical trials¹⁰ for treatment of congestive
79 heart failure. Moreover, other groups, in particular the groups of Prof. Cristalli and Prof. Volpini
80 have kept developing these chemotypes based on adenine as antagonists of AR.^{9,11} In addition,
81 the regulation of purinergic signalling contributes to several important pathologies such as
82 cancer.³

Our group has focused on developing new synthetic methods to produce a wide range of polysubstituted purines. We introduced a one-pot synthesis of trisubstituted 6-alkoxypurines to obtain libraries with structural.^{12,13,14} The use of these preciously developed libraries allowed us to identify key structural features of 6-alkoxy-purine analogues that induce programmed cell death in several types of cancer cell lines, in particular T cell cancer lines¹⁵ and two novel purine-based chemotypes that could be further optimized to generate antiparasitic drugs against both *Plasmodium falciparum* and *Trypanosoma cruzi*.^{12,16} Further to that, a novel synthetic route¹⁷ for the synthesis of polysubstituted adenines was developed. Following this synthetic route, a library of small molecules was prepared and compounds with IC₅₀ values as low as 2.42 ± 0.16 mM, which target specific enzymes involved in purine metabolism within the parasite, were identified.¹⁸

As these compounds were found to target enzymes of the trypanosome purine salvage pathway, whose ligands include adenine and adenosine,¹⁸ we were interested in exploring whether this library might also exhibit affinity for human adenosine receptor subtypes, which also utilize adenosine as a natural ligand. Throughout the course of this project, our design strategy was further inspired by modifications of the adenine scaffold reported for the known hA₁AR antagonist N-0861⁵ and the work of Lambertucci.⁹ Herein, we present our latest findings on the use of modified adenines as ligands of the hAR family, that showed potent binding for the hA₁AR as well as antagonist activity in a cAMP assay. The interaction with the hA₁AR was also simulated through docking computational analysis. We also investigated the cytotoxic effects of these modified adenine ligands on cancer cells, as there is evidence that the adenosine receptors play a role in cancer.^{11,19,20}

2. Material and methods

2.1 Synthesis of purine library

Reaction courses and products mixtures were routinely monitored by TLC on silica gel Merck 60-200 mesh silica gel. Melting points were determined on a Stuart Scientific SMP3 apparatus and are uncorrected. ¹H-NMR spectra were obtained in CDCl₃, solution on a Varian Direct Drive (400 MHz and 500 MHz). Chemical shifts (δ) are given in ppm upfield from tetramethylsilane.

¹³C-NMR spectra were obtained in CDCl₃, on a Varian Direct Drive (125 MHz). All products reported showed ¹H-NMR and ¹³C-NMR spectra in agreement with the assigned structures. Mass spectra were obtained by electrospray (ES) with a LCT Premier XE Micromass Instrument (High resolution mass spectrometry). All final compounds are >95% pure by HPLC analysis (see Supporting Information). The synthesis of compounds **11-15 (a-c)** and their intermediates are described elsewhere.¹⁸

2.1.1. General procedure for the preparation of compounds **7-10**¹⁸

A solution of **1** (7 mmol, 1 equiv.) in CH₂Cl₂ (15 ml) containing TEA (initially 1.5 equivalents of each and in later reactions, when the disubstitution was checked, 2 equivalents). On this solution the different amines (10.54 mmol, 1.5 equiv. and later 2 equivalents) dissolved in CH₂Cl₂ (5 mL) were added at room temperature for half an hour. Evaporation under vacuum gave a solid that was purified by column chromatography eluting with ethyl acetate/petroleum ether solutions.

N⁴,N⁶-di-tert-butyl-5-nitropyrimidine-4,6-diamine (7): Product **7**, is not isolated, it is used as is for the following reaction.**4.1.3.**

N⁴,N⁶-diethyl 5-nitropyrimidine-4,6-diamine (8): Yellow solid, yield 89%, mp: 85°C. δH (400.45 MHz, CDCl₃): 9.29 (2H, bs, NH x2), 8.09 (1H, s, NCHN), 3.63 (4H, q, J=7.2 -CH₂- x2), 1.28 (6H, t, J= 7.3, -CH₃ x2). δC (100.70 MHz, CDCl₃): 159.59, 157.16, 112.59, 36.38, 14.43. ES+HRMS: Calculated M+H=212.1147 C₈H₁₄N₅O₂. Obtained: 212.1148.

N⁴,N⁶-diisobutyl 5-nitropyrimidine-4,6-diamine (9): Yellow solid, yield 83%, mp: 78°C. δH (400.45 MHz, CDCl₃): 9.43 (2H, bs, NH x2), 8.05 (1H, s, NCHN), 3.42 (4H, dd, J=1.2 and 5.6 -CH₂- x2), 1.94 (2H, m, CH x2), 0.97 (12H, d, J= 3.9, -(CH₃)₂ x2). δC (100.70 MHz, CDCl₃): 159.75, 157.67, 112.87, 48.99, 28.28, 20.29. ES+HRMS: Calculated M+H=268.1774 C₁₂H₂₂N₅O₂. Obtained: 268.1774.

N⁴,N⁶-dicyclopentyl-5-nitropyrimidine-4,6-diamine (10): Yellow solid 76%, mp: 79°C. δH (400.45 MHz, CDCl₃): 9.37 (2H, bs, NH x2), 8.10 (1H, s, NCHN), 4.56 (2H, m, -CH cyclopentyl x2), 2.69 (4H, m, -CH₂ cyclopentyl x2), 1.77 (4H, m, -CH₂ cyclopentyl x2), 1.67 (4H, m, -CH₂ cyclopentyl x2), 1.53 (4H, m, -CH₂ cyclopentyl x2). δC (100.70 MHz, CDCl₃): 159.76, 157.05,

137 112.84, 53.29, 33.42, 23.87. ES+HRMS: Calculated M+H=292.1762 C₁₄H₂₂N₅O₂. Obtained:
138 292.1785.

139 2.1.2. General procedure for the preparation of compounds 16-19:

140 A solution of **7-10** (5 mmol, 1 equiv.) in EtOH (10 ml) containing SnCl₂·2H₂O (25 mmol, 5 equiv.),
141 was refluxed for 1 h, monitoring the reaction by TLC. The mixture was then cooled at room
142 temperature and NaHCO₃ was added until pH 8 was reached. After two extractions with EtOAc
143 (10 ml for each one), the organic phase was washed with an aq. saturated solution of NaCl (2x25
144 mL) and dried on Na₂SO₄. Evaporation under vacuum gave a solid that was purified by column
145 chromatography eluting with ethyl acetate/petroleum ether solutions.

146 **N⁴,N⁶-di-tert-butylpyrimidine-4,5,6-triamine (16)**: Pink solid, yield 43%. δH (400.45 MHz,
147 CDCl₃): 8.04 (1H, s, NCHN), 3.88 ((2H, bs, NH x2), 1.44 (18H, s, -C(CH₃)₃ x2).

148 **N⁴,N⁶-diethylpyrimidine-4,5,6-triamine (17)**: Purple solid, yield 32 %, mp: 150°C. δH (400.45
149 MHz, CDCl₃): 8.12 (1H, s, NCHN), 4.81 (2H, bs, NH x2), 3.46 (4H, q, J=7.2, -CH₂- x2), 2.24 (2H,
150 bs, NH₂), 1.22 (6H, t, J= 7.2, -CH₃ x2). δC (100.70 MHz, CDCl₃): 157.84, 153.96, 101.19, 36.09,
151 15.66. ES+HRMS: Calculated M+H=182.1406 C₈H₁₆N₅. Obtained: 182.1393.

152 **N⁴,N⁶-diisobutylpyrimidine-4,5,6-triamine (18)**: Brown oil, yield 59 %. δH (400.45 MHz,
153 CDCl₃): 8.09 (1H, s, NCHN), 5.00 (2H, bs, NH x2), 3.24 (4H, t, J=5.6 -CH₂- x2), 2.14 (2H, bs,
154 NH₂), 1.85 (2H, m, CH x2), 0.95 (12H, d, J= 5.4, -(CH₃)₂ x2). δC (100.70 MHz, CDCl₃): 158.17,
155 153.76, 100.70, 48.76, 28.85, 20.37. ES+HRMS: Calculated M+H=238.2032 C₁₂H₂₄N₅.
156 Obtained: 238.2026.

157 **N⁴,N⁶-dicyclopentylpyrimidine-4,5,6-triamine (19)**: Purple solid, yield 45 %, mp: 265°C. δH
158 (400.45 MHz, CDCl₃): 8.31 (2H, bs, NH x2), 8.12 (1H, s, NCHN), 4.33 (2H, m, -CH cyclopentyl
159 x2), 2.04 (4H, m, -CH₂ cyclopentyl x2), 1.86 (8H, m, -CH₂ cyclopentyl x2), 1.40 (4H, m, -CH₂
160 cyclopentyl x2). δC (100.70 MHz, CDCl₃): 159.76, 157.05, 112.84, 53.29, 33.42, 23.87.
161 ES+HRMS: Calculated M+H=262.2026 C₁₄H₂₄N₅. Obtained: 262.2052.

2.1.3. General procedure for the preparation of compounds 16(a-c), 17c, 18c and 19c:

A solution of **16-19** (1 equiv.), with an excess (500 μ L) of the corresponding trialkylorthoesters or triaryloxyesters and methanesulfonic acid (0.2 equiv) was heated to 110°C for 24h, monitoring the reaction by TLC. The mixture was then cooled at room temperature. After two extractions with CH_2Cl_2 the organic phase was washed with an aq. saturated solution of NaCl and dried on Na_2SO_4 . Evaporation under vacuum gave a solid that was purified by column chromatography eluting with ethyl acetate/petroleum ether solutions.

N,9-Di-tert-butyl-9H-purin-6-amine (16a). White solid, yield 83%, δ H (400.45 MHz, CDCl_3): 8.34 (s, 1H), 7.76 (s, 1H), 5.71 (bs, 1H), 1.76 (s, 9H), 1.55 (s, 9H). δ C (100.70 MHz, CDCl_3): 154.89, 151.62, 149.11, 136.88, 121.68, 57.24, 52.24, 29.30, 29.20. ES+HRMS: Calculated $\text{M}+\text{H}=248.1875$ $\text{C}_{13}\text{H}_{22}\text{N}_5$. Obtained 248.1862.

N,9-Di-tert-butyl-8-methyl-9H-purin-6-amine (16b). Yellow solid, yield 28%, δ H (400.45 MHz, CDCl_3): δ 8.30 (s, 1H), 5.69 (bs, 1H), 2.73 (s, 3H), 1.85 (s, 9H), 1.55 (s, 9H). δ C (100.70 MHz, CDCl_3): 153.90, 151.15, 150.85, 147.99, 123.57, 60.04, 52.20, 30.56, 29.35, 19.99. ES+HRMS: Calculated $\text{M}+\text{H}=262.2032$ $\text{C}_{14}\text{H}_{24}\text{N}_5$. Obtained: 262.2029.

N,9-Di-tert-butyl 8-phenyl-9H-purin-6-amine (16c): Yellow solid, yield 80%, bp: 100°C. δ H (400.45 MHz, CDCl_3): 8.39 (1H, s, NCHN), 7.52-7.40 57 (5H, m, Ph), 5.78 (1H, bs, NH), 1.63 (9H, s), 1.55 (9H, s). δ C (100.70 MHz, CDCl_3): 151.30, 129.95, 129.59, 128.22, 60.46, 52.28, 49.82, 31.03, 29.31. ES+HRMS: Calculated $\text{M}+\text{H}=324.2188$ $\text{C}_{19}\text{H}_{26}\text{N}_5$. Obtained: 324.2164.

N,9-Diethyl-8-phenyl-9H-purin-6-amine (17c): Yellow solid, yield 38 %, bp: 108° C. δ H (400.45 MHz, CDCl_3): 8.41 (1H, s, NCHN), 7.67 (2H, m, Ph), 7.52 (3H, m, Ph), 4.31 (2H, q, J=7.1 and 7.3, N-CH₂), 3.73 (2H, m, NH-CH₂), 1.42 (3H, t, J=7.2, -CH₃), 1.33 (3H, t, J=7.2, -CH₃). δ C (100.70 MHz, CDCl_3): 150.52, 147.33, 132.07, 130.20, 129.09, 127.49, 119.64, 39.00, 29.83, 15.57, 15.17. ES+HRMS: Calculated $\text{M}+\text{H}=268.1774$ $\text{C}_{15}\text{H}_{18}\text{N}_5$. Obtained: 268.1757.

N,9-Diisobutyl-8-phenyl-9H-purin-6-amine (18c): White solid, yield 57 %, bp: 155°C. δ H (400.45 MHz, CDCl_3): 8.41 (1H, s, NCHN), 7.64 (2H, m, Ph), 7.51 (3H, m, Ph), 5.85 (1H, bs, NH), 4.13 (2H, d, J=7.6, N-CH₂), 3.49 (2H, bs, NH-CH₂), 2.06 (1H, m, CH(CH₃)₂), 1.98 (1H, m,

CH(CH₃)₂), 1.02 (6H, d, J=6.7, -CH(CH₃)₂), 0.73 (6H, d, J=6.7, -CH(CH₃)₂). δ C (100.70 MHz, CDCl₃): 155.06, 152.93, 150.94, 130.82, 129.99, 129.03, 119.59, 50.91, 48.34, 28.81, 28.69, 20.40, 19.91. ES+HRMS: Calculated M+H=324.2188 C₁₉H₂₆N₅. Obtained: 324.2169.

N,9-dicyclopentyl-8-phenyl-9H-purin-6-amine (19c): White solid, yield 28%, bp: 67°C. δ H (400.45 MHz, CDCl₃): 8.39 (1H, s, NCHN), 7.59 (2H, m, Ph), 7.52 (3H, m, Ph), 5.80 (1H, bs, NH), 4.69 (2H, m, CH cyclopentyl), 2.57 (2H, m, CH₂ cyclopentyl), 2.07 (4H, m, CH₂ cyclopentyl), 1.98 (2H, m, CH₂ cyclopentyl), 1.63 (8H, m, CH₂ cyclopentyl). δ C (100.70 MHz, CDCl₃): 154.47, 151.11, 150.13, 130.76, 129.96, 129.48, 128.95, 120.30, 58.06, 49.80, 33.53, 31.02, 24.75, 23.81. ES+HRMS: Calculated M+H=348.2182 C₂₁H₂₆N₅. Obtained: 348.

2.2 Biological assays

2.1.4. Competition binding in *hA₁AR*

hA₁AR competition binding experiments were carried out in a multiscreen GF/C 96-well plate (Millipore, Madrid, Spain) pretreated with binding buffer (Hepes 20 mM, NaCl 100 mM, MgCl₂ 10 mM, adenosine deaminase 2 U/mL, pH=7.4). In each well was incubated 5 μ g of membranes from Euroscreen CHO-A₁ cell line and prepared in our laboratory (Lot: A002/13-04-2011, protein concentration=5864 μ g/ml), 1 nM [³H]-DPCPX (140 Ci/mmol, 1 mCi/mL, PerkinElmer NET974001MC) and compounds studied. Non-specific binding was determined in the presence of 10 μ M R-PIA (Sigma P4532). The reaction mixture (Vt: 200 μ L/well) was incubated at 25°C for 60 min, after was filtered and washed four times with 250 μ L wash buffer (Hepes 20 mM, NaCl 100 mM, MgCl₂ 10 mM pH=7.4), before measuring radioactivity in a microplate beta scintillation counter (Microbeta Trilux, PerkinElmer, Madrid, Spain). Data was fitted to a 4-parameter logistic curve with GraphPad Prism 10 and K_i values were derived from the Cheng-Prusoff equation.²¹

2.1.5. Competition binding in *hA_{2A}AR*

hA_{2A}AR competition binding experiments were carried out in a multiscreen GF/C 96-well plate (Millipore, Madrid, Spain) pretreated with binding buffer (Tris-HCl 50 mM, EDTA 1 mM, MgCl₂ 10 mM, adenosine deaminase 2 U/mL, pH=7.4). In each well was incubated 5 μ g of membranes

from Hela-A_{2A} cell line and prepared in our laboratory (Lot: A002/17-04-2018, protein concentration=2058 µg/mL), 3 nM [³H]-ZM241385 (50 Ci/mmol, 1 mCi/mL, ARC-ITISA 0884) and compounds studied. Non-specific binding was determined in the presence of 50 µM NECA (Sigma E2387). The reaction mixture (Vt: 200 µL/well) was incubated at 25°C for 30 min, after was filtered and washed four times with 250 µL wash buffer (Tris-HCl 50 mM, EDTA 1 mM, MgCl₂ 10 mM, pH=7.4), before measuring radioactivity in a microplate beta scintillation counter (Microbeta Trilux, PerkinElmer, Madrid, Spain). Data was fitted to a 4-parameter logistic curve with GraphPad Prism 10 and K_i values were derived from the Cheng-Prusoff equation.²¹

2.1.6. Competition binding in hA_{2B}AR

hA_{2B}AR competition binding experiments were carried out in a multiscreen GF/C 96-well plate. In each well was incubated 25 µg of membranes from Euroscreen HEK-A_{2B} cell line and prepared in our laboratory (Lot: A009/14-02-2020, protein concentration=5254.8 µg/mL), 25 nM [³H]-DPCPX (137 Ci/mmol, 1 mCi/mL, PerkinElmer NET974001MC) and compounds studied. Non-specific binding was determined in the presence of 1000 µM NECA (Sigma E2397). The reaction mixture (Vt: 250 µL/well) was incubated at 25°C for 30 min, 200 µL was transferred to GF/C 96-well plate (Millipore, Madrid, Spain) pretreated with binding buffer (Tris-HCl 50 mM, EDTA 1 mM, MgCl₂ 5 mM, Bacitracin 100 µg/µL, adenosine deaminase 2 U/mL, pH=6.5), after was filtered and washed four times with 250 µL wash buffer (Tris-HCl 50 mM, EDTA 1 mM, MgCl₂ 5 mM, pH=6.5), before measuring radioactivity in a microplate beta scintillation counter (Microbeta Trilux, PerkinElmer, Madrid, Spain). Data was fitted to a 4-parameter logistic curve with GraphPad Prism 10 and K_i values were derived from the Cheng-Prusoff equation.²¹

2.1.7. Competition binding in hA₃AR

hA₃AR competition binding experiments were carried out in a multiscreen GF/B 96-well plate (Millipore, Madrid, Spain) pretreated with binding buffer (Tris-HCl 50 mM, EDTA 1 mM, MgCl₂ 5 mM, adenosine deaminase 2 U/mL, pH=7.4). In each well was incubated 30 µg of membranes from Hela-A₃ cell line and prepared in our laboratory (Lot: A006/17-01-2020, protein concentration=3408 µg/mL), 10 nM [³H]-NECA (22.4 Ci/mmol, 1 mCi/mL, PerkinElmer

NET811250UC) and compounds studied. Non-specific binding was determined in the presence of 100 μ M R-PIA (Sigma P4532). The reaction mixture (Vt: 200 μ L/well) was incubated at 25°C for 180 min, after was filtered and washed six times with 250 μ L wash buffer (Tris-HCl 50mM pH=7.4), before measuring radioactivity in a microplate beta scintillation counter (Microbeta Trilux, PerkinElmer, Madrid, Spain). Data was fitted to a 4-parameter logistic curve with GraphPad Prism 10 and K_i values were derived from the Cheng-Prusoff equation.²¹ The conditions were carried out in triplicate.

2.1.8. Functional study of seven adenine derivatives (Table 2) in hA_1AR

hA_1AR functional experiments were carried out in CHO- A_1 cell line. The day before the assay, the cells were seeded on the 96 well culture plate (Falcon 353072). The cells were washed with wash buffer (Nutrient Mixture F12 Ham's (Sigma N6658), 25mM Hepes; pH=7.4). Wash buffer was replaced by incubation buffer (Mixture F12 Ham's (Sigma N6658), 25mM Hepes, 20 μ M Rolipram (Sigma R6520); pH=7.4). Test compounds and XAC as reference compound (Sigma X103) were added and incubated at 37°C for 15 min. After, a concentration response curve of 5'-(N-ethylcarboxamido)adenosine (NECA) (Sigma E2387) was added and incubated at 37°C for 10 min. 10 μ M FSK (Sigma F3917) was added and incubated at 37°C for 5 min. After incubation, the amount of cAMP is determined using *cAMP Biotrak Enzymeimmunoassay (EIA) System Kit* (GE Healthcare RPN225). Data was fitted to a 4-parameter logistic curve with GraphPad Prism 10 and K_B values were derived from the derivation of Cheng-Prusoff equation proposed by Leff and Dougall.²² The conditions were carried out in triplicate.

2.1.9. Cell based assays

Adherent HCT-116, HKH2 (HCT116 with KRAS knockout), SW480, SW48, A549, MDA-MB-231 and MCF7 cells were maintained in DMEM supplemented with 10% FBS, and 2mM L-Glutamine, and incubated at 5% CO₂ at 37°C in a Heracell 240i tissue culture incubator. Semi-adherent K562 cells were maintained in RPMI 1640 supplemented with 10% FBS, and incubated at 5% CO₂ at 37°C in a Heracell 240i tissue culture incubator. Cells were passaged when 80-90% confluent for maintenance

2.1.10. Phenotypic screening

Cells were seeded into 96-well plates at the following densities with a working volume of 100µL: HCT116, SW480, HKH2, MDA-MB-231, MCF7, and A549 at 1000 cells/well, and SW48 at 4500 cells/well. K562 cells were seeded at 3000 cells/well, in a 95µL working volume. Seeding densities were decided using prior seeding density assays. All plates were incubated for 2 days, prior to treatment, apart from K562 which was seeded and treated on the same day. Drugs were prepared in DMSO with a working volume of 50µL with a concentration range from 100mM – 0.001mM, by serial dilution. To dilute drugs to the desired concentrations before adding to the cell plates, intermediate plates were freshly prepared, using 245µL of DMEM and RPMI media and 5µL of drug at each concentration. 5µL of this solution was then added to 95µL media in the cell plate to achieve a final dilution of 1000x and 0.01% DMSO from the initial drug plates. On day 2, media was aspirated and replaced with 95µL fresh media before adding 5µL of each drug from freshly prepared intermediate plates. Plates were then incubated for a further 5 days for drug treatment. Prestobblue dye was added to an additional seeded cell plate for each cell line to normalize for viability from the 2-day incubation. Prestobblue dye was incubated for 90 minutes. After incubation, absorbance was read on a PerkinElmer Envision 2101 plate reader at an emission wavelength of 580 nm. After 5 days of drug treatment, all plates were incubated with Prestobblue. All drug concentrations were transformed into logarithmic scales, with all conditions being converted into percentage viability compared to untreated cells (100% viability). Percentage viability values were used to calculate EC50 values for each condition, using non-linear regression variable slope (four parameters) in GraphPad Prism 9. The conditions were carried out in triplicate.

2.3 Docking Protocol

Docking analysis was carried out with Autodock 4.2.6 (AD4)²³ on the *hA₁AR* (*hA₁AR*, pdb IDs 5UEN, subunit B) and *hA_{2A}AR* (*hA_{2A}AR*, pdb IDs 4EIY). Ligands structures were built on Avogadro²⁴ and optimized using Gaussian software (HF/6-31G(d,p)) [Frisch et al. 2009].²⁵ Once optimized, ligands PDB files were prepared for docking using the prepare_ligand4.py script

included MGLTools 1.5.4.²³ Protein structure, were prepared for docking using the PDB2PQR tools.²⁶ Water and ligand molecules were removed and charges and non-polar hydrogen atoms were added at pH 7.0. The produced structures were saved as a pdb files and prepared for docking using the prepare_receptor4.py script from MGLTools. AD4 was used to dock the ligands into the *hA*₁ and *hA*_{2A}AR orthosteric binding site. The docking grid was centered on the orthosteric site and set with the following grid parameters: 70 Å × 60 Å × 70 Å with 0.375 Å spacing. In all calculations, AD4 parameter file was set to 100 GA runs, 2,500,000 energy evaluations and a population size of 150. The Lamarckian genetic algorithm local search (GALS) method was used for the docking calculations. All dockings were performed with a population size of 250 and a Solis and Wets local search of 300 rounds was applied with a probability of 0.06. A mutation rate of 0.02 and a crossover rate of 0.8 were used. The docking results from each of the 100 calculations were clustered based on root-mean square deviation (RMSD) solutions differing by less than 2.0 Å between the Cartesian coordinates of the atoms and ranked based on free energy of binding. The obtained conformations were individually inspected and figures were created with UCSF Chimera 1.15 [Pettersen et al. 2004].²⁷

3. Results

3.1 Synthesis of N-6,8,9-trisubstituted adenines

The commercially available compound 4,6-dichloro-5-nitropyrimidine was employed as the starting material to obtain the propargyloxy-substituted pyrimidine **1** in good yield through a reaction with propargyloxy alcohol. Subsequently, pyrimidine **1** was reacted with primary amines at room temperature, giving rise, initially, to the symmetric disubstituted 4,6-bis(alkyl/arylalkyl)amino-5-nitropyrimidines **2-7** via pre-reactive molecular complexes.¹⁷ The reduction of these pyrimidines was performed using tin(II) chloride, resulting in the formation of 4,6-bis(alkyl/arylalkyl)amino-5-aminopyrimidines **11-15**. These compounds were cyclized with three distinct trialkyl orthoesters (trimethyl orthoformate, trimethyl orthoacetate, or trimethyl orthobenzoate) and methanesulfonic acid (Scheme 1) to obtain purines with H (family a), methyl

(family b) and phenyl (family c) at C8 (Scheme 1) to obtain their derivatives **11a-15c**, that were previously presented by our group as antiparasitic compounds.¹⁸ For the purposes of the present study, we expanded the library of compounds to include derivatives with a tert-butyl group, an ethyl group, an isobutyl group and a cyclopentyl group in positions 6 and 9. The synthesis of these novel compounds, specifically **16c**, **17c**, **18c**, and **19c**, was achieved via cyclization with trimethyl orthobenzoate to obtain purines with phenyl (family c) at C8 (Scheme 1). All compounds were purified by column chromatography and characterised by nuclear magnetic resonance (¹H-NMR and ¹³C-NMR), high-resolution mass spectrometry (HRMS) and HPLC. As illustrated in Figure 1, the synthesised compounds share a purine ring, presenting two substituents at positions C6/N9, containing either aromatic or non-aromatic chains, and a substituent at C8.

3.2 Evaluation of the binding affinities of the purine library to hA₁, hA₂A, hA₂B and hA₃ ARs.

A series of seventeen adenine derivatives was evaluated *in vitro* for affinity and selectivity against the four hAR, using radioligand binding assays (Table 1). Biological results are expressed as K_i (nM, n = 3) or as percentage inhibition of specific binding at 10 µM (n = 2, average) for those compounds that did not fully display specific radioligand binding. K_i values were obtained by non-linear regression fitting of the data using Prism 7.0 software (GraphPad, San Diego, CA). Four known hAR ligands (XAC, CGS-15943, ZM241385, and MRS1220) were used as reference compounds, and the results are shown in Table 1.

Out of the seventeen compounds, four compounds stood out due to their affinity for one or two ARs. All these four compounds (**11c**, **12c**, **14c** and **15c**, Figure 1) shared a common structural feature, a phenyl group at position 8 of the adenine scaffold. 6/9-Bisarylalkyl derivatives, such as *N*,9-diphenethyl-8-phenyl-9*H*-purin-6-amine (**12c**) and *N*,9-bis(4-chlorobenzyl)-8-phenyl-9*H*-purin-6-amine (**15c**), proved to be selective against hA₁AR, with low percentages of inhibition of the three other ARs at 10 µM, the highest concentration tested. The dibenzyl derivative without substituents on the aromatic rings at positions C6 and N9, *N*,9-dibenzyl-8-phenyl-9*H*-purin-6-

amine (**11c**) showed a dual profile as hA_1/hA_{2B} AR ligand, with a selectivity index of 2.4. This is the only molecule within the series that can be considered a dual hA_1/hA_{2B} AR ligand ($K_i = 458.8$ nM and 1091 nM, respectively).

Out of the two 6/9-bisalkyl derivatives, *N*,9-diisopropyl-8-phenyl-9H-purin-6-amine (**14c**) proved to be the most active compound within the series, with a hA_1 AR K_i of 2.0 ± 0.3 nM (Table 1). This compound showed an affinity for the hA_3 AR, with a K_i of 211.2 ± 53 nM, meaning a selectivity index of 105.6. Therefore, **14c** was considered as a hA_1 AR selective ligand. Compound **16c**, a similar compound than **14c** but with a *tert*-butyl substituent rather than isopropyl at C6 and N9, was found to be non-active.

3.3 Evaluation of the antagonist potency (K_B) of the best ligands to hA_1 AR.

From the initial screening, it was clear that the most promising compounds had a very specific and selective interaction with hA_1 AR. Therefore, the four most active compounds (**11c**, **12c**, **14c** and **15c**, Figure 1) were selected to further investigate whether they act as agonists or antagonists of hA_1 AR, using a cAMP assay. The results of the hA_1 AR adenosinergic profile are shown in Table 2.

These results clearly show that compound **14c**, with isopropyl substituents, is the most promising candidate due to its low K_B value, indicating higher potency as an antagonist against the hA_1 AR.

3.4 Evaluation of new compounds based on **14c** as lead compound

Adenine **14c** was thus chosen as the lead compound from the library presented above, and the influence of the alkyl substituents at C6 and N9 in this scaffold was further investigated, while keeping the phenyl group at position 8, which is critical for maintaining activity. Derivatives **17c** and **18c** were then synthesized, using the same synthetic pathway shown above (Scheme 1) to introduce ethyl and isobutyl substituents at these positions as less bulky groups than *tert*-butyl. Moreover, the *N*,9-dicyclopentyl-8-phenyl-9H-purin-6-amine (**19c**, Figure 2) was synthesised based on *N*6-endonorbonan-2-yl-9-methyladenine (**N-0861**), which was developed in the early 1990s [Shryock et al. 1992; Pelleg & Hurt 1992];^{5,28} and the most recent work by Lambertucci et

al.,⁹ which reports that these scaffolds, containing a cycloalkylamine substituent at C6 (Figure 2), were found to be antagonists of *hA1AR* with IC_{50} values at the nanomolar level. After successfully synthesizing the three new compounds (Figure 2), they were screened for binding affinity to the four *hARs* (A_1 , A_2A , A_{2B} , and A_3). The three compounds have a K_i for *hA1AR* below 50 nM (Table 1), which is the lowest within the whole library, along with their lead compound **14c**. Furthermore, **19c**, which contains cyclopentyl substituents, exhibits high specificity for *hA1AR* with very low affinity for the three remaining *hARs* at 10 μ M. In contrast, **17c** and **18c** exhibit K_i values in the low micromolar range against some of the other receptors, indicating that **19c** has the highest binding specificity of the compounds presented here towards *hA1AR* among all the adenosine receptors. Finally, their antagonist potency (K_B) against *hA1AR* was measured showing values below 100 nM for **17c**, **18c** and **19c** (Table 2). Having established that the bisalkyl-substituted purines with a phenyl in position 8 of the purine ring have the highest affinity and antagonist activity for *hA1AR*, we decided to phenotypically screen them against several cell lines to determine their antiproliferative activity.

3.5 Phenotypic screening for antiproliferative effects across six different cell lines

To study the antiproliferative effects of the four bisalkyl-substituted purines, we selected six different cell lines shown in Table 3. Each of these cell lines provides a good model for testing the efficacy of anticancer drugs against highly prevalent cancers such as colon, breast, lung and leukaemia.

Table 4 presents the antiproliferative EC_{50} values in micromolar concentrations. **19c** showed significant potency across all tested cell lines, with the lowest EC_{50} value of 7 μ M observed in SW480 cells. The range of EC_{50} values, varying from 7 μ M to 39 μ M, across different cell lines. On the other hand, **18c** showed high EC_{50} values in most cell lines, except for SW-480 and K562, where they had lower EC_{50} values of 13 μ M and 17 μ M, respectively. Finally, **17c** presented very

low or no antiproliferative activity in most cell lines, except in the case of MDA-MB-231, which had a moderately low EC₅₀ value of 30 µM.

3.6 Correlation analysis of mRNA expression and sensitivity to lead compound

19c using RNAseq data from DepMap

To understand whether the antiproliferative effects of the lead compound **19c** are due to its antagonistic action on *hA1ARs*, a correlation analysis was carried out. The analysis looked at the relationship between ADORA1 mRNA expression levels, which encode the adenosine *hA1AR*, across various cell lines (Table 3) and their sensitivity to **19c**. The expression data were sourced from RNAseq datasets provided by the DepMap project.

The results obtained are shown in Figure 3A, which shows scatter plots with linear regression lines, representing the relationship between ADORA1 expression levels and the log-transformed EC₅₀ of **19c**. Although there was a negative correlation observed in both cases ($R = -0.57$, $p = 0.24$; $R = -0.47$, $p = 0.29$), the associations did not reach statistical significance.

On the other hand, Figure 3B shows a boxplot where cell lines are stratified by median expression levels of ADORA1, categorized into 'Low' and 'High' expression groups based on median cutoff values (left = 217, right = 131). T-tests indicate no statistically significant difference in compound sensitivity between the two groups (left p -value = 0.2318, right p -value = 0.1966).

3.7 Molecular Modelling

Based on our screenings, we established that a phenyl ring at the 8th position and alkyl groups at the 6th and 9th positions on the adenine scaffold are significant for selective antagonism of the *hA₁AR*. Specifically, adenine with substituents of either isopropylamine or cyclopentyl at positions 6 and 9 showed high binding affinity and selectivity towards *hA₁AR*. This suggests an optimal fit within the *hA₁AR* binding pocket. To explain the molecular basis of this interaction, we performed docking simulations to investigate the specific interaction between the ligand and receptor.

The best performing compounds, **14c** and **19c**, were thus investigated to assess the molecular interactions of these compounds in the A₁ subtype G-protein-coupled receptor (*hA₁AR*) and the A_{2A} subtype. The crystal structure of *hA₁AR* (PDB ID: 5UEN) was chosen to perform the docking

analysis [Glukhova et al. 2017]. This structure represents an inactive conformation of *hA₁AR* with a covalently-bound selective antagonist (DU172), and exhibits an open site binding cavity capable of accommodating both orthosteric and allosteric ligands.

The preferred binding pose of **14c** on *hA₁AR* (PDB ID: 5UEN) is shown in Figure 4a. The adenine ring is set between Leu250 and Phe171 forming stacking interactions with both residues and matching the binding orientation seen on the adenine moiety of the natural agonist adenosine in the *hA₁AR* active conformation (PDB ID: 7LD4) (Figure 4b) [Draper-Joyce et al. 2018; Draper-Joyce et al. 2021]. Similarly, this orientation allows the formation of a double H-bond interaction between the hydrogen of the 6-amine (hydrogen donor) and the N7 (hydrogen acceptor) in the adenine ring with the side chain of Asn254, as does adenosine in the *hA₁AR* active conformation (see PDB ID: 7LD4). In a similar way, antagonist DU172 in the inactive conformation (PDB ID: 5UEN) H-bonds the side chain of Asn254 (Figure 4c), but in a different orientation when compared with adenine **14c** binding pose. The C8 and N6 substituents at **14c**, are both facing hydrophobic pockets that are closed on the active conformation of *hA₁AR* (Figure 4d). The phenyl ring on C8 is displayed in the same region as the N1-propyl substituent in DU172, in a pocket deeper in the orthosteric site created by Val87, Thr91, Asn184, Thr277, Trp247, and His251, and shows a significant π -stacking interaction with Trp247. The side-chain movement of this residue, together with Val87, Thr91, Asn184, and Thr277, is responsible for the partial closure of this pocket on the *hA₁AR* active conformation. As for the N6-isopropyl substituent, it is displayed in a hydrophobic cleft towards the extracellular end of the receptor (Figure 4a and 4c). This cleft, located between helix5 (H5), helix6 (H6), and helix7 (H7), is also occupied by the cyclohexane moiety of cognate ligand DU172 and is established by residues Glu172, Met180, Leu253, Thr257 and Thr270. This last residue is *hA₁AR* specific and has been suggested to act as a gatekeeper for ligand access to the orthosteric site [Cheng et al. 2017]. On the other hand, the isopropyl substituent at N9 on **14c** occupies the same position as the ribose ring of the adenosine in the *hA₁AR* active conformation (PDB ID: 7LD4), establishing van der Waals interactions with Val87. In an identical manner, the most active compound, ligand **19c**, adopts the same spatial arrangement as **14c** on the inactive conformation of receptor *hA₁AR* (Figure 4e). The difference between these two

ligands is their substituents at N6 and N9 with two isopropyl chains on **14c** and two cyclopentyl rings on **19c**. The two, more voluminous and hydrophobic residues, on **19c** are inserted in the pocket created by Val87, Thr91, Asn184, Thr277, Trp247, and His251, as well as in the hydrophobic cleft between H5, H6, and H7, superimposing the spatial disposition of the isopropyl substituents on **14c**. In both locations, the cyclopentyl rings establish van der Waals interactions with the residues involved (Figure 4e and 4f).

Only minor differences define the active and inactive conformation of *hA₁AR*. These are the inward movement of transmembrane helix1 (H1) and helix2 (H2), upward movement of helix3 (H3), the displacement of H7 toward H6, and side-chain variations of Trp247, His278, Val87, Leu88, Thr91 and Thr277 [Draper-Joyce et al. 2018]. As a result, the inactive conformation shows a more open orthosteric binding site when compared to the active one. As described above, the preferred docking poses of **19c** and **14c** take advantages of this open orthosteric binding site in the inactive conformation, by inserting their N6 substituents in the hydrophobic cleft generated by the extracellular outward movement of H7, and its C8-phenyl ring in the pocket generated by residues Trp247, Val87, Leu88, and Thr91. Hence, this wider and more exposed orthosteric binding site observed in the inactive conformation could explain the higher affinity of ligands **19c** and **14c** toward this conformation that, in combination with the molecular interactions established with important catalytic and conformational-dependent residues, might account for the experimental low nanomolar K_i values observed in this isozyme and its antagonist activity.

As for the differences between *A₁* and *A_{2A}* receptors, they are mainly focused on conformational variations in the extracellular ends of H1, H2, H3, and H7, as well as the orientation of extracellular loop 2 (ECL2). These differences determine that the extracellular region of the orthosteric binding site on *hA₁AR* is more open than that of *hA_{2A}AR*. Some minor differences are also found on their binding pockets, where only four residues change: Val62, Asn70, Glu170, and Thr270 in *hA₁AR* (corresponding to Ala59, Ser67, Leu170, and Met270 in *hA_{2A}AR*) [Cheng et al. 2017]. When ligands **19c** and **14c** were docked on *hA_{2A}AR* (PDB ID: 4EIY), an inactive conformation of the receptor with antagonist ZM241385 bound to its orthosteric binding site, substantially different bonding poses were found compared to those seen on *hA₁AR*. The adenine

ring is displayed with its C8 phenyl ring pointing towards a hydrophobic pocket located between H2 and H3 and enabled by the presence of the less bulky, *hA_{2A}AR* specific, Ala59 residue at H2. Moreover, the inward movement of the extracellular end of H7 in *hA_{2A}AR* also prevents the *hA₁AR* adenine orientation, disallowing the H-bonds with Asn253 (*hA_{2A}AR* numbering) and an effective stacking interaction with Leu249 (Leu250 in *hA₁AR*). In addition, the presence of the *hA_{2A}AR* specific Met270 residue at H7 blocks the cleft that allowed the insertion of the N6-isopropyl and cyclohexyl substituents in *hA₁AR*, forcing the adenine into the new orientation (Figure 5a and 5b). In summary, the different poses observed for ligands **19c** and **14c** on both receptors due to the extracellular conformational variations, as well as the receptor-specific binding site residues, could explain the experimentally observed isozyme selectivity in these ligands.

The predicted binding energies and inhibitory constants of **19c**, **14c** and the natural ligand adenosine against 5UEN (*hA₁AR* inactive), 7LD4 (*hA₁AR* active), 4EIY (*hA_{2A}AR* inactive) and 5G53 (*hA_{2A}AR* active) are presented in Table SI-1. The data indicate that **19c** is the most effective ligand in terms of binding strength and inhibitory potency among the proteins under study. These findings suggest that **19c** may be a promising candidate for further drug development targeting these adenosine receptors.

3.8 Physicochemical and pharmacokinetic characterization of the adenine derivatives and reference standards

Finally, the adenine library and reference standards were characterized for their physicochemical and pharmacokinetic properties using chemical informatics tools to evaluate their potential as drug candidates. The total solvent-accessible surface area (TSPA), partition coefficient (cLogP), were calculated using the SwissADME web-based tools [Daina et al. 2017]. The TSPA and cLogP values provided insight into the molecules' size, shape and lipophilicity. Based on these results, we observed that despite sharing the same TSPA, modification of positions 6 and 9 in the purine ring have a great impact in the lipophilicity of the molecules and therefore can influence the absorption, distribution, metabolism, and excretion (ADME) profile of these compounds.

Next, the compounds and control molecules were further analyzed for their drug metabolism and pharmacokinetics (DMPK) properties using the pkCSM web tool.²⁹ Relevant DMPK parameters, including CaCo2 permeability, intestinal absorption, BBB permeability (log BB), fraction unbound (human) (Fu), and predicted clearance, were calculated. These parameters provided additional information about the compounds' bioavailability, distribution, and elimination characteristics. The parameters obtained for the four most promising compounds within the studied series are shown in Table SI-2.

To analyse these results, we focused on **14c**, the compound with the lowest K_i and K_B for hA_1R , and **19c** the highest selectivity index for hA_1R and the best antiproliferative performance of the entire library presented here. The pharmacokinetic profiles of these two compounds present several similarities. Both compounds exhibit an identical TPSA of 55.63 Å² as well as very similar high intestinal absorption rates. However, there are also notable differences. Compound **14c** present a higher CaCo2 permeability (1.32 Log Papp) in comparison to **19c** (0.64 Log Papp), indicating a superior intestinal permeability. On the other hand, **19c** present markedly elevated blood-brain barrier (BBB) permeability (0.78 Log BB) in comparison to **14c** (-0.03 Log BB), indicating a greater chance for **19c** to go through the BBB. Furthermore, **19c** shows a higher fraction unbound (0.42) in comparison to **14c** (0.17), indicating that a greater proportion of **19c** is present in its free form. Lastly, **19c** has a higher predicted clearance (0.82 log ml/min/kg) than **14c** (0.59 log ml/min/kg), indicating a potentially more rapid clearance from the body

4. Discussion

The described synthetic route provides access to a library of eighteen 6,8,9-trisubstituted adenine derivatives, demonstrating structural diversity generated through an efficient cyclization reaction. To note, the formation of 4,6-bis(alkyl/arylalkyl)amino-5-nitropyrimidines (**2-7**) via pre-reactive molecular complexes, a phenomenon that has rarely been reported in S_NAr reactions. This finding was recently presented by our research.¹⁷

Given that our compounds were initially designed to target enzymes of the trypanosome purine salvage pathway, a metabolic pathway that relies on adenine and adenosine as substrates we hypothesized that they might also have affinity for human adenosine receptors, which also use adenine as a natural ligand. This hypothesis led us to investigate the interaction of these compounds with *h*AR subtypes. Therefore, we began this study by screening our previously published library of compounds, including molecules **11a-15c**, to interrogate their binding affinity toward the four human adenosine receptor subtypes (A_1 , A_{2A} , A_{2B} , and A_3).¹⁸ This initial screen identified several compounds with interesting affinity and selectivity profiles for *h*ARs (Table 1). In particular, the presence of a phenyl group at position C8 was demonstrated to be crucial for obtaining high-affinity ligands for *hA*₁AR. Moreover, the nature of the substituents at positions 6 and 9 was found to be important for achieving selectivity, as bisarylalkyl derivatives such as **12c** and **15c** showed selective binding affinity towards *hA*₁AR. The dibenzyl derivative without substituents on the aromatic rings **11c** showed a dual profile for *hA*₁AR and *hA*_{2B}AR subtypes. Among the bisalkyl derivatives, compound **14c**, with isopropyl substituents at positions 6 and 9, was identified as the most potent binder, with a K_i of 2 nM, lower than that obtained with reference compound XAC, and a selectivity index of 105.6 against the *hA*₃AR receptor subtype. To our knowledge, **14c** is the most potent *hA*₁AR binder described to date. Functional studies of the most promising candidates confirmed that these molecules acted as antagonists at the *hA*₁AR. In addition, using compound **14c** as the lead structure and based on previous work with *hA*₁AR antagonists and inspired by N-0861^{5,28} and the work of Lambertucci,⁹ that presented cycloalkyl groups at N6 of the adenine ring four new derivatives, **16c**, **17c**, **18c** and **19c**, were designed and synthesized. Compound **19c**, containing two cyclopentyl groups showed the highest binding affinity of the three new compounds to the *hA*₁AR, with K_i below 50 nM. Importantly, compound **19c** presented a very high selectivity for the *hA*₁AR over the other AR subtypes, confirming the importance of the substituents at positions 6 and 9 for the selectivity profile. In addition, these new compounds presented K_b values below 100 nM, thus indicating them as very promising lead compounds.

The phenotypic screening (Table 4) of the most promising compounds (**14c**, **17c**, **18c** and **19c**) across six different human cell lines (Table 3) revealed that compound **19c** had an interesting antiproliferative effect, exhibiting the lowest EC₅₀ values, and a particularly strong effect on SW480, a colorectal cancer cell line, for which an EC₅₀ of 7 µM was determined. When comparing the EC₅₀ values of **19c** and **14c** across the six cancer cell lines, both compounds exhibit antiproliferative activity. However, **19c** consistently shows greater potency. The similar proportional effect of **14c** and **19c** across the cell lines could suggest that they target similar pathways, with **19c** being more effective at inhibiting those pathways. The difference in efficacy between **19c** and **14c** may be due to several factors, such as **19c** higher affinity for the target in the cellular environment or better cellular uptake. It is also possible that **19c** engages additional targets or pathways contributing to its enhanced potency. These results indicate that this type of bisalkyl-substituted adenines can be further developed as potential anticancer agents, with a focus on colorectal cancer models. Further mechanistic studies would then be required. In contrast, **18c**, and especially **17c**, had poor or no antiproliferative activity in most of the cell lines. While not achieving statistical significance, a trend towards a negative correlation between ADORA1 mRNA expression and the sensitivity to compound **19c** was observed. This result suggests that ADORA1 expression may potentially serve as a biomarker for responsiveness to these families of compounds, although further investigation with more cell lines and samples would be necessary to confirm this potentiality. Molecular modelling studies suggest that the adenine ring is stabilized by stacking interactions with Leu250 and Phe171, while the N6 and C8 substituents interact with specific residues within the orthosteric pocket. The binding orientation seen on the adenine moiety matches that of the natural agonist adenosine. Importantly, the N6-isopropyl group of **14c** and the N9-isopropyl group of **14c** occupied analogous positions to the ribose and the cyclohexane moiety of known hA₁AR ligands, which suggests a role for these groups in the binding process. The higher activity of **19c**, with cyclopentyl substituents, could be due to the higher hydrophobicity and volume of these

groups, as they occupied the same pocket as the isopropyl substituents in **14c**, but with stronger van der Waals interactions with surrounding residues. Differences in binding poses in the *hA₁*AR and the *hA_{2A}*AR receptors could be attributed to conformational variations and the different specific residues of their orthosteric binding sites. For example, the extracellular end of H7 in *hA_{2A}*AR prevents the adenine orientation seen in the *hA₁*AR, while a more opened hydrophobic pocket, which is specific to the *A_{2A}* subtype, allows the phenyl group to fit. The different binding poses observed are consistent with the observed selectivity profile. While our modeling studies suggest the positioning of the substituents within the receptor binding site, additional experimental data is required to confirm those interactions, as well as the preferred poses of the various molecules. Finally, the *in silico* analysis of the physicochemical and pharmacokinetic profiles of these compounds suggested that both **14c** and **19c** may be potentially useful for drug development, presenting a good balance between their absorption, distribution, metabolism and excretion profiles, and showing high intestinal absorption rates. These properties suggest that **14c** and **19c** may be a potentially useful compound for further development as drug candidates with excellent oral bioavailability.

5. Conclusion

In summary, the results presented here highlight the potential of trisubstituted adenine derivatives as high-affinity and highly selective *hA₁*AR antagonists. The identification of **19c** as a potent and selective *hA₁*AR antagonist, with good bioavailability and significant antiproliferative effects, provides a strong foundation for further optimization and development of a new family of AR ligands. The data obtained in docking simulations also suggest that ligand **19c** may be a promising candidate for further drug development targeting these adenosine receptors. These compounds, and particularly compound **19c**, show promise for future therapeutic applications.

6. Acknowledgments

M.J.M. thanks Ministerio de Ciencia e Innovación (PID2020-116076RJ-I00/AEI/10.13039/501100011033) for the financial support. The authors thank the Centro de Servicios de Informática y Redes de Comunicación (CSIRC), Universidad de Granada, for providing the computing time. Funding for open access charge: Universidad de Granada / CBUA

7. Disclosure

The author(s) report no conflicts of interest in this work.

8. Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, we used DeepL Write and AI Studio (Google) in order to provide language refinement and proofreading purposes during the preparation of this manuscript. These tools were used to improve clarity and coherence without changing the scientific content or interpretations. All intellectual contributions and the final responsibility for the content remain solely with us. After using this tool/service, we reviewed and edited the content as needed and take full responsibility for the content of the publication.

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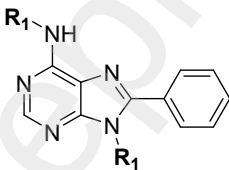
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- Table 1** Binding affinity results for the four *hAR* (A_1 , A_{2A} , A_{2B} and A_3) of the adenine derivatives (see Fig. 1) and reference standards (XAC, CGS-15943, ZM241385, and MRS1220) presented in the manuscript

Compounds	Receptors							
	<i>hA₁</i>		<i>hA_{2A}</i>		<i>hA_{2B}</i>		<i>hA₃</i>	
	% Inhib.	K _i (nM)	% Inhib.	K _i (nM)	% Inhib.	K _i (nM)	% Inhib.	K _i (nM)
11a	43±1		8±3		32±4		45±4	
11b	41±1		4±2		44±3		35±1	
11c		458.8±13	33±4			1091.0±35	28±1	
12a	49±2		7±1		12±1		30±3	
12b	38±1		5±2		18±3		21±3	
12c		620.4±25	8±3		44±1		48±5	
13a	27±2		16±2		2±2		11±3	
13b	29±2		17±4		17±2		24±2	
13c		880.2±31	24±1		3±1		37±4	
14a	55±5		11±3		22±3		21±1	
14b	36±2		4±2		26±4		20±1	
14c		2.0±0.3	33±2		55±5			211.2±53
15a	20±2		23±3		1±1		30±5	
15c		71.9±36.6	27±1		8±3		5±2	

16a	36±5	23±3	2±1	13±4
16b	24±1	11±3	5±3	14±3
16c	20±1	2±2	4±1	7±3
17c	28±4	2664±42	1503±22	1482±34
18c	20±2	30±1	764±60	334±13
19c	46±10	42±2	57 ± 4	42±1
XAC	13.9±2.2 ^a			
CGS-15943		1.4±0.3 ^b		
ZM 241385			28.3±4.4 ^c	
MRS 1220				3.6±0.7 ^d

^a K_i = 29.1 nM, reported by Klotz et al.³⁰ ^b K_i = 4.3 nM, reported by Fredholm et al.³¹ ^c K_i = 32.0 nM, reported by Fredholm et al.³¹ ^d K_i = 1.7 nM, reported by Gao & Jacobson.³² n = 3. Note: Compound **15b** has been synthesized, but the yield has been very low. Therefore, and because its substituents do not belong to the most active series, this molecule was no further evaluated.

Table 2. Antagonist potency (K_B) against hA₁AR of the adenine derivatives **11c**, **12c**, **14c**, **15c**, **17c**, **18c** and **19c** and the reference standard **XAC**

		hA₁AR
Compound	R ₁	K _B (nM)

11c	Bn-	1272.2±268.5
12c	PhEt	550.7±232.6
14c	<i>i</i> Pr	24.9±19.4
15c	pClBn	687.2±32.7
17c	Et	50.7±9.45
18c	<i>i</i> Bu	83.6±28.2
19c	cP	99.7±10.4
XAC^a		37.9±20.2

Table 3. Cell lines used for phenotypic screening of compounds **14c**, **17c**, **18c**, and **19c**.

Cell Line	Origin	Field of use
SW480, SW48	Primary colorectal adenocarcinomas	Colorectal cancer research
HCT116	Colorectal carcinoma	Colorectal cancer research
	Chronic myelogenous leukemia	
K562	(CML)	Hematological research, leukemia,
	Breast adenocarcinoma (triple-	
MDA-MB-231	negative)	TNBC research (highly aggressive)
		Non-small cell lung cancer (NSCLC)
A549	Human lung carcinoma	research

Table 4. EC₅₀ (μM) values of compounds as antiproliferative agents across various cancer cell lines. n = 3

Compound	SW480	SW48	HCT116	K562	MDA-MB-231	A549
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14c	40 ± 12.14	79 ± 35.65	78 ± 37.99	35 ± 0.93	37 ± 11.33	62 ± 33.56
17c	>100	>100	>100	>100	30 ± 0.61	77 ± 40.41
18c	13 ± 3.77	>100	>100	17 ± 8.82	53 ± 40.41	>100
19c	7 ± 0.71	10 ± 0.27	12 ± 2.63	13 ± 9.01	15 ± 5.06	39 ± 9.48

Abbreviations: AR: Adenosine Receptor; GPCR: G protein-coupled receptor; *hA₁*AR: human adenosine A1 receptor; *hA₂*AAR: human adenosine A2A receptor; *hA₂*BAR: human adenosine A2B receptor; *hA₃*AR: human adenosine A3 receptor; N-0861: N6-endo-norbornan-2-yl-9-methyladenine; TLC: Thin-Layer Chromatography; NMR: Nuclear Magnetic Resonance; CDCl₃: Deuterated Chloroform; ES: Electrospray Ionization; HPLC: High-Performance Liquid Chromatography; TEA: Triethylamine; DPCPX: 1,3-dipropyl-8-cyclopentylxanthine; NECA: 5'-(N-ethylcarboxamido)adenosine; R-PIA: R-Phenylisopropyladenosine; Tris-HCl: Tris(hydroxymethyl)aminomethane hydrochloride; EDTA: Ethylenediaminetetraacetic acid; cAMP: cyclic adenosine monophosphate; EIA: Enzyme Immunoassay; FSK: Forskolin; XAC: 8-Cyclopentyl-1,3-dipropylxanthine; DMSO: Dimethyl Sulfoxide; AD4: AutoDock 4 PDB: Protein Data Bank; RMSD: Root-Mean-Square Deviation; TSPA: Total Solvent-Accessible Surface Area cLogP: Calculated octanol-water partition coefficient; ADME: Absorption, Distribution, Metabolism, and Excretion; DMPK: Drug Metabolism and Pharmacokinetics; BBB: Blood-Brain Barrier; Fu: Fraction Unbound in Human Plasma; IC₅₀: Half maximal inhibitory concentration; EC₅₀: Half-maximal effective concentration; mRNA: messenger ribonucleic acid.

Scheme 1. Synthetic methodology. Reagents and conditions: a) Propargyl alcohol, DBU, THF, rt, 1 h; b) R¹-NH₂, TEA, DCM, rt, 30 min.; c) SnCl₂·2H₂O, EtOH, reflux, 1 h, d) (CH₃)₃CR², CH₃SO₃H, 110 °C, 24 h. cP = cyclopentyl. The synthesis of compounds **11-15 (a-c)** and their intermediates is described elsewhere.¹⁸

Figure 1. The first series of adenine **11-16(a-c)** derivatives tested to measure their binding affinity to human adenosine receptors. R² = H (family a), methyl (family b), and phenyl (family c).¹⁸

Figure 2. Bisalkyl compounds synthesized based on **14c** as the lead structure and the well-known hA₁AR inhibitors **N-0861** and **N-cyclopentyl-9-methyl-8-phenyl-adenine**.

Figure 3. A) scatter plot showing the correlation between median-of-ratio normalized ADORA1 mRNA expression and the log-transformed half-maximal inhibitory concentration (EC₅₀) of **19c** across various cancer cell lines. The Pearson correlation coefficient (R = -0.57) indicates a negative correlation, but it is not statistically significant (p = 0.24). The blue line represents the linear regression, and the grey shaded area represents the 95% confidence interval. **B)** Boxplot separating cell lines based on their median ADORA1 expression, with a left cutoff of 217 and a right cutoff of 131. The corresponding log-transformed half-maximal inhibitory concentration (EC₅₀) of **19c** is also shown.

Figure 4. (a) Predicted binding pose of **14c** (blue) on hA₁AR (PDB ID: 5UEN, tan); (b) Superposition of the active conformation of hA₁AR (PDB ID: 7LD4, dark gray) with the natural agonist adenosine (orange) and the predicted binding pose of **14c** (blue) on hA₁AR (PDB ID: 5UEN, tan); (c) superimposition of the covalently bound hA₁AR-selective antagonist DU172 (pink) and the predicted binding pose of **14c** (blue) on the inactive conformation of hA₁AR (PDB ID: 5UEN, tan); (d) Surface superposition of active (PDB ID: 7LD4, dark gray) and inactive (PDB ID: 5UEN, tan) conformations of hA₁AR with the predicted binding pose of **14c** (blue) and the natural agonist adenosine (orange); (e) Predicted binding poses of **14c** (blue) and **19c** (red) on hA₁AR

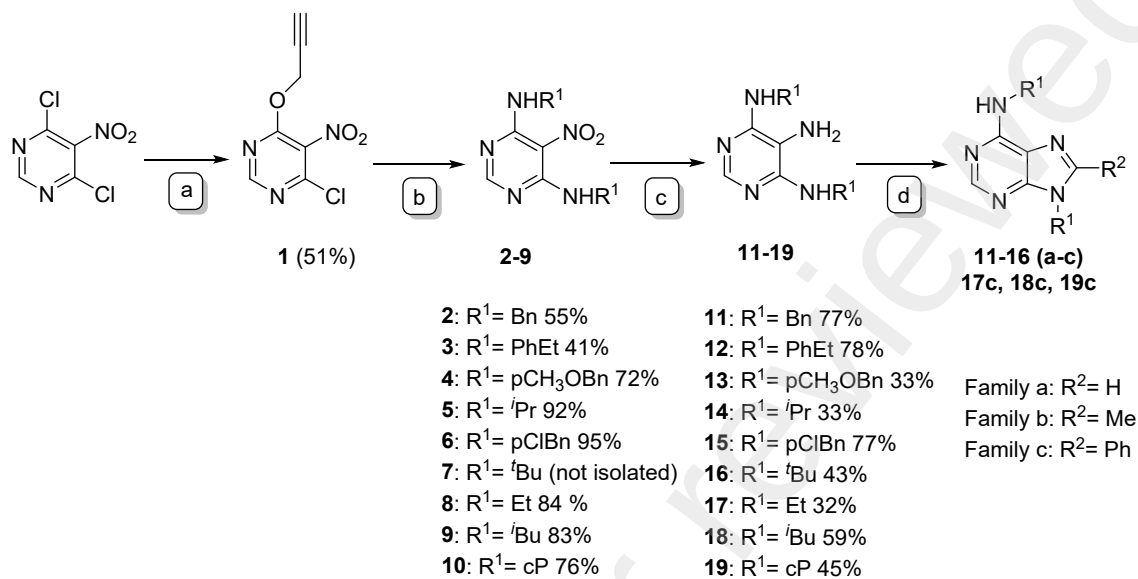
789 (PDB ID: 5UEN, tan); (f) Surface superposition of active (PDB ID: 7LD4, dark gray) and inactive
790 (PDB ID: 5UEN, tan) conformations of *hA₁*AR with the predicted binding pose of **14c** (blue) and
791 **19c** (red). Hydrogen bonds are shown as dashed yellow lines.

792

793 **Figure 5.** (a) Superposition of *hA₁*AR (PDB ID: 5UEN, tan) and *hA_{2A}*AR (PDB ID: 4EIY, purple)
794 with predicted binding pose of **19c** (dark green) and **14c** (light green) on *hA_{2A}*AR; (b) surface
795 representation of the inactive conformation of *hA_{2A}*AR (PDB ID: 4EIY, purple) and *hA₁*AR (PDB
796 ID: 5UEN, tan) with predicted binding poses of **19c** (dark green) and **14c** (light green). Hydrogen
797 bonds are represented by dashed yellow lines.

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Scheme 1.

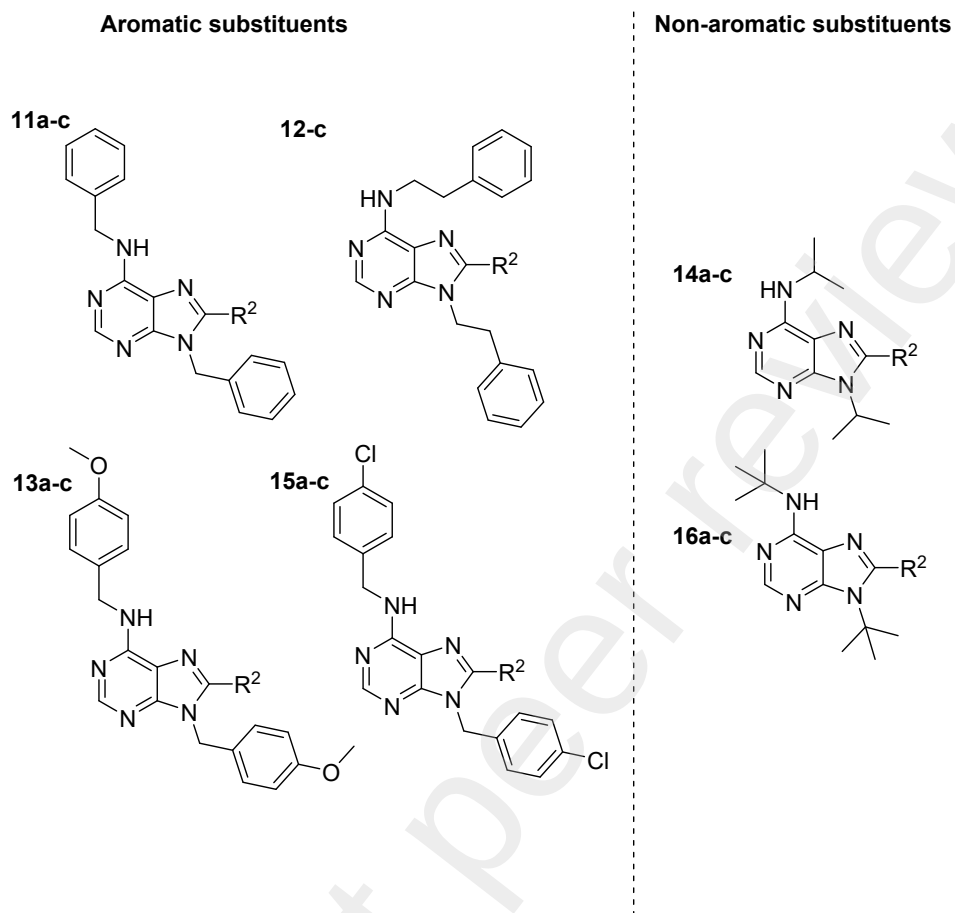


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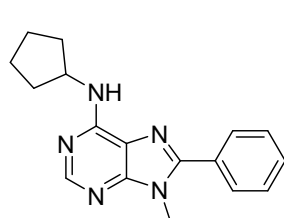
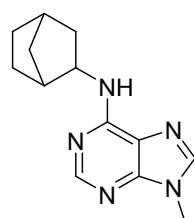
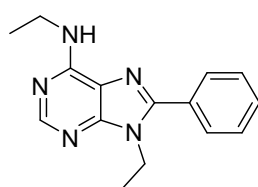
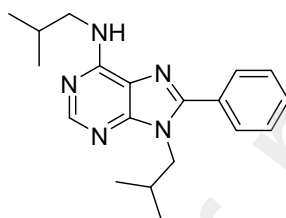
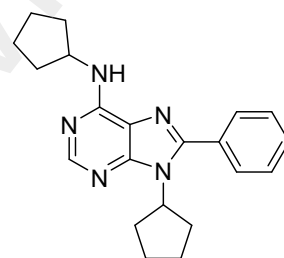
Figure 1.

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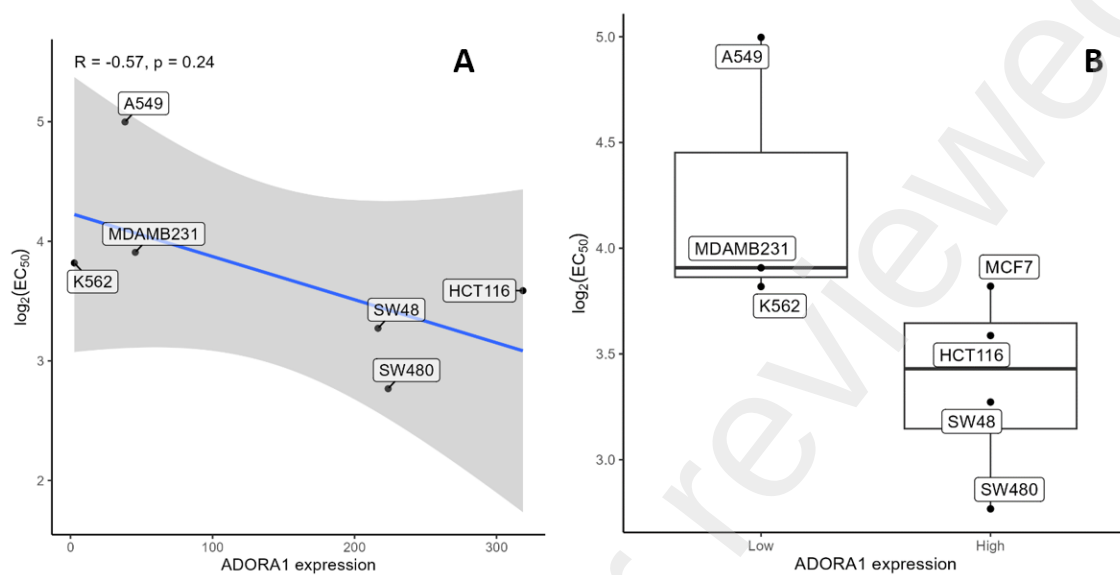
Figure 2**N-cyclopentyl-9-methyl-8-phenyl-adenine****N-0861****17c****18c****19c**

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Figure 3



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Figure 4

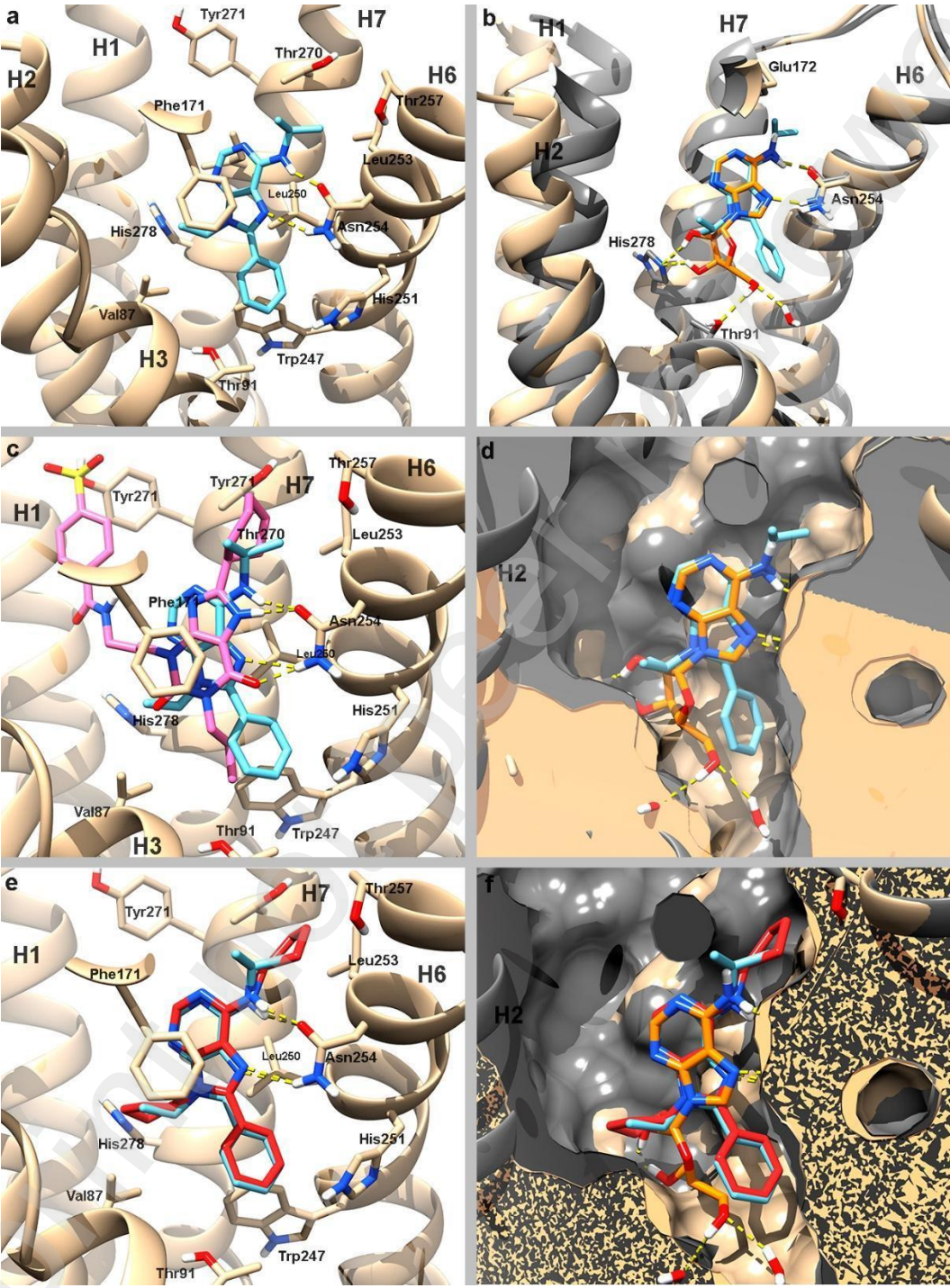
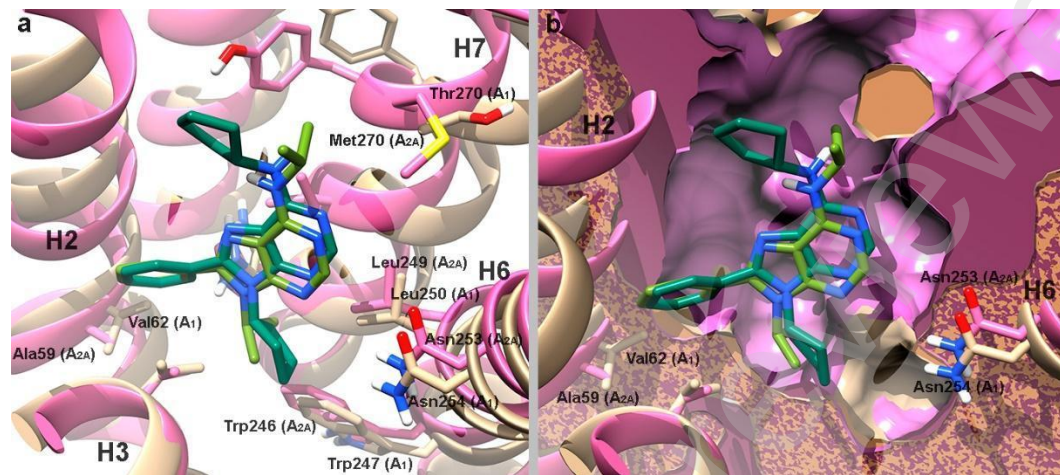
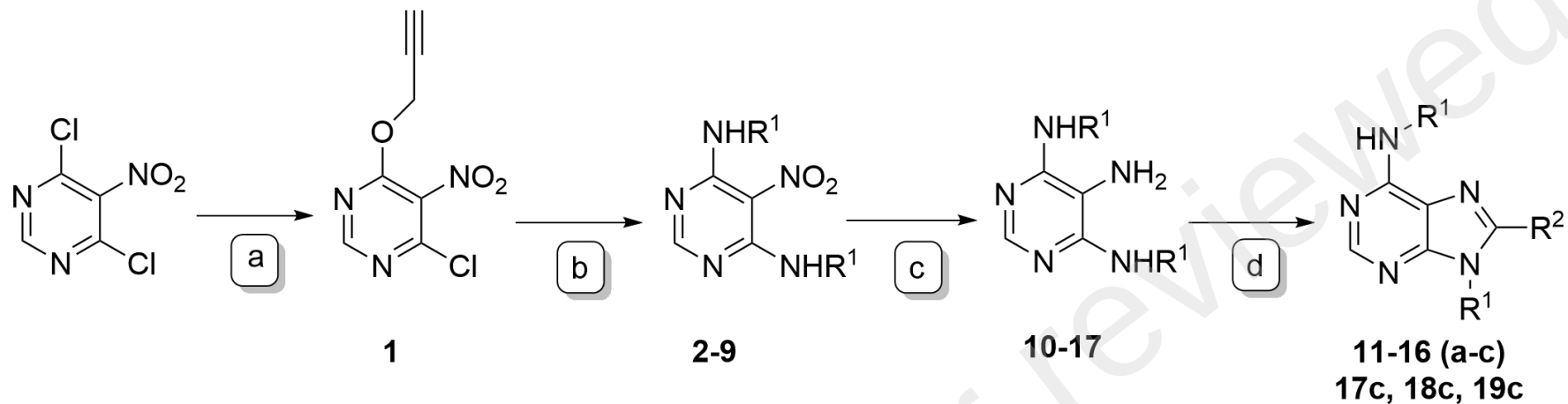


Figure 5





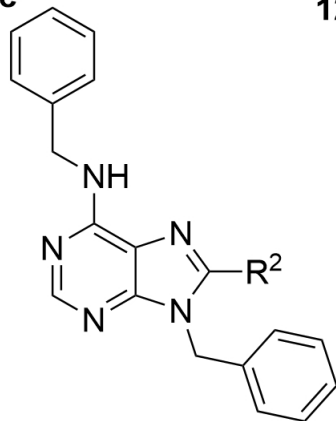
2: R¹ = Bn
3: R¹ = PhEt
4: R¹ = pCH₃OBn
5: R¹ = *i*Pr
6: R¹ = pClBn
7: R¹ = *t*Bu
8: R¹ = Et
9: R¹ = *i*Bu
10: R¹ = cP

11: R¹ = Bn
12: R¹ = PhEt
13: R¹ = pCH₃OBn
14: R¹ = *i*Pr
15: R¹ = pClBn
16: R¹ = *t*Bu
17: R¹ = Et
18: R¹ = *i*Bu
19: R¹ = cP

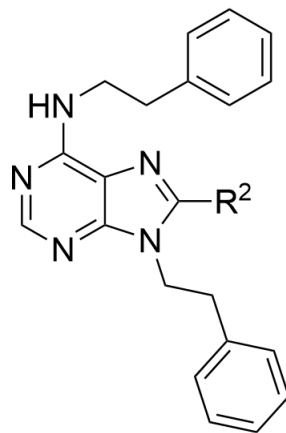
Family a: R² = H
 Family b: R² = Me
 Family c: R² = Ph

Aromatic substituents

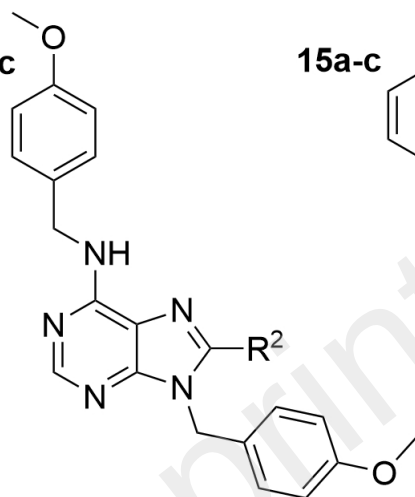
11a-c



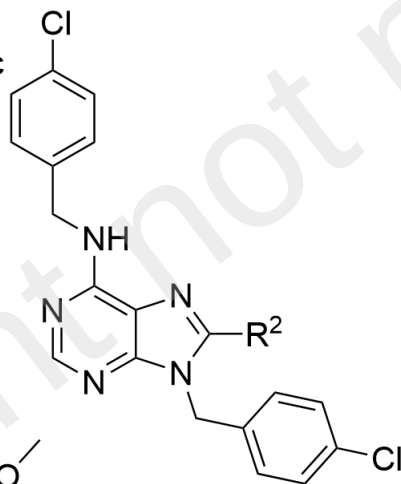
12-c



13a-c

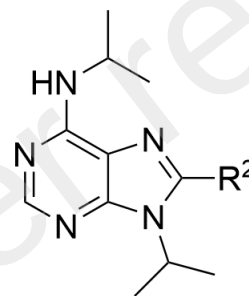


15a-c

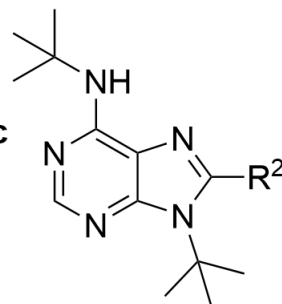


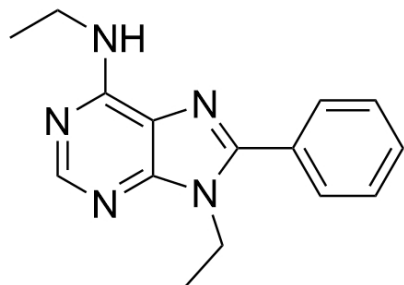
Non-aromatic substituents

14a-c

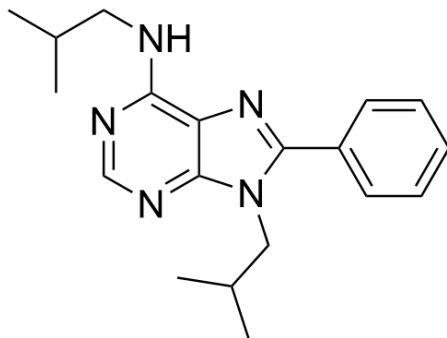


16a-c

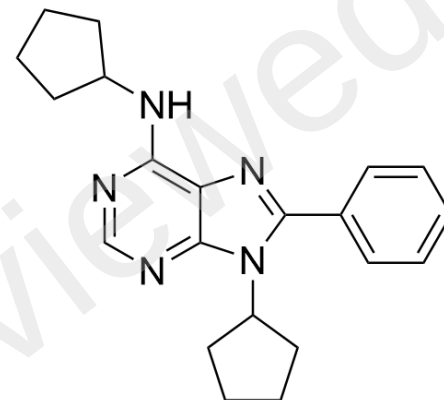




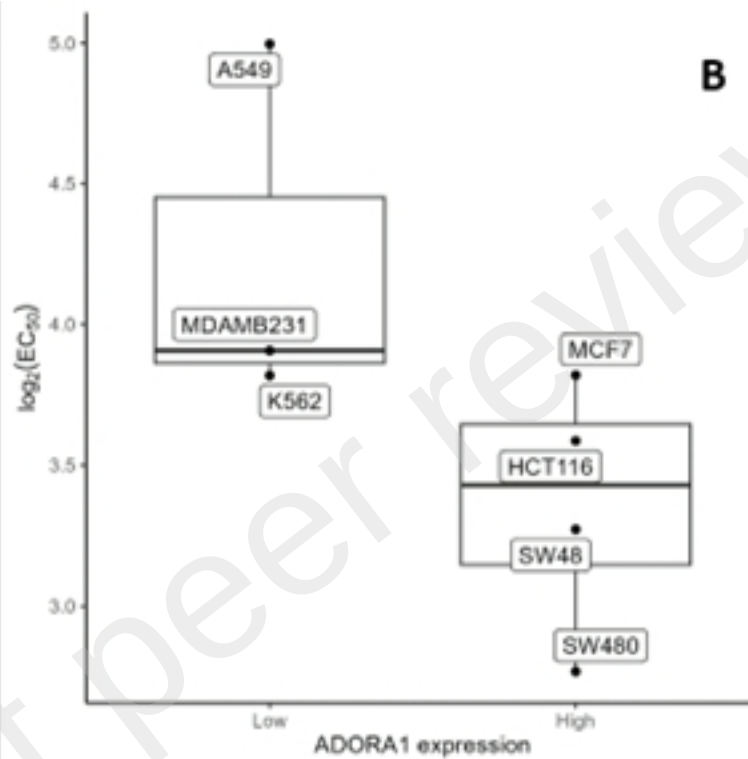
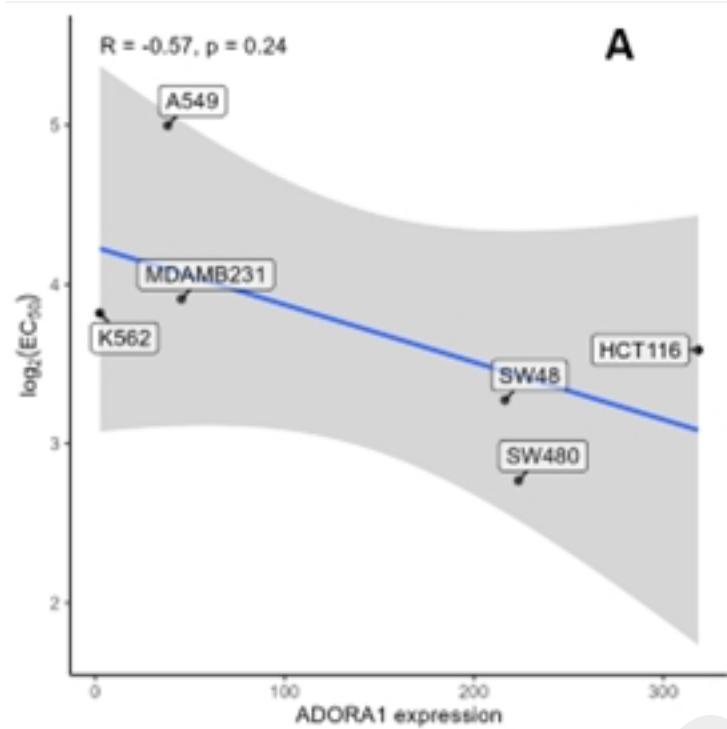
17c

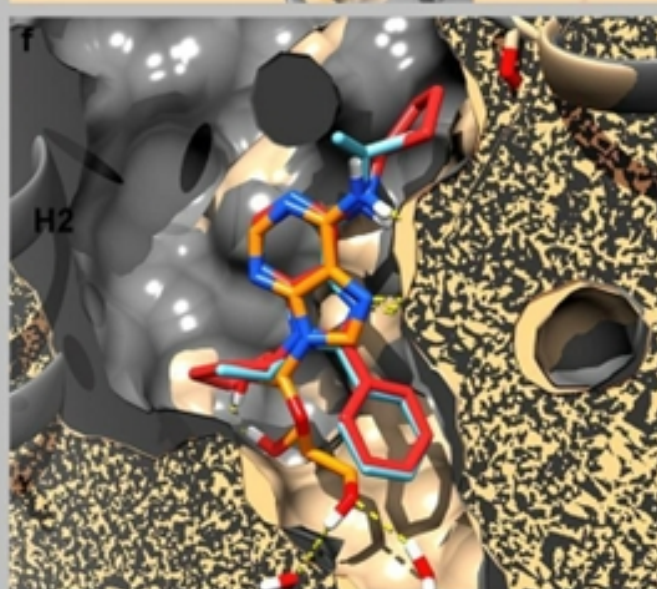
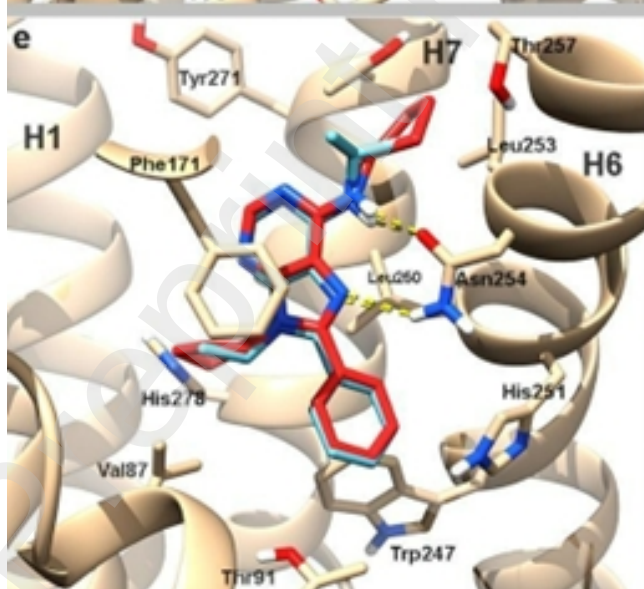
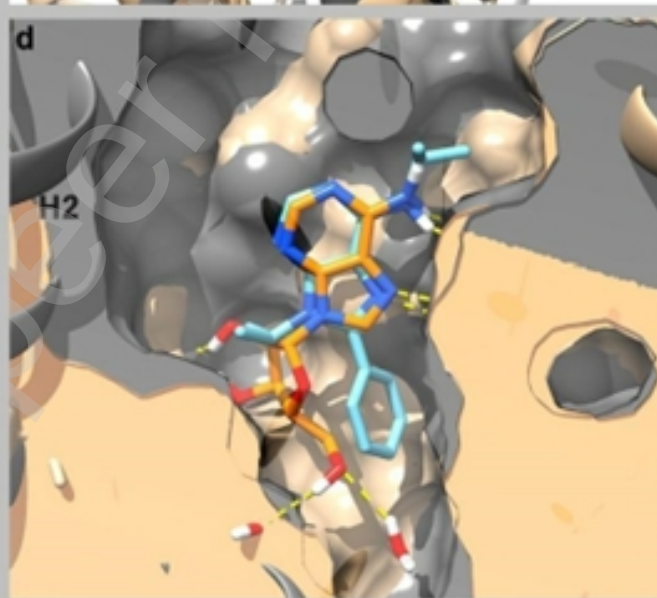
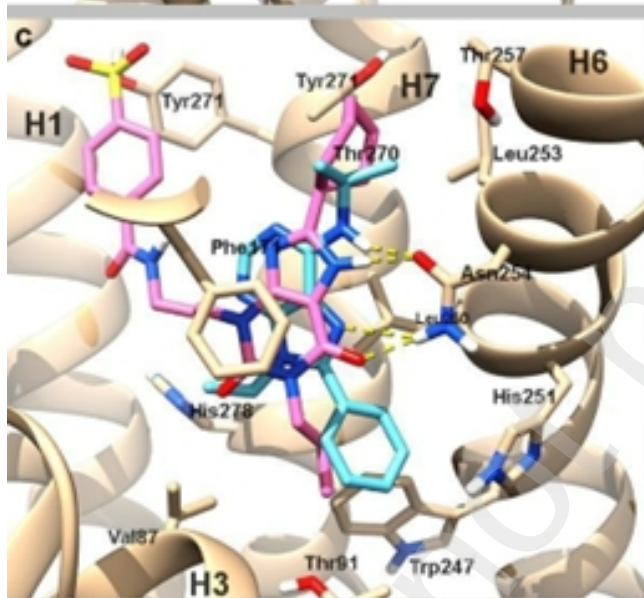
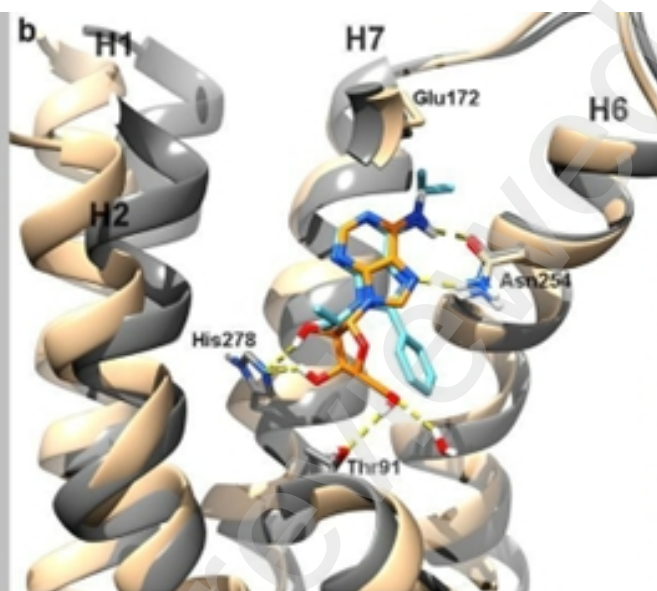
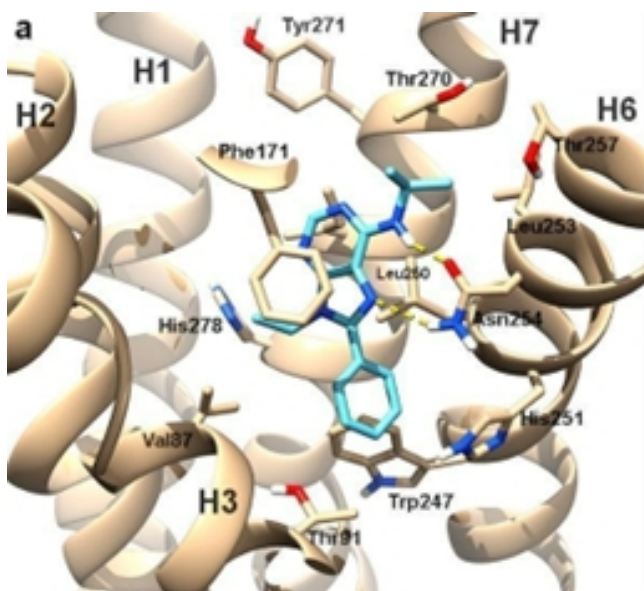


18c



19c





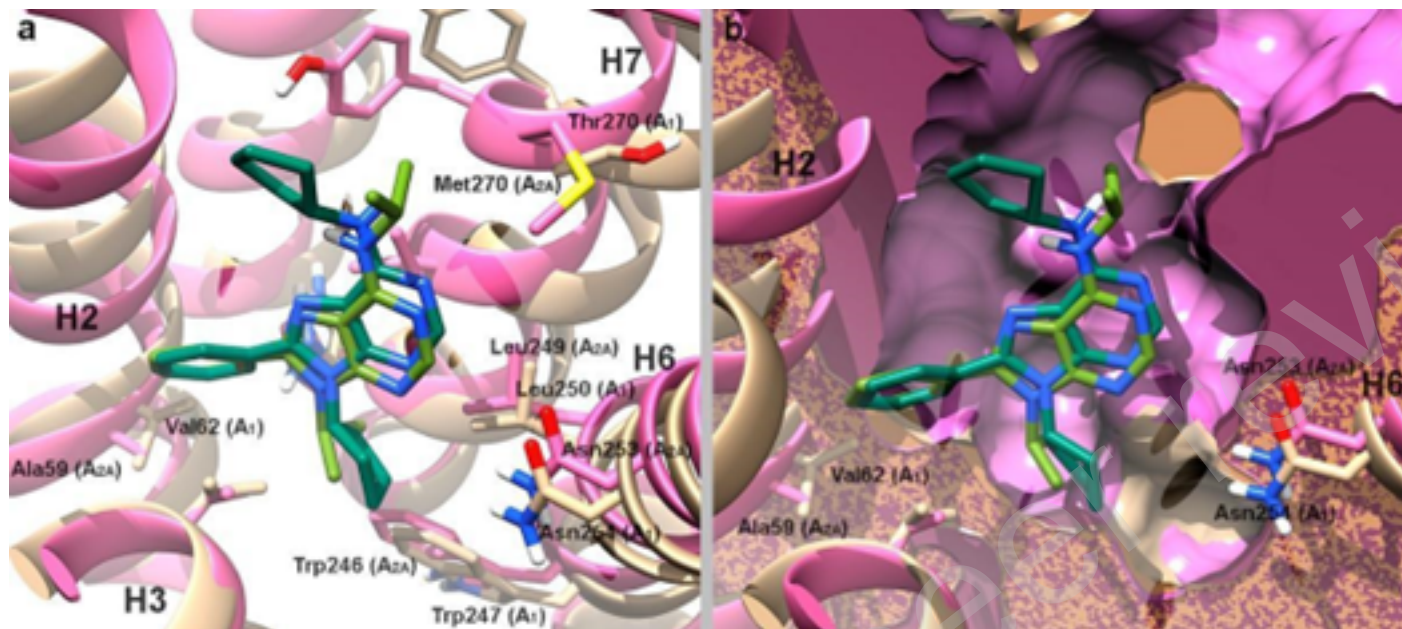


Table 1 Binding affinity results for the four *hAR* (A_1 , A_{2A} , A_{2B} and A_3) of the adenine derivatives (see Fig. 1) and reference standards (XAC, CGS-15943, ZM241385, and MRS1220) presented in the manuscript

Compounds	Receptors							
	hA_1		hA_{2A}		hA_{2B}		hA_3	
	%	K_i (nM)	%	K_i (nM)	%	K_i (nM)	%	K_i (nM)
	Inhib.		Inhib.		Inhib.		Inhib.	
11a	43±1		8±3		32±4		45±4	
11b	41±1		4±2		44±3		35±1	
11c		458.8±13	33±4			1091.0±35	28±1	
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12c		620.4±25	8±3		44±1		48±5	
13a	27±2		16±2		2±2		11±3	
13b	29±2		17±4		17±2		24±2	
13c		880.2±31	24±1		3±1		37±4	
14a	55±5		11±3		22±3		21±1	
14b	36±2		4±2		26±4		20±1	
14c		2.0±0.3	33±2		55±5			211.2±53
15a	20±2		23±3		1±1		30±5	

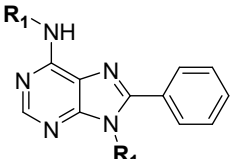
15c		71.9±36.6	27±1	8±3	5±2
16a	36±5		23±3	2±1	13±4
16b	24±1		11±3	5±3	14±3
16c	20±1		2±2	4±1	7±3
17c		28±4	2664±42	1503±22	1482±34
18c		20±2	30±1	764±60	334±13
19c		46±10	42±2	57 ± 4	42±1
XAC		13.9±2.2 ^a			
CGS-15943			1.4±0.3 ^b		
ZM 241385				28.3±4.4 ^c	
MRS 1220					3.6±0.7 ^d

^a K_i = 29.1 nM, reported by Klotz et al.³⁰ ^b K_i = 4.3 nM, reported by Fredholm et al.³¹ ^c K_i = 32.0

nM, reported by Fredholm et al.³¹ ^d K_i = 1.7 nM, reported by Gao & Jacobson.³² n = 3. Note:

Compound **15b** has been synthesized, but the yield has been very low. Therefore, and because its substituents do not belong to the most active series, this molecule was no further evaluated.

Table 2. Antagonist potency (K_B) against hA_1AR of the adenine derivatives **11c**, **12c**, **14c**, **15c**, **17c**, **18c** and **19c** and the reference standard **XAC**



		hA_1AR
Compound	R_1	K_B (nM)
11c	Bn-	1272.2±268.5
12c	PhEt	550.7±232.6
14c	<i>i</i> Pr	24.9±19.4
15c	pClBn	687.2±32.7
17c	Et	50.7±9.45
18c	<i>t</i> Bu	83.6±28.2
19c	cP	99.7±10.4
XAC^a		37.9±20.2

Table 3. Cell lines used for phenotypic screening of compounds **14c**, **17c**, **18c**, and **19c**.

Cell Line	Origin	Field of use
SW480, SW48	Primary colorectal adenocarcinomas	Colorectal cancer research
HCT116	Colorectal carcinoma	Colorectal cancer research
	Chronic myelogenous leukemia	
K562	(CML)	Hematological research, leukemia,
	Breast adenocarcinoma (triple-	
MDA-MB-231	negative)	TNBC research (highly aggressive)
		Non-small cell lung cancer (NSCLC)
A549	Human lung carcinoma	research

Table 4. EC₅₀ (μM) values of compounds as antiproliferative agents across various cancer cell lines. n = 3

Compound	SW480	SW48	HCT116	K562	MDA-MB-231	A549
14c	40 ± 12.14	79 ± 35.65	78 ± 37.99	35 ± 0.93	37 ± 11.33	62 ± 33.56
17c	>100	>100	>100	>100	30 ± 0.61	77 ± 40.41
18c	13 ± 3.77	>100	>100	17 ± 8.82	53 ± 40.41	>100
19c	7 ± 0.71	10 ± 0.27	12 ± 2.63	13 ± 9.01	15 ± 5.06	39 ± 9.48