Simple and non-charged long-lived fluorescent intracellular mitochondria and lysosome trackers based on acridones

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In this work we evaluated by FLIM microscopy the preferential accumulation of long-lived acridone derivatives in mitochondria and lysosomes, based on a new concept of non-protonable and non-charged groups carriers. Thus, thiophene ring has been proved to act as mithocondria carrier whereas pyridine derivative is preferentially accumulated into lisosomes.

Numerous of the current major diseases are related to cell disorders characterized by anomalous physiological processes of the cell cycle. To achieve a better understanding of these pathological progressions an accurate monitoring of subcellular microenvironments, commonly known as organelles, is critical. These structures play important roles in the cell growth, proliferation, signal transduction and apoptosis, being also the main sources of bioactive species. Notably, in the last years high efforts have been devoted to the design and synthesis of organelle targeted fluorescent probes.[1,2] Despite the satisfying results obtained with the developed trackers, some improvements would be welcome. Thus, for example, long fluorescence lifetimes, τ, are interesting in the context of their application in time-resolved methods, as fluorescence lifetime imaging microscopy (FLIM).[3] FLIM is a multidimensional technique that provides not only information on the total fluorescence intensity emitted, but also on the kinetics of the emitted photons. This technique thus provides excellent tools for intracellular sensing, free of interferences such as the cell autofluorescence or inhomogeneity on the excitation source.[4] Current subcellular trackers are characterized by emissions in the range of 3-5 ns. Translation of the actual knowledge to long-lived fluorescent intracellular fluorophores would result in an increase in the sensibility of the technique. A key point is then the selection of the emissive species. It is known that acridones are characterized by unusually long fluorescence lifetimes and also by their ability to be very good DNA intercalating agents, accumulating in the cell nucleus.[5] Due to the interest of this family of fluorophores we have studied the excited-stated dynamics of different 2-Methoxy-9-acridones.[6] Based in this structure we have recently reported how a simple modification of the acridone skeleton allows the qualitative observation of the polarity of different cell microenvironments without affecting their good luminescent properties, as stability in a wide range of pH and long lifetimes.[7] We also observed this functionalization can affect to the distribution of the compound inside the cell. In this sense we thought that the rational inclusion of lateral groups in an alkyl chain joined to the nitrogen atom could result in the control of its subcellular localization.

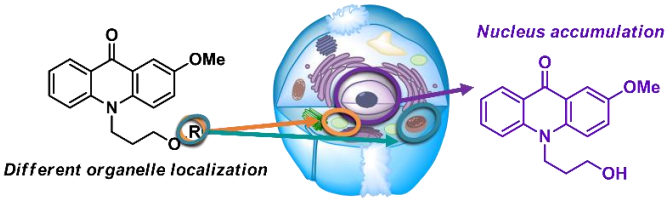

Figure 1. Working hypothesis: functionalization of acridones to promote their accumulation in different organelles.

Thus for example, mitochondria is a key organolle mainly responsible of intracellular respiration.[8] Although its tracking is possible it is usually based on the highly negative mitochondrial transmembrane potential developed during respiration caused by proton transport.[9] In this way, lipophilic cations (triphenylphosphonium (TPP) and ammonium or pyridinium salts)[10] are allowed to act as mitochondria targets. However, these required cationic structures can reduce membrane potential and thus these dyes can easily leak out the organelle. Moreover, cellular toxicity of TPP derivatives is still a problem. On the other hand, lysosomes are the main digestive compartments of cells and play a key role in protein degradation and reparation of plasma membrane.[11] They are characterized by an acidic pH and thus functionalization of probes with basic centers has been the main strategy to lysosome targeting, resulting again charged structures.[12]

Taking into account all those precedents, a smart selection of functionalized acridone structures could yield long-lived fluorescent organelle targets. Here we present the synthesis and intracellular visualization of simple long-lived fluorescent dyes **1**-**4**, targeting mainly to nucleus, mitochondria and lysosomes. Moreover, we have tried to avoid the use of charged structures, which is beneficial from a synthetic point of view and also to reduce the impact of the tracker in cell metabolism. Furthermore, it could be also beneficial to selectively transport therapeutic drugs to the desired organelle, avoiding electrostatic interactions and making easier the coupling between the dye and the tracker.

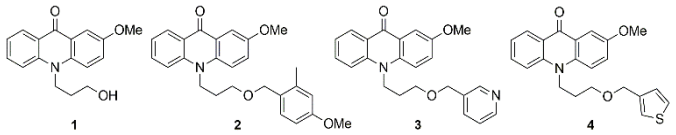

Figure 2. Chemical structure of compounds **1**-**4**

Known compound **1**[6] was used as the starting material for model acridone **2** and rationally designed acridones **3** and **4**. They were prepared by simple and direct alkylation reactions using the corresponding electrophiles (See SI for more details). The use of non-charged species mitochondria targeting is practically unknown. We hypothesized that a pendant group able to interact with ROS (Reactive Oxygen Species) present in mitochondria could change its physicochemical characteristics allowing an accumulation inside the organelle. That is the neutral carrier could be transformed by oxidation of a basic electron pair, with a similar mechanism of action that the proposed by Reshetnikov with the oxidation of ferrocene to ferrocenium cations.[13] Therefore, organic compounds with basic electron pairs prone be oxidized in such environment would be a potential solution for mitochondria targeting. A perfect candidate would be pyridine, a very stable aromatic substrate presenting a basic pair suitable to be oxidized to yield well-known pyridine oxides.[14] Unfortunately, the basicity of such electron pair is high enough to interact with acidic liposomes thus preventing the entry in the mitochondria. Therefore pyridine substituted acridone **3** is likely to respond to acidic environment and therefore preferential lysosome localization is expected. Thiophene is other stable aromatic ring with a well-known oxidation chemistry to thiophene oxides and dioxides by interaction con ROS-type reagents.[15] We then chose compound **4** as a potential mitochondria tracker with long lifetime. Compound **2** was selected as a control owing to the expected redox reaction cannot take place and this aromatic ring presented excellent fluorescent properties in Tokyo Green derivatives.[16] It is worth noting that in this case the logP, being logarithm of the octanol–water partition coefficient, calculated for the two compounds are similar (**4**, 3.67, **2**, 4.39), being ion the expected range (between 0 and 5) for mitochondria localization according to the QSAR theory for organelle localization.[17] Any difference in behaviour should be assigned to the different chemical reactivity of both pendant groups.
The photophysical properties of compounds **1**-**4** were then evaluated in a pH range between 5 and 9, to ensure the stability of acridone derivatives in the different organelles. All of them presented just one emission peak at around 465 nm that remained almost invariant in acid, neutral and basic conditions. We also measured the lifetimes with an excitation source of 440nm, obtaining again negligible differences with the variation of pH. Thus, the analysis of the decay traces fitted to a biexponential function for all the compounds. We also confirmed the presence of a long lifetime in the range of 17-18 ns characteristic of the acridone skeleton. The short lifetime was around 0.8 ns for all the compounds. (see Table S1 in the SI). Moreover, all the compounds presented very good fluorescence quantum yields, between 0.82 and 0.96 in PBS solution at pH= 7.3. After checking their stability and fluorescent properties we studied their localization in breast cancer cells 143B. In general, a rapid incorporation of all the acridones inside cells took place, as we could observe fluorescence immediately after the addition of the dyes. As expected, dual-colour FLIM microscopy (see Table S2 for experimental details) showed that acridone **1** accumulated in the nucleus and the cytoplasm of 143B cells. Noteworthy, compounds **2**-**4** showed a clearly different behaviour (Figure 3B-D). To evaluate the accumulation of compounds **2** and **4** in mitochondria we used MitoTracker Deep Red (MT), carrying out a two-channel detection of the dyes. In Figures 3B and 3D colocalization images are shown in the left whereas the graphs in the right show the intensity traces of the dye (green) and the MT (red), where the asterisks display the common points of both fluorophores. Although both derivatives showed a certain degree of accumulation in mitochondria it is noteworthy that compound **4** exhibited a clearer colocalization, traced in red with MT (Figures 3B and 3D). To check the lysosome targeting of pyridine derivative **3** we used commercially available LysoTracker Deep Red (LT) to mark this organelle, obtaining in this case a good correspondence between the two markers (Figure 3C). Apart from lysosomes, compound **3** was also observed in nucleolus. To check our initial hypothesis concerning that the basicity of pyridine derivative makes this compound preferentially transported to lysosomes and not

to mitochondria we also incubated compound **3** with MT, obtaining in this case no colocalization profiles (See SI). Despite this good results, lysosome and mitchondria accumulation were not completely selective, and all acridones were also observed in the endoplasmic reticulum. An important addition of the dual-colour FLIM microscopy is that the fluorescence lifetime, τ, of the dyes is accessible, providing an extra contrast in the image, and univocal identification of the dyes' properties. Interestingly, the fluorescence lifetime contrast in FLIM images (Figure 2A) showed the clear long τ values for the acridone derivatives, being in all the cases higher than 10 ns (average values inside living cells of 15.9 ns for **1**,14.3 ns for **2**, 15.9 ns for **3** and 14.3 ns for **4**), agreeing with previously observed in *in vitro* measurements, whereas the MT and LT exhibited their characteristic low lifetimes (< 2 ns). These long τ values represent an important tool for contrast filtering through an orthogonal piece of information, especially interesting for intracellular sensing.[18]

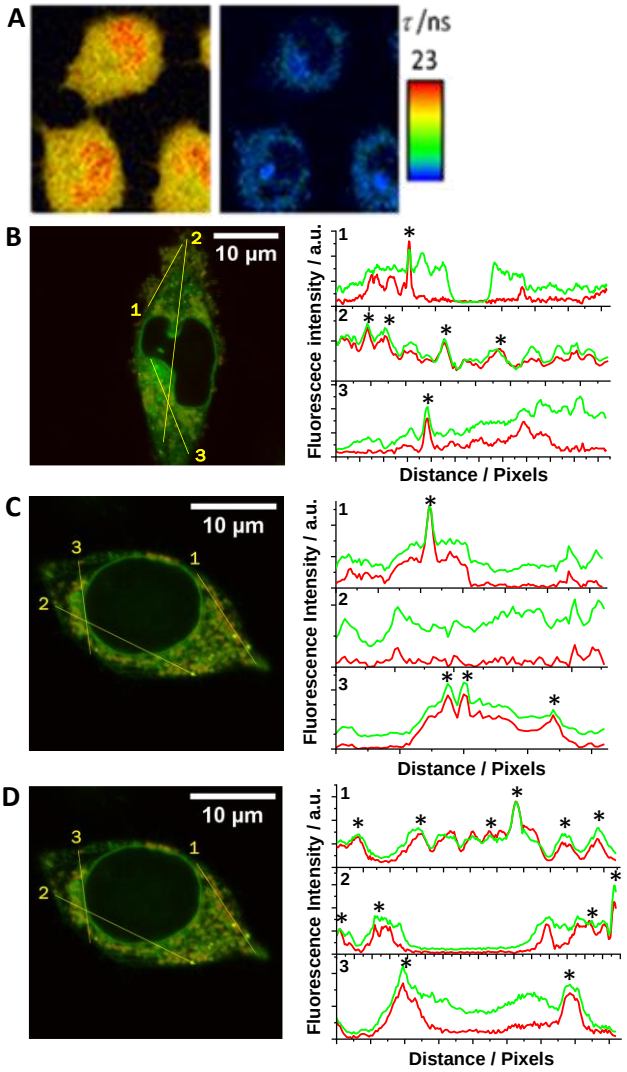

Figure 3. Representative dual-color FLIM image of compound **1** in 143B cells (A). Representative colocalisation images (left) and intensity traces (right) for compounds **2** (B), **3** (C) and **4** (D)., after 20-min incubation with MT (B and D) or LT (C), in the dye's detection channel (green) and the MT or LT detection channel (red).

After checking their behaviour inside cells we decide to examine the accumulation of compounds **2** and **4** in mitochondria isolated from rat liver. The protocol carried out for the isolation of the mitochondria and subsequent cycles with probes including incubation, solution, washing and centrifugation is explained in the SI. After this protocol we obtained three extracts whose fluorescence was compared to the one obtained for the probes before being incubated with mitochondria. In Figure 4A we show the variation of the fluorescence spectra of compound **4**, where it can be seen that a notable decrease of emission was obtained in the first supernatant, due to the accumulation of the probes inside mitochondria. Moreover,

almost no emission was observed in the final extracts, suggesting the probes were not released to the media. These measurements allowed us to calculate an estimation of the percentage of accumulation of both derivatives, being in both cases near 50% and again higher for the tiophene derivative, confirming the results previously observed in FLIM measurements (Figure 4B).

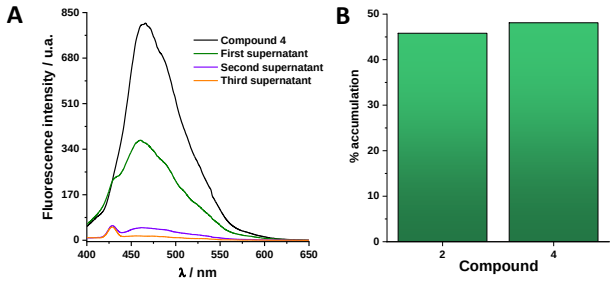

Figure 4. A) Evolution of the fluorescence spectra of compound **4** before and after subsequent incubations with mitochondria isolated from rat liver. B) Estimation of the percentage of accumulation of compounds **2** and **4**

In summary, we have demonstrated here the feasibility of noncharged, neutral carriers for targeting mitochondria and lysosomes. In this sense, tiophene ring has been proved to be a good carrier to mitochondria. In addition, the percentage of accumulation in this organelle isolated from Wistar rats liver has been evaluated. It is worth noting that the absence of cationic structures is a clear advantage not only in the synthetic steps but also considering that mitochondrial transmembrane potential could be kept unaltered after dye addition. On the other hand, fluorescent derivative including a pyridine group, has shown a good accumulation lysosomes. Moreover, we have also demonstrated that none of the targeting groups affect to the lifetimes and fluorescent properties of the acridone. Finally, these compounds have been proved to be non-toxic for 143B cells, which is also a key task when working in biomedical applications. This easy and versatile strategy opens the door to the development of new fluorescent probes with promising applications in organelle delivery of drugs.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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1 P. Gao, W. Pan, N. Li and B. Tang, *Chem. Sci.,* 2019, **10**, 6035-6071.
2 W. Su, Z. Zeng, J.-H. Jiang, Y.-T- Chang and L. Yian, *Angew. Chem. Int. Ed.* **2016**, *55*, 13658-13699.
3 J. A. Smith, R. M. West and M. Allen, *J. Fluorescence* 2004, **14**, 151-171.
4 M. Ruedas-Rama, J. Alvarez-Pez, L. Crovetto, J. M. Paredes, A. Orte, in *Advanced Photon Counting, Vol. 15* (Eds.: P. Kapusta, M. Wahl, R. Erdmann), Springer International Publishing, **2015**, pp. 191-223.
5 K. Thimmaiah, A. G. Ugarkar, E. F. Martis, M. S. Shaikh, E. C. Coutinho and M. C. Yergeri, Nucleosides, Nucleotides and Nucleic Acids 2015, 34, 309-331.
6 M. C. Gonzalez-Garcia, P. Herrero-Foncubierta, S. Castro, S. Resa, J. M. Alvarez-Pez, D. Miguel, J. M. Cuerva, E. Garcia-Fernandez and A. Orte, *Frontiers in Chemistry*, 2019, **7**, 129.
7 Sensors and Actuators
8 J. Zielonka, J. Joseph, A. Siroka, M. Hardy, O. Ouari, J. Vasquez-Vivar, G. Cheng, M. Lopez and B. Kalyanaraman, *Chem. Rev.*, 2017, **117**, 10043-10120.
9 J. Qian and B. Z. Tang, *Chem,* 2017, **3**, 56-91.
a) C. Trapella, R. Voltan, E. Melloni, V. Tisato, C. Celeghini, S. Bianco, A. Fantinati, S. Salvadori, R. Guerrini, P. Secchiero and G. Zauli, J. *Med. Chem.* 2016, **59**, 147-156; b) J. Ripcke, K. Zarse, M. Ristow and M. Birringer, *ChemBioChem* 2009, **10**, 1689-1696; c) R. K. Pathak, S. Marrache, D. A. Harn and S. Dhar, *ACS Chem. Biol.,* 2014, **9**, 1178-1187; d)N. Jiang, J. Fan, F. Xu, X. Peng, H. Mu, J. Wang and X. Xiong, *Angew. Chem. Int. Ed.* 2015, **54**, 2510-2514; e) J. Zielonka, J. Joseph, A. Sikora, M. Hardy, O. Ouari, J. Vasquez-Vivar, G. Cheng, M. Lopez and B. Kalyanaraman *Chem. Soc. Rev.* 2017, **117**, 10043-10120. f) Z. Xue, R. Zhu, S. Wang, J. Li, J. Han,

J. Liu and S. Han *Anal. Chem.*, 2018, **90**, 2954-2962. g) B. Kalyanaraman, G. Cheng, M. Hardy, O. Ouari, M. Lopez, J. Joseph, J. Zielonka and M. B. Dwinell, *Redox Biolol.*, 2018, **14**, 316-327.
10 a) Karp, G. and J.G. Patton, *Cell and molecular biology : concepts and experiments*. 7th ed. 2013, Hoboken, NJ: John Wiley. b) B. Turk and V. Turk, *J. Biolog. Chem.* 2009, **284**, 21783-21787.
11 a) R. A. Firestone and J. M. Pisano, *J. Med. Chem.*, 1979, **22**, 1130-1133. b) Q. Wan, S. Cheng, W. Shi, L. Li and H. Ma, *Angew. Chem. Int. Ed.* 2014, **53,** 10916-10920.
12 V. Reshetnikov, S. Daum, C. Janko, W. Karawacka, R. Tietze, C. Alexiou, S. Paryzhak, T. Dumych, R. Bilyy, P. Tripal, B. Schmid, R. Palmisano, A. Mokhir *Angew. Chem. Int. Ed.* 2018, **57**, 11943.
13 a) D. Limnios and C. G. Kokotos, *Chem., Eur. J.* 2014, **20**, 559-563; b) C. Coperet, H. Adolfsson, T. A. V. Khuong, A. K. Yudin and K. B. Sharpless, *J. Org. Chem.,* 1998, **63**, 1740-1741.
14 a) F. Jalilian, B. Yadollahi, M. R. Farsani, S. Tangestaninejad, H. A. Rudbari and R. Habibi, *Catal. Commun.,* 2015, **66**, 107-110; b) E. Rafiee and F. Mirnezami, *J. Molec. Liq.,* 2014, **199**, 156-161; c) B. C. Ma, W. Zhao, F. M. Zhang, Y. S. Zhang, S. Y. Wu and Y. Ding, *Rsc Advances*, 2014, **4**, 32054-32062; d) A. Tsirk, S. Gronowitz and A. B. Hornfeldt, *Tetrahedron,* 1995, **51**, 7035-7044; e) P. Pouzet, I. Erdelmeier, D. Ginderow, J.-P. Mornon, P. Dansette and D. Mansuy, *J. Chem. Soc. Chem. Commun.* 1995, 473-474.
15 Y. Urano, M. Kamiya, K. Kanda, T. Ueno, K. Hirose and T. Nagano, *J. Am. Chem. Soc.* 2005, **127**, 4888-4894.
16 R. W. Horobin, *Color. Technol.*, 2014, **130**, 155-163.
17 M. J. Ruedas-Rama, J. Alvarez-Pez, L. Crovetto, J. Paredes, A. Orte, in *Advanced Photon Counting*, Vol. 15 (Eds.: P. Kapusta, M. Wahl, R. Erdmann), Springer International Publishing, 2015, pp. 191-223.