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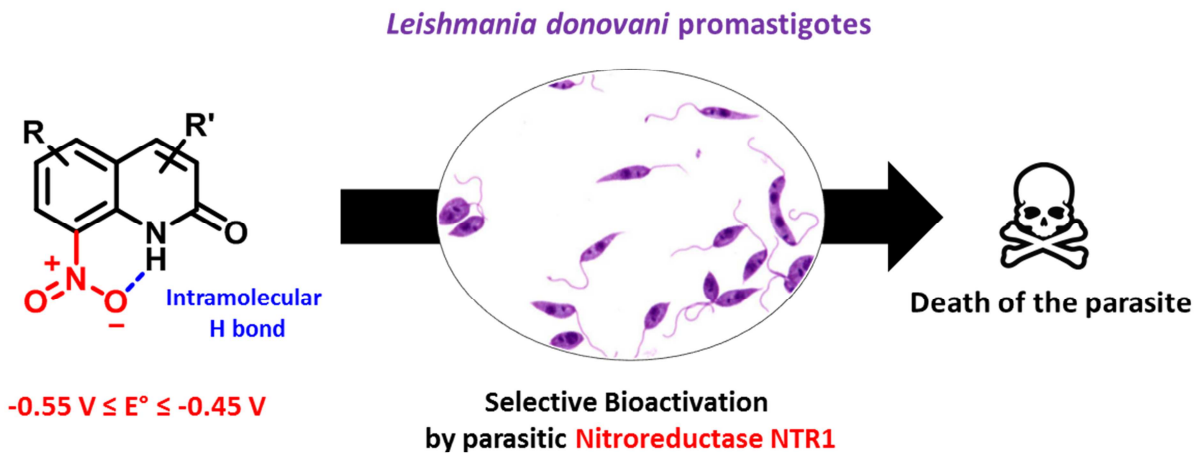

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Novel 8-Nitroquinolin-2(1*H*)-ones as NTR-bioactivated Antikinetoplastid Molecules: Synthesis, Electrochemical and SAR Study

Julien Pedron[1], Clotilde Boudot[2], Sébastien Hutter[3], Sandra Bourgeade-Delmas[4], Jean-Luc Stigliani[1], Alix Sournia-Saquet[1], Alain Moreau[1], Elisa Boutet-Robinet[5], Lucie Paloque[1], Emmanuelle Mothes[1], Michèle Laget[6], Laure Vendier[1], Geneviève Pratviel[1], Susan Wyllie[7], Alan Fairlamb[7], Nadine Azas[3], Bertrand Courtioux[2], Alexis Valentin[4] and Pierre Verhaeghe[1]*

[1] LCC-CNRS Université de Toulouse, CNRS, UPS, Toulouse, France.

[2] Université de Limoges, UMR INSERM 1094, Neuroépidémiologie Tropicale, Faculté de Pharmacie, 2 rue du Dr Marcland, 87025 Limoges, France

[3] IHU Méditerranée Infection, équipe VITROME « Vecteurs, Infections Tropicales et Méditerranéennes », 19-21 boulevard Jean Moulin, 13385 Marseille cedex 05, France.

[4] UMR 152 PharmaDev, Université de Toulouse, IRD, UPS, Toulouse, France.

[5] Toxalim (Research Centre in Food Toxicology), Université de Toulouse, INRA, ENVT, INP-Purpan, UPS, Toulouse, France

[6] UMR MD1, U1261, AMU, INSERM, SSA, IRBA, MCT, Marseille, France.

[7] University of Dundee, School of Life Sciences, Division of Biological Chemistry and Drug Discovery, Dow Street, Dundee DD1 5EH, Scotland, United Kingdom

*Corresponding author: Pierre Verhaeghe (pierre.verhaeghe@univ-tlse3.fr)

Tel: +33 561 333 236

https://orcid.org/0000-0003-0238-2447

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ABSTRACT

To study the antiparasitic 8-nitroquinolin-2(1*H*)-one pharmacophore, a series of 31 derivatives was synthesized in 1-5 steps and evaluated *in vitro* against both *Leishmania infantum* and *Trypanosoma brucei brucei*. In parallel, the reduction potential of all molecules was measured by cyclic voltammetry. Structure-activity relationships first indicated that antileishmanial activity depends on an intramolecular hydrogen bond (described by X-ray diffraction) between the lactam function and the nitro group, which is responsible for an important shift of the redox potential (+0.3 V in comparison with 8-nitroquinoline). With the assistance of computational chemistry, a set of derivatives presenting a large range of redox potentials (from -1.1 to -0.45 V) was designed and provided a list of suitable molecules to be synthesized and tested. This approach highlighted that, in this series, only substrates with a redox potential above -0.6 V display activity toward *L. infantum*. Nevertheless, such relation between redox potentials and *in vitro* antiparasitic activities was not observed in *T. b. brucei*. Compound **22** is a new hit compound in the series, displaying both antileishmanial and antitrypanosomal activity along with a low cytotoxicity on the human HepG2 cell line. Compound **22** is selectively bioactivated by the type 1 nitroreductases (NTR1) of *L. donovani* and *T. brucei brucei*. Moreover, despite being mutagenic in the Ames test, as most of nitroaromatic derivatives, compound **22** was not genotoxic in the comet assay. Preliminary *in vitro* pharmacokinetic parameters were finally determined and pointed out a good *in vitro* microsomal stability (half-life > 40 min) and a 92% binding to human albumin.

KEYWORDS

Anti-kinetoplastids, *Leishmania*, *Trypanosoma*, 8-Nitroquinolin-2(1*H*)-one, Nitroreductases, Electrochemistry.

HIGHLIGHTS

8-nitroquinolin-2(1*H*)-one derivative 22 is bioactivated by *L. donovani* NTR1

They display suitable redox potentials thanks to an intramolecular H-bond

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Introducing a bromine atom at position 3 leads to a new antikinetoplastid hit

INTRODUCTION

Kinetoplastids are protozoan parasites responsible for deadly mammalian infections; *Leishmania spp* and *Trypanosoma spp* being the two main genera encountered in human pathology. These parasites are transmitted to their mammalian hosts by the bite of specific insect vectors. Among the *Leishmania* genus, *L. donovani* and *L. infantum* are the two most important species responsible for visceral leishmaniasis (VL), the most severe and often lethal clinical form of the disease. Briefly, VL occurs in humans after the bite of a sandfly (*Phlebotominae*) which injects the metacyclic flagellated promastigote stage of the parasite into the skin. Parasites are then phagocytized by macrophages and other types of mononuclear phagocytic cells where they transform into the amastigote stage and multiply, bypassing the immune system of the host. Infected cells disseminate in many tissues and organs such as lymph nodes, liver, spleen and bone marrow, leading to death.[1] In the *Trypanosoma* genus, in addition to *T. cruzi* species, causing Chagas disease in South and Central America, the *T. brucei* species (*T. brucei gambiense* or *T. brucei rhodesiense*) are responsible for Human African Trypanosomiasis (HAT), also called “sleeping sickness”. During HAT, the flagellated trypomastigote stage of the parasites penetrates into the blood stream *via* the bite of a “tsetse fly” and disseminates into the whole organism. In a first step of the disease, headaches, anemia, joint pain and various organ damage occur. Then, in a second step, the parasites invade the central nervous system, causing various neurological changes such as sleeping disorders (responsible for the name of the disease), abnormal tone and mobility, ataxia, psychiatric disorders, seizures, coma and finally, death.[2]

It is important to note that all these kinetoplastid infections are lethal if untreated and that very few efficient, safe and affordable drugs are available on the market for the low-income infected patients living in developing countries, which makes these parasitic infections belong to the group of “Neglected Tropical Diseases”.[3] Regarding recent epidemiologic data, the WHO indicates that more than 1 billion people are at risk of contracting VL and that 300.000 new cases occur every year, responsible for about 20.000 deaths.[4] Concerning HAT, 61 million people would be at risk of contracting the disease, no clear data being available about HAT mortality.[5] Approved drugs against

VL are amphotericin B, miltefosine, antimony V derivatives and pentamidine. The liposomal form of amphotericin B, although being quite efficient, is also very expensive and requires parenteral administration, which makes it unsuitable for treating most of infected patients.[6] Pentamidine and pentavalent antimony derivatives are toxic agents which use is limited by parasitic resistances and poor efficacy.[7] Miltefosine (Figure 1) remains the only orally available drug, but it is a teratogenic molecule, preventing its use in women of child-bearing age.[6] The context of anti-HAT drugs is quite similar with only one orally available drug.[8] In addition to pentamidine, suramin is used in the treatment of the first stage of the disease, with nifurtimox in combination with eflornithine (NECT,Figure 1) for treatment of the second (cerebral) stage of HAT. NECT has largely replaced the use of melarsoprol, a highly toxic arsenic-containing drug.[2]

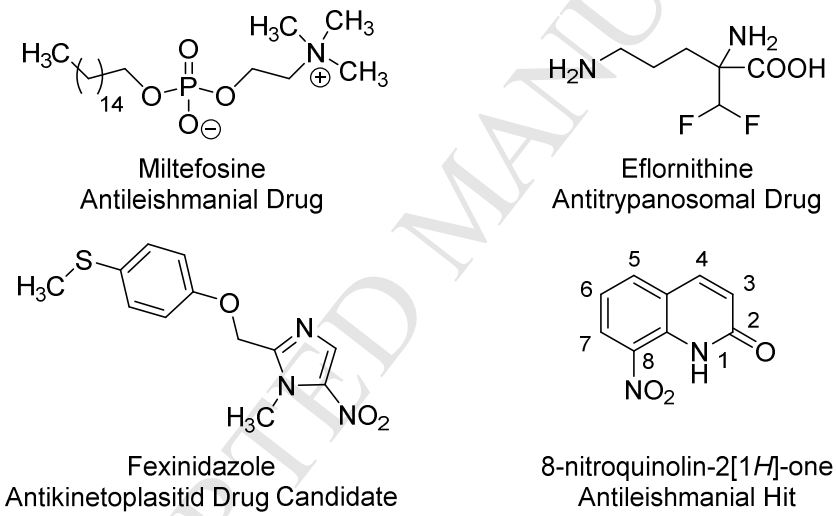


Figure 1: Structures of the antikinetoplastid drugs miltefosine and eflornithine, drug-candidate fexinidazole and antileishmanial hit 8-nitroquinolin-2(1*H*)-one

Unfortunately, there are very few new chemical entities under clinical trial in 2018 against both VL and HAT.[8] In this worrying context, there is a critical need to identify new active molecules and, as observed in the field of tuberculosis, various nitroheterocycles such as delamanid and fexinidazole are re-emerging as key anti-infective molecules.[9, 10] Fexinidazole (Figure 1) is a 5-nitroimidazole derivative that was first evaluated against visceral leishmaniasis in a phase II clinical trial. Although not sufficiently efficient for use as a monotherapy for VL, fexinidazole has now demonstrated efficacy against early and late stages of HAT in a pivotal Phase III clinical trial.[11-12] Fexinidazole is part of

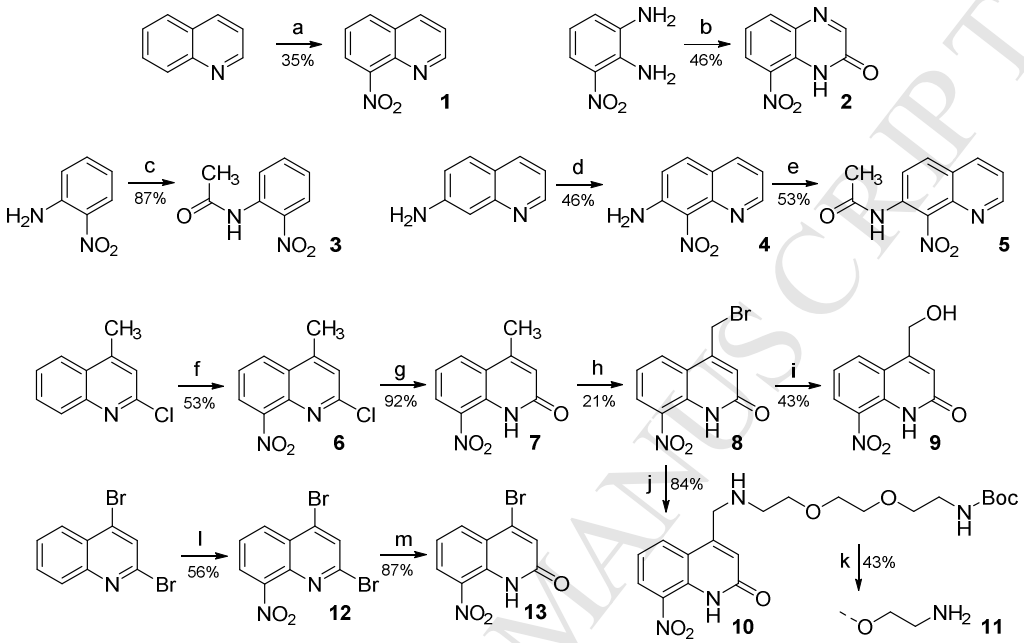
these nitroaromatic molecules which are selectively activated by parasitic nitroreductases (NTRs) to generate electrophilic cytotoxic metabolites.[13,14] Unfortunately, no parasitic NTR X-ray structure is available and most classical rational medicinal chemistry approaches cannot be used for the design of new synthetic substrates of these key enzymes. In this context, our group initiated a research around a class of 8-nitroquinoline derivatives with antiparasitic potential[15] and identified an antileishmanial pharmacophore in 2012, corresponding to the 8-nitroquinolin-2(1*H*)-one scaffold (Figure 1).[16] Some more recent modulations of the position 4 of this pharmacophore failed to achieve more active derivatives.[17, 18] Here, we present a comprehensive study of the structure-activity relationships of this pharmacophore, especially focusing on the modulation of the redox potentials. Thirty one pharmacophore derivatives, substituted at positions 3, 4, 5, 6 and 7, were synthesized and their *in vitro* cytotoxicity and activities against *L. infantum* (axenic amastigotes), and *T. brucei brucei* (trypomastigotes) were first determined. The original pharmacophore possessed poor antitrypanosomal activity, whereas a new derivative (compound **22**) was identified, active against all tested kinetoplastids (*L. donovani* promastigotes, *L donovani* intramacrophage amastigotes, *L. infantum* axenic amastigotes and *T. brucei brucei* trypomastigotes). The antileishmanial mechanism of action of this new hit-compound will be discussed along with its mutagenicity and genotoxicity, a key aspect concerning nitroaromatic compounds. Finally, two preliminary *in vitro* pharmacokinetic properties were measured and appear favorable for further development of this anti-kinetoplastid pharmacophore.

RESULTS AND DISCUSSION

Chemistry

Thirty one derivatives of the initial 8-nitroquinolin-2-(1*H*)-one pharmacophore, including 14 original ones, were prepared. As presented in Scheme 1, compounds **1-5** were obtained by classical nitration, cyclo-condensation or *N*-acetylation reactions. Nitration at position 8 of 2-chloro-4-methylquinoline afforded **6** whose chloroimine moiety reacted with perchloric acid to generate the corresponding lactam **7**, according to a simple and efficient previously reported procedure.[19] Bromination of **7** with

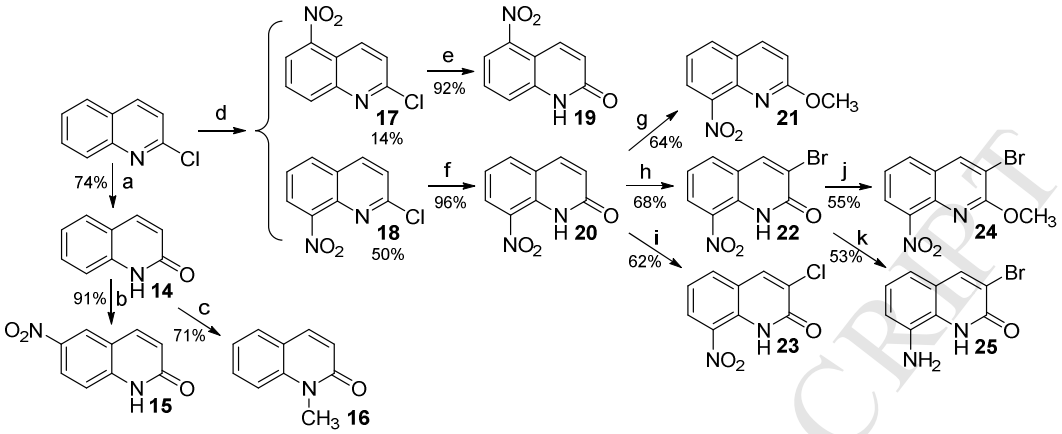
N-bromosuccinimide led to the bromomethyl derivative **8** which was reacted with sodium hydroxide to generate hydroxymethyl derivative **9**. From bromomethyl **8**, by reacting with an amine, it was also possible to access to **10** and its deprotected counterpart **11**. From 2,4-dibromoquinoline, by a sequential nitration/lactamisation procedure, **12** and **13** were obtained in good yields.



Scheme 1: Synthetic routes for the preparation of compounds **1-13**. (a) H_2SO_4, HNO_3, rt, 1 h; (b) glyoxylic acid, EtOH, 80 °C, 6 h; (c) CH_3COOH, Ac_2O, 120 °C, 3 h; (d) H_2SO_4, HNO_3, rt, 4 h; (e) CH_3COOH, Ac_2O, 120 °C, 24 h; (f) H_2SO_4, HNO_3, rt, 3 h; (g) CH_3CN, $HClO_4$, 100 °C, 24 h; (h) NBS, AIBN, CCl_4, 80 °C, 24 h; (i) NaOH, H_2O/THF, 50 °C, 24 h; (j) $NH_2(CH_2CH_2O)_2CH_2CH_2NHBoc$, THF, 50 °C, 24 h; (k) HCl (5N in isopropanol), rt, 4 h; (l) H_2SO_4, HNO_3, rt, 2 h; (m) CH_3CN, $HClO_4$, MW, 100 °C, 1 h.

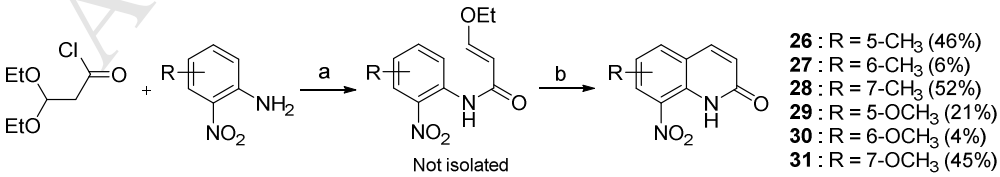
As presented in Scheme 2, the 2-chloroquinoline was the main substrate used in this work. It was first reacted with perchloric acid to generate lactam **14** which was either nitrated at position 6, to afford compound **15** or *N*-methylated with methyl iodide, to afford **16** in good yields. The nitration of 2-chloroquinoline mainly leaded to the 2 position isomers **17** and **18** which were transformed into the corresponding lactams **19** and **20**. Compound **20** was either *O*-methylated with methyl iodide, forming **21**, or selectively halogenated at position 3 by refluxing in HBr or HCl in the presence of sodium bromate or sodium chlorate, to generate derivatives **22** and **23**. Note that this specific halogenation procedure (*in situ* Br_2 or Cl_2 formation and reaction in refluxing aqueous medium), reported by O'Brien and co-workers,[20] is the only one allowing selective halogenation at position 3 among many

others that were tested. The 3-brominated derivative **22** reacted with methyl iodide to form *O*-methylated derivative **24** or was reduced into the amino-derivative **25** by using $SnCl_2$.



Scheme 2: Preparation of compounds **14-25** from 2-chloroquinoline. (a) CH_3CN, $HClO_4$, 100 °C, 72 h; (b) H_2SO_4, HNO_3, rt, 1 h; (c) DMF, K_2CO_3, CH_3I, 80 °C, 48 h; (d) H_2SO_4, HNO_3, rt, 2 h; (e) CH_3CN, $HClO_4$, 100 °C, 72 h ; (f) CH_3CN, $HClO_4$, 100 °C, 72 h; (g) Ar., DMF, NaH, CH_3I, rt, 24 h; (h) $NaBrO_3$, HBr, 100 °C, 5 h; (i) $NaClO_3$, HCl, 100 °C, 45 min; (j) Ar., DMF, NaH, CH_3I, rt, 24 h; (k) EtOH, $SnCl_2$, 80°C, 3 h.

Finally, as presented in Scheme 3, we also used another synthetic pathway allowing the preparation of 8-nitroquinolinone derivatives bearing substituents on the benzene moiety. This strategy was reported by Zaragoza and co-workers.[21] First, commercial 3,3'-diethoxyethylpropionate was saponified into the corresponding carboxylic acid which was then reacted with $SOCl_2$ to form the acyl chloride. This acyl chloride was then reacted with various *ortho*-nitroanilines, to give the corresponding *N*-acylated products which were not isolated. In a final step, cyclization was operated in 98% H_2SO_4, giving the expected products **26-31**. The reaction yields are moderate when using 2-nitroanilines substituted at position 3 or 5 by electron donating groups but turn to be very low when using 2-nitroanilines substituted at position 4 by the same groups.



Scheme 3: Preparation of compounds **26-31** from nitroanilines subtrates. (a) CH_2Cl_2, Pyridine, rt, 24 h; (b) H_2SO_4, rt, 3-4 h.

Compound evaluation

In vitro activity against *L. infantum* and *T. brucei brucei*: importance of the redox potential

All synthesized molecules were screened *in vitro* toward both *L. infantum* axenic amastigotes and *T. brucei brucei* trypomastigotes and compared with commercial reference drugs (amphotericin B and miltefosine against *L. infantum*, suramin and eflornithine against *T. brucei*) along with drug-candidate fexinidazole (Table 1). To assess antiparasitic selectivity, the cytotoxicity of all compounds was also measured on the HepG2 human cell line, using doxorubicin as control. In parallel the LogP of all compounds were calculated and the reduction potentials were measured by cyclic voltammetry in DMSO. The redox potentials measured correspond to a one electron reduction/oxidation regarding the redox couple nitro group / anion radical. Only one new hit compound (**22**) was evaluated in greater depth, as the best compound in the series regarding both activity and selectivity.

Globally, apart from molecule **24**, all synthesized compounds were soluble in aqueous medium and could be evaluated *in vitro*. Out of the 31 tested compounds, none appeared cytotoxic on the HepG2 cell line, nine were active toward *L. infantum* axenic amastigotes (7 µM ≤ IC_{50} ≤ 25 µM) and thirteen were active against *T. brucei brucei* trypomastigotes (2 µM ≤ IC_{50} ≤ 25 µM), molecule **24** being excluded from the analysis because of its lack of solubility. As a first approach, we wanted to see if there was a relationship between activity and lipophilicity. Computed LogP, ranging from 1.15 to 2.5 did not show any correlation, active molecules presenting either low (compound **2**) or high (compound **22**) LogP values.

We then explored the structure-activity relationships (SARs) of the pharmacophore by focusing on the influence of the redox potentials toward the activity against *L. infantum*. Indeed, on the basis that this nitroaromatic pharmacophore could be the substrate of parasitic nitroreductases, it first appeared important to understand which chemical criteria would make this pharmacophore suitable for being efficiently reduced into cytotoxic metabolites. The basic redox potential value of 8-nitroquinoline **1** (-0.84 V) and nitrobenzene (-0.85 V) are quite low and do not seem suitable for the catalytic capabilities

of the parasitic nitroreductases (Figure 2). On the contrary, when the lactam function is combined with the presence of a nitro group at position 8 (initial hit), a significant increase in the value of the redox potential, up to -0.54 V, is observed and the molecule turns active. When keeping the lactam function while moving the nitro group at position 6 or 5 (compounds **15** and **19**), redox potentials become lower and the IC_{50} values of the molecules increased, resulting in a loss of activity against *L. infantum*. The same effect is noted with inactive *O*-methylated analog (compound **21**), which redox potential drastically fell to -0.93 V (Figure 2). Finally, adding an amide function in the *ortho* position of the nitrogroup (compounds **3** and **5**) did not provide an optimal value of redox potential (-0.68 V and -0.86 V, respectively) resulting in poor antileishmanial activity of the compounds. Thus the 8-nitroquinolin-2-(1*H*)-one scaffold definitely appears to be the antileishmanial pharmacophore. Besides, as presented in the X-ray diffraction structures (Figure 3), this pharmacophore presents a noticeable intramolecular hydrogen bond between the lactam function and the nitro group at position 8, responsible for a +0.3 V increase in redox potentials, allowing antileishmanial activity. Nevertheless, molecules **26** and **30**, which present the same redox potential, do not have the same antileishmanial activity (**26** being moderately active and **30** being inactive). By examining their LogP values (respectively 2.05 and 1.38) it could be hypothesized that antileishmanial activity depends on a compromise between redox potential values and lipophilicity.

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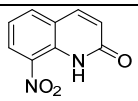
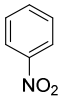
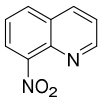
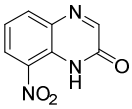
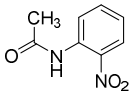
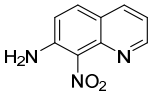
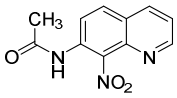
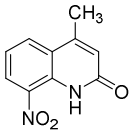
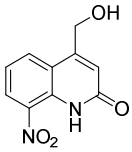
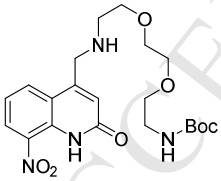
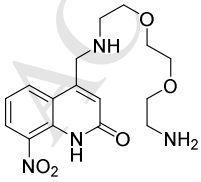
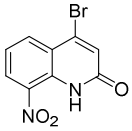
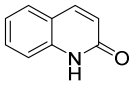
Table 1: Reduction potentials, calculated LogP, anti-kinetoplastid activity and cytotoxicity of the synthesized nitroaromatic compounds **1-31**.

Molecule	Structure	E° (V/NHE)[a]	LogP[b]	*L. infantum* axenic amastigote IC_{50} (µM)	*T. brucei brucei* trypomastigote IC_{50} (µM)	HepG2 *Cytotoxicity* CC_{50} (µM)
Initial hit		- 0.54	1.54	15.5 +/-0.5	23.4 +/-5.7	164 +/-28
Nitro-benzene (NB)		- 0.85	1.91	> 100[d]	> 50[d]	> 100[d]
1		- 0.84	2.07	> 100[d]	17.9 +/-1.8	> 100[d]
2		- 0.46	1.2	11.0 +/-2.1	7.3 +/-0.8	125 +/-19
3		- 0.68	1.15	> 100[d]	> 50[d]	> 100[d]
4		- 1.1	1.89	> 100[d]	> 50[d]	> 100[d]
5		- 0.86[c]	1.31	> 100[d]	10.8 +/-1.4	> 100[d]
7		- 0.53	2.05	10.2 +/-1.0	20.9 +/-8.9	110 +/-9
9		-	-	23.4 +/-4.8	38.3 +/-6.1	> 100[d]
10		-	-	> 100[d]	2.5 +/-1.8	> 100[d]
11		-	-	> 100[d]	7.6 +/-1.8	> 100[d]
13		- 0.51	1.86	20.3 +/-5.0	5.5 +/-1.3	60 +/-2
14		- 1.86[b]	-	> 100[d]	> 50[d]	> 100[d]

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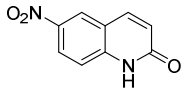
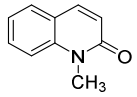
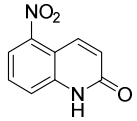
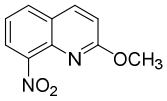
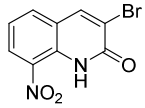
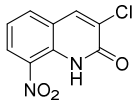
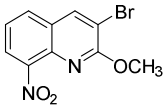
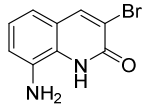
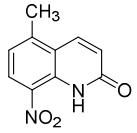
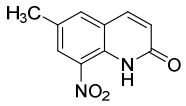
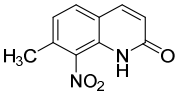
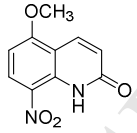
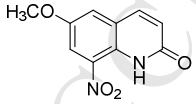
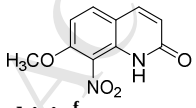
15		- 0.82	1.54	> 100[d]	2.3 +/-0.6	> 100[d]
16		-	-	> 100[d]	> 50[d]	> 100[d]
19		- 0.67	1.54	> 100[d]	> 50[d]	> 100[d]
21		- 0.93	2.51	88.8 +/-13.0	28.5 +/-8.1	> 100[d]
22		**- 0.45**	**2.36**	**7.1 +/-1.5**	**1.9 +/-0.4**	**92 +/-13**
23		- 0.47	2.2	16.0 +/-0.9	1.2 +/-0.2	85 +/-4
24		- 0.86	-	> 6.2[e]	0.4 +/-0.1	> 6.2[e]
25		-	-	> 50[d]	> 50[d]	> 100[d]
26		- 0.58	2.05	25.2 +/-3.7	29.1 +/-4.7	> 100[d]
27		- 0.56	2.05	23.0 +/-5.0	2.1 +/-0.8	> 100[d]
28		- 0.71	2.05	> 100[d]	> 50[d]	> 100[d]
29		- 0.65	1.38	> 100[d]	> 50[d]	> 100[d]
30		- 0.58	1.38	> 100[d]	> 50[d]	> 100[d]
31		- 0.75	1.38	> 100[d]	> 50[d]	> 100[d]
Doxorubicin[f]		-	-	-	-	0.2 +/-0.02
Amphotericin B[g]		-	-	0.06 +/-0.001	-	5.5 +/-0.25
Miltefosine[g]		-	-	0.8 +/-0.2	-	85 +/-8.8
Fexinidazole[g,h]		-	-	3.4 +/-0.8	0.4 +/-0.2	> 200[c]
Suramin[h]		-	-	-	0.02 +/-0.009	> 100[c]
Eflornithine[h]		-	-	-	15.8 +/-2.1	> 100[c]

[a] Cyclic voltammetry conditions: DMSO/TBAPF_6, SCE/GC, 1 electron reversible reduction, values are given in V versus NHE
[b] Weighted logP were computed with Marvin® (ChemAxon)
[c] Irreversible reduction
[d] The IC_{50} or CC_{50} value was not reached at the highest tested concentration
[e] The product could not be tested at higher concentrations due to a poor solubility in aqueous medium
[f] Doxorubicin was used as a cytotoxic reference drug
[g] Amphotericin B, Miltefosine and Fexinidazole were used as antileishmanial reference drugs

[h] Fexinidazole, Suramin and Eflornithine were used as anti-*Trypanosoma brucei* reference drugs

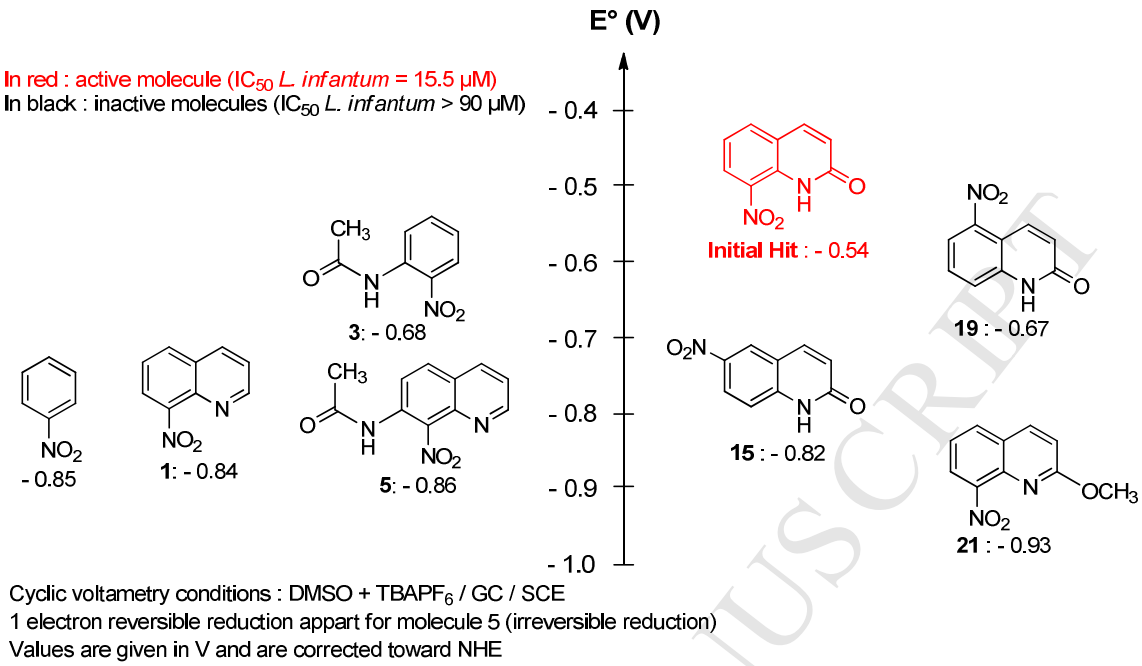


Figure 2: The 8-nitroquinolin-2(1*H*)-one pharmacophore presented a characteristic high redox potential value.

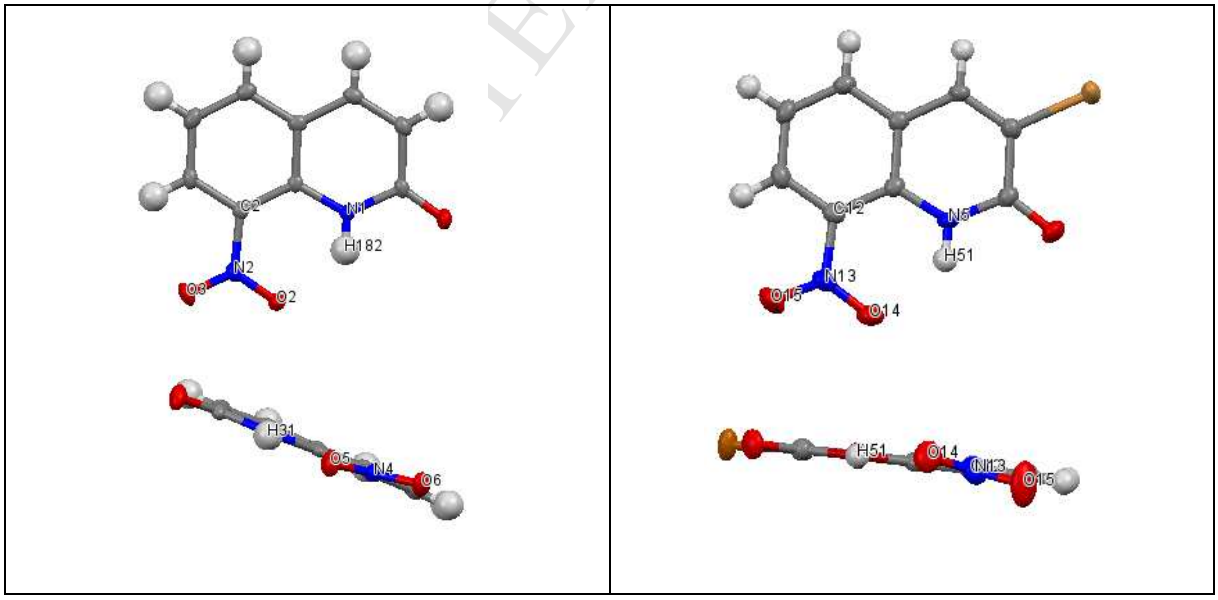


Figure 3: X-ray structures of initial hit and new hit **22** presenting an intramolecular hydrogen bond between the lactam function and the nitro group (Initial hit: N-H distance = 0.89 Å, H-O distance =

2.01 Å, N-H-O angle = 129°; Hit **22** : N-H distance = 0.84 Å, H-O distance = 2.01 Å, N-H-O angle = 131°).

Contrary to what was observed in *Leishmania*, the antitrypanosomal activity did not depend on the redox potential values in the studied series, active molecules presenting either low (compound **15**) or high (compound **22**) redox potential values. It could be hypothesized that the enzymatic capabilities of the *T. brucei* NTR may be compatible with a broader spectrum of redox potentials than the ones of *L. donovani* NTR.

Optimization of redox potentials: computational and *in vitro* studies.

From this point, we investigated the antileishmanial pharmacomodulation of 8-nitroquinolin-2(1*H*)-one by introducing various substituents (or heteroatoms) at all positions of the scaffold and compared the effect of electron-donating and electron withdrawing groups, to gain access to new optimized derivatives. Our goal was to synthesize a series of derivatives presenting a large spectrum of redox potentials, not only to gain a better understanding of the electrochemical process, but also to define as precisely as possible the lowest redox potential value compatible with activity in the series. For that purpose instead of synthesizing molecules randomly, we used ab initio calculations to estimate the redox potential values of many hypothetical derivatives. Previous studies about the computation of reduction potentials of nitroaromatic derivatives showed that the results are very sensitive to the basis set applied. Diffuse s and p-type functions, combined with polarization functions were shown to be important for electron affinity calculations of nitrobenzenes[22,23]. Because accurate results were obtained with the Pople's 6-311++G basis set on several molecules for which computed potentials were compared to experimental ones, the 6-311++G(2d,2p) basis set was selected as a good compromise between accuracy and computational cost for reduction potential calculations. The hybrid M06-2x density functional was chosen for its accuracy to compute thermochemical parameters. The standard redox potentials were computed with the Faraday's law (see equation (1) in the experimental section) from the free energy of the one-electron reduction. Figure 4 shows the correlation between the experimental and theoretical redox potentials. It can be seen that the slope of the correlation is close to 1.00 and the correlation coefficient (r) is 0.97. Therefore, this model was very efficient for predicting

the standard redox potentials and was used to select the most appropriate compounds to be synthesized and evaluated.

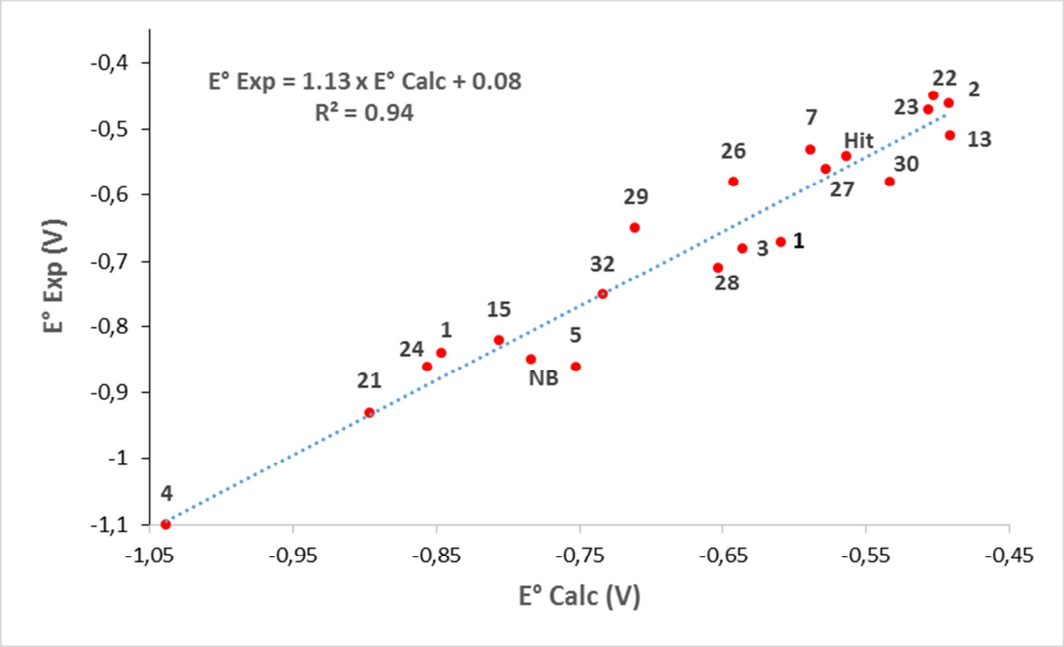


Figure 4: Correlation between theoretical and experimental standard redox potential of nitroquinolines

The studied series was completed with 11 derivatives of the pharmacophore presenting redox potentials ranging from -0.75 V to -0.45 V. Figure 5 shows that introducing a heteroatom at position 4 of the scaffold (quinoxaline analog **2**) or a halogen atom at position 3 (compounds **22** and **23**) increased the redox potential values by about +0.1 V, the corresponding molecules being active against *L. infantum*. When modifying the pyridinone moiety by introducing a methyl group or a bromine atom at position 4 (compounds **7** and **13**, respectively), the redox potential almost remained unchanged and the molecules were active. By substituting the benzene moiety with a methyl group at position 5 or 6 (compounds **26** and **27**), the redox potentials decreased slightly (down to -0.58 V) and the molecules, were less efficient ($IC_{50} \approx 25$ µM). Compounds bearing a methoxy group at positions 5, 6 or 7 (compounds **29-31**) or a methyl group at position 7 (compound **28**) showed lower redox potentials, varying from -0.58 V to -0.75 V, and were totally inactive ($IC_{50} > 100$ µM). Thus, in this

series, it can be noted that the optimal redox potential value to target in order to obtain antileishmanial activity is -0.5 V and that molecules whose redox potential is positioned below -0.6 V lost all activity.

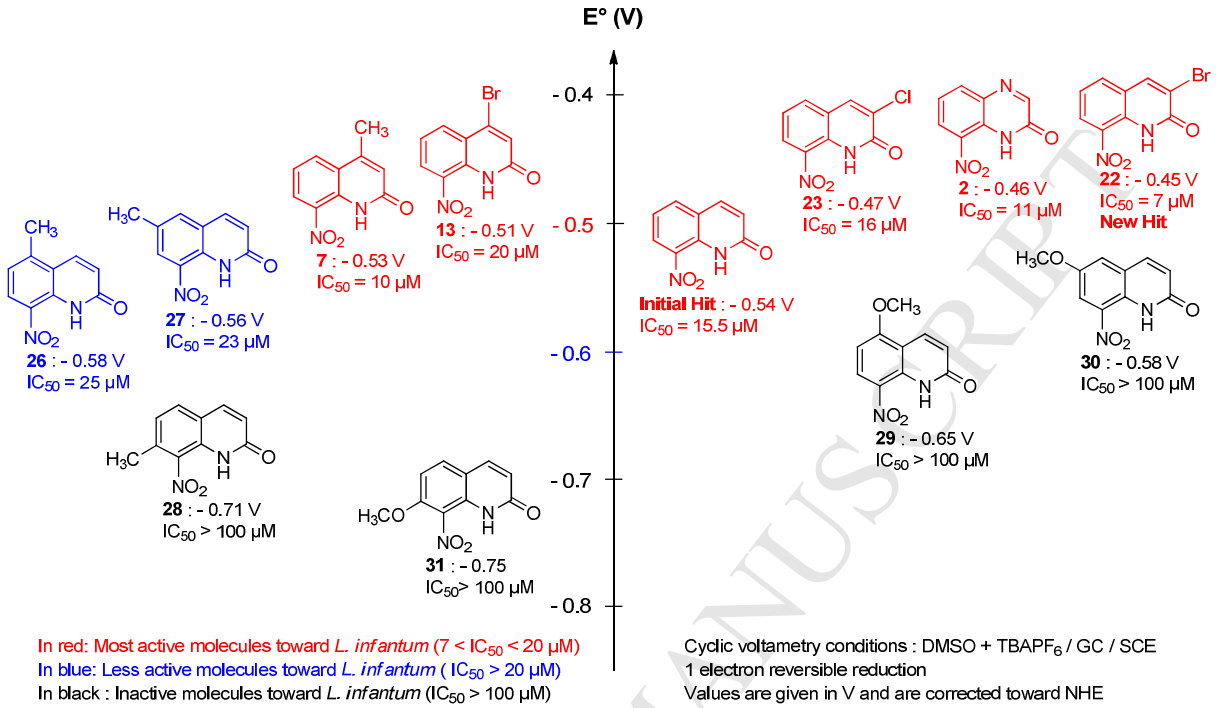


Figure 5: Modulation of the redox potential of the 8-nitroquinolin-2[1*H*]-one pharmacophore.

Mode of action studies, genotoxicity evaluation and preliminary pharmacokinetic properties of 22

Among all synthesized compounds, derivative **22** was highlighted as a new hit (Table1). It is not only active against *L. infantum* (IC_{50} = 7.1 µM), but also against *T. brucei brucei* (IC_{50} = 1.9 µM), which was not the case of the initial hit, displaying modest activity (IC_{50} = 23.4 µM) toward the latter parasite. For comparison with the anti-leishmanial reference drugs, hit compound **22** appears much less active than amphotericin B and about 10 times less active than miltefosine (with equivalent cytotoxicity). Regarding anti-HAT drugs, hit compound **22** appears much less active than suramin, but more active than eflornithine. It is also important to compare hit molecule **22** with drug candidate fexinidazole, as they both belong to the nitroaromatic family: **22** is about 2 times more cytotoxic and, depending on the parasite, between 2 to 5 times less active than this drug candidate, which remains interesting for a hit-compound. It was then necessary to evaluate the *in vitro* activity of **22** against the

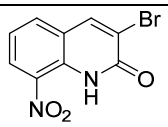
amastigote stage of *Leishmania* in an intramacrophage model, closer to the *in vivo* situation. As presented in Table 2, the test was done on *L. donovani*, the other species responsible for VL. Hit molecule **22**, displays a low cytotoxicity on the THP1 macrophage cell line (CC_{50} = 72 µM) and is about 3 times less active (IC_{50} = 18 µM) on this intramacrophage amastigote *in vitro* model than miltefosine (IC_{50} = 5.4 µM). Fexinidazole is not active on the intramacrophage amastigote stage (IC_{50} > 50 µM) as it first needs to be metabolized into a sulfone derivative.[24] Finally, as expected, compound **14** (non nitrated) and **25** (8-amino derivative), negative controls deriving from hit **22**, did not display any antiparasitic activity.

In order to assess whether this compound series was a substrate of parasitic NTRs, the hit compound **22** was assayed against a wild type strain of *L. donovani* promastigotes and two strains overexpressing NTR1[25] and NTR2[26], respectively (Table 2). Compound **22** was ten times more effective against the strain overexpressing NTR1 (IC_{50} = 0.47 µM) vs WT and NTR2 strains, indicating that it is selectively bioactivated by NTR1 of *L. donovani*. Interestingly, this is the same type 1 nitroreductase that bioactivates fexinidazole and its metabolites.[25] Following the same approach in *T. brucei brucei*, it was also demonstrated that type 1 NTR, the unique NTR enzyme expressed in this parasite, was responsible for the bioactivation of **22** (Table 2).

Nitroaromatic drugs are often suspected to be mutagenic or genotoxic, which has considerably limited the development of such derivatives in the past decades.[27] The most famous *in vitro* test used for evaluating mutagenicity is the Ames test, using *Salmonella typhimurium* strains. Nevertheless, considering that *Salmonella* bacteria possess nitroreductases,[28] the Ames test is usually positive when evaluating nitroaromatics, with no real predicting character for humans, considering that there are no NTRs in mammalian cells and that most of nitroaromatic derivatives only exert a genotoxic character after being bioactivated into reduced metabolites.[27] For that reason, it is nowadays accepted that the comet assay or the micronucleus assay are better *in vitro* tools for evaluating the potential genotoxicity of nitroaromatics.[27] Indeed, metronidazole, one of the most famous anti-infective nitroheterocycles on the market, is mutagenic in the Ames test, but not genotoxic in the comet assay[29] and fexinidazole is not genotoxic in the micronucleus assay despite being mutagenic in the Ames test.[24] Regarding

compound **22**, the Ames test (in metabolizing conditions) and the comet assay were done in parallel and revealed that, although being mutagenic in the Ames test (Table 3) at 0.25 or 2.5 mM, compound **22** was not genotoxic in the comet assay after 2 or 72 h of exposure, at 1, 10, 20 or 40 µM (Figure 6), concentrations chosen from the HepG2 $CC_{10\%}$ (40 µM). This lack of genotoxicity is a key point when thinking about further development of this pharmacophore. Finally, preliminary *in vitro* pharmacokinetic evaluations were done with compound **22** (Table 2). It presented a very good microsomal stability ($T_{1/2}$ > 40 min) and had a strong, but not extreme binding to human albumin (92%), in relation with the relatively high value of its calculated logP (= 2.36).

Table 2: Complementary *in vitro* biological evaluations done on hit compound **22**

***In vitro* studies**	 Compound **22**
Cytotoxicity: CC_{50} THP1 (µM)[a]	72 +/-6
IC_{50} *L. donovani* intramacrophagic amastigotes (µM)[b]	18 +/-2
IC_{50} *L. donovani* promastigotes wild type strain (µM)	5.9 +/-0.12
IC_{50} *L. donovani* promastigotes NTR1 over-expressing strain (µM)	0.47 +/-0.02
IC_{50} *L. donovani* promastigotes NTR2 over-expressing strain (µM)	4.6 +/-0.12
IC_{50} *T. b. brucei* trypomastigotes wild type strain (µM)	17.7 +/-1.0
IC_{50} *T. b. brucei* trypomastigotes NTR1 over-expressing strain (µM)	3.9 +/-0.1
Microsomal stability: $T_{1/2}$ (min)	> 40
Binding % to human albumin	92

[a]Reference compounds: Amphotericin B CC_{50} = 3.6 +/-0.7 µM; Miltefosine CC_{50} > 40 µM; Fexinidazole CC_{50} > 62.5 µM
[b]Reference compounds: Amphotericin B IC_{50} = 0.4 µM; Miltefosine IC_{50} = 5.4 µM; Fexinidazole IC_{50} > 50 µM

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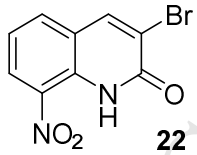
Table 3: Ames test results for hit compound **22**

	 **22**	
	0.25 mM	2.5 mM
TA97a + S9 mix	Positive	Positive
TA98 + S9 mix	Positive	Positive
TA100 + S9 mix	Positive	Positive
TA102 + S9 mix	Negative	Positive

benzo[*a*]pyrene was used as a positive control

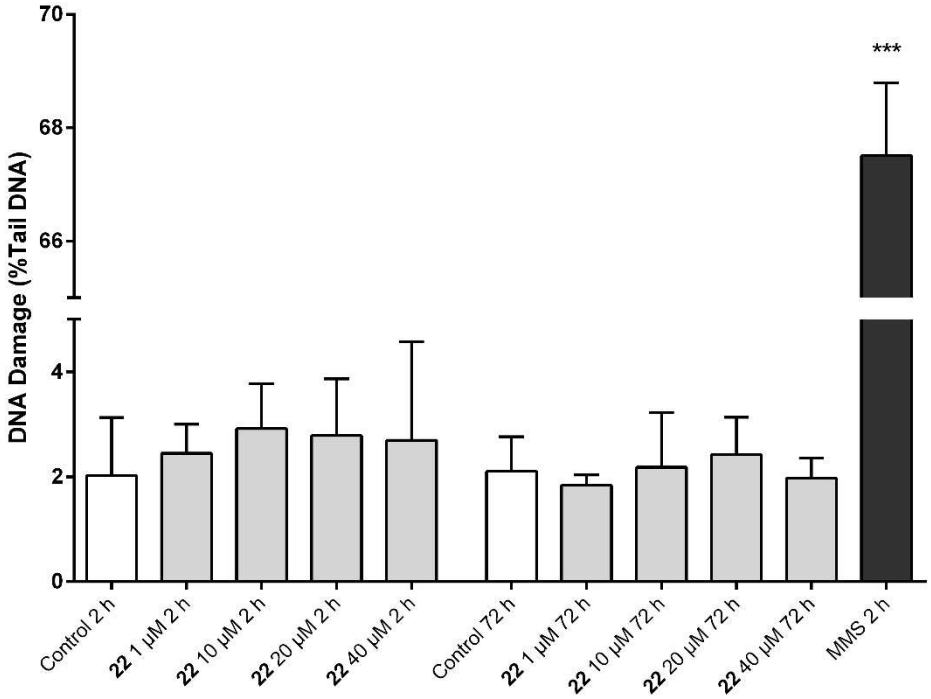


Figure 6: results of the comet assay for hit compound **22**.

Positive control = MMS = 1 mM methylmethanesulfonate

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CONCLUSION

Starting from the previously identified 8-nitroquinolin-2(1*H*)-one antileishmanial pharmacophore, an electrochemistry-guided SAR study revealed that an intramolecular hydrogen bond between the lactam ring and the nitro group is responsible for a +0.3 V increase in the redox potential value (compared with 8-nitroquinoline) resulting in improved antileishmanial activity. A computational model allowed the prediction of the redox potential values of any derivative belonging to this series, and we synthesized a set of derivatives presenting a large range of redox potential values. Then, it was highlighted that, to be active, molecules belonging to this series must display a redox potential value of about -0.5 V and that molecules with a redox potential below -0.6 V were inactive. By introducing electron-withdrawing groups on the pyridone moiety of the scaffold, a new original hit compound was found: **22**. Presenting a low cytotoxicity on the human HepG2 cell line (CC_{50} = 92 µM), it displays activity toward *L. infantum* axenic amastigotes (IC_{50} = 7.1 µM), *L. donovani* promastigotes (IC_{50} = 5.9 µM) and *L. donovani* intramacrophagic amastigotes (IC_{50} = 18 µM), but also toward *T. brucei brucei* (IC_{50} = 1.9 µM). Moreover, we demonstrated that **22** was not genotoxic in the comet assay and that it was selectively bioactivated by the mitochondrial NTR1 of *L. donovani* and *T. brucei brucei*. This whole study, completed with two encouraging preliminary *in vitro* pharmacokinetic parameters (good microsomal stability and 92% binding to human albumin) now opens the way to the rational conception, guided with E° values, of new efficient, selective and safe NTR1 bioactivated 8-nitroquinolin-2(1*H*)-ones against VL and HAT.

ASSOCIATED CONTENT

Supporting Information

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Additional analysis of the hit compound 22 as well as biological methods, Ames test and Comet assay protocols, electrochemistry and computational studies methods, X-Ray data, microsomal stability and plasma protein binding assay results.

AUTHOR INFORMATION

Corresponding author

*****E-mail: pierre.verhaeghe@lcc-toulouse.fr. Phone: +33 561333236.

ORCID

Pierre Verhaeghe: 0000-0003-0238-2447

Notes

The authors declare no competing financial interest

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ABBREVIATIONS

NTR, Nitroreductase; SAR, Structure-Activity Relationship; rt, room temperature; NBS, *N*-bromosuccinimide; AIBN, Azobisisobutyronitrile; THF, Tetrahydrofuran; MW, Microwaves; DMF, N,N-Dimethylformamide, Ar., Argon; NHE, Normal Hydrogen Electrode; IC_{50}, half maximal inhibitory concentration; CC_{50}, half maximal cytotoxic concentration; $TBAPF_6$, Tetrabutylammonium

hexafluorophosphate; SCE, Saturated Calomel Electrode; GC, Glassy Carbon; E°, standard redox potential; E°exp, standard experimental redox potential; E°calc, standard calculated redox potential; HRMS, High-resolution mass spectrometry; NMR, Nuclear Magnetic Resonance; VL, Visceral leishmaniasis; HAT, Human African Trypanosomiasis.

EXPERIMENTAL SECTION

Chemistry

All reagents and solvents were obtained from commercial sources (Fluorochem®, Sigma-Aldrich® or Alfa Aesar®) and used as received. The progress of the reactions was monitored by pre-coated thin layer chromatography (TLC) sheets ALUGRAM® SIL G/UV_{254} from Macherey-Nagel and were visualized by ultraviolet light at 254 nm. The NMR ^{1}H and ^{13}C spectra were recorded on a Bruker UltraShield 300 MHz, a Bruker IconNMR 400 MHz, or a Bruker Avance NEO 600 MHz instrument, at the Laboratoire de Chimie de Coordination and data are presented as follows: chemical shifts in parts per million (δ) using tetramethylsilane (TMS) as reference, coupling constant in Hertz (Hz), multiplicity by abbreviations : (s) singlet, (d) doublet, (t) triplet, (q) quartet, (dd) doublets of doublets, (m) multiplet and (br s) broad singlet. Melting points are uncorrected and were measured on a Stuart Melting Point SMP3 instrument. HRMS measurements were done on a GCT Premier Spectrometer (DCI, CH_4, HRMS) or Xevo G2 QTOF (Waters, ESI+, HRMS) instrument at the Université Paul Sabatier, Toulouse. Microwave reactions were realized in a CEM Discover® microwave reactor. All products were purified by chromatography columns and then recrystallized. Purity of the products was determined by ^{1}H NMR.

General procedure for the preparation of 8-nitroquinoline **1**[30], 7-amino-8-nitroquinoline **4**[31], 2-chloro-4-methyl-8-nitroquinoline **6**[32], 2,4-dibromo-8-nitroquinoline **12**[17], 6-nitroquinolin-2(1*H*)-one **15**[16], 2-chloro-5-nitroquinoline **17**[16] and 2-chloro-8-nitroquinoline **18**[16]:

H_2SO_4 (98%) was added onto 1 equiv. of the quinoline derivatives, cooled with an ice bath. 5 equiv. of 65% HNO_3 were then added dropwise at 0°C and the reaction mixture was stirred at rt for 1-4 h. The

reaction mixture was successively poured into ice, neutralized with NaOH and extracted three times with dichloromethane. The organic layer was washed with water, dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The crude residue was purified by chromatography on silica gel using adapted eluent and recrystallized if necessary to give compounds **1**, **4**, **6**, **12**, **15**, **17** and **18**.

8-nitroquinoline **1** ($C_9H_6N_2O_2$) was purified by chromatography on silica gel using dichloromethane as an eluent, separated from its 5-nitro isomer and isolated to yield a pale yellow solid (35%, 2.7 mmol, 470 mg), mp 90 °C (Lit: 90 °C), ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.53-7.66 (m, 2 H), 8.04 (d, *J* = 8.0 Hz, 2 H), 8.27 (dd, *J* = 1.6 and 8.4 Hz, 1 H, H7), 9.70 (dd, *J* = 1.7 and 4.2 Hz, 1 H, H2). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 122.8 (CH), 123.8 (CH), 125.3 (CH), 129.0 (C), 132.0 (CH), 136.1 (CH), 139.5 (C), 148.2 (C), 152.6 (CH).

7-amino-8-nitroquinoline **4** ($C_9H_7N_3O_2$) was purified by chromatography on silica gel using dichloromethane/ethyl acetate (80/20) as an eluent and isolated to yield an orange solid (46%, 0.16 mmol, 30 mg), mp 193 °C (Lit: 194 °C), ^{1}H NMR (400 MHz, $CDCl_3$) δ: 5.61 (br s, 2 H, NH_2) ; 7.00 (d, *J*=9.0 Hz, 1 H, H6), 7.28 (dd, 1 H, *J*=4.4 and 8.1 Hz, H3), 7.69 (d, *J*= 9.0 Hz, 1H, H5), 8.00 (dd, 1 H, *J*=1.8 and 8.1 Hz, H4), 8.92 (dd, 1 H, *J*=1.8 and 4.4 Hz, H2). ^{13}C NMR (100 MHz, Acetone d6): 118.90 (CH), 119.8 (CH), 120.9 (C), 131.8 (CH), 135.7 (CH), 139.7 (C), 142.8 (C), 143.1 (C), 151.2 (CH).

2-chloro-4-methyl-8-nitroquinoline **6** ($C_{10}H_7ClN_2O_2$) was purified by chromatography on silica gel using cyclohexane/acetone (70/30) as an eluent, separated from its 6-nitro isomer, isolated and recrystallized in isopropanol to yield a white solid in (53%, 14.8 mmol, 3.3 g), mp 143 °C (Lit: 142-143 °C), ^{1}H NMR (400 MHz, $CDCl_3$) δ: 2.74 (d, *J*=1.0 Hz, 3 H, CH_3), 7.35 (d, *J*=1.0 Hz, 1 H, H3), 7.62-7.66 (m, 1 H, H6), 8.00 (dd, *J*= 7.6 and 1.3 Hz, 1 H, H5), 8.18 (dd, *J*= 8.4 and 1.3 Hz, 1 H, H7). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 18.9 (CH_3), 124.2 (CH), 124.5 (CH), 125.4 (CH), 127.8 (CH), 127.9 (C), 138.9 (C), 147.8 (C), 147.9 (C), 153.2 (C).

2,4-dibromo-8-nitroquinoline **12** ($C_9H_4Br_2N_2O_2$) was purified by chromatography on silica gel using cyclohexane/acetone (9/1) as an eluent and isolated to yield a white solid (56%, 6 mmol, 2 g), mp 129 °C (Lit: 129 °C), ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.72-7.76 (m, 1 H, H6), 8.00 (s, 1 H, H3), 8.08 (dd,

J= 7.6 et 1.4 Hz, 1 H, H5), 8.39 (dd, *J*= 8.5 et 1.4 Hz 1 H, H7). ^{13}C NMR (100 MHz, DMSO) δ: 125.3 (CH), 126.9 (CH), 127.5 (C), 130.9 (CH), 131.0 (CH), 135.0 (C), 139.7 (C), 143.6 (C), 147.5 (C).

6-nitroquinolin-2(1*H*)-one **15** ($C_9H_6N_2O_3$) was recrystallized in isopropanol to yield a yellow solid (91%, 6.3 mmol, 1.2 g), mp 279 °C (Lit: 279 °C), ^{1}H NMR (300 MHz, DMSO d6) δ: 6.68 (d, *J*= 9.8 Hz, 1 H, H3), 7.43 (d, *J*= 9.0 Hz, 1 H, H8), 8.12 (d, *J*= 9.8 Hz, 1 H, H4), 8.33 (dd, *J*= 2.6 and 9.0 Hz, 1 H, H7), 8.70 (d, *J*= 2.6 Hz, 1 H, H5), 12,3 (br s, 1 H, NH). ^{13}C NMR (75 MHz, DMSO d6) δ: 116.3 (CH), 118.7 (C), 124.0 (CH), 124.5 (CH), 125.3 (CH), 140.3 (CH), 141.7 (C), 143.5 (C), 162.1 (C).

2-chloro-5-nitroquinoline **17** ($C_9H_5ClN_2O_2$) was purified by chromatography on silica gel using cyclohexane/acetone (8/2) as an eluent, isolated and recrystallized in isopropanol to yield a yellow solid (14% , 4.3 mmol, 0.9 g), mp 134 °C (Lit: 133-134 °C), ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.63 (d, *J*=9,1 Hz, 1 H, H4), 7.82-7.87 (m, 1 H, H7), 8.34 (dd, *J*= 8,6 and 0,7 Hz, 1 H, H8), 8.40 (dd, *J*= 7.6 and 1.2 Hz, 1 H, H6), 8.99 (d, *J*=9.2 Hz, 1 H, H3). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 120.1 (C), 125.0 (CH), 125.6 (CH), 129.0 (CH), 135.1 (CH), 135.7 (CH), 145.6 (C), 148.2 (C), 152.6 (C).

2-chloro-8-nitroquinoline **18** ($C_9H_5ClN_2O_2$) was purified by chromatography on silica gel using cyclohexane/acetone (8/2) as an eluent, isolated and recrystallized in isopropanol to yield a yellow solid (50%, 15.3 mmol, 3.2 g), mp 152 °C (Lit: 152 °C), ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.56 (d, *J*=8.7 Hz, 1 H, H4), 7.65-7.69 (m, 1 H, H6), 8.07 (dd, *J*= 8.3 and 1.4 Hz, 1 H, H5), 8.11 (dd, *J*= 7.6 and 1.4 Hz, 1 H, H7), 8.23 (d, *J*=8.7 Hz, 1 H, H3). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 124.6 (CH), 124.9 (CH), 125.8 (CH), 127.6 (C), 131.8 (CH), 138.6 (CH), 139.0 (C), 147.1 (C), 153.6 (C).

Preparation of 8-nitroquinoxalin-2(1*H*)-one **2**

Ethanol (25 mL) was added onto 1 g of 3-nitro-1,2-phenylenediamine (6.53 mmol, 1 equiv.). 0.6 g of glyoxylic acid monohydrate (6.53 mmol, 1 equiv.) was then added at rt and the reaction mixture was stirred at 80°C for 6 h. The reaction mixture was poured into water and extracted three times with dichloromethane. The organic layer was washed with water, dried over anhydrous $MgSO_4$ and

evaporated *in vacuo*. The crude residue was purified by chromatography on silica gel using dichloromethane/diethyl ether (80/20) as an eluent. 8-nitroquinoxalin-2(1*H*)-one **2** was isolated and recrystallized in acetonitrile to yield an orange solid (46%, 2.98 mmol, 570 mg).

Compound **2** ($C_8H_5N_3O_3$): mp 162 °C, ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.45-7.50 (m, 1 H, H6), 8.25 (dd, *J*=1.5 and 8.2 Hz, 1 H, H5), 8.39 (d, *J*=2.2 Hz, 1 H, H3), 8.55 (dd, *J*=1.5 and 8.2 Hz, 1 H, H7), 11.37 (br s, 1 H, NH). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 122.6 (CH), 127.8 (C), 128.0 (CH), 133.1 (C), 133.8 (C), 137.4 (CH), 152.6 (CH), 153.6 (C). HRMS (DCI CH_4) calcd for $C_8H_6N_3O_3$ $[M+H]^+$ 192.0409, found 192.0412.

Preparation of *N*-(2-Nitrophenyl)-acetamide **3**[33]

Acetic acid (25 mL) was added onto 525 mg of 2-nitroaniline (3.8 mmol, 1 equiv.). The reaction mixture was then stirred at 120°C and 720 µL of acetic anhydride (7.6 mmol, 2 equiv.) were added dropwise. After 3 h under reflux, the reaction mixture was poured into ice, neutralized with Na_2CO_3 and extracted three times with dichloromethane. The organic layer was washed with water, dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. *N*-(2-Nitrophenyl)-acetamide **3** was isolated and recrystallized in acetonitrile to yield a yellow solid (87%, 3.3 mmol, 604 mg).

Compound **3** ($C_8H_8N_2O_3$): mp 93 °C (Lit: 92-94 °C), ^{1}H NMR (400 MHz, $CDCl_3$) δ: 2.29 (s, 3 H, CH_3), 7.16-7.20 (m, 1 H, H4), 7.63-7.67 (m, 1 H, H5), 8.21 (dd, *J*= 8.5 and 1.3 Hz, 1 H, H6), 8,77 (dd, *J*= 8.6 and 1.3 Hz, 1 H, H3), 10.33 (br s, 1H, NH). ^{13}C NMR (100 MHz, $CDCl_3$): 25.60 (CH_3), 122.2 (CH), 123.2 (CH), 125.7 (CH), 134.8 (C), 135.9 (CH), 136.3 (C), 169.0 (C).

Preparation of 7-acetylamino-8-nitroquinoline **5**

Glacial acetic acid (20 mL) was added onto 60 mg of 7-amino-8-nitroquinoline (0.32 mmol, 1 equiv.). The reaction mixture was then stirred at 120°C and 180 µL of acetic anhydride (1.6 mmol, 6 equiv.) were added dropwise. After 24 h under reflux, the reaction mixture was poured into ice, neutralized with K_2CO_3 and extracted three times with dichloromethane. The organic layer was washed with

water, dried over anhydrous $MgSO_4$ and evaporated *in vacuo*. The crude residue was purified by chromatography on silica gel using dichloromethane/ethyl acetate (90/10) as an eluent. 7-acetylamino-8-nitroquinoline **5** was isolated and recrystallized in isopropanol to yield a yellow solid (53%, 0.17 mmol, 39 mg).

Compound **5** ($C_{11}H_9N_3O_2$): mp 189 °C, ^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.28 (s, 3 H, CH_3), 7.49 (dd, *J*=4.3 et 8.3 Hz, 1 H, H3), 7.96 (d, 1H, *J*= 9.2 Hz, H 6), 8.19 (dd, *J*=1.7 et 8.3 Hz, 1 H, H4), 8.35 (br s, 1 H, NH), 8.54 (d, *J*=9.2 Hz, 1 H, H5), 8.99 (dd, *J*=1.7 et 4.3 Hz, 1 H, H2). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 25.0 (CH_3), 121.7 (CH), 121.9 (CH), 125.2 (C), 127.8 (C), 131.4 (CH), 131.8 (C), 135.8 (CH), 140.3 (C), 152.6 (CH), 168.7 (C). HRMS (DCI CH_4) calcd for $C_{11}H_{10}N_3O_2$ $[M+H]^+$ 232.0722, found 232.0719.

General procedure for the preparation of 4-methyl-8-nitroquinolin-2(1*H*)-one **7**[34], quinolin-2(1*H*)-one **14**[35], 5-nitroquinolin-2(1*H*)-one **19**[16] and 8-nitroquinolin-2(1*H*)-one **20**[16].

Acetonitrile (25 mL) and 70 % perchloric acid solution (25 mL) were added onto 1 equiv. of the quinoline derivatives. The reaction mixture was stirred at 100°C overnight. The reaction mixture was then poured into ice, neutralized with KOH and extracted twice with dichloromethane. The organic layer was washed with water, dried over anhydrous $MgSO_4$ and evaporated *in vacuo*. The crude residues were purified by chromatography on silica gel using adapted eluent and recrystallized if necessary to give compounds **7**, **14**, **19**, **20.**

4-methyl-8-nitroquinolin-2(1*H*)-one **7** ($C_{10}H_8N_2O_3$) was isolated and recrystallized in acetonitrile to yield a yellow solid (92%, 11 mmol, 2.2 g), mp 203 °C (Lit: 196 °C), ^{1}H NMR (400 MHz, DMSO) δ: 3.33 (s, 3 H, CH_3), 6.63 (s, 1 H, H3), 7.39-7.44 (m, 1 H, H6), 8.19 (dd, *J*= 8.0 and 1.3 Hz, 1H, H5), 8.42 (dd, *J*= 8.4 and 1.6 Hz, 1 H, H7), 10.98 (br s, 1 H, NH). ^{13}C NMR (DMSO, 100 MHz) δ: 19.6 (CH_3), 121.6 (CH), 122.0 (CH), 122.6 (C), 127.9 (CH), 133.1 (CH), 133.6 (C) 134.6 (C), 148.9 (C), 161.0 (C).

quinolin-2(1*H*)-one **14** (C_9H_7NO) was purified by chromatography on silica gel using dichloromethane/ethyl acetate (8/2) as an eluent and isolated to yield a white solid (74%, 22.7 mmol,

3.3 g), mp 198 °C (Lit: 193 °C), ^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.72 (d, *J*= 9.5 Hz, 1 H, H3), 7.20-7.25 (m, 1 H, H6), 7.40-7,42 (m, 1 H, H5), 7.50-7.54 (m, 1 H, H7), 7.56-7.58 (m, 1 H, H8), 7.82 (d, *J*= 9.5 Hz,1 H, H4), 11.97 (br s, 1 H, NH). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 116.3 (CH), 119.9 (C), 121.3 (CH), 122.7 (CH), 127.7 (CH), 130.7 (CH), 138.5 (C), 141.1 (CH), 164.7 (C).

5-nitroquinolin-2(1*H*)-one **19** ($C_9H_6N_2O_3$) was isolated and recrystallized in acetonitrile to yield a yellow solid (92%, 2.2 mmol, 418 mg), mp 302 °C (Lit: 302 °C), ^{1}H NMR (400 MHz, DMSO d6) δ: 6.76 (d, *J*=10.0 Hz, 1 H, H3), 7.65-7.73 (m, 2 H, H7 H8), 7.88 (dd, *J*= 7.7 and 1.3 Hz, 1 H, H6), 8.25 (d, *J*= 10.0 Hz, 1 H, H4), 12.3 (br s, 1 H, NH). ^{13}C NMR (100 MHz, DMSO d6) δ: 111.9 (C), 118.9 (CH), 121.5 (CH), 125.8 (CH), 130.6 (CH), 134.7 (CH), 140.7 (C), 146.7 (C), 161.3 (C).

8-nitroquinolin-2(1*H*)-one **20** ($C_9H_6N_2O_3$) was isolated and recrystallized in isopropanol to yield a yellow solid (96%, 10.6 mmol, 2.02 g), mp 163 °C (Lit: 163 °C), ^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.79 (dd, *J*= 9.7 and 2.0 Hz, 1H, H3), 7.32-7.36 (m, 1 H, H6), 7.82 (d, *J*= 9.7 and 1.4 Hz, 1 H, H4), 7.91 (dd, *J*= 7.6 Hz, 1 H, H5), 8.53 (dd, *J*= 8.4 and 1.4 Hz, 1 H, H7), 11.3 (br s, 1H, NH). ^{13}C NMR (100 MHz, DMSO d6) δ: 121.4 (CH), 122.1 (C), 123.8 (CH), 127.9 (CH), 133.1 (C), 133.9 (C), 135.6 (CH), 139.9 (CH), 161.6 (C).

Preparation of 4-bromomethyl-8-nitroquinolin-2(1*H*)-one **8**

Carbon tetrachloride (15 mL) was added onto 400 mg of 4-methyl-8-nitroquinolin-2(1*H*)-one (1.9 mmol, 1 equiv.) and 1.7 g of *N*-bromosuccinimide (9.7 mmol, 5 equiv.). The reaction mixture was heated at 80°C. Thent 31 mg of AIBN (0.24 mmol, 0.1 equiv.) were added, and stirred overnight. The reaction mixture was poured into water, extracted twice with dichloromethane and once with ethyl acetate. The combined organic layers were washed with water, dried over anhydrous $MgSO_4$ and evaporated *in vacuo*. The crude residue was purified by chromatography on silica gel using ethyl acetate as an eluent. 4-bromomethyl-8-nitroquinolin-2(1*H*)-one **8** was isolated to yield a yellow solid (21%, 0.42 mmol, 120 mg).

Compound **8** ($C_{10}H_7BrN_2O_3$): mp 161 °C, ^{1}H NMR (400 MHz, $CDCl_3$) δ: 4.60 (s, 2 H, CH_2), 6.87 (d, *J*=2.0 Hz, 1 H, H3), 7.40-7.44 (m, 1 H, H6), 8.18 (dd, *J*= 1.3 and 8.8 Hz, 1 H, H5), 8.57 (dd, *J*= 1.3

and 8.3 Hz, 1 H, H7), 11.44 (br s, 1 H, NH). ^{13}C NMR (100 MHz, $CDCl_3$,) δ: 27.9 (CH_2), 120.2 (C), 121.3 (CH), 123.7 (CH), 128.4 (CH), 132.4 (CH), 133.6 (C), 134.4 (C), 146.1 (C), 160.7 (C). HRMS (DCI CH_4) calcd for $C_{10}H_8BrN_2O_3$ $[M+H]^+$ 282.9718, found 282.9723.

Preparation of 4-hydroxymethyl-8-nitroquinolin-2(1*H*)-one **9**

Fifty millilitres of a mixture H_2O/THF (1/1) were added onto 150 mg of 4-bromomethyl-8-nitroquinolin-2(1*H*)-one (0.53 mmol, 1 equiv). 106 mg of NaOH (2.6 mmol, 5 equiv.) were added and the reaction mixture was stirred at 50°C for 24 h. The reaction mixture was then poured into water, extracted twice with dichloromethane and twice with ethyl acetate. The combined organic layers were washed with water, dried over anhydrous $MgSO_4$ and evaporated *in vacuo*. The crude residue was purified by chromatography on silica gel using ethyl acetate as an eluent. 4-hydroxymethyl-8-nitroquinolin-2(1*H*)-one **9** was isolated to yield a yellow solid (43%, 0.23 mmol, 50 mg).

Compound **9** ($C_{10}H_8N_2O_4$): mp 211 °C, ^{1}H NMR (300 MHz, DMSO) δ: 4.80 (d, *J*=5.5 Hz, 2 H, CH_2), 5.67 (t, *J*=5.5 Hz, 1 H, OH), 6.73 (s, 1 H, H3), 7.37-7.42 (m, 1 H, H6), 8,13 (d, *J*= 7.9 Hz, 1 H, H5), 8.42 (d, *J*= 8.3 Hz, 1 H, H7), 11,01 (br s, 1H, NH). ^{13}C NMR (150 MHz, DMSO) δ: 39.5 (CH_2), 122.0 (CH), 122.1 (C), 123.0 (CH), 128.2 (CH), 133.3 (CH), 133.8 (CH), 136.5 (C), 141.3 (C), 161.6 (C). HRMS (DCI CH_4) calcd for $C_{10}H_9N_2O_4$ $[M+H]^+$ 221.0562, found 221.0558.

Preparation of 4-{[2-(*N*-boc-2-aminoethoxy)ethoxy]ethyl}aminomethyl-8-nitroquinolin-2(1*H*)-one **10**

THF (50 mL) was added onto 500 mg of 4-bromomethyl-8-nitroquinolin-2(1*H*)-one (1.8 mmol, 1 equiv.) and 1.3 g of *N*-Boc-2,2′-(ethylenedioxy)-diethylamine (5.3 mmol, 3 equiv.). Triethylamine (375 µL, 2.7 mmol, 1.5 equiv.) were then added and the reaction mixture was stirred at 50°C overnight. The reaction mixture was evaporated *in vacuo*. The crude product was then resolubilized in ethyl acetate, washed three times with water, dried over anhydrous $MgSO_4$ and evaporated *in vacuo*. The crude residue was purified by chromatography on silica gel using diethyl ether/methanol/ 20% aqueous ammonia solution (8.9/1/0.1) as an eluent. 4-{[2-(*N*-boc-2-

aminoethoxy)ethoxy]ethyl}aminomethyl-8-nitroquinolin-2(1*H*)-one **10** was isolated to yield an orange oil (84%, 1.5 mmol, 680 mg).

Compound **10** ($C_{21}H_{30}N_4O_7$): ^{1}H NMR (600 MHz, DMSO) δ: 1.36 (s, 9H, *t*Bu), 2.5 (s, 1 H, NH amine), 2.75-2.77 (m, 2 H, CH_2 3'), 3.03-3.06 (m, 2 H, CH_2 10'), 3.36-3.39 (m, 2 H, CH_2 4'), 3.51-3.54 (m, 6 H, 3xCH_2 6' 7' 9'), 4.02 (s, 2 H, CH_2 1'), 6.73 (br s, 1 H, NH carbamate), 6.76 (s, 1 H, H3), 7.38-7.42 (m, 1 H, H6), 8.31 (dd, *J*= 8.1 and 1.3 Hz, 1H, H5), 8.42 (dd, *J*= 8.1 and 1.3 Hz, 1 H, H7), 11.03 (br s, 1 H, NH).

^{13}C NMR (150 MHz, DMSO) δ: 28.7 (3 CH_3, *t*Bu), 40.1 (CH_2), 48.7 (CH_2), 49.9 (CH_2), 69.6 (CH_2), 70.0 (CH_2), 70.1 (CH_2), 70.5 (CH_2), 78.0 (C), 120.5 (CH), 121.4 (C), 121.6 (CH), 127.8 (CH), 132.9 (CH), 133.6 (C), 134.5 (C), 150.6 (C), 156.0 (C), 161.1 (C). HRMS (ESI) calcd for $C_{21}H_{31}N_4O_7$ $[M+H]^+$ 450.2114, found 450.2118.

Preparation of 4-{[2-(2-aminoethoxy)ethoxy]ethyl}aminomethyl-8-nitroquinolin-2(1*H*)-one **11**

Hydrochloric acid (15 mL of 5 N HCL in isopropanol) was added onto 600 mg of 4-{[2-(*N*-boc-2-aminoethoxy)ethoxy]ethyl}aminomethyl-8-nitroquinolin-2(1*H*)-one (1.33 mmol, 1 equiv.) and the reaction mixture was then stirred at rt for 4 h. The reaction mixture was successively poured into water, neutralized with K_2CO_3 and extracted several times with dichloromethane. The organic layer was dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. 4-{[2-(2-aminoethoxy)ethoxy]ethyl}aminomethyl-8-nitroquinolin-2(1*H*)-one **11** was isolated to yield an oil in (43%, 0.57 mmol, 200 mg).

Compound **11** ($C_{16}H_{22}N_4O_5$): ^{1}H NMR (400 MHz, DMSO) δ: 2.5 (s, 1 H, NH amine), 2.63-2.66 (m, 2 H, CH_2 10'), 2.74-2.77 (m, 2 H, CH_2 3'), 3.35-3.38 (m, 2 H, CH_2 9'), 3.49-3.55 (m, 6 H, 3xCH_2 4' 6' 7'), 4.01 (d, *J*= 1.9 Hz, 2 H, CH_2 1'), 6.76 (br s, 1 H, H3), 7.38-7.42 (m, 1 H, H6), 8.30 (dd, *J*= 8.1 and 1.3 Hz, 1 H, H5), 8.40 (dd, *J*= 8.1 and 1.3 Hz, 1 H, H7), 11.0 (br s, 1 H, NH lactame). ^{13}C NMR (150 MHz, DMSO) δ: 41.7 (CH_2), 48.7 (CH_2), 50.0 (CH_2), 70.0 (CH_2), 70.1 (CH_2), 70.6 (CH_2), 73.2 (CH_2), 120.4 (CH), 121.4 (C), 121.5 (CH), 127.7 (CH), 132.8 (CH), 133.7 (C), 134.6 (C), 150.7 (C), 161.2 (C). HRMS (ESI) calcd for $C_{16}H_{23}N_4O_5$ $[M+H]^+$ 351.1668, found 351.1674.

Preparation of 4-bromo-8-nitroquinolin-2(1*H*)-one **13**[17]

Acetonitrile (10 mL) and 500 µL of a 70% perchloric acid solution (5.4 mmol, 6 equiv.) were added onto 300 mg of 2,4-dibromo-8-nitroquinoline (0.90 mmol, 1 equiv.) in a sealed tube. The reaction mixture was then heated at 100°C in a microwave reactor during 1 h. The reaction mixture was poured into ice, neutralized with K_2CO_3 and extracted three times with dichloromethane. The organic layer was washed with water, dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. 4-bromo-8-nitroquinolin-2(1*H*)-one **13** was isolated and recrystallized in acetonitrile to yield a yellow solid (87%, 0.78 mmol, 211 mg).

Compound **13** ($C_9H_5BrN_2O_3$): mp 239 °C (Lit: 240 °C), ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.20 (d, *J*= 2.0 Hz, 1 H, H3), 7.37-7.42 (m, 1 H, H6), 8.36 (dd, *J*= 8.0 and 1.4 Hz, 1 H, H5), 8.58 (dd, *J*= 8.3 and 1.4 Hz, 1 H, H7), 11.42 (br s, 1 H, NH). ^{13}C NMR (100 MHz, DMSO) δ: 121.0 (C), 122.6 (CH), 126.9 (CH), 129.4 (CH), 132.7 (C), 134.8 (C), 135.5 (CH), 136.5 (C), 159.5 (C).

Preparation of *N*-methylquinolin-2(1H)-one **16**[36]

DMF (30 mL) was added onto 1 g of quinoline-2(1*H*)-one (6.9 mmol, 1 equiv.), 1.43 g of K_2CO_3 (10.3 mmol, 1.5 equiv.). The reaction mixture was heated at 80°C and 430 µL of methane iodide (6.9 mmol, 1 equiv.) were added dropwise. After 48 h, the reaction mixture was poured into water and extracted three times with dichloromethane. The organic layer was washed 5 times with water, dried over anhydrous Na_2SO_4 and evaporated in vacuo. The crude residue was purified by chromatography on silica gel using dichloromethane/ethyl acetate (7.5/2.5) as eluent. *N*-methylquinolin-2(1H)-one **16** was isolated and recrystallized in isopropanol to yield a white solid (71%, 4.90 mmol, 780 mg).

Compound **16** ($C_{10}H_9NO$): mp 76 °C (Lit: 74 °C), ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.73 (s, 3 H, CH_3), 6.71 (d, *J*=9.4 Hz, 1 H, H3), 7.21-7.25 (m, 1 H, H6), 7.37 (dd, *J*= 8.1 and 1.0 Hz, 1 H, H5), 7.55-7.59 (m, 2 H, H7 - H8), 7.67 (d, *J*=9.5 Hz, 1 H, H4). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 29.4 (CH3), 114.1 (CH), 120.6 (C), 121.7 (CH), 122.1 (CH), 128.7 (CH), 130.6 (CH), 138.9 (CH), 140.0 (C), 162.3 (C).

Preparation of 2-methoxy-8-nitroquinoline **21**[16]

Under Argon atmosphere, 100 mg of 8-nitroquinolin-2(1*H*)-one (0.53 mmol, 1 equiv.) were solubilized in 5 mL of DMF and were added onto a DMF solution (5 mL) of 42 mg of 60% sodium hydride (1.05 mmol, 2 equiv.). After 10 min of stirring at rt, 65 µL of methyl iodide (1.05 mmol, 2 equiv.) were added dropwise. After one night stirring at rt, the reaction mixture was poured into ice. The resulting precipitate was filtered and dried *in vacuo*. The crude residue was purified by chromatography on silica gel using diethyl ether as eluent. 2-methoxy-8-nitroquinoline **21** was isolated and recrystallized in cyclohexane to yield a pale white solid (64%, 0.34 mmol, 70 mg).

Compound **21** ($C_{10}H_8N_2O_3$): mp 125 °C (Lit: 114 °C), ^{1}H NMR (400 MHz, $CDCl_3$) δ: 4.09 (s, 3 H, CH_3), 7.05 (d, *J*= 8.9 Hz, 1 H, H3), 7.42-7.46 (m, 1 H, H6), 7.93 (dd, *J*= 8.1 and 1.4 Hz, 1 H, H5), 7.99 (dd, *J*= 7.6 and 1.4 Hz, 1 H, H7), 8.07 (d, *J*= 8.9 Hz, 1 H, H4). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 54.1 (CH_3), 115.1 (CH), 122.6 (CH), 124.0 (CH), 126.2 (C), 131.5 (CH), 138.2 (C), 138.5 (CH), 146.6 (C), 163.7 (C).

Preparation of 3-bromo-8-nitroquinolin-2(1*H*)-one **22**

Fifty milliliters of a 48% hydrobromic acid solution were added onto 1.34 g of 8-nitroquinolin-2(1*H*)-one (7.05 mmol, 1 equiv.). The reaction mixture was then stirred at 100°C. Then 3.2 g of sodium bromate (21.1 mmol, 3 equiv.) were added with precaution (Br_2 formation). After 5 h under reflux, the reaction mixture was left for 2 h under the hood to evacuate remaining Br_2 vapors, and then poured into ice, neutralized with K_2CO_3 and extracted three times with dichloromethane. The organic layer was washed with water, dried over anhydrous Na_2SO_4 and evaporated *in vacuo*.

The crude residue was purified by chromatography on silica gel using cyclohexane/ethyl acetate (1/1) as an eluent. 3-bromo-8-nitroquinolin-2(1*H*)-one **22** was isolated and recrystallized in acetonitrile to yield a yellow solid (68%, 4.8 mmol, 1.3 g).

Compound **22** ($C_9H_5BrN_2O_3$): mp 216 °C, ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.36-7.40 (m, 1 H, H6), 7.89 (dd, *J*= 8.4 and 1,4 Hz, 1 H, H5), 8.6 (s, 1 H, H4), 8.56 (dd, *J*= 7,7 and 1,6 Hz, 1 H, H7), 11.51 (br s, 1 H, NH). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 119.9 (C), 122.1 (CH), 122.3 (C), 128.0 (CH), 132.9 (C), 133.2 (C), 134.8 (CH), 141.0 (CH), 157.5 (C). HRMS (DCI CH_4) calcd for $C_9H_6BrN_2O_3$ $[M+H]^+$ 268.9562, found 268.9554.

Preparation of 3-chloro-8-nitroquinolin-2(1*H*)-one **23**

Fifty milliliters of a 37% hydrochloric acid solution were added onto 1 g of 8-nitroquinolin-2(1*H*)-one (5.3 mmol, 1 equiv.). The reaction mixture was then stirred at 100°C before 1.7 g of sodium chlorate (15.8 mmol, 3 equiv.) were added with precaution (Cl_2 formation). After 45 min under reflux, the reaction mixture was left for 2 h under the hood to evacuate remaining Cl_2 vapors, and then was poured into ice, neutralized with K_2CO_3 and extracted three times with dichloromethane. The organic layer was washed with water, dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The crude residue was purified by chromatography on silica gel using diethyl ether as an eluent. 3-chloro-8-nitroquinolin-2(1*H*)-one **23** was isolated and recrystallized in acetonitrile to yield a yellow solid (62%, 3.3 mmol, 738 mg).

Compound **23** ($C_9H_5ClN_2O_3$): mp 195 °C, ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.37-7.41 (m, 1 H, H6), 7.88 (dd, *J*= 7.7 and 1.4 Hz, 1 H, H5), 8.04 (s, 1 H, H4), 8.55 (dd, *J*=8.4 and 1.4 Hz, 1 H, H7), 11.56 (br s, 1 H, NH). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 121.8 (C), 122.2 (CH), 127.9 (CH), 129.2 (C), 132.3 (C), 133.1 (C), 134.9 (CH), 137.0 (CH), 157.5 (C). HRMS (DCI CH_4) calcd for $C_9H_6ClN_2O_3$ $[M+H]^+$ 225.0067, found 225.0070.

Preparation of 3-bromo-2-methoxy-8-nitroquinoline **24**

Under Argon atmosphere, 250 mg of 3-bromo-8-nitroquinolin-2(1*H*)-one (0.93 mmol, 1 equiv.) were solubilized in 5 mL of dry DMF and were added onto a DMF solution (5 mL) of 74 mg of 60% sodium hydride (1.86 mmol, 2 equiv.). After 10 min of stirring at rt, 115 µL of methyl iodide (1.86 mmol, 2 equiv.) were added dropwise. The reaction mixture was stirred at rt overnight, before being

poured into ice. The resulting precipitate was filtered and dried *in vacuo*. The crude residue was purified by chromatography on silica gel using cyclohexane/acetone (9/1) as eluent. 3-bromo-2-methoxy-8-nitroquinoline **24** was isolated to yield a pale white solid (55%, 0.51 mmol, 146 mg).

Compound **24** ($C_{10}H_7BrN_2O_3$): mp 208 °C, ^{1}H NMR (300 MHz, $CDCl_3$) δ: 4.15 (s, 3 H, CH_3), 7.43-7.49 (m, 1 H, H6), 7.86 (dd, *J*= 8.1 and 1.4 Hz, 1 H, H5), 8.02 (dd, *J*= 7.7 and 1.4 Hz, 1 H, H7), 8.35 (s, 1 H, H4). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 55.4 (CH_3), 110.4 (C), 123.5 (CH), 124.4 (CH), 127.1 (C), 130.6 (CH), 136.7 (C), 140.6 (CH), 146.2 (C), 159.2 (C). HRMS (DCI CH_4) calcd for $C_{10}H_7BrN_2O_3$ $[M+H]^+$ 282.9718, found 282.9722.

Preparation of 8-amino-3-bromoquinolin-2(1*H*)-one **25**

Ethanol (20 mL) was added onto 300 mg of 3-bromo-8-nitroquinolin-2(1*H*)-one (1.11 mmol, 1 equiv.) and 1.1 g of tin (II) chloride (5.55 mmol, 5 equiv.). The reaction mixture was refluxed for 3 h. The reaction mixture was then neutralized into an aqueous solution of Na_2CO_3, filtered on celite, extracted three times with dichloromethane and once with ethyl acetate. The combined organic layers were washed with water, dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The crude residue was purified by chromatography on silica gel using dichloromethane/methanol (95/5) as eluent. 8-amino-3-bromoquinolin-2(1*H*)-one **25** was isolated to yield a pale brown solid (53%, 0.59 mmol, 140 mg).

Compound **25** ($C_9H_7BrN_2O$): Dec. 248 °C, ^{1}H NMR (400 MHz, DMSO-d6) δ: 5.60 (br s, 2 H, NH_2), 6.84 (dd, *J*= 7.5 and 1.5 Hz, 1 H, H7), 6.90 (dd, *J*= 7.7 and 1.5 Hz, 1 H, H5), 6.95-6.99 (m, 1 H, H6), 8.40 (s, 1 H, H4), 11.35 (br s, 1 H, NH). ^{13}C NMR (100 MHz, DMSO-d6) δ: 115.2 (CH), 115.5 (CH), 116.6 (C), 120.6 (C), 123.5 (CH), 125.6 (C), 135.4 (C), 143.2 (CH), 158.3 (C). HRMS (DCI CH_4) calcd for $C_{10}H_7BrN_2O_3$ $[M+H]^+$ 238.9820, found 238.9820.

General procedure for the preparation of 5-methyl-8-nitroquinolin-2(1*H*)-one **26**, 6-methyl-8-nitroquinolin-2(1*H*)-one **27**[37], 7-methyl-8-nitroquinolin-2(1*H*)-one **28**, 5-methoxy-8-nitroquinolin-2(1*H*)-one **29**, 6-methoxy-8-nitroquinolin-2(1*H*)-one **30**[38] and 7-methoxy-8-nitroquinolin-2(1*H*)-one **31**.

In a first step, according to a previously reported procedure,[21] 1.2 equiv. of 3,3'-diethoxyacryloyl chloride (prepared from ethyl 3,3'-diethoxypropionate by successive saponification and reaction with $SOCl_2$) was reacted at rt with 1 equiv. of the appropriate nitroaniline derivative in dichloromethane, in the presence of 2 equiv. of pyridine. The reaction mixtures were then poured into water and extracted three times with dichloromethane. The organic layers were washed with brine, dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. In a second step, each crude residue was reacted with 98% sulfuric acid and stirred at rt for 3-4 h (monitored by TLC). The reaction mixtures were then poured into ice, neutralized with K_2CO_3 and extracted three times with dichloromethane. The organic layers were washed with water, dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The final crude residues were purified by chromatography on silica gel.

5-methyl-8-nitroquinolin-2(1*H*)-one **26** ($C_{10}H_8N_2O_3$) was purified by chromatography on silica gel using cyclohexane/ethyl acetate (90/10) as eluent, isolated and recrystallized in acetonitrile to yield a pale brown solid (46%, 2.39 mmol, 490 mg), mp 213 °C, ^{1}H NMR (400 MHz, DMSO-d6) δ: 2.67 (s, 3 H, CH_3), 6.78 (dd, *J*= 10.0 and 2.2 Hz, 1 H, H3), 7.15 (dd, *J*= 8.5 and 0.6 Hz, 1 H, H6), 7.99 (d, *J*= 10.0 Hz, 1 H, H4), 8.40 (d, *J*= 8.5 Hz, 1 H, H7), 11.5 (br s, 1 H, NH). ^{13}C NMR (100 MHz, DMSO-d6) δ: 19.8 (CH_3), 120.1 (C), 123.1 (CH), 123.3 (CH), 127.8 (CH), 131.7 (C), 134.1 (C) 136.6 (CH), 145.4 (C), 161.3 (C). HRMS (DCI CH_4) calcd for $C_{10}H_9N_2O_3$ $[M+H]^+$ 205.0613, found 205.0608.

6-methyl-8-nitroquinolin-2(1*H*)-one **27** ($C_{10}H_8N_2O_3$) was purified by chromatography on silica gel using cyclohexane/ethyl acetate (90/10) as eluent and isolated to yield a brown solid (6%, 0.34 mmol, 70 mg), mp 200 °C (Lit: 199-200), ^{1}H NMR (400 MHz, $CDCl_3$) δ: 2.50 (s, 3H, CH_3), 6.74 (dd, *J*=9.7 and 2.0 Hz, 1 H, H3), 7.69 (s, 1 H, H5), 7.74 (d, *J*=9.7 Hz, 1 H, H4), 8.33 (d, *J*=2.0 Hz, 1H, H7), 11.22 (br s, 1 H, NH). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 20.5 (CH_3), 122.0 (C), 123.7 (CH), 128.4 (CH), 131.7 (C), 131.8 (C), 132.8 (C), 135.9 (CH) 139.7 (CH), 161.6 (C).

7-methyl-8-nitroquinolin-2(1*H*)-one **28** ($C_{10}H_8N_2O_3$) was purified by chromatography on silica gel using cyclohexane/ethyl acetate (90/10) as eluent, isolated and recrystallized in acetonitrile to yield a

yellow solid (52%, 3.42 mmol, 700 mg), mp 233 °C, ^{1}H NMR (400 MHz, $CDCl_3$) δ: 2.64 (s, 3 H, CH_3), 6.69 (d, *J*= 9.6 Hz, 1 H, H6), 7.15 (dd, *J*= 8.0 and 0.6 Hz, 1 H, H3), 7.64 (d, *J*= 8.0 Hz, 1 H, H4), 7.74 (d, *J*= 9.6 Hz, 1 H, H5), 10.12 (br s, 1 H, NH). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 21.2 (CH_3), 119.7 (C), 122.7 (CH), 125.5 (CH), 132.2 (CH), 132.6 (C), 135.6 (C) 137.6 (C), 139.9 (CH), 161.7 (C). HRMS (DCI CH_4) calcd for $C_{10}H_9N_2O_3$ $[M+H]^+$ 205.0613, found 205.0615.

5-methoxy-8-nitroquinolin-2(1*H*)-one **29** ($C_{10}H_8N_2O_4$) was purified by chromatography on silica gel using cyclohexane/ethyl acetate (90/10) as eluent, isolated and recrystallized in acetonitrile to yield a dark orange solid (21%, 0.61 mmol, 135 mg), mp 246 °C, ^{1}H NMR (400 MHz, $CDCl_3$) δ: 4.09 (s, 3 H, OCH_3), 6.68 (dd, *J*= 10.0 and 2.1 Hz, 1 H, H3), 6.73 (d, *J*= 9.4 Hz, 1 H, H6), 8.17 (d, *J*= 9.9 Hz, 1 H, H4), 8.52 (d, *J*= 9.4 Hz, 1 H, H7), 11.45 (br s, 1H, NH). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 56.8 (OCH_3), 102.8 (CH), 110.7 (C), 121.8 (CH), 127.0 (C), 130.6 (CH), 134.6 (CH) 135.6 (C), 161.7 (C), 161.8 (C). HRMS (DCI CH_4) calcd for $C_{10}H_9N_2O_3$ $[M+H]^+$ 221.0562, found 221.0561.

6-methoxy-8-nitroquinolin-2(1*H*)-one **30** ($C_{10}H_8N_2O_4$) was purified by chromatography on silica gel using cyclohexane/ethyl acetate (90/10) as eluent and isolated to yield a pale brown solid (4%, 0.05 mmol, 12 mg), mp 210 °C (Lit : 210-211 °C), ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.93 (s, 3 H, OCH_3), 6.76 (dd, *J*= 9.7 and 1.8 Hz, 1 H, H3), 7.42 (d, *J*= 2.9 Hz, 1 H, H5), 7.73 (d, *J*= 9.7 Hz, 1 H, H4), 8.07 (d, *J*= 2.9 Hz, 1 H, H7), 11.15 (br s, 1H, NH). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 56.3 (OCH_3), 113.5 (CH), 113.9 (C), 120.7 (CH), 122.9 (C), 124.4 (CH), 128.3 (C) 139.4 (CH), 153.5 (C), 161.4 (C).

7-methoxy-8-nitroquinolin-2(1*H*)-one **31** ($C_{10}H_8N_2O_4$) was purified by chromatography on silica gel using dichloromethane/ethyl acetate (90/10) as eluent, isolated and recrystallized in acetonitrile to yield a yellow solid (45%, 0.79 mmol, 175 mg), mp 244 °C, ^{1}H NMR (400 MHz, $CDCl_3$) δ: 4.04 (s, 3 H, OCH_3), 6.57 (d, *J*= 9.6 Hz, 1 H, H3), 6.93 (d, *J*= 8.9 Hz, 1 H, H6), 7.68 (d, *J*= 9.3 Hz, 2 H, H4 H5), 9.9 (br s, 1H, NH). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 57.2 (OCH_3), 105.0 (C), 106.9 (CH), 114.3 (C),

120.6 (CH), 133.5 (CH), 133.7 (C) 139.9 (CH), 155.9 (C), 161.8 (C). HRMS (DCI CH_4) calcd for $C_{10}H_9N_2O_3$ $[M+H]^+$ 221.0562, found 221.0556.

Electrochemistry

Voltammetric measurements were carried out with a potentiostat Autolab PGSTAT100 (ECO Chemie, The Netherlands) controlled by GPES 4.09 software. Experiments were performed at room temperature in a homemade airtight three–electrode cell connected to a vacuum/argon line. The reference electrode consisted of a saturated calomel electrode (SCE) separated from the solution by a bridge compartment. The counter electrode was a platinum wire of approximately 1cm² apparent surface. The working electrode was GC microdisk (1.0 mm of diameter – Bio-logic SAS). The supporting electrolyte $(nBu_4N)[PF_6]$ (Fluka, 99% puriss electrochemical grade) and the solvent DMSO (Sigma-Aldrich puriss p.a. dried<0.02% water) were used as received and simply degassed under argon. The solutions used during the electrochemical studies were typically 10^{-3} M in compound and 0.1 M in supporting electrolyte. Before each measurement, the solutions were degassed by bubbling Ar and the working electrode was polished with a polishing machine (Presi P230). Under these experimental conditions employed in this work, the half-wave potential ($E_{1/2}$) of the ferrocene Fc+/Fc couple in DMSO was $E_{1/2}$ = 0.45 V vs SCE. Experimental peak potentials have been measured versus SCE and converted to NHE by adding 0.241 V.

Computational studies

All the calculations were carried out with the GAUSSIAN 09 suite[39]. Geometry optimizations were performed with the M06-2x[40] density functional and the 6-311++G (2d,2p) basis set. Previous studies have indicated that extended basis sets, with diffuse s- and p-type functions and polarization functions were important for the description of the electronic affinity of nitrobenzene derivatives[22,23]. The effect of solvation were described with the self-consistent reaction field (SCRF) method using the integral

equation formalism polarizable continuum Model (IEFPCM) with DMSO as solvent[41]. Vibrational frequency calculation were used to confirm the convergence to local minima and to calculate the unscaled zero-point-energy (ZPE) and the entropy corrections at 298 K. The standard variation of the Gibb's free energies were calculated as the energy difference between anion-radical and neutral forms after full geometry optimization according to the following equation: $R\text{-}NO_2 + 1\ e^- \rightarrow .\ R\text{-}NO_2^{\cdot -}$. Reduction potentials of the nitro-compounds were calculated using the equation based on Faraday's law:

$E° = -\frac{\Delta G_{red}}{nF} + E_H^0$ (1) where ΔG_{red} is the Gibbs free energy of reduction, n is the number of electrons transferred (i.e. one electron), F is the Faraday constant which equals 23.06 $kcal.mol^{-1}.V^{-1}$ and E_H^0 is the absolute potential of the normal hydrogen electrode. The E_H^0 value was taken as -4.28 V.[42]

Biology

Antileishmanial activity on L. infantum axenic amastigotes.[43]

L. infantum promastigotes (MHOM/MA/67/ITMAP-263, CNR Leishmania, Montpellier, France, expressing luciferase activity) in logarithmic phase cultivated in RPMI 1640 medium supplemented with 5% foetal calf serum (FCS), 2 mM L-glutamine and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin), were centrifuged at 900 g for 10 min. The supernatant was removed carefully and was replaced by the same volume of RPMI 1640 complete medium at pH 5.4 and incubated for 24 h at 24 °C. The acidified promastigotes were incubated for 24 h at 37 °C in a ventilated flask. Promastigotes were then transformed into axenic amastigotes. The effects of the tested compounds on the growth of *L. infantum* axenic amastigotes were assessed as follows. *L. infantum* amastigotes were incubated at a density of 2.10^6 parasites/mL in sterile 96-well plates with various concentrations of compounds dissolved in DMSO (final concentration less than 0.5% v/v), in duplicate. Appropriate controls DMSO, amphotericin B, miltefosine and fexinidazole (reference drugs purchased from Sigma Aldrich) were added to each set of experiments. After a 48 h incubation period at 37 °C, each plate-well was then microscope-examined for detecting any precipitate formation. To estimate the luciferase

activity of axenic amastigotes, 80 µl of each well are transferred to white 96-well plates, Steady Glow® reagent (Promega) was added according to manufacturer's instructions, and plates were incubated for 2 min. The luminescence was measured in Microbeta Luminescence Counter (PerkinElmer). Inhibitory concentration 50% (IC_{50}) was defined as the concentration of drug required to inhibit by 50% the metabolic activity of *L. infantum* amastigotes compared to control. IC_{50} values were calculated by non-linear regression analysis processed on dose response curves, using TableCurve 2D V5 software. IC_{50} values represent the mean of three independent experiments.

Antileishmanial activity on L. donovani promastigotes.

The effects of the tested compounds on the growth of *L. donovani* promastigotes (MHOM/IN/00/DEVI) were assessed by MTT assay.[44] Briefly, promastigotes in logarithmic phase in Schneider's medium supplemented with 20% foetal calf serum (FCS), 2 mM L-glutamine and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin), were incubated at an average density of 10^6 parasites/mL in sterile 96-well plates with various concentrations of compounds dissolved in DMSO (final concentration less than 0.5% v/v), in duplicate. Appropriate controls treated by DMSO, miltefosine or amphotericin B (reference drugs purchased from Sigma Aldrich) were added to each set of experiments. After a 72 h incubation period at 27 °C, parasite metabolic activity was determined. Each plate-well was then microscope-examined for detecting possible precipitate formation. 10µL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (10 mg/mL in PBS) were added to each well followed by incubation for another 4 h. The enzyme reaction was then stopped by addition of 100 µL of 50% isopropanol–10% sodium dodecyl sulfate. Plates were shaken vigorously (300 rpm) for 10 min. The absorbance was finally measured at 570 nm in a BIO-TEK ELx808 Absorbance Microplate Reader. Inhibitory concentration 50% (IC_{50}) was defined as the concentration of drug required to inhibit by 50% the metabolic activity of *L. donovani* and promastigotes compared to the control. IC_{50} were calculated by nonlinear regression analysis processed on dose–response curves, using TableCurve 2D V5.0 software. IC_{50} values represent the mean value calculated from three independent experiments.

Antileishmanial activity on L. donovani intracellular amastigotes.

The effects of the tested compounds on the growth of *Leishmania donovani* intracellular amastigotes (MHOM/IN/00/DEVI) were assessed according to the method of Da Luz *et al*.[45] 400 µL of THP-1 cells activated with Phorbol 12-Myristate 13-Acetate (final concentration: 50 ng/ml) were seeded in sterile chamber-slides at an average density of 10^5 cells/mL and incubated for 48 h at 37 °C and 6% CO_2. *L. donovani* promastigotes were centrifuged at 900g for 10 min and the supernatant replaced by the same volume of Schneider 20% FCS pH 5.4 and incubated for 24 h at 27 °C. THP-1 cells were then infected by acidified promastigotes at an average density of 10^6 cells/mL (10:1 ratio) and chamber-slides incubated for 24 h at 37 °C. Then, in duplicate, the medium containing various concentrations of tested-compounds was added (final DMSO concentration being inferior to 0.5% v/v). Appropriate controls treated with or without solvent (DMSO), and various concentrations of miltefosine and amphotericin B (reference drugs purchased from Sigma Aldrich) were added to each set of experiments. After 120 h incubation at 37 °C and 6% CO_2, well supernatant was removed. Cells were fixed with analytical grade methanol and stained with 10% Giemsa. The percentage of infected macrophages in each assay was determined microscopically by counting at least 200 cells in each sample. IC_{50} was defined as the concentration of drug necessary to produce a 50% decrease of infected macrophages compared to the control. IC_{50} were calculated by non-linear regression analysis processed on dose–response curves, using TableCurve 2D V5.0 software. IC_{50} values represent the mean value calculated from three independent experiments.

Antileishmanial activity on L. donovani promastigotes NTR1 and NTR2 over-expressing strain.

Cell lines and culture conditions: The clonal *Leishmania donovani* cell line LdBOB (derived from MHOM/SD/62/1S-CL2D) was grown as promastigotes at 26 °C in modified M199 media, as previously described[46]. LdBOB promastigotes overexpressing NTR1 (LinJ.05.0660)[47] and NTR2 (LinJ.12.0730)[48] were grown in the presence of nourseothricin (100 µg ml^{-1}). ***In vitro* drug sensitivity assays:** To examine the effects of test compounds on growth, triplicate promastigote cultures were seeded with 5×10^4 parasites ml^{-1}. Parasites were grown in 10 ml cultures in the presence of drug for 72 h, after which 200 µL aliquots of each culture were added to 96-well plates, 50 µM resazurin was

added to each well and fluorescence (excitation of 528 nm and emission of 590 nm) measured after a further 4 h incubation[49]. Data were processed using GRAFIT (version 5.0.4; Erithacus software) and fitted to a 2-parameter equation, where the data are corrected for background fluorescence, to obtain the effective concentration inhibiting growth by 50% (EC_{50}): $y = \frac{100}{1+\left(\frac{[I]}{EC_{50}}\right)^m}$. In this equation [I] represents inhibitor concentration and m is the slope factor. Experiments were repeated at least two times and the data is presented as the mean plus standard deviation.

Antitrypanosomal activity on T. brucei brucei trypomastigotes.

Assays were performed on *Trypanosoma brucei brucei* AnTat 1.9 strain (IMTA, Antwerpen, Belgium). It was cultured in MEM with Earle's salts, supplemented according to the protocol of Baltz et *al.*[50] with the following modifications, 0.5 mM mercaptoethanol (Sigma Aldrich®, France), 1.5 mM L-cysteine (Sigma Aldrich®), 0.05 mM bathocuproïne sulfate (Sigma Aldrich®) and 20% heat-inactivated horse serum (Gibco®, France), at 37°C in an atmosphere of 5% CO_2. The parasites were incubated at an average density of 2000 parasites/well in sterile 96-wells plates (Mc2®, France) with various concentrations of compounds dissolved in DMSO (Sigma Aldrich®), in duplicate. Reference drugs suramin, pentamidine, eflornithine, and fexinidazole (purchased from Sigma Aldrich, France and Fluorochem, UK) suspended in NaCl 0.9% or DMSO, were added to each set of experiments. The effects of the tested compounds were assessed by the viability marker Alamar Blue® (Fisher, France) assay described by Räz et *al.*[51] After a 69 h incubation period at 37 °C, 10 µL of Alamar Blue® was then added to each well, and the plates were incubated for 5h.[52] The plates were read in a PerkinElmer ENSPIRE (Germany) microplate reader using an excitation wavelength of 530 nm and an emission wavelength of 590 nm. IC_{50} were calculated by nonlinear regression analysis processed on dose-response curves, using GraphPad Prism software (USA). IC_{50} was defined as the concentration of drug necessary to inhibit by 50% the viability of *T. brucei brucei* compared to the control. IC_{50} values were calculated from three independent experiments in duplicate.

Antitrypanosomal activity on T. b. brucei trypomastigotes NTR1 over-expressing strain.

Trypanosoma brucei bloodstream-form 'single marker' S427 (T7RPOL TETR NEO) and drug-resistant cell lines were cultured at 37°C in HMI9-T medium[53] supplemented with 2.5 µg.mL^{-1} G418 to maintain expression of T7 RNA polymerase and the tetracycline repressor protein. Bloodstream trypanosomes overexpressing the *T. brucei* nitroreductase (NTR1)[54] were grown in medium supplemented with 2.5 µg.mL^{-1} phleomycin and expression of NTR was induced by the addition of 1 µg.mL^{-1} tetracycline. Cultures were initiated with 1 × 10^5 cells.mL^{-1} and sub-cultured when cell densities approached 1–2 (× 10^6).mL^{-1}.

In order to examine the effects of inhibitors on the growth of these parasites, triplicate cultures containing the inhibitor were seeded at 1 × 10^5 trypanosomes.mL^{-1}. Cells overexpressing NTR were induced with tetracycline 48 h prior to EC_{50} analysis. Cell densities were determined after culture for 72 h, as previously described[55]. EC_{50} values were determined using the following two-parameter equation by non-linear regression using GraFit:

$$y = \frac{100}{1 + \left(\frac{[I]}{EC_{50}} \right)^m}$$

where the experimental data were corrected for background cell density and expressed as a percentage of the uninhibited control cell density.In this equation [I] represents inhibitor concentration and m is the slope factor.

Cytotoxic evaluation on HepG2 cell line.

The evaluation of the tested molecules cytotoxicity on the HepG2 (hepatocarcinoma cell line from ECACC purchased from Sigma-Aldrich, ref 85011430-1VL certificated without mycoplasma) was done according to the method of Mosman with slight modifications.[44] Briefly, cells (5 x 10^4 cells/mL) in 100 µL of complete medium, [Alpha MEM Eagle from PAN BIOTECH supplemented with 10%

foetal bovine serum, 2 mM L-glutamine and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin)] were seeded into each well of 96-well plates and incubated at 37 °C and 5% CO_2. After a 24 h incubation, 100 µL of medium with various product concentrations and appropriate controls were added and the plates were incubated for 72 h at 37 °C and 5% CO_2. Each plate-well was then microscope-examined for detecting possible precipitate formation before the medium was aspirated from the wells. 100 µL of MTT solution (0.5 mg/mL in Alpha MEM Eagle) were then added to each well. Cells were incubated for 2 h at 37 °C and 5% CO_2. After this time, the MTT solution was removed and DMSO (100 µL) was added to dissolve the resulting formazan crystals. Plates were shaken vigorously (300 rpm) for 5 min. The absorbance was measured at 570 nm with a microplate spectrophotometer (Eon BioTek). DMSO was used as blank and doxorubicin (purchased from Sigma Aldrich) as positive control. CC_{50} were calculated by non-linear regression analysis processed on dosee response curves, using TableCurve 2D V5 software. CC_{50} values represent the mean value calculated from three independent experiments.

Cytotoxic evaluation on THP1 cell line.

The evaluation of the tested molecules cytotoxicity on the differenciated THP-1 cell line (acute monocytic leukemia cell line purchased from ATCC, ref TIB-202) was performed according to the method of Mosman with slight modifications.[44] Briefly, cells in 100 µL of complete RPMI medium with Phorbol 12-Myristate 13-Acetate (final concentration: 50 ng/ml) were incubated at an average density of 10^6 cells/mL and in sterile 96-well plates. After 48 h incubation, 100 µL of medium with various product concentrations dissolved in DMSO (final concentration less than 0.5% v/v) were added and the plates were incubated for 72 h at 37 °C. Each plate-well was then microscope-examined for detecting possible precipitate formation before the medium was aspirated from the wells. 100 µL of MTT solution (0.5 mg/mL in medium without FCS) were then added to each well. Cells were incubated for 2 h at 37 °C. After this time, the MTT solution was removed and DMSO (100 µL) was added to dissolve the resulting blue formazan crystals. Plates were shaken vigorously (300 rpm) for 10 min. The absorbance was measured at 570 nm with 630 nm as reference wavelength

spectrophotometer using a BIO-TEK ELx808 Absorbance Microplate Reader. DMSO was used as blank and doxorubicin (purchased from Sigma Aldrich) as positive control. Cell viability was calculated as percentage of control (cells incubated without compound). The 50% cytotoxic concentration was determined from the dose–response curve by using the TableCurve 2D V5.0 software.

Ames test method

Mutagenicity test was carried out by using a modified version[56] of the liquid incubation assay of the classical Ames test. *S. typhimurium* tester strains (TA97a, TA98, TA100 and TA102) were grown overnight in a Nutrient Broth n°2 (Oxoid, France). After this period, 5 - 50 mM DMSO solutions of the tested drugs were added to 0.1 mL of culture and incubated with 4% S9 mix for 1 h at 37 °C with shaking. Each sample was assayed in duplicate. After incubation, 2 mL of molten top agar were mixed gently with the pre-incubated solution and poured onto Vogel-Bonner minimal agar plates. After 48 h at 37 °C in the dark, the number of spontaneous and drug induced revertants per plate was determined for each dose with a laser bacterial colony counter. A product was considered mutagenic when it induces a two-fold increase of the number of revertants, compared with the spontaneous frequency (negative control). Benzo[a]pyrene was used as a positive control with all *Salmonella* strains in presence of S9 mix.

Comet assay

The alkaline comet assay was used to detect DNA strand breaks and alkali-labile sites. Trypsinized HepG2 cells were embedded in 0.7% low-melting point agarose (Sigma “Low Gelling Temperature”) and laid on pre-cut sheets of polyester film (Gelbond® film) to perform minigel deposits as previously described[57]. Film were then placed in lysis solution (NaCl 2.5 M, Na_2EDTA 0.1 M, Tris 10 mM, 1 % Triton X-100, 10 % DMSO pH 10) for 18h at 4 °C. Electrophoresis (with a solution which contained 0.3 M NaOH, 1 mM Na_2EDTA, pH>13) was processed for 24 min in a tank with a power supply

giving 28 V (resulting in 0.8 V/cm). After electrophoresis, films were immersed 2 x 5 min in PBS for neutralization, followed by fixation in 100% ethanol for 1.5h and drying. After staining with SYBR® Gold (Life Technologies) at 10 000 X dilution for 20 min, films were observed at 20x magnification with an epifluorescence microscope equipped with an automated platform (Nikon NiE) and coupled to a camera (DS-Q1Mc) and the software Nikon NiS Element Advanced Research to automatically capture images. In these images, for each cell, the level of DNA damage was evaluated using a semi-automated scoring system, by measurement of the intensity of all tail pixels divided by the total intensity of all pixels in head and tail of comet, by means of the software "Lucia comet assay" (Laboratory Imaging, Prague Czech Republic). Fifty cells per deposit and four deposits *per* sample were analysed. The median from these 200 values was calculated, and named "% tail DNA".

Microsomal stability and plasma protein binding assays

Microsomal stability protocol.

The tested product and propranolol, used as reference, are incubated in duplicate (reaction volume of 0.5 mL) with female mouse microsomes (CD-1, 20 mg / ml, BD Gentest™) at 37 °C in a 50 mM phosphate buffer, pH 7.4, in the presence of $MgCl_2$ (5 mM), NADP (1 mM), glucose-6-phosphate dehydrogenase (0.4 U/mL) and glucose-6-phosphate (5 mM). For the estimation of the intrinsic clearance: 50 µL aliquot at 0, 5, 10, 20, 30 and 40 min are collected and the reaction is stopped with 4 volumes of acetonitrile (ACN) containing the internal standard. After centrifugation at 10000 g, 10 min, 4 °C, the supernatants are kept at 4 °C for immediate analysis or placed at -80 °C in case of postponement of the analysis. Controls (t_0 and t_{final}) in triplicate are prepared by incubation of the internal standard with microsomes denatured by acetonitrile. The LC-MS used for this study is a Waters® Acquity I-Class / Xevo TQD, equipped with a Waters® Acquity BEH C18 column, 50 x 2.1 mm, 1.7 µm. The mobile phases are (A) ammonium acetate 10 mM and (B) acetonitrile with 0.1% formic acid. The injection volume is 1 µL and the flow rate is 600 µL/min. The chromatographic analysis, total duration of 4 min, is made with the following gradient: $0 < t < 0.2$ min, 2% (B); $0.2 < t < 2$ min, linear increase to 98% (B); $2 < t < 2.5$ min, 98% (B); $2.5 < t < 2.6$ min, linear decrease to 2% (B); $2.6 < t < 4$ min, 2% (B). 8-Bromo-6-chloro-3-nitro-2-(phenylsulfonylmethyl)imidazo[1,2-

a]pyridine is used as internal standard. The quantification of each compound is obtained by converting the average of the ratios of the analyte/internal standard surfaces to the percentage of consumed product. The ratio of the control at t_0 corresponds to 0% of product consumed. The calculation of the half-life ($t_{1/2}$) of each compound in the presence of microsomes is done according to the equation: $t_{\frac{1}{2}} = \frac{\ln(2)}{k}$. Where k is the first-order degradation constant (the slope of the logarithm of compound concentration versus incubation time). The intrinsic clearance *in vitro* (Cl_{int} expressed in µL/min/mg) is calculated according to the equation:

$Cl_{int} = \frac{dose}{AUC_{\infty}} / [microsomes]$. Where dose is the initial concentration of product in the sample, AUC_{∞} is the area under the concentration-time curve extrapolated to infinity and [microsomes] is the microsome concentration expressed in mg/µL.

Plasma protein binding procedure.

The plasma doped with the tested compound is incubated at 37 °C in triplicate in one of the compartments of the insert, the other compartment containing a phosphate buffer solution at pH 7.2. After stirring for 4 hours at 300 rpm, a 25 µL aliquot of each compartment is taken and diluted; the dilution solution is adapted to obtain an identical matrix for all the compartments after dilution. In parallel, the reprocessing of a plasma doped but not incubated will allow to evaluate the recovery of the study. The LC-MS used for this study is a Waters® Acquity I-Class / Xevo TQD, equipped with a Waters® Acquity BEH C18 column, 50 x 2.1 mm, 1.7 µm. The mobile phases are (A) ammonium acetate 10 mM and (B) acetonitrile with 0.1% formic acid. The injection volume is 1 µL and the flow rate is 600 µL/min. The chromatographic analysis, total duration of 4 min, is made with the following gradient: 0 < t < 0.2 min, 2% (B); 0.2 < t < 2 min, linear increase to 98% (B); 2 < t < 2.5 min, 98% (B); 2.5 < t < 2.6 min, linear decrease to 2% (B); 2.6 < t < 4 min, 2% (B). Carbamazepine, oxazepam, warfarine and diclofenac are used as reference drugs and Propranolol is used as internal standard. The unbound fraction (fu) is calculated according to the following formula: $\mathbf{f_u} = \frac{\mathbf{A_{Plasma,4h} - A_{PBS,4h}}}{\mathbf{A_{Plasma,4h}}} \times \mathbf{100}$. The percentage of recovery is calculated according to the following formula: $\% \mathbf{Recovery} =$

$\frac{(V_{PBS} \times A_{PBS,4h}) + (V_{Plasma} \times A_{Plasma,4h})}{(V_{Plasma} \times A_{Plasma,0h})}$. Where A is the ratio of the area under peak of the studied molecule and the area under peak of the internal standard (propranolol 200 nM). V is the volume of solution present in the compartments (VPBS = 350 μL and Vplasma = 200 μL).

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