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1 **Antikinetoplastid SAR study in 3-nitroimidazopyridine series: identification of**
2 **a novel non-genotoxic and potent anti-*T. b. brucei* hit-compound with improved**
3 **pharmacokinetic properties.**

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35

36 **Abstract:**

37 To study the antikinoplastid 3-nitroimidazo[1,2-*a*]pyridine pharmacophore, a structure-activity
38 relationship study was conducted through the synthesis of 26 original derivatives and their *in vitro*
39 evaluation on both *Leishmania spp* and *Trypanosoma brucei brucei*. This SAR study showed that
40 the antitrypanosomal pharmacophore was less restrictive than the antileishmanial one and
41 highlighted positions 2, 6 and 8 of the imidazopyridine ring as key modulation points. None of the
42 synthesized compounds allowed improvement in antileishmanial activity, compared to previous
43 hit molecules in the series. Nevertheless, compound **8**, the best antitrypanosomal molecule in this
44 series ($EC_{50} = 17$ nM, $SI = 2650$ & $E^{\circ} = -0.6$ V), was not only more active than all reference drugs
45 and previous hit molecules in the series but also displayed improved aqueous solubility and better
46 *in vitro* pharmacokinetic characteristics: good microsomal stability ($T_{1/2} > 40$ min), moderate
47 albumin binding (77 %) and moderate permeability across the blood brain barrier according to a
48 PAMPA assay. Moreover, both micronucleus and comet assays showed that nitroaromatic
49 molecule **8** was not genotoxic *in vitro*. It was evidenced that bioactivation of molecule **8** was
50 operated by *T. b. brucei* type 1 nitroreductase, in the same manner as fexinidazole. Finally, a mouse
51 pharmacokinetic study showed that **8** displayed good systemic exposure after both single and
52 repeated oral administrations at 100 mg/kg (NOAEL) and satisfying plasmatic half-life ($T_{1/2} = 7.7$
53 h). Thus, molecule **8** appears as a good candidate for initiating a hit to lead drug discovery program.

54

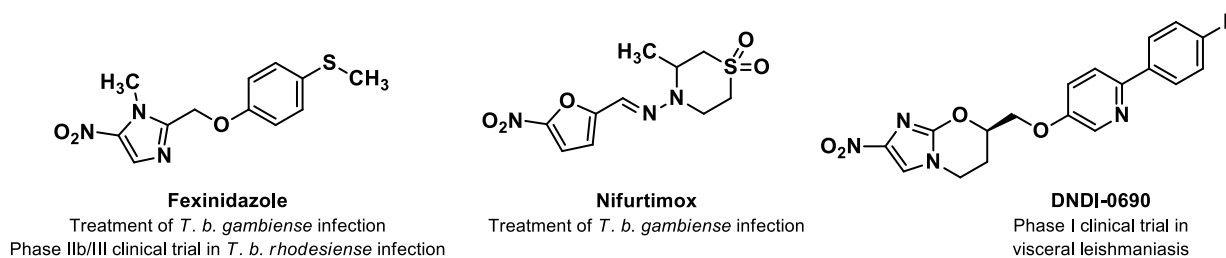
55 **Keywords:** Imidazo[1,2-*a*]pyridine; Nitroaromatic; Nitroreductases; Kinetoplastids; Redox
56 potentials; SARs.

57 1. Introduction

58 Kinetoplastid diseases are vectorial parasitoses caused by flagellated blood protozoa and
59 transmitted to their hosts by specific insect vectors. These diseases are found in developing
60 countries in intertropical regions, especially where health facilities are inadequate [1]. They
61 threaten nearly one billion people, causing an estimated 30 000 deaths annually [2]. The World
62 Health Organization (WHO) thereby classifies them as Neglected Tropical Diseases (NTDs) [3].
63 These infectious diseases are caused by two parasitic genera: *Leishmania* spp. and *Trypanosoma*
64 spp. The first is responsible for leishmaniasis (caused by several *Leishmania* species) which can
65 evolve in different forms (visceral, cutaneous or cutaneomucous) [4].
66 The second genus contains most of *Trypanosoma* species especially *T. cruzi* causing Chagas
67 disease in South and Central America, and *T. brucei* spp. responsible for Human African
68 Trypanosomiasis (HAT, or sleeping sickness) [5,6]. HAT is found in 36 countries throughout sub-
69 Saharan Africa. It is estimated that 70 million people would be at risk of contracting it [7]. Two
70 subspecies, *T. brucei gambiense* (*T.b.g.*) and *T. brucei rhodesiense* (*T.b.r.*), are at the origin of
71 different symptoms and two clinical forms: chronic disease for *T.b.g.* and acute disease for *T.b.r.*
72 Following the bite of the blood sucking fly (tsetse fly or glossina), the parasite penetrates into the
73 derma and disseminates into the whole organism. In a first step, blood and lymph are affected by
74 the bloodstream form, and patients show headaches, anemia, joint pain and various organ damages
75 occur. This stage is called hemolymphatic stage. Then, without treatment, the parasites are able to
76 cross the blood brain barrier (BBB) and invade the central nervous system (stage 2, or nervous
77 stage), causing various neurological changes such as sleeping disorders (responsible for the name
78 of the disease), abnormal tone and mobility, ataxia, psychiatric disorders, seizures, coma and
79 finally, death, as all kinetoplastid diseases are lethal if untreated [7,8]. Nevertheless, very few
80 efficient, safe and affordable drugs are proposed to treat the infected populations. Regarding HAT,
81 treatments are complex because they are stage and species dependent. For early stage HAT,
82 suramin and pentamidine, molecules presenting severe side effects, must be given by injection (IV

83 or IM) for a prolonged period against *T.b.r.* and *T.b.g.* infections, respectively. For the late-stage,
84 melarsoprol is given in *T.b.r.* infections despite the high toxicity of this arsenic derivative (it can
85 cause encephalopathies in 10% of cases). Against *T.b.g.* infections, the NECT (Nifurtimox
86 Eflornithine Combination Therapy) is prescribed despite the genotoxic character of nifurtimox [9].
87 Since the end of 2018, a new safer nitroaromatic drug, fexinidazole, is available to treat HAT
88 [10,11]. Concerning the new chemical entities that are clinically studied by the Drug for Neglected
89 Disease initiative (DNDi), only acoziborole (benzoxaborole derivative) is in phase IIb/III against
90 HAT [10]. This quite limited drug portfolio calls for the research of new molecules that could
91 strengthen the therapeutic arsenal and reduce the risk of emergence of resistant parasites.
92 Concerning fexinidazole, a 2-substituted-5-nitroimidazole derivative, it is a prodrug which is
93 activated through the reduction of its nitro group by a NADH-specific type 1 nitroreductase (NTR),
94 to generate electrophilic cytotoxic metabolites (nitroso and hydroxylamine derivatives) able to
95 form covalent adducts with DNA and that are cytotoxic for the parasite [9,12]. Only one
96 mitochondrial type 1 nitroreductase was identified in *Trypanosoma brucei* [13]. The particularity
97 of nitroreductases is that they are absent in mammalian cells and then, constitute important targets
98 for the development of new selective antiparasitic treatments. In this context, nitroaromatic
99 compounds are key derivatives that have been and remain studied to fight against kinetoplastids,
100 as shown with the structures of nifurtimox, fexinidazole and DNDI-0690, a novel 4-nitroimidazole
101 derivative that is clinically studied against visceral leishmaniasis [14] (**Figure 1**).

102



103

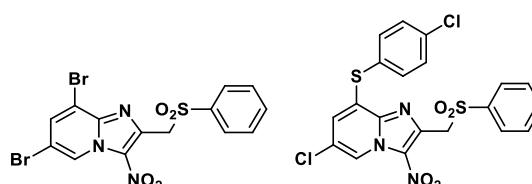
104 **Figure 1.** Structures of nitroheterocyclic drug-compounds that are marketed or clinically studied
105 against *Trypanosoma brucei* and *Leishmania* infections.

106

107 Within the framework of our research program, focused on the development of original
 108 nitroheterocyclic antikinoplastid molecules, we formerly reported a first antileishmanial hit
 109 compound in 8-halogeno-3-nitroimidazo[1,2-*a*]pyridine series (hit A, **Figure 2**) [15].
 110 Pharmacomodulation studies at position 8 of the scaffold were conducted and showed that
 111 introducing a 4-chlorophenylthio moiety improved *in vitro* antikinoplastid activity (hit B, **Figure**
 112 **2**) [16]. However, hit-molecule A displayed limited water solubility while hit B showed a poor
 113 mouse liver microsomal stability ($T_{1/2} = 3$ min), even if some probable sulfoxide and sulfone
 114 metabolites remained active. Thus, to improve hydrosolubility, microsomal stability and
 115 antikinoplastid activity of this nitroheterocyclic series, we explored deeper the structure activity
 116 relationships by studying the influence of substituents at position 2 and 6 of the imidazo[1,2-*a*]
 117 *a*]pyridine ring and comparing to the activities of hits A and B.

118

119 **Figure 2.** Structures and biological profiles of previously identified antikinoplastid hit-
 120 compounds in 3-nitroimidazo[1,2-*a*]pyridine series [15–17].



	Hit A	Hit B
EC ₅₀ <i>L. donovani</i> promastigotes (μM)	1.8	1.0
EC ₅₀ <i>L. donovani</i> amastigotes (μM)	5.5	1.3
EC ₅₀ <i>L. infantum</i> axenic amastigotes (μM)	4.4	1.7
EC ₅₀ <i>T. b. brucei</i> trypomastigotes (μM)	2.9	1.3
CC ₅₀ HepG2 (μM)	>31	>100

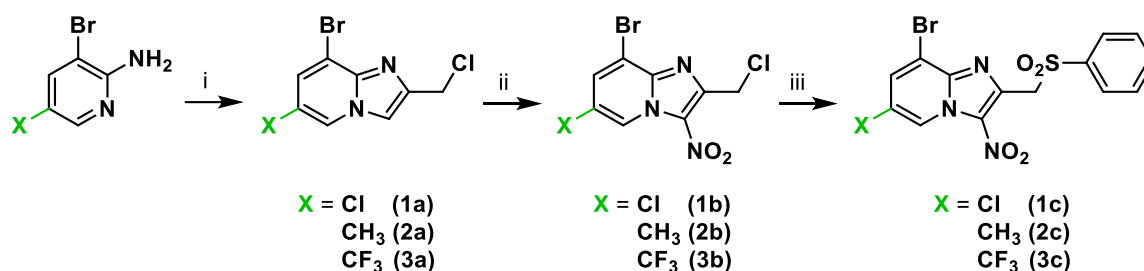
121

122 2. Results and discussion

123 To evaluate if redox potentials had an influence on the bioactivation of this chemical series by
 124 parasitic NTRs, analogs of hit A bearing a methyl group (electron donating) or a trifluoromethyl
 125 group (electron withdrawing) at position 6 of the imidazo[1,2*a*]pyridine ring were synthesized. A
 126 bromine atom was not included in the pharmacomodulation study considering that we previously
 127 noted that molecules belonging to this series and bearing a bromine atom at position 6 showed the

128 same level of antiparasitic activity as their chlorinated analogs, these latter presenting the
129 advantage to show a better aqueous solubility. Thus, in parallel with the previously described 6-
130 chloro analogue **1c** of hit A, new derivatives **2c** and **3c** were obtained through a 3-steps synthesis
131 procedure (**Scheme 1**) that was previously reported [18,19].

132



136

137

138 **Scheme 1.** Synthesis of compounds **1a-c** to **3a-c**.

139 *Reagents and conditions:* (i) 1,3-Dichloroacetone 1.1 equiv, EtOH or DME, 80°C, 31% to 60%;

140 (ii) HNO₃ 6 equiv, H₂SO₄, 0°C→RT, 51% to 85%; (iii) Sodium benzenesulfinate 3 equiv, DMSO,

141 RT, 3 h, 57% to 89%.

142

143 To study the influence of the substituent at position 2 of the imidazo[1,2-*a*]pyridine ring, the

144 phenylsulfonylmethyl group encountered in the structures of hit A and B was modulated (**Scheme**

145 **2**). First, a 2-unsubstituted derivative (**4b**) was obtained through a cyclocondensation reaction of

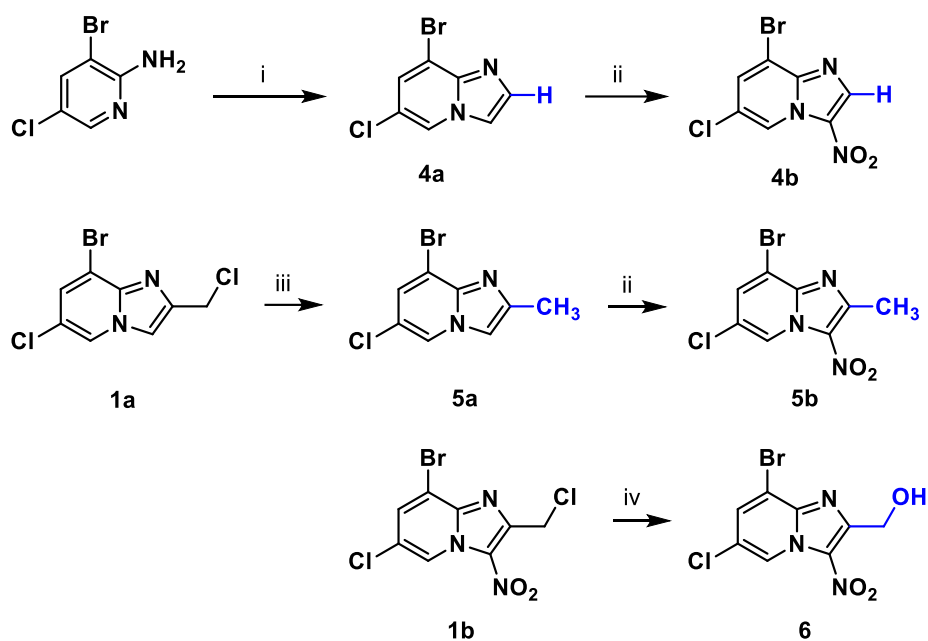
146 2-amino-3-bromo-5-chloropyridine with chloroacetaldehyde, followed by a nitration reaction.

147 Then, the 2-methyl-substituted derivative **5b** was synthesized by dehalogenation of **1a** followed

148 by a nitration reaction of **5a** at position 3. Finally, the 2-hydroxymethyl-derivative **6** was obtained

149 by hydrolysis of **1b** in the presence of copper sulfate in aqueous DMSO [20].

150



148

149 **Scheme 2.** Synthesis of compounds **4a-b**, **5a-b** and **6**.

150 *Reagents and conditions:* (i) Chloroacetaldehyde 1.5 equiv, NaHCO₃ 2 equiv, EtOH, 80 °C, 31%;

151 (ii) HNO₃ 6 equiv, H₂SO₄, 0°C→RT, 78% to 93%; (iii) NaBH₄ 1 equiv, DMSO, RT, 63%; (iv)

152 CuSO₄ 1 equiv, DMSO/H₂O (7.5:2.5), 100 °C, 32%.

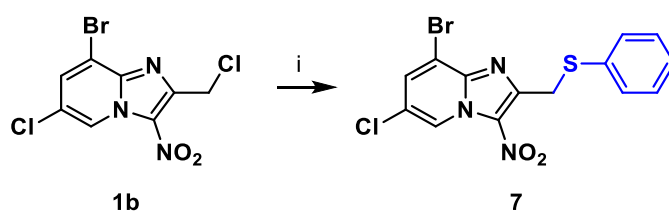
153

154 In order to explore the role of the sulfone moiety, an original 2-phenylthiomethyl derivative **7** was

155 prepared from **1b** by a substitution reaction with sodium thiophenolate, formed *in situ* with NaH

156 in DMSO at RT (**Scheme 3**), according to a protocol that we previously reported [21].

157



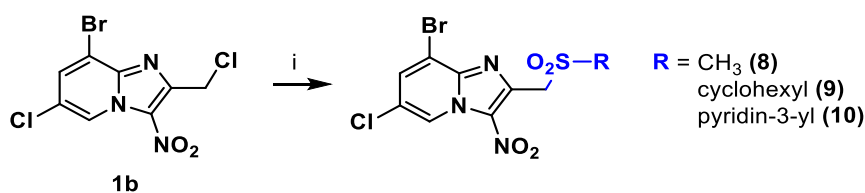
158

159 **Scheme 3.** Synthesis of compound **7**.

160 *Reagents and conditions:* (i) Thiophenol 1 equiv, NaH 1.1 equiv, DMSO, N₂, RT, 28%.

161

162 Then, the phenyl ring of the phenylsulfonylmethyl moiety was replaced by a methyl, cyclohexyl
 163 or pyridin-3-yl group, using a previously described reaction [15] and leading to original derivatives
 164 **8-10** respectively, according to **Scheme 4**.



165

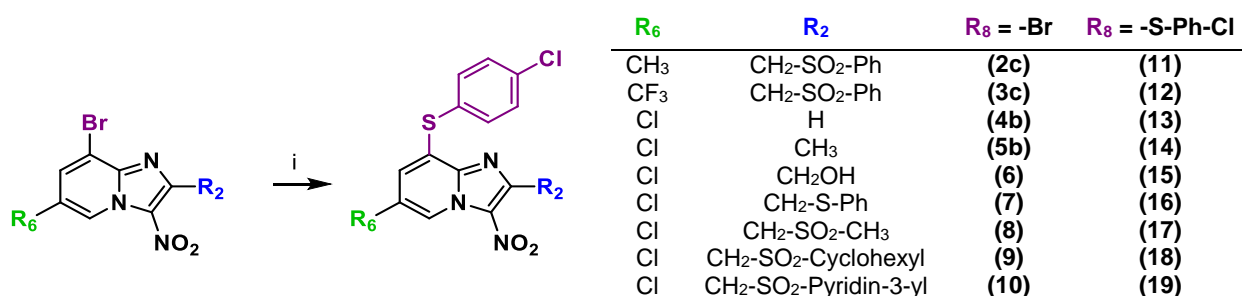
166 **Scheme 4.** Synthesis of compounds **8-10**.

167 *Reagents and conditions:* (i) Appropriate sulfonyl chloride 2 equiv, NaHCO₃ 2 equiv, Na₂SO₃ 2
 168 equiv, EtOH/H₂O (1:1), N₂, 120 °C, MW, 22% to 47%.

169

170 To complete SAR data and allow comparison with hit B, nine derivatives (**11-19**) bearing a 4-
 171 chlorophenylthio moiety at position 8 were synthesized, applying a protocol that we previously
 172 reported in various nitroaromatic series with antiparasitic potential [22,23] (**Scheme 5**).

173



174

175 **Scheme 5.** Synthesis of compounds **11-19**.

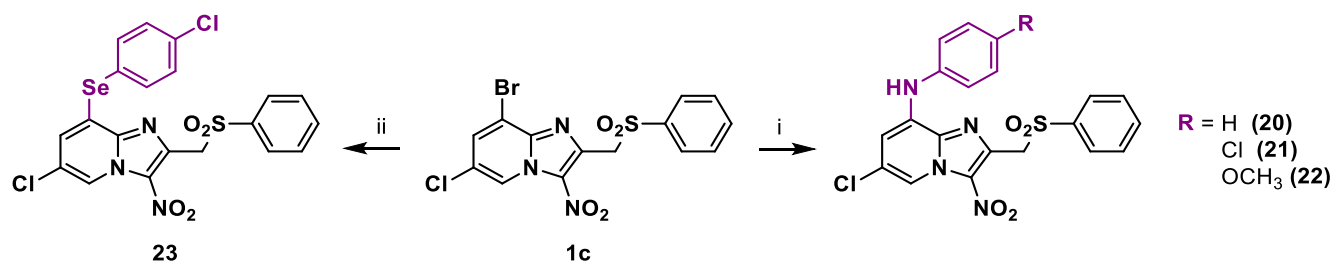
176 *Reagents and conditions:* (i) 4-Chlorothiophenol 1 equiv, NaH 1 equiv, DMSO, N₂, RT, 20% to 90%.

177

178 Still by analogy with hit B, the influence of the heteroatom at position 8 was investigated deeper.
 179 First, the synthesis of 8-anilino derivatives *via* a Buchwald-Hartwig cross-coupling reaction was
 180 investigated (**Scheme 6**). The reaction conditions were studied by varying several parameters such
 181 as nature of base, nature and amount of catalyst and ligand, nature of solvent, and reaction

182 temperature. Three derivatives bearing an aniline moiety at position 8 were synthesized (**Scheme**
183 **6**) in moderate yields (42% to 52%). Then, a selenium analogue (**23**) of hit B was also prepared
184 according to a previously described protocol [24], as presented in **Scheme 6**.

185



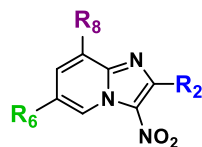
188 **Scheme 6.** Synthesis of 8-anilino derivatives **20-22** and 8-phenylselenanyl derivative **23**.

189 *Reagents and conditions:* (i) Appropriate aniline 1.2 equiv, Pd(OAc)₂ 0.08 equiv, *rac*-BINAP 0.15
190 equiv, K₂CO₃ 1.8 equiv, toluene, N₂, 140 °C, MW, 42% to 52 %; (ii) Bis(4-
191 chlorophenyl)diselenide 0.5 equiv, NaBH₄ 2 equiv, PEG-400, N₂, 60 °C, 9 %.

192

193 Globally, ten molecules (analogues of hit A) bearing a bromine atom at position 8 of the
194 imidazo[1,2-*a*]pyridine ring and 13 molecules (analogues of hit B) bearing a phenylthio,
195 phenylamino or phenylselenanyl group at the same position were evaluated *in vitro*. Their influence
196 on cell viability (cytotoxic concentration 50% = CC₅₀) was assessed on the HepG2 cell line, using
197 doxorubicin as a positive control. *In vitro* antileishmanial activity (measured through effective
198 concentration 50% = EC₅₀) was also measured on both the promastigote and axenic amastigote
199 form of *L. donovani* and *L. infantum*, respectively. In parallel, the *in vitro* antitrypanosomal
200 activity (EC₅₀) of these derivatives was determined on the trypomastigote blood stream form (BSF)
201 of *T. b. brucei*. For all molecules, selectivity indices (SI) were calculated. Their antikinoplastid
202 activity was compared to previous hit molecules and to commercially available reference drugs
203 amphotericin B, miltefosine, fexinidazole, suramine, eflornithine and nifurtimox. The results are
204 summarized in **Table 1**.

205 **Table 1.** *In vitro* evaluation of molecules **1c-23** on *L. donovani* promastigotes, *L. infantum* axenic
 206 amastigotes, *T. b. brucei* trypomastigotes (BSF) and on the human hepatocyte HepG2 cell line.



Compd	R ₂	R ₆	R ₈	Cell viability		Activity				
				CC ₅₀ HepG2 (μM)	EC ₅₀ <i>L. donovani</i> pro. (μM)	SI <i>L. donovani</i> pro.	EC ₅₀ <i>L. infantum</i> axenic ama. (μM)	SI <i>L. infantum</i> axenic ama.	EC ₅₀ <i>T. b. brucei</i> BSF (μM)	SI <i>T. b. brucei</i>
1c	-CH ₂ -SO ₂ -Ph	-Cl	-Br	>15.6 ^a	5.3 ± 0.3	>2.9	16.4 ± 0.2	>1	1.4 ± 0.4	>11.1
2c	-CH ₂ -SO ₂ -Ph	-CH ₃	-Br	>31.2 ^a	5.6 ± 1.4	>5.6	>3.1	-	1.2 ± 0.5	>26
3c	-CH ₂ -SO ₂ -Ph	-CF ₃	-Br	>15.6 ^a	6.2 ± 0.7	>2.5	>3.1	-	4.0 ± 2.7	>3.9
4b	-H	-Cl	-Br	16.2	9.3 ± 1.7	1.7	15.9 ± 0.7	1	0.4 ± 0.01	40.5
5b	-CH ₃	-Cl	-Br	>125 ^a	>12.5 ^c	-	32.1 ± 0.7	>3.9	>50 ^e	-
6	-CH ₂ -OH	-Cl	-Br	>125 ^a	>12.5 ^c	-	46.3 ± 1.4	>5.4	>50 ^e	-
7	-CH ₂ -S-Ph	-Cl	-Br	>31.2 ^a	>12.5 ^c	-	17.8 ± 0.8	>1.8	>50 ^e	-
8	-CH₂-SO₂-CH₃	-Cl	-Br	45	5.7 ± 1.4	7.9	>25^c	<1.8	0.017 ± 0.002	2647
9	-CH ₂ -SO ₂ -Cyclohexyl	-Cl	-Br	>15.6 ^a	0.9 ± 0.3	>17.3	>100 ^e	-	0.5 ± 0.07	>31.2
10	-CH ₂ -SO ₂ -Pyridin-3-yl	-Cl	-Br	>62.5 ^a	0.8 ± 0.3	>78	77.8 ± 0.8	>0.8	0.025 ± 0.005	2500
11	-CH ₂ -SO ₂ -Ph	-CH ₃	4-Cl-Ph-S-	>62.5 ^a	2.6 ± 0.4	>24	5.0 ± 0.3	12.5	1.8 ± 0.5	>34.7
12	-CH ₂ -SO ₂ -Ph	-CF ₃	4-Cl-Ph-S-	>7.8 ^a	1.9 ± 0.2	>4.1	-	-	0.17 ± 0.02	>45.9
13	-H	-Cl	4-Cl-Ph-S-	>7.8 ^a	2.5 ± 0.2	>3.1	-	-	0.085 ± 0.02	>92.8
14	-CH ₃	-Cl	4-Cl-Ph-S-	>7.8 ^a	>12.5 ^c	-	-	-	-	-
15	-CH ₂ -OH	-Cl	4-Cl-Ph-S-	>15.6 ^a	4.0 ± 0.3	>3.9	7.9 ± 0.3	>2	>50	-
16	-CH ₂ -S-Ph	-Cl	4-Cl-Ph-S-	>15.6 ^a	7.4 ± 0.4	>2.1	-	-	>50	-
17	-CH ₂ -SO ₂ -CH ₃	-Cl	4-Cl-Ph-S-	>31.2 ^a	1.2 ± 0.1	>26	10.3 ± 0.4	>3	0.067 ± 0.007	>465.7
18	-CH ₂ -SO ₂ -Cyclohexyl	-Cl	4-Cl-Ph-S-	>31.2 ^a	1.9 ± 0.1	>16.4	1.6 ± 0.2	>19.5	1.5 ± 0.1	>20.8
19	-CH ₂ -SO ₂ -Pyridin-3-yl	-Cl	4-Cl-Ph-S-	>31.2 ^a	0.5 ± 0.1	>62.4	0.8 ± 0.2	>39	1.2 ± 0.2	>26
20	-CH ₂ -SO ₂ -Ph	-Cl	Ph-NH-	>3.9 ^a	-	-	-	-	-	-
21	-CH ₂ -SO ₂ -Ph	-Cl	4-Cl-Ph-NH-	>3.9 ^a	-	-	-	-	-	-
22	-CH ₂ -SO ₂ -Ph	-Cl	4-CH ₃ O-Ph-NH-	>3.9 ^a	-	-	-	-	-	-
23	-CH ₂ -SO ₂ -Ph	-Cl	4-Cl-Ph-Se-	>7.8 ^a	0.7 ± 0.1	>11.1	0.9 ± 0.1	>8.7	0.3 ± 0.03	>26
Ref. 1	Hit A molecule			>31 ^a	1.8 ± 0.8	>17.2	4.4 ± 0.8	>7.1	2.9 ± 0.5	>10.7
Ref. 2	Hit B molecule			>100	1.0 ± 0.3	>100	1.7 ± 0.3	>58.8	1.3 ± 0.1	>76.9
Ref. 3	Doxorubicin ^b			0.2 ± 0.02	-	-	-	-	-	-
Ref. 4	Amphotericin B ^d			8.8 ± 0.3	0.07 ± 0.01	125.7	0.06 ± 0.001	146.7	-	-
Ref. 5	Miltefosine ^d			85 ± 8.8	3.1 ± 0.2	27.4	0.8 ± 0.2	106.3	-	-
Ref. 6	Fexinidazole ^{d,e}			>200 ^c	1.2 ± 0.2	>166.7	3.4 ± 0.8	>58.8	0.6 ± 0.2	>333
Ref. 7	Suramin ^e			>100 ^c	-	-	-	-	0.03 ± 0.009	>3333
Ref. 8	Eflornithine ^e			>100 ^c	-	-	-	-	13.3 ± 2.1	>7.5
Ref. 9	Nifurtimox ^e			45.2 ± 1.3	-	-	-	-	2.6 ± 0.8	17.4

207 ^a The product could not be tested at higher concentrations due to a lack solubility in the culture medium

208 ^b Doxorubicin was used as a cytotoxic reference drug

209 ^c The EC₅₀ or CC₅₀ value was not reached at the highest tested concentration

210 ^d Amphotericin B, Miltefosine and Fexinidazole were used as antileishmanial reference drugs

211 ^e Fexinidazole, Suramin, Eflornithine and Nifurtimox were used as anti-*Trypanosoma brucei* reference drugs

212 ^f SI = CC₅₀ HepG2 / EC₅₀ *L. infantum*

213 ^g SI = CC₅₀ HepG2 / EC₅₀ *T. brucei brucei*

214 **Bold:** New antitrypanosomal hit

215 Out of the 23 tested molecules, 7 showed a poor aqueous solubility ($< 10 \mu\text{M}$) limiting their *in*
216 *vitro* evaluation. Among them, 8-anilino-derivatives **20-22** were particularly insoluble in
217 biological culture media, indicating that the aniline moiety should be avoided at position 8 of the
218 antitrypanosomal scaffold. Regarding the effect on the cell viability of the HepG2 human cells, all
219 molecules appeared as weakly cytotoxic ($15.6 \leq \text{CC}_{50} < 125 \mu\text{M}$) in comparison with doxorubicin
220 ($\text{CC}_{50} = 0.2 \mu\text{M}$).

221 Then, 20 molecules were screened *in vitro* for their activity against *L. donovani* promastigotes and
222 compared both to previous hit molecules and to amphotericin B, miltefosine and fexinidazole.
223 Among them, only compounds **9**, **10**, **17**, **18** and **19**, bearing an alkyl- or pyridin-3-yl-
224 sulfonylmethyl group at position 2, showed both good EC_{50} values ($0.5 \leq \text{EC}_{50} \leq 1.9 \mu\text{M}$) and
225 good selectivity indices ($16 < \text{SI} < 78$). This suggests that a sulfonyl group in position 2 is needed
226 to reach a good antileishmanial activity level on *L. donovani* promastigotes.

227 When considering the screening against *L. infantum* axenic amastigotes, out of the 5 molecules
228 that were active on *L. donovani* promastigotes, only compounds **18** and **19** remained active ($0.8 \leq$
229 $\text{EC}_{50} \leq 1.6 \mu\text{M}$) and selective ($19.5 < \text{SI} < 39$). These results confirmed that, in addition to a
230 sulfonylmethyl substituent at position 2 of the imidazopyridine ring, antileishmanial activity was
231 also favored when substituting position 8 by a thiophenol moiety. Unfortunately, compounds **18**
232 and **19** were then evaluated on the intramacrophage amastigote stage of *L. donovani* and were not
233 active ($\text{EC}_{50} > 10 \mu\text{M}$). Consequently, hit B still remains the most active antileishmanial molecule
234 in this chemical series.

235 Regarding antitrypanosomal activity, molecules were tested on the blood stream form of *T. b.*
236 *brucei* and compared to reference drugs fexinidazole, suramin, eflornithine and nifurtimox. Out of
237 the 20 tested molecules, 8 displayed submicromolar activities ($0.017 \leq \text{EC}_{50} \leq 0.5 \mu\text{M}$), quite
238 significantly better than the ones of hit A and B ($\text{EC}_{50} = 2.9$ and $1.3 \mu\text{M}$ respectively). Contrary to
239 what was noted for antileishmanial activity, these 8 antitrypanosomal molecules are equally
240 distributed between hit A (8-Br) and hit B (8-thiophenol) derivatives, showing that the

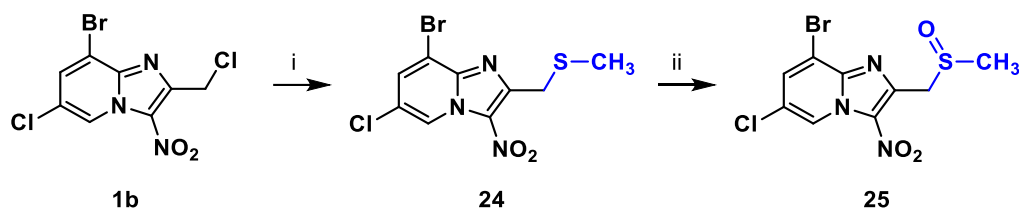
241 antitrypanosomal pharmacophore is less restrictive than the antileishmanial pharmacophore.
242 Among these submicromolar active molecules, **8**, **9**, **10**, **12**, **17** and **23** presented a sulfonylmethyl
243 moiety at position 2 whereas molecules **4b** and **13** were not substituted. Nevertheless, **4b** was the
244 most cytotoxic derivative in the series and **13** showed a poor aqueous solubility. Then, as with
245 antileishmanial activity, the sulfonylmethyl moiety seems to play a favorable role toward
246 antitrypanosomal activity. When looking at hit A derivatives, methyl-, cyclohexyl- and pyridin-3-
247 yl-sulfonylmethyl groups provided good to excellent activities (molecules **8-10**), showing that the
248 substituent of the sulfonylmethyl moiety is a key modulation zone. Thus, molecule **8** appeared as
249 the most active ($EC_{50} = 17$ nM) and selective ($SI = 2647$) antitrypanosomal molecule in the series.
250 It was a lot more active than hits A and B ($EC_{50} = 2.9$ and 1.3 μ M respectively) but also more
251 active than fexinidazole ($EC_{50} = 0.6$ μ M), suramin ($EC_{50} = 30$ nM), eflornithine ($EC_{50} = 13.3$ μ M)
252 and nifurtimox ($EC_{50} = 2.6$ μ M). The selenium containing analog **23** of hit B appeared a lot less
253 soluble in aqueous biological media than hit B despite being 4 times more active. Because of the
254 same lack of water solubility, the 6-trifluoromethylated analogue **12** of hit B appeared less
255 promising than other derivatives. Nevertheless, the comparison of the close activities of **11** and **12**
256 toward hit B but also **2c** and **3c** toward **1c** and hit A, permitted to conclude that the
257 antitrypanosomal pharmacophore allows small electron-donating or electron-withdrawing
258 substituents such as CH_3 , Cl, Br or CF_3 at position 6.

259 From antitrypanosomal hit-molecule **8**, to deeper evaluate the influence of the sulfonylmethyl
260 moiety, a 2-methylthiomethyl analog (**24**) was synthesized from substrate **1b**, using sodium
261 methanethiolate as reagent (Table 2). Partial oxidation of the sulfur atom of **24** using *m*-CPBA in
262 dichloromethane led to the sulfoxide derivative **25** (Table 2). The activities of **24** and **25** were
263 evaluated *in vitro* for their activity against *T. b. brucei* and for their effect on cell viability on the
264 HepG 2 cell line (Table 2). Thioether derivative **24** showed limited water solubility and modest
265 antitrypanosomal activity ($EC_{50} = 2.2$ μ M) whereas sulfoxide derivative **25** was very active against
266 *T. b. brucei* ($EC_{50} = 20$ nM) and displayed excellent selectivity index ($SI = 2570$). These results
267 confirmed that position 2 was key to the antiparasitic activity and indicated that the

268 antitrypanosomal pharmacophore included either a sulfone or a sulfoxide functional group at
269 position 2 of the imidazopyridine ring.

270

271 **Table 2.** Synthesis and biological activities of compounds **24** and **25**.



Compound	<i>T. brucei brucei</i> BSF EC ₅₀ (μM)	HepG2 CC ₅₀ (μM)	SI <i>T. b. brucei</i>
24	2.20 ± 0.24	>15.6 ^a	>7.1
25	0.020 ± 0.006	51.4 ± 8.3	2570

^aThe product could not be tested at higher concentrations due to a poor solubility in aqueous medium.

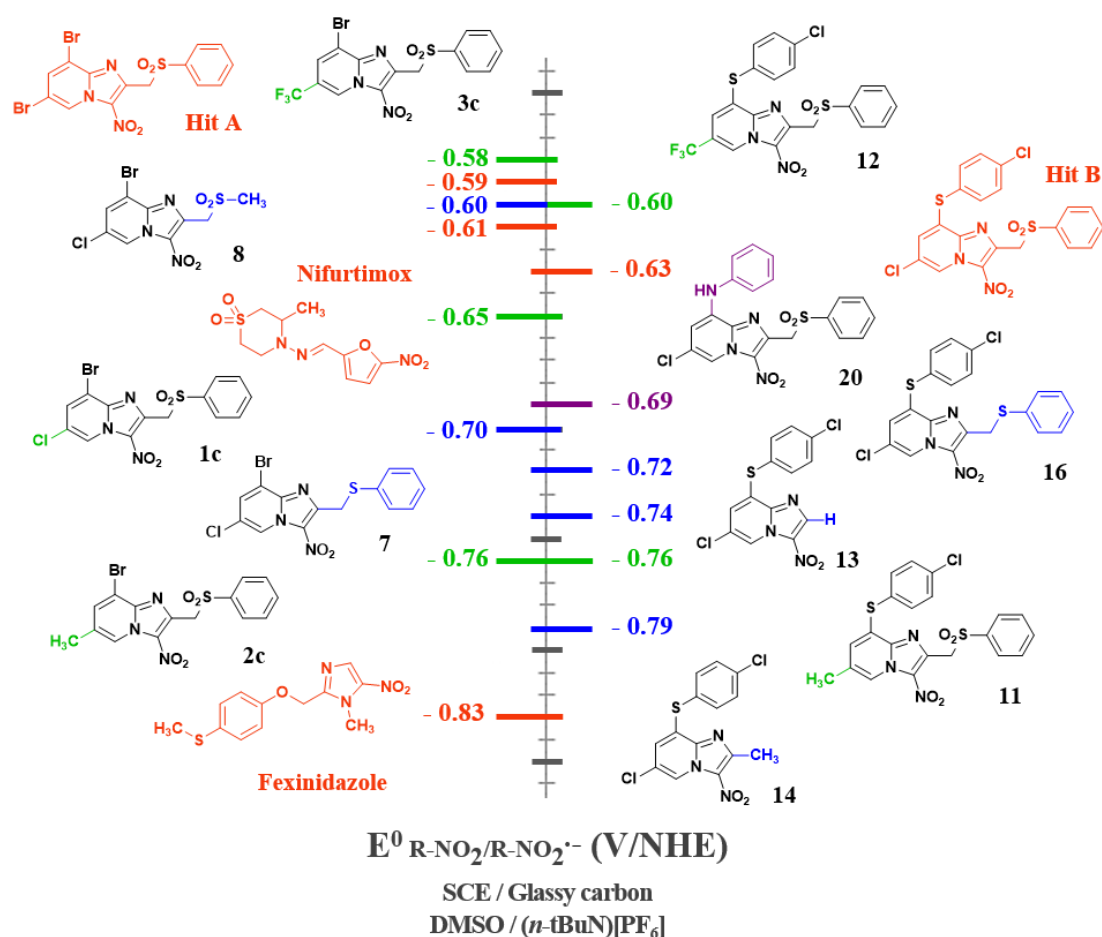
Reagents and conditions: (i) CH₃SNa 1 equiv, DMSO, RT, 52% (ii) *m*CPBA 1 equiv, CH₂Cl₂, 0 °C, 36%.

272

273 To assess the influence of the aforementioned modulations at position 2, 6 and 8 on the reduction
274 potential of this nitroaromatic scaffold, an electrochemical study was conducted on several 3-
275 nitroimidazo[1,2-*a*]pyridine derivatives using cyclic voltammetry. Experimental reduction
276 potentials (**Figure 3**) were compared to the ones of fexinidazole, nifurtimox, and hit A (for 8-Br-
277 derivatives) or hit B (for 8-phenylthio-derivatives). For all tested compounds, a reversible single
278 electron reduction was observed and attested the formation of a nitro radical anion. The redox
279 potentials were measured and the values were corrected versus Normal Hydrogen Electrode
280 (NHE). These values ranged from -0.58 V to -0.79 V, in comparison with the ones of nifurtimox
281 ($E^0 = -0.61$ V) and fexinidazole ($E^0 = -0.83$ V). There was almost no difference in redox potential
282 values when comparing hit A with hit B analogues, bearing the same substituents at positions 2
283 and 6: the electronic effect of the bromine atom or of the *p*-chlorothiophenol group at position 8
284 were almost the same. Introducing an aniline moiety at position 8 of the imidazopyridine ring

285 (molecule **20**, $E^0 = -0.69$ V) had the same effect toward the redox potential value as a phenyl
286 moiety [17] but allowed to reach a lower E^0 value than with a *p*-chlorothiophenol moiety (hit B,
287 $E^0 = -0.63$ V). Modifying the substituent at position 6 of the imidazopyridine ring had a strong
288 influence toward the reducibility of the nitro group: 6-trifluoromethyl-derivatives (**3c** and **12**)
289 displayed high E^0 values (-0.60 to -0.58 V) whereas 6-methyl-derivatives (**2c** and **11**) presented
290 the lowest E^0 values (-0.76 V) among the derivatives presenting a phenylsulfonylmethyl side chain
291 at position 2. There was also an important influence of the substituent at position 2 toward E^0
292 values. Switching from phenylsulfonylmethyl derivatives **1c** or **hit B** (E^0 values = -0.65 V and -
293 0.63 V) to their phenylthiomethyl analogues **7** or **16** provided molecules with lower E^0 values (-
294 0.70 and -0.72 V respectively). This effect that was rather comparable to the one noted with the
295 hydrogen atom when comparing **16** to **13** ($E^0 = -0.74$ V). This decrease in E^0 value was even more
296 acute when introducing a methyl group at position 2 (molecule **14**, $E^0 = -0.79$ V). Finally, by
297 comparing the E^0 values of compounds **1c** and **8**, it appeared that replacing the
298 phenylsulfonylmethyl group by a methylsulfonylmethyl one was responsible for a significant
299 increase in the redox potential values from -0.65 to -0.60 V. Nevertheless, no correlation between
300 redox potential values and antiparasitic EC_{50} values could be noted in the studied series.

301

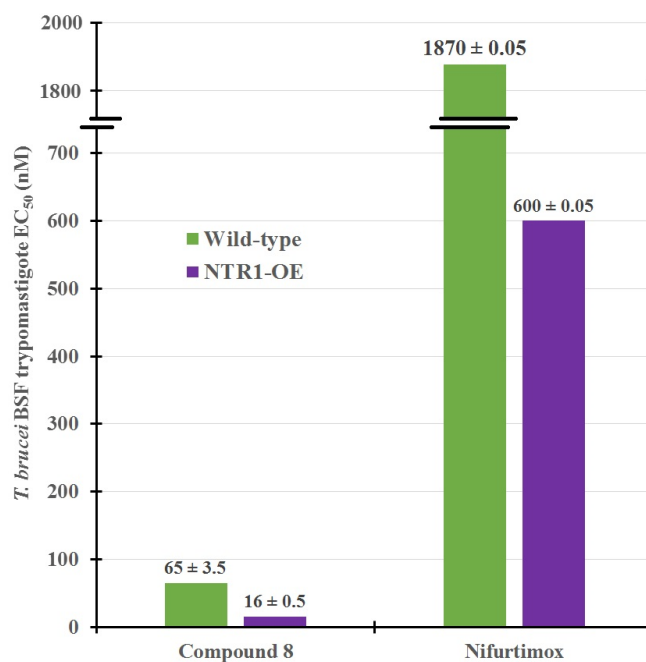


302

303 **Figure 3.** Redox potentials (E^0) of Hit A (left) and Hit B (right) derivatives determined by cyclic
 304 voltammetry and given versus NHE. Conditions: selected compounds (10^{-3} mol.L $^{-1}$) in non-
 305 aqueous medium (DMSO + 0.1 mol.L $^{-1}$ (*n*-Bu₄N)[PF₆]) on GC microdisk ($r = 0.5$ mm) at room
 306 temperature. Scan rate: 0.2 V.s $^{-1}$.

307

308 These molecules being probable substrates of parasitic nitroreductases, the mechanism of
 309 bioactivation of molecule **8** in the *Trypanosoma* cell was investigated by comparing the EC₅₀
 310 values measured on a wild-type strain with the one measured on a NTR-overexpressing strain.
 311 This assay confirmed that, like for nifurtimox and for all previous hit molecules studied in 3-
 312 nitroimidazopyridine series, molecule **8** was bioactivated by *T. b. brucei* type 1 NTR as the EC₅₀
 313 value of **8** on the NTR-overexpressing strain was 4 times lower than on the wild-type strain.
 314 (**Figure 4**). Noticeably, compound **8** was almost thirty times more active on *T. b. brucei* wild-type
 315 strain than nifurtimox.



316

317 **Figure 4.** Sensitivity of wild-type and NTR-overexpressing *T. brucei* BSF trypanomastigotes strains
 318 toward hit molecule **8** and nifurtimox control.

319

320 The 3-amino-analogue **26** of hit compound **8** was also synthesized and evaluated *in vitro* to confirm
 321 the essentiality of the nitro group for antikinoplastid activity. Molecule **26** was prepared by
 322 reduction of **8** in the presence of iron powder in refluxing acetic acid (**Table 3**). As expected, **26**
 323 did not show any antileishmanial activity and displayed a poor antitrypanosomal activity (EC₅₀ =
 324 7.2 μM). These results are consistent with the hypothesis that these 3-nitroimidazo[1,2-*a*]pyridines
 325 require bioactivation by parasitic NTRs, making the nitro group an essential element of the
 326 antikinoplastid pharmacophore.

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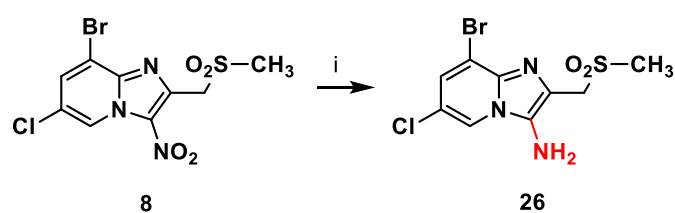
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Compound	<i>L. donovani</i> promastigotes EC ₅₀ (μM)	<i>L. infantum</i> axenic amastigotes EC ₅₀ (μM)	<i>T. brucei brucei</i> BSF EC ₅₀ (μM)	HepG2 CC ₅₀ (μM)
26	>12.5 ^a	>100 ^a	7.2 ± 0.8	>125 ^b

^a The EC₅₀ or CC₅₀ value was not reached at the highest tested concentration.

^b The product could not be tested at higher concentrations due to a poor solubility in aqueous medium.

Reagents and conditions: (i) Fe⁰ powder 10 equiv, AcOH, reflux, 30 min, 40 %.

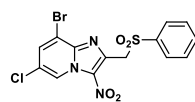
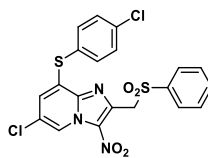
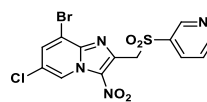
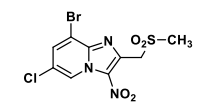
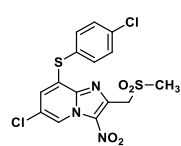
334

335 The poor microsomal stability of hit molecules A and B was a limiting PK parameter for further
 336 development of the series (hit A T_{1/2} = 9 min and hit B T_{1/2} = 3 min; T_{1/2} of propranolol control =
 337 26 min), although some metabolites of hit B remained active *in vitro* against *Leishmania* spp [16].
 338 For that reason, more polar, ionizable or less lipophilic pyridin-3-yl- or methyl-sulfonylmethyl-
 339 derivatives **8**, **10** and **17** of hit B and **1c** were evaluated *in vitro* for their microsomal stability
 340 (**Table 4**). As expected, microsomal stability was greatly improved when changing the phenyl ring
 341 (**1c**, T_{1/2} = 12 min) with a pyridine one (**10**, T_{1/2} = 27 min) and even better with a methyl group (**8**,
 342 T_{1/2} > 40 min). Effectively, molecules **10** and **8** were less lipophilic and more water-soluble than
 343 compound **1c**, but some microsomal *N*-oxidation can possibly be anticipated on the pyridine
 344 moiety of **10**. Even if real, this improvement in microsomal stability was less concluding
 345 comparing hit B to **17**, most probably because the sulfur atom of the thiophenol moiety remains
 346 actively oxidized by microsomes in **17**. Thus, positions 2 and 6 of the imidazopyridine ring
 347 appeared as essential modulation sites for increasing microsomal stability. Besides, the binding of
 348 molecule **8** to human albumin was logically much lower than all other derivatives as the two
 349 aromatic rings that substitute the imidazopyridine ring in hit B (thiophenol and
 350 phenylsulfonylmethyl moieties) were avoided, leading to a decarbonated scaffold with lower

351 molecular weight. Reducing the binding to human albumin could be an important parameter to
352 control so as to optimize future *in vivo* activity that can be restricted by insufficient distribution.

353

354 **Table 4.** *In vitro* pharmacokinetic data of selected key compounds.

					
	1c	Hit B	10	8	17
Binding to human albumin (%)	96.5	99.9	87.0	76.9	98.9
Microsomal stability: T _{1/2} (min)	12	3	27	>40	12

355

356

357 Some important physicochemical parameters of hit molecule **8** were also calculated or measured
358 (**Table 5**): molecule **8** showed average lipophilicity (cLogP = 1.14) and was 20 times more soluble
359 in water than hit B (thermodynamic solubility of **8** = 26 μ M versus 1.4 μ M for hit B) [16].

360 The mutagenicity of many nitroheterocyclic molecules towards bacteria or parasites has been
361 known for many years [25], the reduction of the nitro group being at the origin of this property.
362 For fexinidazole, nitroreductase-dependent mutagenic activity was observed in the Ames test.
363 However, its genotoxicity evaluation in the micronucleus assay was negative for the native
364 molecule and its two metabolites [26]. This result suggests that the Ames test, the international
365 reference test for measuring the mutagenicity of a molecule *in vitro* [27], is not suitable for the
366 evaluation of nitroheterocyclic molecules. Indeed, since they undergo metabolic activation by
367 *Salmonella* type 1 NTRs during the test, it appears difficult to extrapolate their genotoxicity profile
368 to humans. Thus, to overcome the presence of bacterial nitroreductases expressed by the
369 *Salmonella* strains used in the Ames test, a study of the genotoxicity of compound **8** was carried
370 out using two complementary assays. First, a micronucleus test was conducted on the CHO-K1

371 cell line, both with and without addition of the metabolizing S9 mixture (see supplementary
372 material). Molecule **8** showed no significant increase in micronucleated cell rates for all the tested
373 concentrations (0.01 mM to 0.5 mM), either in the absence or presence of S9 mix, indicating that
374 molecule **8** does not exert cytogenetic effects in CHO cells in culture and does not produce
375 metabolites with cytogenetic effects. Then a comet assay was carried out on the HepG2 cell line
376 at 5, 10 and 20 μ M, exposing cells with compound **8** for 2, 24 or 72 h and using methyl
377 methanesulfonate as a positive control (supplementary material): the values of % DNA in tail were
378 similar to the ones obtained in control cells. The negative results of these 2 assays are consistent
379 with those obtained with previously studied hit molecules in the series and also with those of
380 fexinidazole that is also devoid of mutagenic properties.

381 Finally, an *in vivo* toxicity study of molecule **8** was carried out to determine its maximal tolerated
382 dose. A group of 4 mice received a once daily repeated oral (intra-gastrical gavage) dose of
383 molecule **8** at 100 mg/kg for 5 days, and a follow-up was performed. As no side effect was
384 detected, the No Observed Adverse Event Level (NOAEL) in mice was set at 100 mg/kg/day. A
385 post-mortem examination revealed no lesion on different organs (brain, lung, heart, liver and
386 kidney). Thereafter, a pharmacokinetic analysis was performed to identify the behavior of
387 molecule **8** after oral administration. The main pharmacokinetic parameters are presented in **Table**
388 **5**. Compound **8** was orally absorbed and showed good systemic exposure. It displayed a long half-
389 time (7.7 h) and, with a T_{max} of 5 h, a single daily administration could be considered for further
390 experiments. A PAMPA BBB assay finally showed the possibility for molecule **8** to cross the
391 blood-brain barrier by a moderate passive diffusional way, in accordance with its CNS MPO score
392 (5.8) [28].

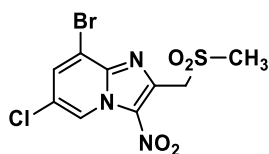
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396

397 **Table 5.** Physicochemical, pharmacokinetic and genotoxic data regarding hit compound **8**.



cLogP ^a	1.14
Thermodynamic solubility (μM)	25.8 ± 0.6
PAMPA blood-brain barrier permeability assay : P _e (nm/s)	84.2 ± 2.9
CNS MPO score	5.8
C _{max} in mouse (ng/mL)	741 ± 580
T _{max} in mouse (h)	5.0 ± 5.2
Plasma half-life in mouse (h)	7.7 ± 1.3
AUC _{0-inf} (ng.h/mL)	7060 ± 693
Clearance (mL/h)	0.51 ± 0.05
Micronucleus assay (@ 10, 50, 100 and 500 μM +/- S9mix)	Negative
Comet assay (@ 5, 10 and 20 μM for 2, 24 and 72 h)	Negative

^a Weighted clogP was computed by Marvin[®] (ChemAxon)

398

399

400 **3. Conclusion**

401 This antikinoplastid SAR study was conducted through the synthesis and evaluation of 26
402 original imidazo[1,2-*a*]pyridine derivatives. Although most of these molecules displayed modest
403 to good *in vitro* antileishmanial activity on the promastigote form of *L. donovani*, they were
404 ineffective on the axenic amastigote form of *L. infantum* and/or the intramacrophage amastigote
405 form of *L. donovani*. However, nine of these compounds showed submicromolar EC₅₀ values on
406 *T. b. brucei* bloodstream form and low cytotoxicities on the HepG2 cell line, including 3 molecules
407 with a selectivity index ≥2500. In this series, positions 2 and 6 appeared as key positions for
408 modulating the reduction potential of these nitroheterocycles while maintaining their
409 antitrypanosomal activity. Position 2 also allowed to improve mouse liver microsomal stability, as
410 illustrated by the best molecule in this series (**8**) bearing a methylsulfonylmethyl group instead of
411 the phenylsulfonylmethyl group found in previous hit molecules A and B. Indeed, in addition to
412 its very good *in vitro* activity on *T. b. brucei* BSF (better than the ones of all reference compounds),
413 its *in vitro* microsomal stability was >40 min and therefore greatly increased in comparison with

414 previous imidazopyridine hit molecules that we reported. Compound **8** also displayed increased
415 water solubility, appeared BBB-permeable in a PAMPA assay and was shown to be bioactivated
416 by type 1 parasitic NTR. Moreover, this derivative was not genotoxic, neither the micronucleus
417 assay nor in the comet assay, which could represent a significant improvement over nitroaromatic
418 antitypanosomal drugs like nifurtimox. In the mouse, molecule **8** was orally absorbed and well
419 tolerated after repeated administrations of 100 mg/kg for 5 days. Its long plasma half-life (7.7 h)
420 is encouraging and opens the way for a hit to lead program searching for novel antikinoplastid
421 drug candidates.

422

423 **4. Experimental section**

424 **4.1. Organic synthesis and characterizations**

425 Commercial reagents were used as received without additional purification. Melting points were
426 determined in open capillary tubes with a Büchi apparatus and are uncorrected. Elemental analysis
427 and HRMS were carried out at the Spectropole, Faculté des Sciences et Techniques de Saint-
428 Jérôme, Marseille, France. NMR spectra were recorded on a Bruker ARX 200 spectrometer or a
429 Bruker AV 250 spectrometer at the Faculté de Pharmacie de Marseille, or a BRUKER Avance III
430 nanobay 400 spectrometer at the the Spectropole, Faculté des Sciences et Techniques de Saint-
431 Jérôme, Marseille, or on a Bruker UltraShield 300 MHz or a Bruker IconNMR 400 MHz
432 spectrometer at the Laboratoire de Chimie de Coordination, Toulouse (^1H -NMR: 200, 250, 300 or
433 400 MHz, ^{13}C -NMR: 50, 63, 75 or 100 MHz). NMR references were the following: ^1H : CHCl_3 δ
434 = 7.26, $\text{DMSO-}d_6$ δ = 2.50 and ^{13}C : CHCl_3 δ = 76.9, $\text{DMSO-}d_6$ δ = 39.5. Solvents were dried by
435 conventional methods. The following adsorbent was used for column chromatography: silica gel
436 60 (Merck, particle size 0.063–0.200 mm, 70–230 mesh ASTM). TLC was performed on 5 cm \times
437 10 cm aluminum plates coated with silica gel 60F-254 (Merck) in an appropriate eluent.
438 Visualization was made with ultraviolet light (254 nm). HRMS spectra were recorded on QStar

439 Elite (Applied Biosystems SCIEX) spectrometer. PEG was the matrix for HRMS. The
440 experimental exact mass was given for the ion which has the maximum isotopic abundance. Purity
441 of synthesized compounds was checked with LC-MS analyses which were realized at the Faculté
442 de Pharmacie de Marseille with a Thermo Scientific Accela High Speed LC System[®] coupled with
443 a single quadrupole mass spectrometer Thermo MSQ Plus[®]. The RP-HPLC column used is a
444 Thermo Hypersil Gold[®] 50 × 2.1 mm (C18 bounded), with particles of 1.9 μm diameter. The
445 volume of sample injected on the column was 1 μL. The chromatographic analysis, total duration
446 of 8 min, is made with the gradient of following solvents: t = 0 min, water/methanol 50/50; 0 < t
447 < 4 min, linear increase in the proportion of methanol to a ratio water/methanol 5/95; 4 < t < 6
448 min, water/methanol 5/95; 6 < t < 7 min, linear decrease in the proportion of methanol to return to
449 a ratio 50/50 water/methanol; 6 < t < 7 min, water/methanol 50/50. The water used was buffered
450 with 5 mM ammonium acetate. The retention times (*t_R*) of the molecules analyzed are indicated in
451 min.

452 Compounds **1a**, **1b** and **1c** were prepared as previously described [17].

453 **4.1.1. 8-Bromo-2-chloromethyl-6-methylimidazo[1,2-*a*]pyridine (2a)**

454 To a solution of 3-bromo-5-methylpyridin-2-amine (2 g, 10.1 mmol, 1 equiv.) in ethanol (100 mL),
455 1,3-dichloroacetone (1.46 g, 11.8 mmol, 1.1 equiv.) was added. The reaction mixture was stirred
456 and heated under reflux for 72 h. The solvent was then evaporated *in vacuo*. Compound **2a** was
457 obtained, after purification by chromatography on silica gel (eluent: cyclohexane–ethyl acetate
458 6.5:3.5) and recrystallization from propan-2-ol as a beige solid in 31% yield (0.82 g). mp: 151 °C.
459 ¹H NMR (300 MHz, CDCl₃) δ: 2.31 (3H, s), 4.79 (2H, s), 7.33 (1H, d, *J* = 1.4 Hz), 7.63 (1H, s),
460 7.84 (1H, t, *J* = 1.2 Hz). ¹³C NMR (100 MHz, CDCl₃) δ: 18.0 (CH₃), 39.7 (CH₂), 111.0 (C), 112.53
461 (CH), 122.9 (C), 123.0 (CH), 130.7 (CH), 142.4 (C), 143.9 (C). LC/MS ESI⁺ *t_R* 1.77 min, (m/z)
462 [M+H]⁺ 259.08/261.09/263.12. HRMS (+ESI): 260.9609 (M + H⁺). Calcd for C₉H₈BrClN₂:
463 260.9610.

464 **4.1.2. 8-Bromo-2-chloromethyl-6-methyl-3-nitroimidazo[1,2-*a*]pyridine (2b)**

465 To a solution of 8-bromo-2-chloromethyl-6-methylimidazo[1,2-*a*]pyridine **2a** (0.8 g, 3.09 mmol,
466 1 equiv.) in concentrated sulfuric acid (8 mL) cooled by an ice-water bath, nitric acid 65% (850
467 μL , 18.5 mmol, 6 equiv.) was added. The reaction mixture was stirred for 1 h at room temperature.
468 Then, the mixture was slowly poured into an ice-water mixture and the desired product
469 precipitated. The beige solid was collected by filtration, dried under reduced pressure and
470 recrystallized from propan-2-ol to give the expected product **2b** in 85% yield (0.8 g). mp 179 °C.
471 ^1H NMR (400 MHz, CDCl_3) δ : 2.51 (3H, d, $J = 0.8$ Hz), 5.10 (2H, s), 7.79 (1H, d, $J = 1.1$ Hz),
472 9.24–9.25 (1H, m). ^{13}C NMR (100 MHz, $\text{DMSO-}d_6$) δ : 17.6 (CH_3), 31.1 (CH_2), 110.8 (C), 125.4
473 (CH), 126.1 (C), 128.0 (C), 136.5 (CH), 140.8 (C), 146.5 (C). LC/MS ESI⁺ t_{R} 2.04 min, (m/z)
474 $[\text{M}+\text{H}]^+$ 304.44/305.80/307.06. HRMS (+ESI): 305.9461 (M + H⁺). Calcd for $\text{C}_9\text{H}_7\text{BrClN}_3\text{O}_2$:
475 305.9461.

476 **4.1.3. 8-Bromo-6-methyl-3-nitro-2-(phenylsulfonylmethyl)imidazo[1,2-*a*]pyridine**
477 **(2c)**

478 To a solution of 8-bromo-2-chloromethyl-6-methyl-3-nitroimidazo[1,2-*a*]pyridine **2b** (0.5 g, 1.64
479 mmol, 1 equiv.) in dimethylsulfoxide (30 mL), sodium benzenesulfinate (0.81 g, 4.93 mmol, 3
480 equiv.) was added. The reaction mixture was stirred for 3 h at room temperature. The reaction
481 mixture was then slowly poured into an ice-water mixture, making the desired product precipitate.
482 The pale yellow solid was collected by filtration, dried under reduced pressure and recrystallized
483 from acetonitrile to give the expected product **2c** in 89% yield (0.6 g). mp 202 °C. ^1H NMR (200
484 MHz, CDCl_3) δ : 2.50 (3H, d, $J = 0.8$ Hz), 5.17 (2H, s), 7.52–7.57 (2H; m), 7.67 (1H, tt, $J = 1.3$
485 and 7.4 Hz), 7.76 (1H, d, $J = 1.5$ Hz), 7.86–7.89 (2H, m), 9.19–9.20 (1H, m). ^{13}C NMR (50 MHz,
486 CDCl_3) δ : 18.6 (CH_3), 57.0 (CH_2), 112.4 (C), 124.9 (CH), 128.0 (C), 128.7 (2CH), 129.3 (2CH),
487 131.7 (C), 134.3 (CH), 136.1 (CH), 139.3 (C), 139.4 (C), 141.7 (C). LC/MS ESI⁺ t_{R} 1.81 min,
488 (m/z) $[\text{M}+\text{H}]^+$ 409.90/411.89/412.98. HRMS (+ESI): 411.9750 (M + H⁺). Calcd for
489 $\text{C}_{15}\text{H}_{12}\text{BrN}_3\text{O}_4\text{S}$: 411.9785.

490 **4.1.4. 8-Bromo-2-chloromethyl-6-(trifluoromethyl)imidazo[1,2-*a*]pyridine (3a)**

491 To a solution of 3-bromo-5-trifluoromethylpyridin-2-amine (1 g, 4.15 mmol, 1 equiv.) in 1,2-
492 dimethoxyethane (60 mL), 1,3-dichloroacetone (0.58 g, 4.56 mmol, 1.1 equiv.) was added. The
493 reaction mixture was stirred and heated under reflux for 72 h. The solvent was then evaporated *in*
494 *vacuo* and the product **3a** was directly involved in the next synthesis step, without being isolated.
495 ¹H NMR (200 MHz, CDCl₃) δ: 5.55 (2H, s), 7.62–7.71 (1H, m), 7.84–7.95 (1H, m), 8.22–8.32
496 (1H, m). LC/MS ESI⁺ t_R 2.49 min, (m/z) [M+H]⁺ 312.9/314.99/316.93.

497 **4.1.5. 8-Bromo-2-chloromethyl-3-nitro-6-(trifluoromethyl)imidazo[1,2-*a*]pyridine**
498 **(3b)**

499 To a solution of 8-bromo-2-chloromethyl-6-(trifluoromethyl)imidazo[1,2-*a*]pyridine **3a** (1 g, 3.19
500 mmol, 1 equiv.) in concentrated sulfuric acid (12 mL) cooled by an ice-water bath, nitric acid 65%
501 (1 mL, 19.14 mmol, 6 equiv.) was added. The reaction mixture was stirred for 1 h at room
502 temperature. Then, the mixture was slowly poured into an ice-water mixture with sodium
503 carbonate and extracted three times with dichloromethane. The organic layer was washed three
504 times with brine, dried over MgSO₄, filtered and evaporated. The crude residue was purified by
505 column chromatography on silica gel (eluent: dichloromethane) and compound **3b** was isolated as
506 a white solid in 51% yield (0.58 g). mp 143 °C. ¹H NMR (200 MHz, CDCl₃) δ: 5.11 (2H, s), 8.06
507 (1H, d, *J* = 1.5 Hz), 9.75–9.77 (1H, m). ¹³C NMR (50 MHz, CDCl₃) δ: 38.1 (CH₂), 114.3 (C),
508 121.6 (C, q, *J* = 35.5 Hz), 122.1 (C, q, *J* = 272.9 Hz), 125.6 (CH, q, *J* = 5.5 Hz), 129.1 (CH, d, *J* =
509 2.6 Hz), 130.5 (C), 142.5 (C), 148.8 (C). LC/MS ESI⁺ t_R 2.83 min, (m/z) [M+H]⁺ 357.97/358.95.
510 HRMS (+ESI): 357.9202 (M + H⁺). Calcd for C₉H₄BrClF₃N₃O₂: 357.9200.

511 **4.1.6. 8-Bromo-3-nitro-2-(phenylsulfonylmethyl)-6-(trifluoromethyl)imidazo[1,2-*a*]**
512 **pyridine (3c)**

513 To a solution of 8-bromo-2-chloromethyl-3-nitro-6-(trifluoromethyl)imidazo[1,2-*a*]pyridine **3b**
514 (0.5 g, 1.4 mmol, 1 equiv.) in dimethylsulfoxide (40 mL), sodium benzenesulfinate (0.69 g, 4.18

515 mmol, 3 equiv.) was added. The reaction mixture was stirred for 2 h at room temperature. Then,
516 the mixture was slowly poured into an ice-water mixture and extracted three times with
517 dichloromethane. The organic layer was washed five times with brine, dried over MgSO₄, filtered
518 and evaporated. The crude residue was recrystallized from acetonitrile to obtain the expected
519 product **3c** as a white solid in 57% yield (0.37 g). mp 210 °C. ¹H NMR (200 MHz, CDCl₃) δ: 5.20
520 (2H, s), 7.55–7.62 (2H, m), 7.68–7.72 (1H, m), 7.88–7.93 (2H, m), 8.05–8.06 (1H, m), 9.74–9.75
521 (1H, m). ¹³C NMR (50 MHz, CDCl₃) δ: 56.6 (CH₂), 114.1 (C), 121.5 (C, q, *J* = 35.5 Hz), 122.1
522 (C, q, *J* = 273.4 Hz), 125.3 (CH, q, *J* = 5.4 Hz), 128.5 (2CH), 129.1 (CH, d, *J* = 2.5 Hz), 129.3
523 (2CH), 132.5 (C), 134.4 (CH), 139.0 (C), 140.6 (C), 142.4 (C). LC/MS ESI⁺ t_R 2.76 min, (m/z)
524 [M+H]⁺ 463.74/465.78/468.23. HRMS (+ESI): 465.9499 (M + H⁺). Calcd for C₁₅H₉BrF₃N₃O₄S:
525 465.9502.

526 **4.1.7. 8-Bromo-6-chloroimidazo[1,2-*a*]pyridine (4a)**

527 To a solution of 3-bromo-5-chloropyridin-2-amine (10 g, 48.2 mmol, 1 equiv.) in ethanol (150
528 mL), chloroacetaldehyde (6.69 g, 72.3 mmol, 1.5 equiv.) and sodium bicarbonate (6.86 g, 81.6
529 mmol, 2 equiv.) were added. The reaction mixture was stirred and heated under reflux for 96 h.
530 Then, the mixture was slowly poured into an ice-water mixture and extracted three times with
531 dichloromethane. The organic layer was washed three times with brine, dried over MgSO₄, filtered
532 and evaporated. Compound **4a** was obtained, after purification by chromatography on silica gel
533 (eluent: dichloromethane) and recrystallization from propan-2-ol as a pale beige solid in 31% yield
534 (3.48 g). mp: 131 °C. ¹H NMR (300 MHz, DMSO-*d*₆) δ: 7.68 (1H, d, *J* = 1.2 Hz), 7.74 (1H, d, *J*
535 = 1.8 Hz), 8.05 (1H, d, *J* = 1.2 Hz), 8.90 (1H, d, *J* = 1.8 Hz). ¹³C NMR (75 MHz, DMSO-*d*₆) δ:
536 110.9 (C), 111.8 (C), 116.0 (CH), 118.3 (C), 124.8 (CH), 127.3 (CH), 134.4 (CH). LC/MS ESI⁺
537 t_R 1.21 min, (m/z) [M+H]⁺ 231.09/233.09/235.10. HRMS (+ESI): 232.9300 (M + H⁺). Calcd for
538 C₇H₄BrClN₂: 232.9297.

539

540 **4.1.8. 8-Bromo-6-chloro-3-nitroimidazo[1,2-*a*]pyridine (4b)**

541 To a solution of 8-bromo-6-chloroimidazo[1,2-*a*]pyridine **4a** (0.5 g, 2.16 mmol, 1 equiv.) in
542 concentrated sulfuric acid (5 mL) cooled by an ice-water bath, nitric acid 65% (500 μ L, 13 mmol,
543 6 equiv.) was added. The reaction mixture was stirred for 1 h at room temperature. Then, the
544 mixture was slowly poured into an ice-water mixture with potassium carbonate and extracted three
545 times with dichloromethane. The organic layer was washed three times with brine, dried over
546 MgSO₄, filtered and evaporated. Compound **4b** was obtained, after purification by
547 chromatography on silica gel (eluent: dichloromethane) and recrystallization from propan-2-ol as
548 a beige solid in 93% yield (0.56 g). mp 145 °C. ¹H NMR (400 MHz, CDCl₃) δ : 7.90 (1H, d, *J* =
549 1.8 Hz), 8.65 (1H, s), 9.46 (1H, d, *J* = 1.8 Hz). ¹³C NMR (100 MHz, CDCl₃) δ : 113.4 (C), 124.3
550 (CH), 125.0 (C), 130.2 (C), 133.4 (CH), 138.1 (CH), 148.0 (C). LC/MS ESI⁺ *t*_R 1.42 min, (m/z)
551 [M+H]⁺ 275.98/276.96/277.58. HRMS (+ESI): 277.9149 (M + H⁺). Calcd for C₇H₃BrClN₃O₂:
552 277.9148.

553 **4.1.9. 8-Bromo-6-chloro-2-methylimidazo[1,2-*a*]pyridine (5a)**

554 To a solution of sodium borohydride (0.07 g, 1.79 mmol, 1 equiv.) in dimethylsulfoxide (60 mL),
555 8-bromo-6-chloro-2-chloromethylimidazo[1,2-*a*]pyridine **1a** (0.5 g, 1.79 mmol, 1 equiv.) was
556 added. The reaction mixture was stirred for 72 h at room temperature. Then, the mixture was
557 slowly poured into an ice-water mixture and extracted three times with dichloromethane. The
558 organic layer was washed five times with brine, dried over MgSO₄, filtered and evaporated.
559 Compound **5a** was obtained, after purification by chromatography on silica gel (eluent:
560 dichloromethane/cyclohexane/diethyl ether 7:2.5:0.5) and recrystallization from acetonitrile as a
561 white solid in 63% yield (0.28 g). mp 140 °C. ¹H NMR (300 MHz, CDCl₃) δ : 2.48 (3H, s), 7.39–
562 7.41 (2H, m), 8.70 (1H, d, *J* = 1.8 Hz). ¹³C NMR (75 MHz, CDCl₃) δ : 14.7 (CH₃), 111.1 (C), 112.0
563 (CH), 119.5 (C), 122.5 (CH), 127.5 (CH), 141.8 (C), 145.5 (C). LC/MS ESI⁺ *t*_R 1.71 min, (m/z)
564 [M+H]⁺ 245.05/247.04/249.07. HRMS (+ESI): 246.9453 (M + H⁺). Calcd for C₈H₆BrClN₂:
565 246.9453.

566 **4.1.10. 8-Bromo-6-chloro-2-methyl-3-nitroimidazo[1,2-*a*]pyridine (5b)**

567 To a solution of 8-bromo-6-chloro-2-methylimidazo[1,2-*a*]pyridine **5a** (0.3 g, 1.22 mmol, 1
568 equiv.) in concentrated sulfuric acid (5 mL) cooled by an ice-water bath, nitric acid 65% (500 μ L,
569 7.3 mmol, 6 equiv.) was added. The reaction mixture was stirred for 30 min at room temperature.
570 Then, the mixture was slowly poured into an ice-water mixture and the desired product
571 precipitated. The yellow solid was collected by filtration, dried under reduced pressure and
572 recrystallized from acetonitrile to give the expected product **5b** in 78% yield (0.28 g). mp 192 °C.
573 ¹H NMR (300 MHz, DMSO-*d*₆) δ : 2.74 (3H, s), 8.36 (1H, d, *J* = 1.8 Hz), 9.34 (1H, d, *J* = 1.8 Hz).
574 ¹³C NMR (75 MHz, DMSO-*d*₆) δ : 14.0 (CH₃), 111.3 (C), 122.6 (C), 125.2 (CH), 131.2 (C), 133.5
575 (CH), 140.7 (C), 150.1 (C). LC/MS ESI⁺ *t*_R 2.13 min, (m/z) [M+H]⁺ 289.90/291.90/293.90. HRMS
576 (+ESI): 291.9302 (M + H⁺). Calcd for C₈H₅BrClN₃O₂: 291.9304.

577 **4.1.11. (8-Bromo-6-chloro-3-nitroimidazo[1,2-*a*]pyridin-2-yl)methanol (6)**

578 To a solution of copper sulfate pentahydrate (0.77 g, 3.08 mmol, 1 equiv.) in a
579 dimethylsulfoxide/water mixture (7.5/2.5, 80 mL), 8-bromo-6-chloro-2-chloromethyl-3-
580 nitroimidazo[1,2-*a*]pyridine **1b** (1 g, 3.08 mmol, 1 equiv.) was added. The reaction mixture was
581 stirred and heated at 100 °C for 24 h. Then, the mixture was slowly poured into an ice-water
582 mixture with sodium carbonate and extracted three times with dichloromethane. The organic layer
583 was washed five times with brine, dried over MgSO₄, filtered and evaporated. The crude residue
584 was purified by column chromatography on silica gel (eluent: dichloromethane/ethyl acetate
585 9.5:0.5) and compound **6** was isolated as a white solid in 32% yield (0.3 g). mp 202 °C. ¹H NMR
586 (300 MHz, DMSO-*d*₆) δ : 4.92 (2H, d, *J* = 6.3 Hz), 5.57 (1H, t, *J* = 6.3 Hz), 8.38 (1H, d, *J* = 1.8
587 Hz), 9.36 (1H, d, *J* = 1.8 Hz). ¹³C NMR (75 MHz, DMSO-*d*₆) δ : 58.3 (CH₂), 111.9 (C), 122.9 (C),
588 125.2 (CH), 129.7 (C), 133.6 (CH), 141.0 (C), 153.2 (C). LC/MS ESI⁺ *t*_R 1.01 min, (m/z) [M+H]⁺
589 305.84/307.83/309.91. HRMS (+ESI): 307.9255 (M + H⁺). Calcd for C₈H₅BrClN₃O₃: 307.9253.

590

591 **4.1.12. 8-Bromo-6-chloro-3-nitro-2-(phenylthiomethyl)imidazo[1,2-*a*]pyridine (7)**

592 To a sealed 20 mL flask containing a solution of NaH 60% (0.07 g, 0.98 mmol, 1.1 equiv.) in
593 dimethylsulfoxide (3 mL), thiophenol (0.17 g, 1.54 mmol, 1 equiv.) was added under N₂
594 atmosphere. The reaction mixture was stirred for 30 min at room temperature. Then, a solution of
595 8-bromo-6-chloro-2-chloromethyl-3-nitroimidazo[1,2-*a*]pyridine **1b** (0.5 g, 1.54 mmol, 1 equiv.)
596 in dimethylsulfoxide was injected. The reaction mixture was stirred at room temperature for 45
597 min. Then, the mixture was slowly poured into an ice-water mixture and extracted three times with
598 dichloromethane. The organic layer was washed five times with brine, dried over MgSO₄, filtered
599 and evaporated. The crude residue was purified by column chromatography on silica gel (eluent:
600 dichloromethane/cyclohexane 6:4) and compound **7** was isolated as a yellow solid in 28% yield
601 (0.17 g). mp 136 °C. ¹H NMR (250 MHz, CDCl₃) δ: 4.66 (2H, s), 7.19–7.31 (3H, m), 7.45–7.48
602 (2H, m), 7.86 (1H, d, *J* = 1.7 Hz), 9.46 (1H, d, *J* = 1.1 Hz). ¹³C NMR (62.5 MHz, CDCl₃) δ: 33.4
603 (CH₂), 112.9 (C), 124.7 (C), 124.9 (CH), 127.3 (CH), 129.1 (2CH), 131.0 (2CH), 131.9 (C), 133.9
604 (CH), 134.8 (C), 141.0 (C), 150.5 (C). LC/MS ESI⁺ t_R 4.01 min, (m/z) [M+H]⁺ 398.81/399.84.
605 HRMS (+ESI): 399.9336 (M + H⁺). Calcd for C₁₄H₉BrClN₃O₂S: 399.9338.

606 **4.1.13. General procedure for the preparation of sulfone derivatives from the 8-**
607 **bromo-6-chloro-2-chloromethyl-3-nitroimidazo[1,2-*a*]pyridine (1b) :**

608 To a 20 mL flask containing a solution of 8-bromo-6-chloro-2-chloromethyl-3-nitroimidazo[1,2-
609 *a*]pyridine **1b** (0.8 g, 2.46 mmol, 1 equiv.) in a water/ethanol mixture (1:1, 12 mL), sodium
610 bicarbonate (0.42 g, 4.92 mmol, 2 equiv.), sodium sulfite (0.62 g, 4.92 mmol, 2 equiv.) and the
611 appropriate sulfonyl chloride (4.92 mmol, 2 equiv.) were added. The flask was sealed and the
612 mixture was heated at 120 °C under microwave irradiation until complete disappearance of the
613 starting material (as monitored by LC/MS or TLC). Water was then added and the mixture was
614 extracted three times with dichloromethane. The organic layer was dried over MgSO₄, filtered and
615 evaporated. The crude residue was purified by column chromatography on silica gel and
616 recrystallized from the appropriate solvent, affording compounds **8** to **10**.

617 **4.1.13.1. 8-Bromo-6-chloro-2-(methylsulfonylmethyl)-3-nitroimidazo[1,2-**
618 **a]pyridine (8)**

619 Compound **8** was obtained after purification by chromatography (eluent: dichloromethane/ethyl
620 acetate 9:1) and recrystallization from propan-2-ol as a pale brown solid in 47% yield (0.43 g). mp
621 233 °C. ¹H NMR (400 MHz, CDCl₃) δ: 3.20 (3H, t, *J* = 0.9 Hz), 5.09 (2H, q, *J* = 0.9 Hz), 7.96
622 (1H, d, *J* = 1.8 Hz), 9.51 (1H, d, *J* = 1.8 Hz). ¹³C NMR (100 MHz, CDCl₃) δ: 41.9 (CH₃), 54.9
623 (CH₂), 113.3 (C), 125.0 (CH), 125.6 (C), 131.1 (C), 134.5 (CH), 140.3 (C), 141.3 (C). LC/MS
624 ESI⁺ t_R 0.86 min, (m/z) [M+H]⁺ 367.76/369.89/370.94. HRMS (+ESI): 369.9077 (M + H⁺). Calcd
625 for C₉H₇BrClN₃O₄S: 369.9079.

626 **4.1.13.2. 8-Bromo-6-chloro-2-(cyclohexylsulfonylmethyl)-3-nitroimidazo[1,2-**
627 **a]pyridine (9)**

628 Compound **9** was obtained after purification by chromatography (eluent: dichloromethane) and
629 recrystallization from acetonitrile as a yellow solid in 29% yield (0.31 g). mp 214 °C. ¹H NMR
630 (400 MHz, CDCl₃) δ: 1.22–1.40 (3H, m), 1.60–1.77 (3H, m), 1.96–2.01 (2H, m), 2.29–2.33 (2H,
631 m), 3.28 (1H, tt, *J* = 3.4 and 12.2 Hz), 5.02 (2H, s), 7.92 (1H, d, *J* = 1.8 Hz), 9.47 (1H, d, *J* = 1.8
632 Hz). ¹³C NMR (100 MHz, CDCl₃) δ: 25.1 (2CH₂), 25.2 (CH₂), 25.3 (2CH₂), 50.1 (CH₂), 61.8 (CH),
633 113.2 (C), 125.0 (CH), 125.4 (C), 127.0 (C), 134.3 (CH), 140.5 (C), 141.2 (C). LC/MS ESI⁺ t_R
634 2.92 min, (m/z) [M+H]⁺ 435.79/437.85/437.00. HRMS (+ESI): 435.9723 (M + H⁺). Calcd for
635 C₁₄H₁₅BrClN₃O₄S: 435.9728.

636 **4.1.13.3. 8-Bromo-6-chloro-3-nitro-2-(pyridin-3-ylsulfonylmethyl)imidazo[1,2-**
637 **a]pyridine (10)**

638 Compound **10** was obtained after purification by chromatography (eluent:
639 dichloromethane/cyclohexane/diethyl ether 5:3:2) and recrystallization from acetonitrile as a
640 yellow solid in 21% yield (0.22 g). mp 220 °C. ¹H NMR (400 MHz, CDCl₃) δ: 5.21 (2H, s), 7.53–
641 7.56 (1H, m), 7.91 (1H, d, *J* = 1.8 Hz), 8.21 (1H, dt, *J* = 1.7 and 8.1 Hz), 8.90 (1H, bd, *J* = 3.9 Hz),

642 9.01 (1H, bs), 9.45 (1H, d, $J = 1.8$ Hz). ^{13}C NMR (100 MHz, CDCl_3) δ : 56.9 (CH_2), 113.4 (C),
643 124.0 (CH), 124.9 (CH), 125.8 (C), 128.3 (C), 134.5 (CH), 135.8 (C), 136.9 (CH), 139.3 (C), 141.2
644 (C), 149.7 (CH), 154.6 (CH). LC/MS ESI⁺ t_{R} 1.21 min, (m/z) $[\text{M}+\text{H}]^+$ 430.61/432.29/433.31.
645 HRMS (+ESI): 430.9210 (M + H⁺). Calcd for $\text{C}_{13}\text{H}_8\text{BrClN}_4\text{O}_4\text{S}$: 430.9211.

646 **4.1.14. General procedure for the preparation of 8-(4-chlorophenylthio)imidazo[1,2-**
647 ***a*]pyridine derivatives (11 to 19):**

648 To a sealed 20 mL flask containing NaH 60% (9.4 mg, 0.47 mmol, 1 equiv.) in dimethylsulfoxide
649 (1 mL), 4-chlorothiophenol (68 mg, 0.47 mmol, 1 equiv.) was added under N_2 atmosphere. The
650 reaction mixture was stirred at room temperature for 30 min. Then a solution of the appropriate 8-
651 bromo-3-nitro-imidazo[1,2-*a*]pyridine derivative (0.47 mmol, 1 equiv.) in dimethylsulfoxide (5
652 mL) was injected. The reaction mixture was stirred at room temperature until complete
653 disappearance of the starting material (as monitored by LC/MS or TLC). The reaction mixture was
654 then poured into an ice-water mixture. The mixture was extracted three times with
655 dichloromethane, then the organic layer was washed five times with brine, dried over MgSO_4 ,
656 filtered and evaporated. The crude residue was purified by column chromatography on silica gel
657 and/or recrystallized from the appropriate solvent, affording compounds **11** to **19**.

658 **4.1.14.1. 8-[4-(Chlorophenyl)thio]-6-methyl-3-nitro-2-**
659 **(phenylsulfonylmethyl)imidazo[1,2-*a*]pyridine (11)**

660 Compound **11** was obtained after purification by chromatography (eluent: petroleum ether/ethyl
661 acetate 7:3) and recrystallization from acetonitrile as a yellow solid in 90% yield (0.18 g). mp 184
662 °C. ^1H NMR (200 MHz, CDCl_3) δ : 2.34 (3H, s), 5.17 (2H, s), 6.77 (1H, d, $J = 1.1$ Hz), 7.44–7.48
663 (2H, m), 7.51–7.57 (4H, m), 7.64–7.70 (1H, m), 7.87–7.90 (2H, m), 8.96–8.97 (1H, m). ^{13}C NMR
664 (50 MHz, CDCl_3) δ : 19.0 (CH_3), 56.9 (CH_2), 122.7 (C), 127.5 (C), 127.8 (CH), 128.7 (2CH), 129.2
665 (C), 129.3 (2CH), 130.5 (CH), 130.6 (2CH), 131.1 (C), 134.2 (CH), 136.6 (C), 136.7 (2CH), 138.7

666 (C), 139.4 (C), 141.0 (C). LC/MS ESI⁺ t_R 3.80 min, (m/z) [M+H]⁺ 473.83/475.84. HRMS (+ESI):
667 474.0340 (M + H⁺). Calcd for C₂₁H₁₆ClN₃O₄S₂: 474.0344.

668 **4.1.14.2. 8-[4-(Chlorophenyl)thio]-3-nitro-2-(phenylsulfonylmethyl)-6-**
669 **(trifluoromethyl)imidazo[1,2-*a*]pyridine (12)**

670 Compound **12** was obtained after recrystallization from propan-2-ol as a yellow solid in 70% yield
671 (0.17 g). mp 191 °C. ¹H NMR (250 MHz, CDCl₃) δ: 5.18 (2H, s), 6.91 (1H, d, *J* = 1.2 Hz), 7.47–
672 7.63 (6H, m), 7.71 (1H, t, *J* = 7.4 Hz), 7.90 (2H, dd, *J* = 1.4 and 7.2 Hz), 9.44 (1H, s). ¹³C NMR
673 (62.5 MHz, CDCl₃) δ: 56.7 (CH₂), 120.5 (CH, d, *J* = 2.7 Hz), 121.6 (C, q, *J* = 34.8 Hz), 122.5 (CH,
674 d, *J* = 5.6 Hz), 122.5 (C, q, *J* = 272.8 Hz), 125.6 (CH), 128.6 (2CH), 129.4 (2CH), 131.0 (C), 132.0
675 (2CH), 134.0 (C), 134.5 (C), 137.1 (2CH), 137.6 (C), 139.2 (C), 139.9 (C), 141.4 (C). LC/MS
676 ESI⁺ t_R 4.24 min, (m/z) [M+H]⁺ 528.11/529.15. HRMS (+ESI): 528.0061 (M + H⁺). Calcd for
677 C₂₁H₁₃ClF₃N₃O₄S₂: 528.0061.

678 **4.1.14.3. 6-Chloro-8-[4-(chlorophenyl)thio]-3-nitroimidazo[1,2-*a*]pyridine (13)**

679 Compound **13** was obtained after purification by chromatography (eluent:
680 dichloromethane/cyclohexane 7:3) and recrystallization from acetonitrile as a yellow solid in 69%
681 yield (0.11 g). mp 192 °C. ¹H NMR (400 MHz, CDCl₃) δ: 6.78 (1H, d, *J* = 1.8 Hz), 7.49–7.53 (2H,
682 m), 7.57–7.60 (2H, m), 8.59 (1H, s), 9.20 (1H, d, *J* = 1.8 Hz). ¹³C NMR (100 MHz, CDCl₃) δ:
683 121.6 (CH), 125.7 (CH), 125.8 (C), 126.2 (C), 130.9 (2CH), 133.0 (C), 133.3 (C), 137.0 (2CH),
684 137.4 (C), 137.5 (CH), 142.1 (C). LC/MS ESI⁺ t_R 4.10 min, (m/z) [M+H]⁺ 340.10/341.11/342.08.
685 HRMS (+ESI): 339.9705 (M + H⁺). Calcd for C₁₃H₇Cl₂N₃O₂S: 339.9709.

686 **4.1.14.4. 6-Chloro-8-[4-(chlorophenyl)thio]-2-methyl-3-nitroimidazo[1,2-**
687 ***a*]pyridine (14)**

688 Compound **14** was obtained after purification by chromatography (eluent: dichloromethane) and
689 recrystallization from acetonitrile as a yellow solid in 75% yield (0.13 g). mp 199 °C. ¹H NMR
690 (400 MHz, CDCl₃) δ: 2.89 (3H, s), 6.75 (1H, d, *J* = 1.8 Hz), 7.48–7.60 (4H, m), 9.23 (1H, d, *J* =

691 1.8 Hz). ^{13}C NMR (100 MHz, CDCl_3) δ : 17.5 (CH_3), 122.2 (CH), 124.8 (C), 125.9 (CH), 126.4
692 (C), 130.9 (2CH), 131.2 (C), 131.6 (C), 137.0 (2CH), 137.3 (C), 140.0 (C), 150.5 (C). LC/MS
693 ESI⁺ t_{R} 4.50 min, (m/z) $[\text{M}+\text{H}]^+$ 354.08/355.19/356.31. HRMS (+ESI): 353.9864 (M + H⁺). Calcd
694 for $\text{C}_{14}\text{H}_9\text{Cl}_2\text{N}_3\text{O}_2\text{S}$: 353.9865.

695 **4.1.14.5. {6-Chloro-8-[4-(chlorophenyl)thio]-3-nitroimidazo[1,2-*a*]pyridin-2-
696 yl}methanol (15)**

697 Compound **15** was obtained after purification by chromatography (eluent:
698 dichloromethane/methanol 9.8:0.2) as a pale yellow solid in 40% yield (70 mg). mp 227 °C. ^1H
699 NMR (400 MHz, $\text{DMSO-}d_6$) δ : 4.92 (2H, s), 5.54 (1H, sl), 6.99 (1H, d, $J = 1.8$ Hz), 7.61–7.68
700 (4H, m), 9.17 (1H, d, $J = 1.8$ Hz). ^{13}C NMR (100 MHz, $\text{DMSO-}d_6$) δ : 58.2 (CH_2), 122.9 (CH),
701 123.3 (C), 126.6 (CH), 127.2 (C), 129.4 (C), 129.7 (C), 130.5 (2CH), 135.1 (C), 136.2 (2CH),
702 140.0 (C), 152.5 (C). LC/MS ESI⁺ t_{R} 3.68 min, (m/z) $[\text{M}+\text{H}]^+$ 369.86/371.12/372.06. HRMS
703 (+ESI): 369.9811 (M + H⁺). Calcd for $\text{C}_{14}\text{H}_9\text{Cl}_2\text{N}_3\text{O}_3\text{S}$: 369.9814.

704 **4.1.14.6. 6-Chloro-8-[4-(chlorophenyl)thio]-3-nitro-2-
705 (phenylthiomethyl)imidazo[1,2-*a*]pyridine (16)**

706 Compound **16** was obtained after purification by chromatography (eluent: petroleum
707 ether/dichloromethane 5.5:4.5) as a yellow solid in 21% yield (46 mg). mp 142 °C. ^1H NMR (200
708 MHz, CDCl_3) δ : 4.67 (2H, s), 6.76 (1H, d, $J = 1.8$ Hz), 7.22–7.25 (1H, m), 7.29–7.33 (1H, m),
709 7.48–7.58 (7H, m), 9.21 (1H, d, $J = 1.8$ Hz). ^{13}C NMR (50 MHz, CDCl_3) δ : 33.3 (CH_2), 122.1 (C),
710 125.4 (C), 126.2 (CH), 126.3 (C), 127.1 (CH), 129.1 (2CH), 130.6 (2CH), 130.9 (2CH), 132.3 (C),
711 135.2 (C), 135.9 (CH), 137.0 (2CH), 137.3 (C), 139.8 (C), 149.6 (C). LC/MS ESI⁺ t_{R} 5.36 min,
712 (m/z) $[\text{M}+\text{H}]^+$ 461.81/463.92. HRMS (+ESI): 461.9897 (M + H⁺). Calcd for $\text{C}_{20}\text{H}_{13}\text{Cl}_2\text{N}_3\text{O}_2\text{S}_2$:
713 461.9899.

714

715 **4.1.14.7. 6-Chloro-8-[4-(chlorophenyl)thio]-2-(methylsulfonylmethyl)-3-nitro-**
716 **imidazo[1,2-*a*]pyridine (17)**

717 Compound **17** was obtained after purification by chromatography (eluent: dichloromethane) and
718 recrystallization from acetonitrile as a beige solid in 46% yield (93 mg). mp 213 °C. ¹H NMR (400
719 MHz, DMSO-*d*₆) δ: 3.07 (3H, s), 5.11 (2H, q, *J* = 0.8 Hz), 7.18 (1H, d, *J* = 1.8 Hz), 7.58–7.67 (4H,
720 m), 9.22 (1H, d, *J* = 1.8 Hz). ¹³C NMR (100 MHz, DMSO-*d*₆) δ: 40.9 (CH₃), 54.1 (CH₂), 123.5
721 (CH), 123.9 (C), 127.5 (C), 128.0 (CH), 129.3 (C), 130.3 (2CH), 131.5 (C), 134.9 (C), 135.7
722 (2CH), 139.4 (C), 140.0 (C). LC/MS ESI⁺ t_R 3.42 min, (m/z) [M+H]⁺ 432.70/433.72/434.89.
723 HRMS (+ESI): 431.9640 (M + H⁺). Calcd for C₁₅H₁₁Cl₂N₃O₄S₂: 431.9641.

724 **4.1.14.8. 6-Chloro-8-[4-(chlorophenyl)thio]-2-(cyclohexylsulfonylmethyl)-3-**
725 **nitroimidazo[1,2-*a*]pyridine (18)**

726 Compound **18** was obtained after purification by chromatography (eluent: dichloromethane) and
727 recrystallization from cyclohexane as a brown solid in 47% yield (0.11 g). mp 82 °C. ¹H NMR
728 (400 MHz, CDCl₃) δ: 1.21–1.39 (3H, m), 1.61–1.78 (3H, m), 1.97–2.01 (2H, m), 2.31–2.34 (2H,
729 m), 3.31 (1H, tt, *J* = 3.4 and 12.1 Hz), 5.02 (2H, s), 6.79 (1H, d, *J* = 1.8 Hz), 7.50–7.59 (4H, m),
730 9.22 (1H, d, *J* = 1.8 Hz). ¹³C NMR (100 MHz, CDCl₃) δ: 25.1 (2CH₂), 25.2 (CH₂), 25.3 (2CH₂),
731 50.0 (CH₂), 61.6 (CH), 113.1 (C), 122.0 (CH), 126.0 (C), 126.3 (CH), 130.1 (C), 131.0 (2CH),
732 132.8 (C), 137.0 (2CH), 137.5 (C), 139.5 (C), 140.0 (C). LC/MS ESI⁺ t_R 4.57 min, (m/z) [M+H]⁺
733 499.81/501.85/503.88. HRMS (+ESI): 500.0271 (M + H⁺). Calcd for C₂₀H₁₉Cl₂N₃O₄S₂: 500.0267.

734 **4.1.14.9. 6-Chloro-8-[4-(chlorophenyl)thio]-3-nitro-2-(pyridin-3-**
735 **ylsulfonylmethyl)imidazo[1,2-*a*]pyridine (19)**

736 Compound **19** was obtained after purification by chromatography (eluent: dichloromethane) and
737 recrystallization from acetonitrile as a yellow solid in 20% yield (47 mg). mp 176 °C. ¹H NMR
738 (400 MHz, CDCl₃) δ: 5.21 (2H, s), 6.74 (1H, d, *J* = 1.7 Hz), 7.50–7.59 (5H, m), 8.23 (1H, dt, *J* =
739 1.7 and 7.9 Hz), 8.91 (1H, d, *J* = 4.1 Hz), 9.01 (1H, s), 9.17 (1H, d, *J* = 1.7 Hz). ¹³C NMR (100

740 MHz, CDCl₃) δ : 56.9 (CH₂), 121.8 (CH), 123.9 (CH), 125.6 (C), 126.1 (CH), 126.4 (C), 131.0
741 (2CH), 132.0 (C), 133.4 (C), 135.8 (C), 136.8 (CH), 137.2 (2CH), 137.6 (C), 138.4 (C), 139.9 (C),
742 149.8 (CH), 154.8 (CH). LC/MS ESI⁺ t_R 3.63 min, (m/z) [M+H]⁺ 494.77/496.71. HRMS (+ESI):
743 494.9750 (M + H⁺). Calcd for C₁₉H₁₂Cl₂N₄O₄S₂: 494.9750.

744 **4.1.15. General procedure for the preparation of 8-arylamino-imidazo[1,2-**
745 **a]pyridine derivatives (20 to 22):**

746 A mixture of 8-bromo-6-chloro-3-nitro-2-(phenylsulfonylmethyl)imidazo[1,2-*a*]pyridine **1c** (400
747 mg, 0.93 mmol, 1 equiv.), palladium diacetate (17 mg, 0.07 mmol, 0.08 equiv.), potassium
748 carbonate (0.23 g, 1.7 mmol, 1.8 equiv.), *rac*-BINAP (87 mg, 0.14 mmol, 0.15 equiv.) and
749 appropriate aniline (1.1 mmol, 1.2 equiv.) in toluene (12 mL) under N₂ atmosphere was heated at
750 140 °C under microwave irradiation until complete disappearance of the starting material (as
751 monitored by LC/MS or TLC). Water was then added and the mixture was extracted three times
752 with dichloromethane. The organic layer was washed three times with water, dried over MgSO₄,
753 filtered and evaporated. The crude residue was purified by column chromatography on silica gel
754 with 0.5% triethylamine and recrystallized from the appropriate solvent, affording compounds **20**
755 to **22**.

756 **4.1.15.1. 6-Chloro-3-nitro-*N*-phenyl-2-(phenylsulfonylmethyl)imidazo[1,2-**
757 **a]pyridin-8-amine (20)**

758 Compound **20** was obtained after purification by chromatography (eluent:
759 dichloromethane/cyclohexane 7:3) and recrystallization from propan-2-ol as an orange solid in
760 50% yield (0.21 g). mp 254 °C. ¹H NMR (400 MHz, CDCl₃) δ : 5.18 (2H, s), 7.12–7.16 (2H, m),
761 7.40–7.42 (4H, m), 7.60–7.64 (2H, m), 7.74–7.80 (3H, m), 8.72 (1H, d, *J* = 1.3 Hz), 9.09 (1H, bs).
762 ¹³C NMR (100 MHz, CDCl₃) δ : 56.4 (CH₂), 107.6 (CH), 114.6 (CH), 121.9 (2CH), 124.0 (CH),
763 125.6 (C), 128.0 (2CH), 129.4 (4CH), 131.5 (C), 134.3 (CH), 134.5 (C), 136.3 (C), 136.9 (C),
764 138.8 (C), 139.4 (C). LC/MS ESI⁺ t_R 4.08 min, (m/z) [M+H]⁺ 442.91/444.90. HRMS (+ESI):
765 443.0577 (M + H⁺). Calcd for C₂₀H₁₅ClN₄O₄S: 443.0575.

766 **4.1.15.2. 6-Chloro-*N*-(4-chlorophenyl)-3-nitro-2-**
767 **(phenylsulfonylmethyl)imidazo[1,2-*a*]pyridin-8-amine (21)**

768 Compound **21** was obtained after purification by chromatography (eluent: dichloromethane) and
769 recrystallization from propan-2-ol as an orange solid in 52% yield (0.23 g). mp 255 °C. ¹H NMR
770 (400 MHz, DMSO-*d*₆) δ: δ 5.17 (2H, s), 7.18–7.20 (1H, m), 7.39–7.48 (4H, m), 7.59–7.63 (2H,
771 m), 7.74–7.79 (3H, m), 7.76–7.76 (1H, m), 9.23 (1H, bs). ¹³C NMR (100 MHz, DMSO-*d*₆) δ: 56.4
772 (CH₂), 108.5 (CH), 115.2 (CH), 123.1 (2CH), 125.5 (C), 127.3 (C), 128.0 (2CH), 129.3 (2CH),
773 129.4 (2CH), 130.6 (C), 131.5 (C), 134.0 (C), 134.3 (CH), 137.0 (C), 138.7 (C), 138.8 (C). LC/MS
774 ESI⁺ t_R 4.99 min, (m/z) [M+H]⁺ 476.69/478.60. HRMS (+ESI): 477.0186 (M + H⁺). Calcd for
775 C₂₀H₁₄Cl₂N₄O₄S: 477.0186.

776 **4.1.15.3. 6-Chloro-*N*-(4-methoxyphenyl)-3-nitro-2-**
777 **(phenylsulfonylmethyl)imidazo[1,2-*a*]pyridin-8-amine (22)**

778 Compound **22** was obtained after purification by chromatography (eluent:
779 dichloromethane/cyclohexane 9:1) and recrystallization from propan-2-ol as a red solid in 42%
780 yield (0.19 g). mp 228 °C. ¹H NMR (400 MHz, CDCl₃) δ: 3.78 (3H, s), 5.17 (2H, s), 6.81 (1H, d,
781 *J* = 1.4 Hz), 6.99–7.01 (2H, m), 7.30–7.32 (2H, m), 7.60–7.64 (2H, m), 7.74–7.80 (3H, m), 8.64
782 (1H, d, *J* = 1.4 Hz), 8.93 (1H, s). ¹³C NMR (100 MHz, CDCl₃) δ: 55.3 (CH₃), 56.4 (CH₂), 106.0
783 (CH), 113.6 (CH), 114.7 (2CH), 125.0 (2CH), 125.8 (C), 128.0 (2CH), 129.4 (2CH), 131.4 (C),
784 131.7 (C), 134.3 (CH), 135.9 (C), 136.0 (C), 136.8 (C), 138.8 (C), 156.5 (C). LC/MS ESI⁺ t_R 4.62
785 min, (m/z) [M+H]⁺ 472.88/474.74. HRMS (+ESI): 473.0680 (M + H⁺). Calcd for C₂₁H₁₇ClN₄O₅S:
786 473.0681.

787 **4.1.16. 6-Chloro-8-(4-chlorophenylselenanyl)-3-nitro-2-**
788 **(phenylsulfonylmethyl)imidazo[1,2-*a*]pyridine (23)**

789 To a solution of 1,2-bis(4-chlorophenyl)diselenide [24] (0.4 g, 1.05 mmol, 1 equiv.) in PEG-400
790 (4 mL) under N₂ atmosphere, sodium borohydride (0.16 g, 4.2 mmol, 4 equiv.) was added. The
791 reaction mixture was stirred 1 h at 60 °C. 8-bromo-6-chloro-3-nitro-2-
792 (phenylsulfonylmethyl)imidazo[1,2-*a*]pyridine **1c** (0.9 g, 2.1 mmol, 2 equiv.) was then introduced.
793 The resulting mixture was diluted with H₂O and acidified with 1 N hydrochloric acid. The mixture

794 was extracted three times with dichloromethane, dried over MgSO₄, filtered and evaporated. The
795 crude residue was purified by column chromatography on silica gel (eluent: dichloromethane) as
796 a yellow solid in 9% yield (0.11 g). mp 209 °C. ¹H NMR (250 MHz, CDCl₃) δ: 5.15 (2H, s), 6.87
797 (1H, d, *J* = 1.7 Hz), 7.42-7.75 (7H, m), 7.90 (2H, d, *J* = 7.3 Hz), 9.20 (1H, d, *J* = 1.7 Hz). ¹³C NMR
798 (63 MHz, CDCl₃) δ: 56.7 (CH₂), 122.2 (CH), 122.5 (C), 126.3 (C), 128.6 (C), 128.7 (2CH), 128.9
799 (C), 129.4 (2CH), 131.0 (2CH), 131.3 (CH), 134.4 (C), 137.3 (CH), 138.6 (2CH), 139.1 (C), 139.3
800 (C), 140.7 (C). LC/MS ESI⁺ t_R 4.31 min, (m/z) [M+H]⁺ 541.68/543.53/546.10. HRMS (+ESI):
801 540.9169 (M + H⁺). Calcd for C₂₀H₁₃Cl₂N₃O₄SSe: 540.9171.

802 **4.1.17. 8-Bromo-6-chloro-2-(methylthiomethyl)-3-nitroimidazo[1,2-*a*]pyridine (24)**

803 To a solution of 8-bromo-6-chloro-2-chloromethyl-3-nitroimidazo[1,2-*a*]pyridine **1b** (0.8 g, 2.46
804 mmol, 1 equiv.) in DMSO (10 mL), a solution of sodium thiomethoxide (0.17 g, 2.46 mmol, 1
805 equiv.) in DMSO (5 mL) was added dropwise. The reaction mixture was stirred for 45 min at room
806 temperature. Then, the mixture was slowly poured into an ice-water mixture and the desired
807 product precipitated. The yellow solid was collected by filtration, dried under reduced pressure
808 and purified by column chromatography on silica gel (eluent: dichloromethane/cyclohexane 5:5)
809 as a pale yellow solid in 52% yield (0.43 g). mp 186 °C. ¹H NMR (400 MHz, DMSO-*d*₆) δ: 2.13
810 (3H, s), 4.13 (2H, s), 8.40 (1H, d, *J* = 1.8 Hz), 9.37 (1H, d, *J* = 1.8 Hz). ¹³C NMR (100 MHz,
811 DMSO-*d*₆) δ: 14.9 (CH₃), 31.1 (CH₂), 111.7 (C), 123.0 (CH), 125.5 (C), 130.0 (C), 133.8 (CH),
812 140.7 (C), 150.4 (C). LC/MS ESI⁺ t_R 2.76 min, (m/z) [M+H]⁺ 334.64/337.82/339.91. HRMS
813 (+ESI): 337.9182 (M + H⁺). Calcd for C₉H₇BrClN₃O₂S: 337.9181.

814 **4.1.18. 8-Bromo-6-chloro-2-(methylsulfinylmethyl)-3-nitroimidazo[1,2-*a*]pyridine**

815 **(25)**

816 To a solution of 8-bromo-6-chloro-2-(methylthiomethyl)-3-nitroimidazo[1,2-*a*]pyridine **24** (0.4 g,
817 1.19 mmol, 1 equiv.) in dichloromethane (40 mL) cooled by an ice-water bath, mCPBA (~70%)
818 (0.29 g, 1.19 mmol, 1 equiv.) was added. The reaction mixture was stirred for 1 h at 0 °C. Then,

819 the mixture was washed three times with H₂O, dried over MgSO₄, filtered and evaporated. The
820 crude residue was purified by column chromatography on silica gel (eluent: dichloromethane/ethyl
821 acetate 8:2) as a yellow solid in 36% yield (0.15 g). mp 164 °C. ¹H NMR (400 MHz, DMSO-*d*₆)
822 δ: 2.70 (3H, s), 4.55-4.81 (2H, m), 8.42 (1H, d, *J* = 1.8 Hz), 9.38 (1H, d, *J* = 1.8 Hz). ¹³C NMR
823 (100 MHz, DMSO-*d*₆) δ: 38.6 (CH₃), 53.3 (CH₂), 111.8 (C), 123.3 (CH), 125.4 (C), 132.0 (C),
824 134.0 (CH), 141.0 (C), 142.7 (C). LC/MS ESI⁺ t_R 0.84 min, (m/z) [M+H]⁺ 351.83/353.92/355.94.
825 HRMS (+ESI): 353.9127 (M + H⁺). Calcd for C₉H₇BrClN₃O₃S: 353.9130.

826 **4.1.19. 8-Bromo-6-chloro-2-(methylsulfonylmethyl)imidazo[1,2-*a*]pyridin-3-amine**

827 **(26)**

828 To a solution of 8-bromo-6-chloro-2-(methylsulfonylmethyl)-3-nitroimidazo[1,2-*a*]pyridine **8**
829 (0.2 g, 0.54 mmol, 1 equiv.) in acetic acid (30 mL), iron powder (0.3 g, 5.4 mmol, 10 equiv.) was
830 added. The reaction mixture was stirred and heated under reflux for 30 min. The mixture was then
831 filtered through celite and the solvent was removed *in vacuo*. The resulting residue was diluted
832 with H₂O and basified with sat. aq. NaHCO₃. The mixture was extracted three times with
833 dichloromethane, dried over MgSO₄, filtered and evaporated. The crude residue was purified by
834 column chromatography on silica gel (eluent: dichloromethane/diethyl ether 6:4) as a yellow solid
835 in 40% yield (0.07 g). mp 215 °C. ¹H NMR (250 MHz, DMSO) δ: 3.02 (3H, s), 4.62 (2H, s), 5.62
836 (2H, s), 7.50 (1H, d, *J* = 1.7 Hz), 8.33 (1H, d, *J* = 1.7 Hz). ¹³C NMR (62.5 MHz, DMSO) δ: 40.1
837 (CH₃), 52.7 (CH₂), 110.8 (C), 117.4 (CH), 117.6 (C), 119.9 (C), 123.7 (CH), 133.0 (C), 134.2 (C).
838 LC/MS ESI⁺ t_R 1.03 min, (m/z) [M+H]⁺ 337.90/339.92/341.95. HRMS (+ESI): 339.9336 (M +
839 H⁺). Calcd for C₉H₉N₃O₂SBrCl: 339.9337.

840

841 **4.2. Electrochemistry**

842 Voltammetric measurements were carried out with a potentiostat Autolab PGSTAT100 (ECO
843 Chemie, The Netherlands) controlled by GPES 4.09 software. Experiments were performed at

844 room temperature in a homemade airtight three-electrode cell connected to a vacuum/argon line.
845 The reference electrode consisted of a saturated calomel electrode (SCE) separated from the
846 solution by a bridge compartment. The counter electrode was a platinum wire of 1 cm² apparent
847 surface. The working electrode was GC microdisk (1.0 mm of diameter – Bio-logic SAS). The
848 supporting electrolyte (nBu₄N)[PF₆] (Fluka, 99% puriss electrochemical grade) and the solvent
849 DMSO (Sigma-Aldrich puriss p.a. dried <0.02% water) were used as received and simply degassed
850 under argon. The solutions used during the electrochemical studies were typically 10⁻³ M in
851 compound and 0.1 M in supporting electrolyte. Before each measurement, the solutions were
852 degassed by bubbling Ar and the working electrode was polished with a polishing machine (Presi
853 P230). Under these experimental conditions employed in this work, the half-wave potential (E_{1/2})
854 of the ferrocene Fc⁺/Fc couple in DMSO was E_{1/2} = 0.45 V vs SCE. Experimental peak potentials
855 have been measured versus SCE and converted to NHE by adding 0.241 V.

856

857 **4.3. Biology**

858 **4.3.1. Antileishmanial activity against *L. donovani* promastigotes**

859 *Leishmania* species used in this study were *L. donovani* (MHOM/IN/00/DEVI) purchased from
860 CNR Leishmania (Montpellier, France). *Leishmania* promastigotes forms were grown in
861 Schneider's Drosophila medium (Life Technologies, Saint-Aubin, France) supplemented with 100
862 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine and 20% FCS (Life Technologies,
863 Saint-Aubin, France) at 27 °C. The *in vitro* evaluation of the antileishmanial activity on
864 promastigote forms of the tested compound was carried out by an MTT assay according to the
865 Mosmann protocol [29] with some modifications. Briefly, promastigotes in log-phase were
866 incubated at an average density of 10⁶ parasites/mL in sterile 96-well plates with various
867 concentrations of compound dissolved in DMSO (final concentration less than 0.5% v/v), in
868 duplicate. Appropriate controls treated by DMSO, miltefosine, amphotericin B, fexinidazole and

869 doxorubicin (reference drugs purchased from Sigma-Aldrich, Saint-Louis, Missouri, USA) were
870 added to each set of experiments. After a 72h incubation period at 27 °C, parasitic metabolic
871 activity was determined. Each plate-well was then microscope-analyzed for detecting possible
872 precipitate formation. 20 µL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
873 bromide) (Sigma-Aldrich, Saint-Louis, Missouri, USA) solution (5 mg/mL in PBS) were added to
874 each well followed and incubated 4 h at 27 °C. The enzyme reaction was then stopped by addition
875 of 100 µL of 50% isopropanol – 10% sodium dodecyl sulfate. Plates were shaken vigorously at
876 300 rpm for 10 min. The absorbance was finally measured at 570 nm in a BIO-TEK Elx808
877 (Biotek, Colmar, France) absorbance microplate reader. Inhibitory concentration 50% (EC₅₀) was
878 defined as the concentration of drug required to inhibit by 50% the metabolic activity of
879 *Leishmanial* promastigotes forms compared to the control. EC₅₀ were calculated by non-linear
880 regression analysis processed dose-response curves, using TableCurve 2D V5.0 software. EC₅₀
881 values represent the mean value calculated from at least three independent experiments.

882

883 **4.3.2. Antileishmanial activity on *L. infantum* axenic amastigotes [29]**

884 *L. infantum* promastigotes (MHOM/MA/67/ITMAP-263, CNR Leishmania, Montpellier, France,
885 expressing luciferase activity) were cultivated in RPMI 1640 medium supplemented with 10%
886 foetal calf serum (FCS), 2 mM L-glutamine and antibiotics (100 U/mL penicillin and 100 µg/mL
887 streptomycin) and harvested in logarithmic phase of growth by centrifugation at 900 g for 10 min.
888 The supernatant was removed carefully and was replaced by the same volume of RPMI 1640
889 complete medium at pH 5.4 and incubated for 24 h at 24 °C. The acidified promastigotes were
890 then incubated for 24 h at 37 °C in a ventilated flask to transform promastigotes into axenic
891 amastigotes. The amastigote stage was checked both by electron microscopy (short flagellum with
892 small bulbous tip extending beyond a spherical cell body) and RT-PCR for confirming the
893 overexpression of ATG8 and amastin genes in amastigotes, compared to promastigotes. The
894 effects of the tested compounds on the growth of *L. infantum* axenic amastigotes were assessed as

895 follows. *L. infantum* amastigotes were incubated at a density of 2×10^6 parasites/mL in sterile 96-
896 well plates with various concentrations of compounds dissolved in DMSO (final concentration
897 less than 0.5% v/v), in duplicate. Appropriate controls DMSO, amphotericin B, miltefosine and
898 fexinidazole (reference drugs purchased from Sigma Aldrich) were added to each set of
899 experiments. After a 48 h incubation period at 37 °C, each plate-well was then microscopically-
900 examined to detect any precipitate formation. To estimate the luciferase activity of axenic
901 amastigotes, 80 µL of each well were transferred to white 96-well plates, Steady Glow® reagent
902 (Promega) was added according to manufacturer's instructions, and plates were incubated for 2
903 min. The luminescence was measured in Microbeta Luminescence Counter (PerkinElmer).
904 Efficient concentration 50% (EC₅₀) was defined as the concentration of drug required to inhibit by
905 50% the metabolic activity of *L. infantum* amastigotes compared to control. EC₅₀ values were
906 calculated by non-linear regression analysis processed on dose response curves, using TableCurve
907 2D V5 software. EC₅₀ values represent the mean of three independent experiments.

908

909 **4.3.3. Antileishmanial activity on *L. donovani* intracellular amastigotes**

910 **4.3.3.1. Cells culture**

911 The undifferentiated THP1 (ATCC) human monocyte cells were grown in RPMI 1640 medium
912 (Life Technologies, Saint-Aubin, France) supplemented with 10% FCS (Life Technologies, Saint-
913 Aubin, France), 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine at 37 °C,
914 5% CO₂. The culture was maintained between $3 \cdot 10^5$ and $1 \cdot 10^6$ cells/ml.

915 **4.3.3.2. Antileishmanial evaluation**

916 The *in vitro* evaluation of the antileishmanial activity on intracellular amastigotes forms of the
917 tested compound was assessed according to the da Luz *et al* protocol [30] with some modifications.
918 Briefly, 400 µL of THP-1 cells with Phorbol 12-Myristate 13-Acetate (final concentration 50
919 ng/ml) were seeded in sterile chamber-slides at an average density of 5×10^4 cells/ml and
920 incubated for 48 h at 37 °C, 5 µ CO₂. Leishmanial promastigotes forms were centrifuged at 3000

921 rpm for 10 min and the supernatant replaced by the same volume of Schneider 20% FCS pH 5.4
922 and incubated for 24 hours at 27 °C. Differentiated THP-1 cells were then infected by acidified
923 promastigotes with an infection ratio of ten parasites for one macrophage and incubated for 24
924 hours at 37 °C, 5% CO₂. Then, in duplicate, the medium containing various concentrations of
925 tested-compounds was added (final DMSO concentration being inferior to 0.5% v/v). Appropriate
926 control treated with or without solvent (DMSO), and various concentration of miltefosine,
927 Amphotericin B and Fexinidazole (reference drugs purchased from Sigma-Aldrich, Saint-Louis,
928 Missouri, USA) were added to each set of experiments. After 120 hours incubation at 37 °C, 5%
929 CO₂, well supernatant was removed. Cells were then fixed with analytical grade methanol and
930 stained with 10% Giemsa (Sigma-Aldrich, Saint-Louis, Missouri, USA). The percentage of
931 infected macrophages in each assay was determined microscopically by counting at least 200 cells
932 in each sample. IC₅₀ was defined as the concentration of drug necessary to produce 50% decrease
933 of infected macrophages compared to the control. IC₅₀ were calculated by non-linear regression
934 analysis processed on dose-response curves, using Table-Curve 2D V5.0 software. IC₅₀ values
935 represent the mean value calculated from at least three independent experiments.

936

937 **4.3.4. Antitrypanosomal evaluation on *T. b. brucei* BSF trypomastigotes**

938 The effects of the tested compounds on the growth of *T. b. brucei* were assessed by Alamar Blue[®]
939 assay described by Rüz *et al.* [31] *T. b. brucei* AnTat 1.9 (IMTA, Antwerpen, Belgium) was
940 cultured in MEM with Earle's salts, supplemented according to the protocol of Baltz *et al.* [32]
941 with the following modifications: 0.5 mM mercaptoethanol (Sigma Aldrich[®], France), 1.5 mM L-
942 cysteine (Sigma Aldrich[®]), 0.05 mM bathocuproïne sulfate (Sigma Aldrich[®]) and 20% heat-
943 inactivated horse serum (Gibco[®], France), at 37 °C and 5% CO₂. They were incubated at an
944 average density of 2000 parasites/100 µL in sterile 96-wells plates (Fisher[®], France) with various
945 concentrations of compounds dissolved in DMSO, in duplicate. Appropriate controls treated by
946 DMSO on sterile water, suramin, eflornithine and fexinidazole (reference drugs purchased from

947 Sigma Aldrich, France and Fluorochem, UK) were added to each set of experiments. After a 69 h
948 incubation period at 37 °C, 10 µL of the viability marker Alamar Blue® (Fisher, France) was then
949 added to each well, and the plates were incubated for 5 h. The plates were read in a ENSPIRE
950 microplate reader (PerkinElmer) using an excitation wavelength of 530 nm and an emission
951 wavelength of 590 nm. EC₅₀ was defined as the concentration of drug necessary to inhibit by 50%
952 the activity of *T. b. brucei* compared to the control. EC₅₀ were calculated by nonlinear regression
953 analysis processed on dose-response curves, using GraphPad Prism software (USA). EC₅₀ values
954 were calculated from three independent experiments.

955

956 **4.3.5. Antitrypanosomal activity on *T. brucei* NTR1 over-expressing strain.**

957 *Trypanosoma brucei* bloodstream-form 'single marker' S427 (T7RPOL TETR NEO) and drug-
958 resistant cell lines were cultured at 37 °C in HMI9-T medium [33] supplemented with 2.5 µg/mL
959 G418 to maintain expression of T7 RNA polymerase and the tetracycline repressor protein.
960 Bloodstream trypanosomes overexpressing the *T. brucei* nitroreductase (NTR1) [34] were grown
961 in medium supplemented with 2.5 µg/mL phleomycin and expression of NTR was induced by the
962 addition of 1 µg/mL tetracycline. Cultures were initiated with 1×10^5 cells/mL and sub-cultured
963 when cell densities approached $1-2 (\times 10^6)/\text{mL}$.

964 In order to examine the effects of inhibitors on the growth of these parasites, triplicate cultures
965 containing the inhibitor were seeded at 1×10^5 trypanosomes/mL. Cells overexpressing NTR were
966 induced with tetracycline 48 h prior to EC₅₀ analysis. Cell densities were determined after culture
967 for 72 h, as previously described [35]. EC₅₀ values were determined using the following two-
968 parameter equation by non-linear regression using GRAFIT:

969 where the experimental data were corrected for background cell density and expressed as a
970 percentage of the uninhibited control cell density.

971
$$y = \frac{100}{1 + \left(\frac{[I]}{EC_{50}} \right)^m}$$

972 In this equation, [I] represents inhibitor concentration and m is the slope factor.

973

974 **4.3.6. Cytotoxicity evaluation on HepG2 cell line**

975 HepG2 cell line was maintained at 37 °C, 6% CO₂ with 90% humidity in RPMI supplemented
976 with 10% foetal bovine serum, 1% L-glutamine (200 mM) and penicillin (100 U/mL)/streptomycin
977 (100 mg/mL) (complete RPMI medium). The evaluation of the tested molecules cytotoxicity on
978 the HepG2 (hepatocarcinoma cell line purchased from ATCC, ref HB-8065) cell line was
979 performed according to the method of Mosmann [36] with slight modifications. Briefly, 5 × 10³
980 cells in 100 mL of complete medium were inoculated into each well of 96-well plates and
981 incubated at 37 °C in a humidified 6% CO₂. After 24 h incubation, 100 mL of medium with various
982 product concentrations dissolved in DMSO (final concentration less than 0.5% v/v) were added
983 and the plates were incubated for 72 h at 37 °C. Triplicate assays were performed for each sample.
984 Each plate-well was then microscope-examined for detecting possible precipitate formation before
985 the medium was aspirated from the wells. 100 mL of MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-
986 diphenyl-2*H*-tetrazolium bromide) solution (0.5 mg/mL in medium without FCS) were then added
987 to each well. Cells were incubated for 2 h at 37 °C. After this time, the MTT solution was removed
988 and DMSO (100 mL) was added to dissolve the resulting blue formazan crystals. Plates were
989 shaken vigorously (700 rpm) for 10 min. The absorbance was measured at 570 nm with 630 nm
990 as reference wavelength using a BIO-TEK ELx808 Absorbance Microplate Reader. DMSO was
991 used as blank and doxorubicin (purchased from Sigma Aldrich) as positive control. Cell viability
992 was calculated as percentage of control (cells incubated without compound). The 50% cytotoxic
993 concentration (CC₅₀) was determined from the dose–response curve by using the TableCurve 2D

994 v.5.0 software (Jandel scientific). CC₅₀ values represent the mean value calculated from three
995 independent experiments.

996

997 **4.3.7. Cytotoxicity on THP-1 cell line**

998 The evaluation of the tested molecules cytotoxicity on the THP-1 cell line (acute monocytic
999 leukemia cell line purchased from ATCC, ref TIB-202) was performed according to the method
1000 of Mosman [36] with slight modifications. Briefly, cells in 100 µL of complete RPMI medium,
1001 were incubated at an average density of 5×10^4 cells/mL in sterile 96-well plates with various
1002 concentrations of compounds dissolved in DMSO (final concentration less than 0.5% v/v), in
1003 duplicate. The plates were incubated for 72 h at 37 °C. Each well plate was then microscope-
1004 examined for detecting possible precipitate formation before the medium was aspirated from the
1005 wells. 100 µL of MTT solution (0.5 mg/mL in medium without FCS) were then added to each
1006 well. Cells were incubated for 2 h at 37 °C. After this time, the MTT solution was removed and
1007 DMSO (100 µL) was added to dissolve the resulting blue formazan crystals. Plates were shaken
1008 vigorously (300 rpm) for 10 min. The absorbance was measured at 570 nm with 630 nm as
1009 reference wavelength spectrophotometer using a BIO-TEK ELx808 Absorbance Microplate
1010 Reader. DMSO was used as blank and doxorubicin (purchased from Sigma Aldrich) as positive
1011 control. Cell viability was calculated as percentage of control (cells incubated without compound).
1012 The 50% cytotoxic concentration (CC₅₀) was determined from the dose–response curve by using
1013 the TableCurve 2D v.5.0 software. CC₅₀ values represent the mean value calculated from three
1014 independent experiments.

1015

1016 **4.3.8. Plasma protein binding**

1017 Plasma doped with the tested compound is incubated at 37 °C in triplicate in one of the
1018 compartments of the insert, the other compartment containing a phosphate buffer solution at pH

1019 7.2. After stirring for 4 h at 300 rpm, a 25 μL aliquot of each compartment is taken and diluted;
1020 the dilution solution is adapted to obtain an identical matrix for all the compartments after dilution.
1021 In parallel, the reprocessing of a plasma doped but not incubated will allow to evaluate the recovery
1022 of the study. The LC-MS used for this study is a Waters[®] Acquity I-Class / Xevo TQD, equipped
1023 with a Waters[®] Acquity BEH C18 column, 50×2.1 mm, $1.7 \mu\text{M}$. The mobile phases are (A)
1024 ammonium acetate 10 mM and (B) acetonitrile with 0.1% formic acid. The injection volume is 1
1025 μL and the flow rate is $600 \mu\text{L}/\text{min}$. The chromatographic analysis, total duration of 4 min, is made
1026 with the following gradient: $0 < t < 0.2$ min, 2% (B); $0.2 < t < 2$ min, linear increase to 98% (B);
1027 $2 < t < 2.5$ min, 98% (B); $2.5 < t < 2.6$ min, linear decrease to 2% (B); $2.6 < t < 4$ min, 2% (B).
1028 Carbamazepine, oxazepam, warfarin and diclofenac are used as reference drugs and propranolol
1029 is used as internal standard. The unbound fraction (f_u) is calculated according to the following
1030 formula: $f_u = \frac{A_{\text{Plasma},4\text{h}} - A_{\text{PBS},4\text{h}}}{A_{\text{Plasma},4\text{h}}} \times 100$. The percentage of recovery is calculated according to the
1031 following formula:

$$1032 \quad \% \text{ Recovery} = \frac{(V_{\text{PBS}} \times A_{\text{PBS},4\text{h}}) + (V_{\text{Plasma}} \times A_{\text{Plasma},4\text{h}})}{(V_{\text{Plasma}} \times A_{\text{Plasma},0\text{h}})}$$

1033 Where A is the ratio of the area under peak of the studied molecule and the area under peak of the
1034 internal standard (propranolol 200 nM). V is the volume of solution present in the compartments
1035 ($V_{\text{PBS}} = 350 \mu\text{L}$ and $V_{\text{plasma}} = 200 \mu\text{L}$).

1036

1037 **4.3.9. Microsomal stability**

1038 The tested product and propranolol, used as reference, are incubated in duplicate (reaction volume
1039 of 0.5 mL) with female mouse microsomes (CD-1, 20 mg/mL, BD Gentest[™]) at 37 °C in a 50
1040 mM phosphate buffer, pH 7.4, in the presence of MgCl_2 (5 mM), NADP (1 mM), glucose-6-
1041 phosphate dehydrogenase (0.4 U/mL) and glucose-6-phosphate (5 mM). For the estimation of the
1042 intrinsic clearance: 50 μL aliquot at 0, 5, 10, 20, 30 and 40 min are collected and the reaction is

1043 stopped with 4 volumes of acetonitrile (ACN) containing the internal standard. After
1044 centrifugation at 10000 g, 10 min, 4 °C, the supernatants are kept at 4 °C for immediate analysis
1045 or placed at -80 °C in case of postponement of the analysis. Controls (t_0 and t_{final}) in triplicate are
1046 prepared by incubation of the internal standard with microsomes denatured by acetonitrile. The
1047 LC-MS used for this study is a Waters® Acquity I-Class / Xevo TQD, equipped with a Waters®
1048 Acquity BEH C18 column, 50 × 2.1 mm, 1.7 μm. The mobile phases are (A) ammonium acetate
1049 10 mM and (B) acetonitrile with 0.1% formic acid. The injection volume is 1 μL and the flow rate
1050 is 600 μL/min. The chromatographic analysis, total duration of 4 min, is made with the following
1051 gradient: 0 < t < 0.2 min, 2% (B); 0.2 < t < 2 min, linear increase to 98% (B); 2 < t < 2.5 min, 98%
1052 (B); 2.5 < t < 2.6 min, linear decrease to 2% (B); 2.6 < t < 4 min, 2% (B). 8-Bromo-6-chloro-3-
1053 nitro-2-(phenylsulfonylmethyl)imidazo[1,2-*a*]pyridine **1c** is used as internal standard. The
1054 quantification of each compound is obtained by converting the average of the ratios of the
1055 analyte/internal standard surfaces to the percentage of consumed product. The ratio of the control
1056 at t_0 corresponds to 0% of product consumed. The calculation of the half-life ($t_{1/2}$) of each
1057 compound in the presence of microsomes is done according to the equation: $t_{1/2} = \frac{\ln(2)}{k}$, where k is
1058 the first-order degradation constant (the slope of the logarithm of compound concentration versus
1059 incubation time). The intrinsic clearance *in vitro* (Cl_{int} expressed in μL/min/mg) is calculated
1060 according to the equation:

$$1061 \quad Cl_{\text{int}} = \frac{\text{dose}}{AUC_{\infty}} / [\text{microsomes}]$$

1062 Where dose is the initial concentration of product in the sample, AUC_{∞} is the area under the
1063 concentration-time curve extrapolated to infinity and [microsomes] is the microsome
1064 concentration expressed in mg/μL.

1065

1066 **4.3.10. Parallel artificial membrane permeability assay (PAMPA)**

1067 The Pampa-BBB experiments were conducted using the Pampa Explorer Kit (Pion Inc) according
1068 to manufacturer's protocol. Briefly, the stock compound solution (20 mM in DMSO) was diluted
1069 in Prisma HT buffer pH 7.4 (pION) to 100 μ M. 200 μ L of this solution (n = 6) was added to donor
1070 plate (P/N 110243). 5 μ L of the BBB-1 Lipid (P/N 110672) was used to coat the membrane filter
1071 of the acceptor plate (P/N 110243). 200 μ L of the Brain Sink Buffer (P/N 110674) was added to
1072 each well of the acceptor plate. The sandwich was incubated at room temperature for 4 h, without
1073 stirring. After the incubation the UV-visible spectra were measured with the microplate reader
1074 (Tecan infinite M200) and the permeability value (P_e) was calculated by the PAMPA Explorer
1075 software v.3.7 (pION). Corticosterone ($P_e = 130.3 \pm 7.1$ nm/s), and theophylline ($P_e = 4.7 \pm 0.6$
1076 nm/s) were used as high and low permeability standards, respectively. Each measure was
1077 performed in sixplicate.

1078

1079 **4.4. Thermodynamic solubility at pH 7.4 of compound 15**

1080 Thermodynamic solubility at pH 7.4 of compounds was determined according to
1081 a miniaturized shake-flask method (Organisation for Economic Cooperation and Development
1082 guideline n°105) [37]. Phosphate Buffer solutions (pH 7.4, 10 μ M, ionic strength 150 μ M) were
1083 prepared from Na_2HPO_4 , KH_2PO_4 and KCl (Sigma Aldrich, Saint Quentin Fallavier, France); 10
1084 μ L of 20 mM stock solution were added to 5 mL glass tube containing 990 mL buffer (n =
1085 4). Tubes were briefly sonicated and shaken by inversion during 24 h at room temperature. Then,
1086 tube contents were put in a microtube which was centrifuged at 12,225 g for 10 min; 100 μ L
1087 supernatant was mixed with 100 μ L acetonitrile in a Greiner UV microplate. Standard solutions
1088 were prepared extemporaneously diluting 20 mM DMSO stock solutions at 0, 2 and 5 mM; 5 μ L
1089 each working solution was diluted with 995 μ L buffer and 100 μ L was then mixed in microplate
1090 with 100 μ L acetonitrile to keep unchanged the final proportions of each solvent in standard
1091 solutions and samples. Determination of solubility at pH 7.4 was calculated from UV spectra (230
1092 to 450 nm) obtained using a Synergy 2 (Biotek, Colmar, France) microplate reader. The calibration

1093 curve was obtained from absorbance measures at 370 nm of the three standard solutions at 0, 10
1094 and 25 μ M in a 50:50 (vol/vol) mixture of buffer with acetonitrile/DMSO (99:1;
1095 vol/vol). Calibration curves were linear with $R^2 > 0.99$.

1096

1097 **4.5. Micronucleus assay method**

1098 **Cell line:** the micronucleus assay was performed on a Chinese Hamster Ovary cell line CHO-K1
1099 (ATCC, United States, ATCC[®] CCL-61, n^o6574112, Lot 12516, low passage number (<50).

1100 **Culture medium:** McCoy's 5A medium (PAN BIOTECH, Lot 4487521) supplemented with 1
1101 mM glutamine, 10 μ g/mL of a mixture of penicillin-streptomycin (PAN BIOTECH, Lot 784561)
1102 and 10% of inactivated calf serum (PAN BIOTECH, Lot P114526), pH 7.2, freshly prepared,
1103 stored no longer than 1 week.

1104 **Dilutions of test substances:** the test material was dissolved into DMSO. Due to its low solubility
1105 in the culture medium, the maximal soluble concentration to be tested was 0.5 mM.

1106 **Controls:** solvent control: PBS; Positive controls: mitomycin C (0.6 μ g/mL) and benzo[*a*]pyrene
1107 (5 μ g/mL), diluted in DMSO and stored at -80 °C.

1108 **Test procedure:** all the assays were conducted in duplicate. The CHO-K1 cells, suspended in Mac
1109 Coys'5A medium, were transferred into Labteck wells at a concentration of 100,000 cells/ml, and
1110 incubated for 24 h at 37°C in CO₂ (5%). When the test was performed without metabolic
1111 activation, the test substances were added into cell cultures at concentrations previously defined.
1112 A negative control containing culture medium, a solvent control containing 1% DMSO and a
1113 positive control containing 0.6 μ g/mL of mitomycin C were added. When the assay was performed
1114 in the presence of metabolic activation, S9 mix metabolizing mixture was added to cell cultures at
1115 a concentration of 10%. Then the test substances were added to the cell cultures at concentrations
1116 previously defined. A negative control containing culture medium, a solvent control containing
1117 1% DMSO and a positive control containing 5 μ g/ml of benzo[*a*]pyrene were added. After 3 hours
1118 of incubation at 37 °C in CO₂ (5%), the culture medium was removed, the cells were rinsed with

1119 phosphate buffered saline (PBS), and then returned to culture in McCoy's 5A medium containing
1120 3 µg/ml of cytochalasin B. After a 21-hour incubation period at 37°C, cells were rinsed with
1121 phosphate buffered saline (PBS), fixed with methanol and stained with 10% Giemsa for 20
1122 minutes.

1123 **Analysis of results:** the analysis of results was performed under a microscope at ×1000
1124 magnification. The antiproliferative activity of test substances was estimated by counting the
1125 number of binucleated cells relative to the number of mononucleated cells on a total of 500 cells
1126 for each dose (250 cells counted per well). The proliferation index (Cytokinesis Blocked
1127 Proliferative Index CBPI) was calculated using the following formula:

$$1128 \qquad \qquad \qquad \text{CBPI} = (2 \times BI + MONO)/500$$

1129 BI : number of binucleated cells

1130 $MONO$: number of mononucleated cells

1131 The cytostasis index (CI%), i.e. the percentage of cell replication inhibition, was calculated using
1132 the following formula:

$$1133 \qquad \qquad \qquad \text{CI\%}: 100 - \{100 \times (\text{CBPI}_{\text{test material}} - 1)/(\text{CBPI}_{\text{solvent control}} - 1)\}$$

1134 After this step, only the doses inducing a decrease of less than $55 \pm 5\%$ of CI% as compared to the
1135 negative control were taken into account for counting micronuclei. The rates of micronuclei were
1136 evaluated for the presence of independent nuclear core entities in 1000 binucleated cells per well,
1137 which corresponds to 2000 cells examined by test substance dose. Micronuclei were identified as
1138 small nuclei well differentiated from cell nucleus, stained in the same manner and having a
1139 diameter less than one third of that of the cell nucleus.

1140

1141 Micronuclei rates obtained for different doses of test substances were compared to the negative
1142 control by a χ^2 test. The assay was considered positive if:

1143 - A dose-response relationship was obtained between the rate of micronuclei and the doses tested,

1144 - At least one of these doses induced a statistically significant increase ($P < 0.05$) in the number of
1145 micronucleated cells as compared to the negative control.

1146

1147 **4.6. Comet assay**

1148 **4.6.1. Cell culture and treatment**

1149 The human hepatocarcinoma cell line HepG2 was obtained from the American Type Culture
1150 Collection (ATCC, ref. HB-8065). Cells were cultured in Eagle's Minimum Essential Medium
1151 (EMEM, ref. ATCC® 30-2003TM) supplemented with 10% heat-inactivated fetal bovine serum,
1152 100 U/mL penicillin and 0.1 mg/mL streptomycin (all from Gibco). Cells were maintained at 37
1153 °C in a humidified atmosphere with 5% CO₂. Cells were used in passage number 5-8.

1154 Compound 8 was tested at 3 concentrations (5, 10 and 20 µM) for 3 different times of incubation
1155 (2, 24 and 72 h). Briefly, HepG2 cells were seeded at 1.13×10^5 cells/mL in 6-well plates (3 mL
1156 of cell suspension per well) and incubated at 37 °C in a humidified atmosphere with 5% CO₂. After
1157 24, 72 and 94 h of incubation, cells were treated with different concentrations of the compound or
1158 the vehicle (0.5% dimethylsulfoxide, DMSO) for 72, 24 and 2 h respectively. Additionally, in the
1159 2 h treatment plate, cell in an additional well were treated with 1mM methyl methanesulfonate
1160 (MMS) as positive control for the comet assay.

1161 After treatment, medium was removed from the wells and cells were washed with phosphate
1162 buffered saline (PBS). Finally, cells were trypsinized and trypsin was neutralized with fresh
1163 medium. From this point, cells were kept ice-cold to avoid DNA repair.

1164 **4.6.2. Comet assay**

1165 The standard alkaline comet assay was employed for the detection of DNA strand breaks (SBs)
1166 and alkali-labile sites (ALS) in cell treated with compound 8. Trypsinized HepG2 cells were
1167 centrifuged at 125 g for 5 min at 4 °C and resuspended in cold PBS at 1×10^6 cells/mL. For the
1168 preparation of the agarose gels, 30 µL of cell suspension were mixed with 140 µL of 1 % low
1169 melting point agarose in PBS at 37 °C and 2 aliquots of 70 µL of cell/agarose mixture were placed

1170 on agarose-precoated microscope slides. Each droplet was covered with a 20 x 20 mm coverslip
1171 and after 2-3 min on a cold metal plate the coverslips were removed. Then, slides were immersed
1172 in lysis solution (2.5 M NaCl, 0.1 M Na₂EDTA, 0.01 M Tris base, pH 10 and 1% Triton X-100)
1173 at 4 °C for 1 h. After lysis, slides were transferred to the electrophoresis tank and incubated for 40
1174 min at 4 °C in the electrophoresis solution (0.3 M NaOH, 1 mM Na₂EDTA, pH > 13) to allow
1175 DNA unwinding. After that, electrophoresis was carried out at 1.2 V/cm for 20 min (4 °C). Then,
1176 gels were neutralized and washed by immersing the slides in PBS for 10 min and distilled water
1177 for another 10 min (both at 4 °C). Gels were then air dried at room temperature.

1178 Comets were stained by adding 30 µL of 1 µg/mL of 4,6-diamidino-2-phenylindole (DAPI) on top
1179 of each gel and placing 22 x 22 mm coverslips on top. Slides were incubated with DAPI at room
1180 temperature for 30 mins before the analysis. The semi-automated image analysis system Comet
1181 Assay IV (Instem) was used to evaluate 50 comets per gel (100/condition). The percentage of
1182 DNA in tail was the descriptor used for each comet.

1183 **4.6.3. Statistics**

1184 The median percentage of DNA in tail for 50 comets was calculated for each of the duplicate gels
1185 in each experiment and the mean of the two medians was then calculated. The mean percentage of
1186 DNA in tail of 3 independent experiments and the standard deviation (SD) were calculated.

1187

1188 **4.7. *In vivo* analysis**

1189 Female Swiss mice of 8 weeks (weight 30-32 g) are used. Mice were housed and procedures were
1190 conducted in agreement with european directive 2010/63/EU on animals used for scientific
1191 purposes applied in France as the 'Décret n°2012-118 du 1er février 2013 relatif à la protection des
1192 animaux utilisés à des fins scientifiques'. Accordingly, the present project was APAFIS#19730-
1193 2019031215178087 v1 authorized by the 'Ministère de l'Education Nationale, de l'Enseignement
1194 Supérieur et de la Recherche'.

1195 The determination of maximal tolerated dose used one group of 4 mice which received an oral
1196 administration of **8** at 100 mg/kg. **8** was prepared as a suspension comprising 5% Tween 80/ 95%
1197 carboxymethylcellulose 0.5% in water.

1198 Observations of side effects were codified. The same protocol was used with repeated dose during
1199 5 days.

1200 **4.7.1. *In vivo* pharmacokinetics parameters**

1201 **Chemicals:** The internal standard (IS), ornidazole, was obtained from Sigma Aldrich. LC-MS
1202 Optima grade acetonitrile (ACN) and methanol (MeOH), acetic acid and formic acid (FA) were
1203 purchased from Fisher Scientific. Ready-to-use QuEChERS salts (6 g MgSO₄/1.5 g NaCl/1.5 g
1204 sodium citrate dihydrate/750 mg sodium citrate sesquihydrate) were supplied by VWR.

1205 **Sample preparation:** samples were stored at -20 °C until extraction. 200 µL of ACN containing
1206 ornidazole (internal standard; IS) at a concentration of 62.5 ng/mL were added to 100 µL blood
1207 samples. The mixture was vortexed during 30 sec. After 10 min, 40 mg of QuEChERS salts were
1208 added. Samples were briefly vortexed and centrifuged at 16,000 g for 10 min. Ten microliters of
1209 the upper layer was directly transferred in an injection vial before being diluted (1/10; v/v) in a 0.1
1210 % formic acid in water. Finally, 5 µL were injected in the LC-MS-MS system. Calibrations
1211 standards (9 levels, from 5 to 1,000 ng/mL) and quality controls (QC) (10, 75 and 625 ng/mL)
1212 were obtained by adding appropriate 20 × working standard solutions in blank whole blood.

1213 **LC-MS/MS conditions:** the chromatographic system consisted in two Shimadzu LC-30 AD
1214 pumps (NexeraX2), a CTO 20AC oven, and a SIL-30AC autosampler (Shimadzu, Marne-la-
1215 Vallée, France). Chromatographic separation was performed using a EC-C8 column (Poroshell
1216 120, 2.1 mm × 75 mm, 2.7 µM; Agilent) at a flow rate of 0.25 mL/min using a gradient of 0.1%
1217 acetic acid in water (A) and 0.1% acetic acid in MeOH/ACN 50:50 (B) programmed as follows:
1218 0.0–0.1, 20% (B); 0.1–1.0, 20 to 70% (B); 1.0–4.0, 70% to 100% (B); 4.0–5.5, 100% (B); 5.5–6.0,

1219 100 to 20% (B); and 6.0–8.0 column equilibration with 20% (B). Oven temperature was set at 60
1220 °C.

1221 A Shimadzu 8060 triple quadrupole mass spectrometer was used in the positive electrospray
1222 ionization mode. The main common parameter settings were as follows: interface voltage, 1.5 kV;
1223 nebulizing gas flow, 3 L/min; heating gas flow, 10 L/min; interface temperature, 300 °C;
1224 desolvation line (DL) temperature, 250 °C; heat block temperature, 400 °C; and drying gas flow,
1225 10 L/min. All parameters (collision energy, Q1/Q3 pre-bias) were optimized from standard flow
1226 injection analysis. Dwell time was set at 100 ms per transition.

1227 **Validation procedure for whole blood:** validation protocol and the set of acceptance criteria
1228 were as follows:

1229 - Linearity: Calibration curve was generated by plotting the peak area ratios (analyte/internal
1230 standard) vs the expected concentration. Linearity of the calibration curve was evaluated by a
1231 quadratic regression analysis using a $1/x^2$ weighting. A value greater than 0.99 was expected for
1232 the coefficient of determination (r^2).

1233 - Precision and accuracy of the method were assessed at lower limit of quantitation (LLOQ; 5
1234 ng/mL) and at the three quality control concentrations (10, 75 and 625 ng/mL). Precision is
1235 calculated as the coefficient of variation (CV%) within a single run (intra-assay; $n = 5$) and
1236 between different assays (inter-assay; $n = 5$), and accuracy is the percentage of deviation between
1237 nominal and found concentration with the established calibration curve. Acceptance criteria were
1238 intra-assay and inter-assay precision (CV%) and an accuracy (bias) less than 20%.

1239 - The lower limit of quantification (LLOQ) was estimated to be the minimal concentration with
1240 accuracy and precision within $\pm 20\%$. The lower limit of detection (LLOD) was calculated based
1241 on a signal-to-noise ratio >3 .

1242 - Extraction recoveries were determined by comparing the LLOQ and the quality controls samples
1243 (n = 5) with their extracted blank whole blood counterparts spiked at the correct concentration
1244 after extraction (n = 3). CV% in the extraction recovery had to be less than 20%.

1245 - The effect of dilution was investigated on samples spiked at MQC and HQC then analyzed after
1246 1.25-, two- and four-fold dilutions and on samples spiked at 150% of ULOQ (1500 ng/ml) for a
1247 two-fold dilution. Precision CV and bias were set less than 25% to successfully validate.

1248 - The absence of carryover was checked by injecting blank samples just after the analysis of the
1249 most concentrated sample (1,000 ng/mL).

1250 **Pharmacokinetics:** the same samples used for the validation were used for the analysis. Monolix
1251 Lixoft software was used to analyze data by noncompartmental model to fit pharmacokinetics
1252 parameters.

1253

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