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Original article

1-Aryl-4-nitro-1*H*-imidazoles, a new promising series for the treatment of human African trypanosomiasis

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ABSTRACT

Nitroimidazoles are a well-known class of antibacterial and antiprotozoal drugs but in spite of the widespread clinical and veterinary use of these drugs, this family has been stigmatized in part due to associated genotoxicity problems. Here we report the synthesis, the anti-trypanosomal activity and a structure–activity relationship (SAR) study of a series of about fifty 1-aryl-4-nitro-1*H*-imidazoles, with an emphasis on selected *in vivo* active molecules. Compounds 4-nitro-1-{4-(trifluoromethoxy)phenyl}-1*H*-imidazole and 1-(3,4-dichlorophenyl)-4-nitro-1*H*-imidazole are curative in mouse models of both acute and chronic African trypanosomiasis when given orally at doses of 25–50 mg/kg for 4 days for the acute infection, and 50–100 mg/kg (bid) for 5 days in the chronic model. While both compounds are bacterial mutagens, activity is lost in strains lacking bacterial specific nitro-reductases. Mammalian nitro-reductases do not reduce nitroaromatic compounds with low redox potentials with same avidity as their bacterial counterparts and these compounds were shown to be devoid of genotoxicity in mammalian cells. Both compounds are promising leads for the treatment of human African trypanosomiasis (HAT or sleeping sickness), including the fatal stage 2 of the disease, for which new treatments are urgently needed.

1. Introduction

Classified among the most neglected diseases, human African trypanosomiasis (HAT), also known as sleeping sickness and caused by the two pathogenic parasite subspecies *Trypanosoma brucei rhodesiense* and *T. b. gambiense*, is a major health problem in sub-Saharan Africa [1,2]. Treatment of HAT is difficult [3], especially in its advanced fatal stage when the parasites have crossed the blood–brain barrier (BBB) for which only two drugs are currently registered and neither is adequate. Melarsoprol, an old arsenical drug in use for the treatment of HAT since 1949, is toxic (5–10% associated risk of mortality due to drug induced encephalopathy) and requires painful intravenous injections. Eflornithine, originally developed as an anticancer drug and registered for the treatment of HAT in 1981, has a narrow therapeutic window and requires 14 days of 6-hourly slow infusions, which is an impractical regimen for many patients in the countries where the disease is prevalent. Recently, a simplified co-administration of oral Nifurtimox and intravenous Eflornithine (NECT) has proven good safety and efficacy and provides an improved first line treatment for stage 2 HAT, although as above it remains a severe challenge to implement in remote and rural settings where HAT is endemic [4,5]. With a renewed interest in neglected diseases, a new drug candidate has also emerged in recent years: Fexinidazole is today in phase I clinical development with potential for the advanced stage of HAT [6,7]. Despite this progress, and taking into account the inevitable attrition rates in drug development, there is still a long way to go before an improved treatment could become available for patients.

Nitroimidazoles are a well-known family of antibacterial and antiprozoal drugs [8], including anti-trypanosomal drugs or compounds with known anti-trypanosomal activity [9–11]. Metronidazole, the first drug to be introduced for this purpose and probably also the best-known drug in this class, has been in use for more than 50 years [12]. Other well-known examples include

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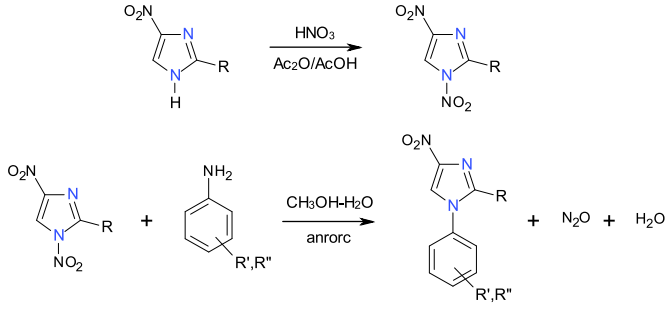
Tinidazole [13] closely related to Metronidazole and the anti-trypanosomal drug Benznidazole indicated for the treatment of Chagas disease [14,15]. In spite of extensive use, this class of compounds has often been stigmatized for reasons of perceived genotoxic risks associated with the nitroaromatic group [16]. A well-known case here is the anti-trypanosomal drug candidate Megazol [10,11] which was abandoned because of clear mammalian cell genotoxicity [17,18]. In recent years however, several new nitroimidazole drug candidates have emerged, for instance PA-824 [19] and OPC-67683 [20], both in clinical development for the treatment of tuberculosis, and Fexinidazole for HAT [6]. In each of these cases, a detailed analysis of the genotoxic properties of the compounds concluded that they did not pose a genotoxic risk to humans.

One electron reduction potentials of nitroimidazoles as well as other nitroazoles mainly depend on the position of the nitro group on the azole ring and for nitroimidazoles generally increase in the following order 4-NO_2 < 5-NO_2 < 2-NO_2 [21,22]. It is believed that generally genotoxicity problems increase in a similar order. Examples of non-genotoxic anti-infective 4-nitroimidazoles have been described [19,20], but while 1-alkyl derivatives have been largely explored, few 1-aryl-4-nitro-1*H*-imidazoles have been studied due to serious synthetic limitations. We have developed a general method which allowed the synthesis of several 1-aryl-4-nitro-1*H*-imidazoles from a very simple coupling reaction between 1,4-dinitro-1*H*-imidazole and the corresponding anilines [23]. Several compounds in this series have shown anti-tuberculosis activity [24,25]. Here we report the anti-trypanosomal activity and a structure–activity relationship (SAR) study, with an emphasis on selected *in vivo* active molecules, with evidence for lack of mammalian cell genotoxicity. Several 1-aryl-4-nitro-1*H*-pyrazoles [26] were also prepared but proved inactive and are not included in the present paper.

2. Results and discussion

2.1. Chemistry

This work has further validated and demonstrated the wide applicability of the coupling reaction between 1,4-dinitro-1*H*-imidazoles and anilines for the synthesis of 1-aryl-4-nitro-1*H*-imidazoles: the forty-three 1-aryl-4-nitro-1*H*-imidazoles and six 1-aryl-2-methyl-4-nitro-1*H*-imidazoles of the present study were obtained by this method in relatively good yields (40–90%), with substituents on the benzene ring ranging from strong electron-withdrawing (e.g. NO_2, CO_2R) to electron-donating groups (e.g. Me, MeO) (Scheme 1). For a detailed description of the method, see [23,25]. The mechanism of the reaction involves a degenerated ring transformation reaction also called *anrorc* reaction (***a**ddition of **n**ucleophile*, ***r**ing **o**pening*, ***r**ing closure*); on the *anrorc* reaction, see [27] and references cited herein. The reaction is generally performed at room temperature though a higher temperature is often required for completion of the reaction. Water and gaseous nitrogen(I) oxide are the single easy to separate by-products; the so called 'atom economy' of this reaction [28], describing the conversion efficiency of a chemical process in terms of all atoms involved, exceeds 80%. Alternative approaches exist, nevertheless none of them competes with the present *anrorc* coupling reaction between 1,4-dinitro-1*H*-imidazoles and anilines. Nitration of 1-phenyl-1*H*-imidazole is not selective, compounds from nitration of both aromatic rings are usually obtained [29], and to our knowledge, selective reduction of the resulting dinitro- or trinitro-compounds has not been reported yet. 1-Arylation of 4(5)-nitro-1*H*-imidazole anions with 1-fluoro(or chloro)-2(or/and 4)-nitrobenzenes is limited to these substrates only [24] (and references cited herein). Finally, attempts to replace the 1-nitro substituent in 1,4-dinitro-1*H*-imidazoles by another electron withdrawing group (e.g. −CN, $-SO_2Ar$, $-SO_2NR_2$) were either unsuccessful [30] or only partly successful [31,32]. A similar degenerated ring transformation process mechanism was observed in these cases but the reaction was generally not clean, not reproducible and yields of the desired 1-aryl-4-nitro-1*H*-imidazoles were usually low. Only a few known compounds have been prepared following this approach [30–32].



Scheme 1. Synthesis of 1-aryl-4-nitro-1*H*-imidazoles. R = H, CH_3; R′, R″ = H, electron donating or electron withdrawing group, one or two substituents in *ortho*, *meta* or/and *para* positions.

2.2. Anti-trypanosomal activity

An overview of the *in vitro* assays is shown in Table 1. Of the 43 1-aryl-4-nitro-1*H*-imidazoles evaluated, 28 compounds showed anti-trypanosomal activity with good selectivity against *T. b. rhodesiense* (STIB900) with an IC_{50} in the micromolar range or below 1 μM for 15 compounds. Potent activity was observed for two compounds in particular, 4-nitro-1-{4-(trifluoromethoxy)phenyl}-1*H*-imidazole (**16**) and 1-(3,4-dichlorophenyl)-4-nitro-1*H*-imidazole (**31**) (IC_{50} 0.16 and 0.10 μM, respectively), comparable to the activity known for Megazol (IC_{50} 0.10 μM). Several other compounds showed similar activity as compared to the new drug candidate Fexinidazole (IC_{50} 2.57 μM). A number of analogs with a methyl substituent in position C2 on the imidazole ring (1-aryl-2-methyl-4-nitro-1*H*-imidazoles) did not show activity; similarly none of the pyrazole analogs showed any activity (results not shown).

From these *in vitro* data, the following structure/anti-trypanosomal activity relationships can be drawn (Scheme 2): 1-good activity is observed with the presence of an electron withdrawing substituent in the meta or para position at the phenyl group, as compared to the parent compound namely 4-nitro-1-phenyl-1*H*-imidazole (**1**); the activity is about the same with F, Cl, Br or NO_2. In contrast, except in a few exceptions, the activity is decreased or lost with an electron donating substituent in the *meta* and *para* positions. 2-The activity is preserved with the addition of a second substituent in the para or other meta-position, independently on the nature of the substituent (electron withdrawing or electron donating group). 3-No to low activity is observed with a substituent present in the ortho position or the addition of a methyl substituent on the imidazole ring. A possible explanation for the loss of activity in the two latter cases lies in the lack of co-planarity of the two aromatic rings in those compounds; other derivatives can be almost planar (from quantum chemical calculations and x-ray measurements). 4-No to low activity is observed with a carboxyl ester substituent though we could expect some activity due to the electron withdrawing character of the substituent.

The two potent compounds 4-nitro-1-{4-(trifluoromethoxy) phenyl}-1*H*-imidazole (**16**) and 1-(3,4-dichlorophenyl)-4-nitro-1*H*-imidazole (**31**) were further evaluated in mouse models of HAT. In an acute infection model, both compounds were shown to be

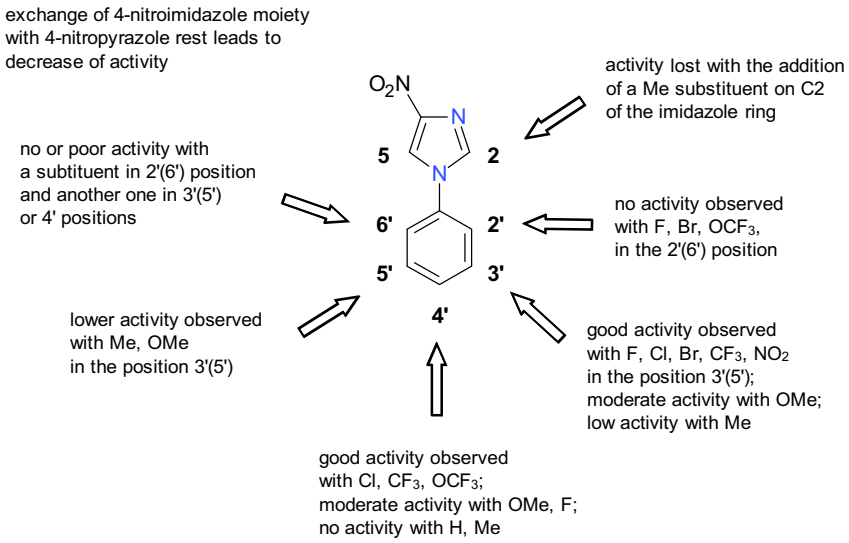
Table 1
IC50s of 1-aryl-4-nitro-1*H*-imidazoles and reference molecules against *T. b. rhodesiense* (STIB900) and in L-6 rat myoblast cells.

Compound No	Subtituents	MW	*T. b. rhodesiense* (STIB900) [μg/mL] [a]	*T. b. rhodesiense* (STIB900) [μM] [a]	Cytotoxicity (L-6 rat myoblast cells) [μg/mL]	Reference to synthesis
(***I***) *1-ary1-4-nitro-1H-imidazoles monosubtituted on the benzene ring*						
1	H	189.17	0.85	4.49	>90	23
2	2-F	207.16	0.59	2.85	>90	25
3	3-F	207.16	0.15	0.72	>90	25
4	4-F	207.16	0.52	2.53	>90	25
5	2-Cl	223.62	n.a.	n.a.	no data	25
6	3-Cl	223.62	0.16	0.72	>90	23, 25
7	4-Cl	223.62	0.16	0.72	35.68	23
8	2-Br	268.07	n.a.	n.a.	no data	[b]
9	3-Br	268.07	0.24	0.90	>90	[b]
10	4-Br	268.07	0.26	0.97	17.88	24
11	3-NO_2	234.17	0.18	0.77	>90	[b]
12	3-CF_3	257.17	0.37	1.45	>90	[b]
13	4-CF_3	257.17	0.18	0.70	>90	[b]
14	2-CF_3O	273.17	5.67	20.76	>90	[b]
15	3-CF_3O	273.17	0.48	1.76	>90	[b]
16	4-CF_3O	273.17	0.04	0.16	>90	[b]
17	3-Me	203.20	0.37	1.82	>90	23
18	4-Me	203.20	2.43	11.96	>90	23
19	3-MeO	219.20	0.42	1.92	>90	[b]
20	4-MeO	219.20	0.70	3.19	66.80	23,24
21	4-CO_2Bu	289.29	8.11	28.03	>90	[b]
(***II***) *1-aryl-4-nitro-1H-imidazoles disubstituted on the benzene ring*						
22	2,3-diF	225.15	0.49	2.18	>90	25
23	2,4-diF	225.15	n.a.	n.a.	no data	25
24	2,5-diF	225.15	0.37	1.64	>90	25
25	2,6-diF	225.15	n.a.	n.a.	no data	25
26	3,4-diF	225.15	0.17	0.76	>90	25
27	3,5-diF	225.15	0.11	0.49	>90	25
28	2,3-diCl	258.06	0.49	1.90	38.66	25
29	2,4-diCl	258.06	n.a.	n.a.	no data	25
30	2,5-diCl	258.06	n.a.	n.a.	no data	25
31	3,4-diCl	258.06	0.03	0.10	>90	25
32	3,5-diCl	258.06	0.18	0.70	40.55	25
33	3-Cl-4-F	241.61	0.17	0.70	>90	[b]
34	2-Br-4-Me	282.10	n.a.	n.a.	no data	[b]
35	3-Me-4-Br	282.10	0.28	0.99	15.54	[b]
36	3-Br-4-Me	282.10	0.56	1.99	>90	[b]
37	2,4-diMe	217.23	n.a.	n.a.	no data	[b]
38	3,4-diMe	217.23	0.46	2.12	>90	[b]
39	2,4-diMeO	249.20	n.a.	n.a.	no data	[b]
40	3,4-diMeO	249.20	n.a.	n.a.	no data	[b]
41	3,4-O_2CH_2	233.18	0.22	0.94	>90	[b]
42	3-CO_2Bu-4-Cl	323.74	45.75	141.32	>90	[b]
43	3-CO_2H-4-Cl	267.63	25.57	95.54	>90	[b]
(***III***) *1-aryl-2-methyl-4-nitro-1H-imidazoles mono or disubstituted on the benzene ring*						
44	4-CF_3O	287.20	3.42	11.89	>90	[b]
45	4-CF_3	271.20	3.06	11.28	>90	[b]
46	2-Br-4-Me	296.12	36.9	124.6	>90	[b]
47	3-Cl-4-F	255.64	6.91	27.03	>90	[b]
48	4-Br-3-Me	296.12	5.97	20.16	>90	[b]
49	3-Br-4-Me	296.12	6.97	23.54	>90	[b]
Reference molecules						
Fexinidazole			0.72	2.57	>90	–
Megazol			0.02	0.10	57	–
Melarsoprol			0.004	0.009	1.3	–
Eflornithine			0.90	3.80	12	–

[a] n.a. is for not active.
[b] Prepared for the present study, synthetic details are provided in the experimental part.

curative with a 100% cure rate at an oral dose of 25 mg/kg/day **16** or 50 mg/kg/day **31** administered for 4 days (Table 2). In the stage 2 HAT infection model involving brain infection (also known as the "chronic CNS model"), 100% cure was achieved at an oral dose of 50 mg/kg **16** or 100 mg/kg **31** administered twice a day (bid) for five days, while a daily oral dose of 100 mg/kg administered for five days was partially curative (Table 3). Very few compounds are known to cure this established chronic CNS model except some arsenicals and selected experimental diamidines [33], and the relatively low curative dose of 50 mg/kg/day given bid over 5 days is quite remarkable. In fact the curative capacity of these compounds is comparable or even slightly better than Fexinidazole, also a nitroimidazole, currently in phase I clinical development for HAT [6]. The lowest curative dose for Fexinidazole in the late stage model is 5 × 100 (bid) mg/kg/day.

Interestingly, in this series compound **14** showed *in vitro* activity against *Trypanosoma cruzi*, the causative agent of Chagas disease; compound **33** showed *in vitro* activity against both *trypanosoma*



Scheme 2. Qualitative structure antiparasitic activity relationship (SAR) analysis of substituted 1-aryl-4-nitro-1*H*-imidazoles.

Table 2
Efficacy of selected 1-aryl-4-nitro-1*H*-imidazoles in the treatment of experimental acute infections with *T. b. rhodesiense* (STIB900) in mice.

Compound	Dose (days × mg/kg)	Route[a]	Cured/infected	Mean survival days (MSD)
Control[b]	–	–	0/4	8
31	4 × 25	p.o	0/4	27.75
31	4 × 50	p.o	4/4	>60
16	4 × 25	p.o	4/4	>60
16	4 × 50	p.o	4/4	>60

[a] p.o. = oral application.
[b] Negative control: mice were infected but not treated.

species (*Trypanosoma brucei rhodesiense* and *Trypanosoma cruzi*) (IC_{50} against *T. cruzi* respectively 2.7 μM and 7.0 μM). Both compounds were further evaluated in animal models for Chagas disease; they are described here in relation to their genotoxicity profile only (see further below).

Table 3
Efficacy of selected 1-aryl-4-nitro-1*H*-imidazoles in the treatment of experimental chronic CNS infection with *T. b. brucei* (GVR35) in mice.

Study	Compound	Dose (days × mg/kg)	Route[a]	Cured/infected	Mean survival days (MSD)
1	Control[b]	1 × 40	i.p.	0/5	55.8
	31	5 × 50	p.o.	1/5	>120.4
	31	5 × 100	p.o.	2/5	>149.2
	16	5 × 50	p.o.	0/5	95.2
	16	5 × 100	p.o.	1/5	>156.8
2	Control[b]	1 × 40	i.p.	0/5	57.2
	31	5 × (50 bid)	p.o.	3/4	>155
	31	5 × (100 bid)	p.o.	5/5	>180
	16	5 × (50 bid)	p.o.	5/5	>180
	16	5 × (100 bid)	p.o.	3/3	>180

[a] i.p. = intraperitoneal application; p.o. = oral application. bid: twice per day at a 8 h interval.
[b] Negative control: mice were infected and treated on day 21 with a single dose of diminazene aceturate (see further below paragraph 4.2.3).

2.3. Genotoxicity profile of four selected active compounds

2.3.1. Bacterial mutagenicity—Ames test

Bacterial mutagenicity of the four compounds **14**, **16**, **31** and **33** was assessed in the standard *Salmonella* tester strains recommended for mutagenicity screening by international guidelines for

Table 4
Ames study.

Lowest concentration at which mutagenic effect is observed (μg/plate)[a]	Compound No			
	31	**16**	**33**	**14**
TA98, *detects frameshift mutations*				
TA98 −S-9	100	N	20	N
TA98NR −S-9	N	N	N	N
TA98 +S-9	100	N	100	N
TA98NR+S-9	N	N	N	N
TA100, *detects base-pair substitutions*				
TA100 −S-9	100	350	20	700
TA100NR −S-9	N	N	N	N
TA100 +S-9	20	350	20	700
TA100NR +S-9	N	N	N	N
TA102, *detects base-pair substitutions*				
TA102 −S-9	N*	N*	N	N
TA102NR −S-9	Not treated	Not treated	Not treated	Not treated
TA102 +S-9	N*	N*	N	N
TA102NR +S-9	Not treated	Not treated	Not treated	Not treated
TA1535, *detects base-pair substitutions*				
TA1535−S-9	N	N	N	N
TA1535NR −S-9	Not treated	Not treated	Not treated	Not treated
TA1535 +S-9	N	N	N	N
TA1535NR +S-9	Not treated	Not treated	Not treated	Not treated
TA1537, *detects frameshift mutations*				
TA1537 −S-9	100	N	N	N
YG7167 (NR) −S-9	350	Not treated	Not treated	Not treated
TA1537 +S-9	N	N	N	N
YG7167 (NR) +S-9	Not treated	N	Not treated	Not treated

* Statistically significant increases in revertant numbers were observed at one or more concentrations although as they were not concentration related and were of a small magnitude, they were not considered to be indicative of mutagenic activity.
All positive controls used gave the expected significant increases in revertant colonies.
[a] 'N' for 'negative', meaning there no mutagenic activity was observed at any dose tested (0.16, 0.8, 4, 20, 100, 350, 700 and 5000 μg/plate).

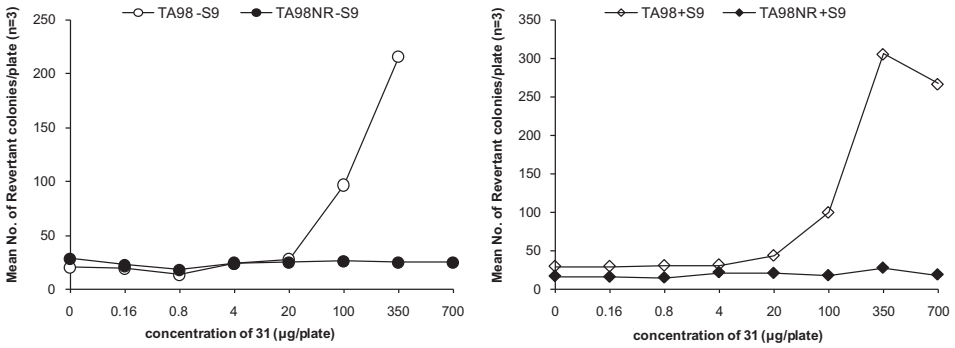


Fig. 1. Mutagenic activity of compound **31** in the Ames test using *Salmonella typhymurium* strain TA98 and its nitro-reductase-deficient variant TA98NR, in the presence and absence of metabolic activation (+/− S9).

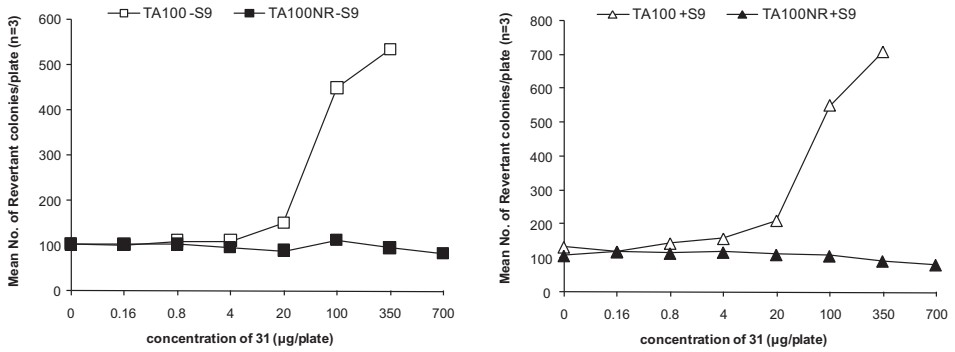


Fig. 2. Mutagenic activity of compound **31** in the Ames test using *Salmonella typhymurium* strain TA100 and its nitro-reductase-deficient variant TA100NR, in the presence and absence of metabolic activation (+/− S9).

genotoxicity assessment: TA1535, TA100 and TA102, to detect base substitution point mutations and TA1537 and TA98 to detect frameshift point mutations. In addition, the assays were repeated in the corresponding strains lacking one or both of the bacterial nitro-reductase genes (NR-deficient strains). By checking the difference in response between the normal Ames strains and its corresponding NR-deficient counterpart, the contribution of bacterial nitro-reduction to the observed mutagenicity can be estimated.

A summary of the Ames tests results is shown in Table 4; as an example, dose responses for compound **31** in two tester strains are shown in Fig. 1 and Fig. 2.

All four test compounds induced mutations in one or more of the standard tester strains used. However, in each case, mutagenicity was abolished in the corresponding nitro-reductase deficient strains, with the exception of compound **31** in TA1537, where mutagenic activity was reduced but not eliminated by the removal of the classical nitro-reductase in strain YG7167 (TA1537NR) (Table 4). This provides strong evidence that the bacterial mutagenicity observed for **16**, **14** and **33** is due entirely to the action of bacterial specific nitro-reductases. Mutagenicity induced by **31** is also due, at least in part to bacterial nitro-reduction, as confirmed by the lack of activity in TA98NR, which lacks both the standard nitro-reductase and a supplementary nitro-reductase [34]. Frameshift mutagenicity induced by **31** in strain TA1537 is influenced by the classical nitro-reductase but the residual activity seen in its sister strain lacking this nitro-reductase may be due to the action of the supplementary nitro-reductase, which is active in this strain.

2.3.2. *In vitro micronucleus test*

Each test compound was also screened in the human peripheral lymphocyte micronucleus test that detects chromosomal damage and aneugenicity. Compound **16** did not induce significant increases in micronuclei in human peripheral lymphocytes under the conditions of these assays, at concentrations that induced 57% cytotoxicity or less. The positive controls did induce statistically significant increases in the proportion of cells with micronuclei. Full data for **16** is shown in Table 5A–C.

Preliminary data for the other three compounds in this series are shown in Tables 6–8. Compound **31** showed no activity at

Table 5
In vitro micronucleus test of compound **16**.

Test concentration µg/mL	Cytotoxicity (%)	Mean MNBN[a] (%)
A) −S9, 3 h exposure, 21 h recovery		
Vehicle	–	0.60
120.0	0	0.15
140.0	0	0.30
160.0	0	0.40
MMC[b], 0.08	ND	11.25[c]
B) −S9, 24 h exposure, no recovery		
Vehicle	–	0.50
12.5	10	0.55
22.5	40	0.50
30.0	57	0.40
VIN[d], 0.03	ND	6.06[c]
C) +S9, 3 h exposure, 21 h recovery		
Vehicle	–	0.40
80.0	0	0.05
100.0	0	0.20
120.0	0	0.65
CPA[e], 12.5	ND	2.45[c]

[a] 2000 cells scored for the vehicle control and 1000 cells scored for the test compound groups and the positive control.
[b] Mitomycin C (MMC), positive control.
[c] Statistically significant $p < 0.001$. MNBN = micronucleated binucleate cells, ND = not done.
[d] Vinblastine (VIN), positive control.
[e] Cyclophosphamide (CPA), positive control that requires metabolic activation by S9.

Table 6
In vitro micronucleus test of compound **31**.

Test concentration μg/mL	Cytotoxicity (%)	Mean MNBN[a] (%)
A) −S9, 20 h exposure, 28 h recovery		
Vehicle	–	0.3
343.6	3	0.3
536.9	62	0.7
2048	60	0.1
4NQO[b], 2.5	ND	4.8[c]
B) +S9, 3 h exposure, 45 h recovery		
Vehicle	–	0.5
100.0	5	0.4
200.0	20	0.3
250.0	56	0.3
CPA[d], 6.25	ND	7.8[c]

[a] 2000 cells scored for the vehicle control and 1000 cells scored for the test compound groups and the positive control.
[b] 4-Nitroquinoline-*N*-oxide (4NQO), positive control.
[c] Statistically significant $p < 0.001$. MNBN = micronucleated binucleate cells, ND = not done.
[d] Cyclophosphamide (CPA), positive control.

Table 8
In vitro micronucleus test of compound **33**.

Test concentration μg/mL	Cytotoxicity (%)	Mean MNBN[a] (%)
A) −S9, 20 h exposure, 28 h recovery		
Vehicle	–	0.4
150.0	25	0.6
200.0	27	0.7
300.0	44	0.4
4NQO[b], 5.0	ND	3.5[c]
B) +S9, 3 h exposure, 45 h recovery		
Vehicle	–	0.8
150.0	16	0.5
200.0	0	1.3[e]
300.0	10	0.6
CPA[d], 6.25	ND	10.0[e]

[a] 2000 cells scored for the vehicle control and 1000 cells scored for the test compound groups and the positive control.
[b] 4-Nitroquinoline-*N*-oxide (4NQO), positive control.
[c] Statistically significant $p < 0.001$. MNBN = micronucleated binucleate cells, ND = not done.
[d] Cyclophosphamide (CPA), positive control.
[e] Statistically significant $p < 0.05$. MNBN = micronucleated binucleate cells, ND = not done.

concentrations inducing up to 62% cytotoxicity (Table 6A and B). Compound **14** showed no activity in the absence of S9. In the presence of S9 there were apparent small increases at 25 and 45 μg/mL compared to the concurrent vehicle control (Table 7A and B). However, the values for the number of micronucleated cells at these concentrations were at the lower end of the normal historical control range (0–1.5%) for the testing laboratory concerned (as can be seen from comparisons with the data in Tables 5–8) and were attributed to an abnormally low vehicle control response.

Data for compound **33** are given in Table 8A and B. In the absence of S9 a negative response was obtained. In the presence of S9, a small increase was seen at the middle test concentration of 200 μg/mL, but no increases were seen at the lower of higher test concentrations i.e. the increase was not dose-related. In addition the response was not consistent between the two replicate cultures. Thus this increase was not regarded as biologically significant.

In conclusion all four compounds were deemed negative in human peripheral lymphocyte micronucleus tests.

Table 7
In vitro micronucleus test of compound **14**.

Test concentration μg/mL	Cytotoxicity (%)	Mean MNBN[a] (%)
A) −S9, 20 h exposure, 28 h recovery		
Vehicle	–	0.2
175.0	9	0.2
400.0	31	0.3
475.0	69	0.3
4NQO[b], 2.5	ND	5.4[c]
B) +S9, 3 h exposure, 45 h recovery		
Vehicle	–	0.0
400.0	9	0.0
525.0	25	0.20[e]
550.0	45	0.30[e]
CPA[d], 12.5	ND	18.1[e]

[a] 2000 cells scored for the vehicle control and 1000 cells scored for the test compound groups and the positive control.
[b] 4-Nitroquinoline-*N*-oxide (4NQO), positive control.
[c] Statistically significant $p < 0.001$. MNBN = micronucleated binucleate cells, ND = not done.
[d] Cyclophosphamide (CPA), positive control.
[e] Statistically significant $p < 0.05$. MNBN = micronucleated binucleate cells, ND = not done.

2.3.3. Redox potential

The single electron redox potential of compound **16** was −575 mV, which is substantially more negative than mammalian redox systems. For comparison, it is −516 mV for Metronidazole, −511 mV for Fexinidazole and significantly higher at −422 mV for Megazol. This low redox potential is consistent with the lack of activity observed in the *in vitro* micronucleus tests, indicating that mammalian cells cannot nitro-reduce these compounds to produce genotoxic chemical species under normal aerobic conditions.

2.4. Preliminary further evaluation of the lead compound *16*

Considering the LogP and PSA physicochemical parameters alone, the lead compound **16** fits well to the selection criteria in terms of drugability and blood brain barrier permeability (logP (calc) = 2.3 (target value ~2); PSA(calc) = 72.9 (target value 60–80)). A preliminary further evaluation of the ADME (Absorption, Distribution, Metabolism and Excretion) and safety profile of the lead compound **16** was performed in *in vitro* assays and animal studies including *in vitro* liver microsomal (mouse and human) metabolic stability assays, *in vitro* permeability assays (Caco-2, MDR1-MDCK), *in vitro* safety pharmacology/receptor binding assays, a pharmacokinetics study in mice and a five-day repeated oral toxicity study in rats (all studies are listed in an annex, data are available on request); so far no critical issue or toxicity alerts could be identified which would preclude the further evaluation of this lead compound toward preclinical development. This assessment also confirmed the ability of the compound to cross the blood brain barrier which is a prerequisite for drugs intended to treat stage 2 HAT and is consistent with the high efficacy of the drug observed in the stage 2 HAT mouse model involving brain infection.

3. Conclusion

This series of 1-aryl-4-nitro-1*H*-imidazoles has demonstrated potent and selective anti-trypanosomal activity, including the exceptional capacity to cure a stringent model of second stage HAT, the chronic CNS model. Taken together with the absence of mammalian mutagenicity and the ADME and safety profile investigated so far, this confirms these compounds and in particular the lead compound **16**, as promising leads for further development into a new oral treatment of human African trypanosomiasis. It also

corroborates the findings that it is possible to select compounds within the nitroimidazoles family that are pharmacologically active yet are unlikely to pose a genotoxic hazard to patients.

Although bacterial mutagenicity was observed in the standard Ames strains used, as is often the case for compounds containing nitroaromatic groups, this mutagenic activity was lost in strains lacking the classical bacterial nitro-reductase for three of the four compounds evaluated. No mutagenic activity was observed in the *in vitro* micronucleus test using human peripheral lymphocytes for any of the compounds tested, indicating that mammalian cells are unable to reduce these nitroaromatic groups to mutagenic products. To confirm the conclusion that these compounds are unlikely to represent a genotoxic risk for humans, a full regulatory genotoxicity assessment needs to be completed including *in vivo* mammalian genotoxicity assays (e.g. *in vivo* micronucleus or chromosome aberration test).

Finally, it is clear that this family of 1-aryl-4-nitro-1*H*-imidazoles merits further exploration for anti-microbial drug discovery, including the synthesis of new molecules with different substituents on the phenyl group ($-SO_2R$, $-CO_2R$) or a different aryl group (e.g. quinoline, pyridine, indole) or a different position of the nitro group on the imidazole ring (e.g. 2-methyl-5-nitroimidazole), which may lead to new antiparasitic activity of interest without genotoxic activity. For instance, several compounds already showed activity of interest against *Trypanosoma cruzi*, which may need further optimization before a drug lead for Chagas disease can be selected in this series.

4. Material and methods

4.1. Chemistry

4.1.1. General

Melting points (not corrected) were determined in an open capillary or with a Boetius HMK apparatus; ^{1}H and ^{13}C NMR spectra were recorded on a Varian XL-300 (300 MHz for ^{1}H, 75.5 MHz for ^{13}C) or on a Varian 600 (600 MHz for ^{1}H, 150 MHz for ^{13}C) in DMSO-d_6 (unless otherwise specified) and with tetramethylsilane as the internal reference. The chemical shifts (δ) are reported in parts per million and the coupling constants (J) in hertz. Elementary analyses (EA) were performed using a Perkin–Elmer CHN automatic analyzer. Mass spectra were recorded using HPLC-MS Integrity Systems with a Termabeam Mass Detector (EI, 70 eV) (with introduction of samples in methanol) or on a GC/MS Perkin–Elmer Clarus 600T (with injection of samples in acetone). UV–vis spectra were recorded on a Hitachi U-2910 spectrometer in water containing 2.5–6% methanol as the solvent.

4.1.2. Synthesis

4.1.2.1. 1,4-Dinitro-1H-imidazoles. 1,4-Dinitro-1*H*-imidazoles were obtained by nitration of imidazoles following a known general procedure [35]. CAUTION: 1,4-dinitro-1*H*-imidazoles are potential self-reacting/explosive substances; for the risk associated with their synthesis and handling, see Ref. [30,36].

4.1.2.1.1. 1,4-Dinitro-1H-imidazole. Yield 70%, white prisms, m. p. 92–94 °C, ^{1}H NMR (300 MHz) 8.97 (d, 1H, J = 1.5 Hz, H-2$_{imid}$), 9.40 (d, 1H, J = 1.5 Hz, H-5$_{imid}$), ^{13}C NMR (75.5 MHz): 115.9 (s, C-5$_{imid}$), 132.6 (s, C-2$_{imid}$), 144.3 (s, C-4$_{imid}$).

4.1.2.1.2. 2-Methyl-1,4-dinitro-1H-imidazole. Yield 70%, white needles, m. p. 122–124 °C, ^{1}H NMR (300 MHz) 2.67 (s, 3H, $-CH_3$ $_{imid}$), 9.26 (s, 1H, H-5$_{imid}$), ^{13}C NMR (75.5 MHz) 16.2 (s, $-CH_3$ $_{imid}$), 116.9 (s, C-5$_{imid}$), 141.4 (s, C-2$_{imid}$), 142.7 (s, C-4$_{imid}$).

4.1.2.2. Anilines. Commercially available anilines were used without purification except in a few cases of dark liquids or darkish solids which were distilled or recrystallized prior to use. Other anilines were prepared according to published procedures. Preparation of butyl 5-amino-2-chlorobenzoate [CAS No: 135813-38-6] is described below.

4.1.2.2.1. 4-Bromo-3-methylaniline [37]. Yield 85%, m. p. 81–82 °C, white powder, ^{1}H NMR (300 MHz) 2.18 (s, 3H, $-CH_3$), 5.13 (s, 2H, $-NH_2$), 6.33 (dd, 1H, J = 8.7 Hz, J = 2.1 Hz, H-6), 6.52 (d, 1H, J = 2.1 Hz, H-2), 7.12 (d, 1H, J = 8.7 Hz, H-2), ^{13}C NMR (75.5 MHz) 22.5, 108.8, 113.5, 116.3, 132.0, 136.8, 148.2.

4.1.2.2.2. 3-Bromo-4-methylaniline [38,39]. Yield 87%, dark yellow oil, ^{1}H NMR (300 MHz) 2.15 (s, 3H, $-CH_3$), 5.11 (s, 2H, $-NH_2$), 6.46 (dd, 1H, J = 8.1 Hz, J = 2.4 Hz, H-6), 6.78 (d, 1H, J = 2.4 Hz, H-2), 6.93 (d, 1H, J = 8.1 Hz, H-5), ^{13}C NMR (75.5 MHz) 21.2, 113.4, 116.8, 122.9, 124.2, 131.0, 148.1.

4.1.2.2.3. 3-Bromo-5-methylaniline [40]. Yield 45%, after distillation colorless oil, b.p. 124–125 °C$_{(6\ mm\ Hg)}$ (lit. 150–154 °C$_{(4\ mm\ Hg)}$), ^{1}H NMR (300 MHz) 2.13 (s$_b$, 3H, $-CH_3$), 5.26 (s$_b$, 2H, $-HN_2$), 6.33–6.34 (m, 1H, Ar–H), 6.45–6.46 (m, 1H, Ar–H), 6.53–6.55 (m, 1H, Ar–H), ^{13}C NMR (75.5 MHz) 20.8, 113.3, 113.3, 118.6, 121.9, 140.2, 150.2.

4.1.2.2.4. Butyl 5-amino-2-chlorobenzoate. A mixture of 2-chloro-5-aminobenzoic acid (3.0 g, 11 mmol), 1-butanol (20.25 g, 270 mmol) and sulfuric acid (1 mL) was heated under reflux for 5 h in a flask equipped with a Dean–Stark apparatus. After addition of diethyl ether (60 mL), the resulting solution was washed with 3 × 15 mL of a saturated solution of sodium bicarbonate and then with water to neutral pH. 3-Butoxycarboxy-4-chloroaniline was extracted by washing with 5 × 15 mL of hydrochloric acid 10%. The aqueous layer was neutralized with solid sodium bicarbonate and the aniline extracted with diethyl ether 4 × 20 mL. The ethereal layer was separated, dried over magnesium sulfate and evaporated to dryness. 3-Butoxycarbonyl-4-chloroaniline was obtained as a yellowish oil. Yield 24%, ^{1}H NMR (300 MHz, $CDCl_3$) 0.97 (t, 3H, J = 7.2 Hz, $-CH_3$), 1.44–1.51 (m, 2H, $-CH_2-CH_3$), 1.72–1.77 (m, 2H, $-CH_2-CH_2-CH_3$), 3.67 (s$_b$, 2H, $-NH_2$), 4.32 (t, 2H, J = 6.6 Hz $-O-CH_2-CH_2-CH_2-CH_3$), 6.70 (dd, 1H, J = 8.6 Hz, J = 3.0 Hz, H-6), 7.09 (d, 1H, J = 3.0 Hz, H-2), 7.18 (d, 1H, J = 8.6 Hz, H-5). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 13.7, 19.3, 30.7, 65.3, 117.2, 118.9, 122.2, 130.9, 131.6, 145.0, 166.1. MS (m/z): 227 (M^+, 42%), 171 (100%), 154 (69%).

*4.1.2.3. 1-Aryl-4-nitro-1H-imidazoles (general procedure). For compounds **1**–**7**, **10**, **17**, **18** and **22**–**32**, see reference to synthesis given in Table 1.* 1-Aryl-4-nitro-1*H*-imidazoles were prepared following the general procedure we have developed in our laboratory [23] with some slight modifications. Equimolar amount of aniline derivative was treated with 1,4-dinitro-1*H*-imidazole in aqueous methanol at ambient temperature in the dark for several hours until complete disappearance of 1,4-dinitro-1*H*-imidazole monitored by TLC. In some cases, the mixture was heated under reflux to complete the reaction. On cooling, the desired crude 1-aryl-4-nitro-1*H*-imidazoles separated from the mixture by precipitation. After filtration and recrystallization the pure product was obtained. Yields, solvents used for recrystallization and specific data are given below in the respective sections. Further details are available on request.

*4.1.2.3.1. 1-(2-Bromophenyl)-4-nitro-1H-imidazole (**8**).* Yield 61%, light brown powder, m. p. 134–135.5 °C (ethyl acetate/hexane); ^{1}H NMR (300 MHz) 7.54 (td, 1H, J = 7.5 Hz, J = 1.5 Hz, Ar–H), 7.62 (td, 1H, J = 7.5 Hz, J = 1.5 Hz, Ar–H), 7.71 (dd, 1H, J = 7.5 Hz, J = 1.5 Hz, Ar–H), 7.79 (dd, 1H, J = 7.5 Hz, J = 1.5 Hz, Ar–H), 8.15 (d, 1H, J = 1.5 Hz, H-2$_{imid.}$), 8.76 (d, 1H, J = 1.5 Hz, H-5$_{imid.}$); ^{13}C NMR (75.5 MHz) 119.5, 122.7, 128.9, 129.0, 131.8, 133.5, 134.8, 138.0, 147.4 (s, 1C, C-4$_{imid.}$); EA: calcd. for $C_9H_6BrN_3O_2$ C 40.32, H 2.26, N 15.68; found C 40.24, H 2.30, N 15.68.

*4.1.2.3.2. 1-(3-Bromophenyl)-4-nitro-1H-imidazole (**9**).* Yield 85%, yellowish powder, m. p. 113–114 °C (methanol/water); ^{1}H NMR (300 MHz) 7.53 (t, 1H, J = 8.1 Hz, H-5′), 7.68 (ddd, 1H, J = 8.1 Hz, J = 1.8 Hz, J = 0.9 Hz, Ar–H), 7.85 (ddd, 1H, J = 8.1 Hz, J = 1.8 Hz, J = 0.9 Hz, Ar–H), 8.14 (t, 1H, J = 1.8 Hz, H-2′), 8.53 (d, 1H, J = 1.5 Hz, H-2$_{imid.}$), 9.06 (d, 1H, J = 1.5 Hz, H-5$_{imid.}$); ^{13}C NMR (75.5 MHz) 119.7, 120.2, 122.5, 124.0, 131.3, 131.6, 135.6, 136.7, 148.1 (s, 1C, C-4$_{imid.}$); UV–vis λ_{max} = 300 nm; EA: calcd. for $C_9H_6BrN_3O_2$ C 40.32, H 2.26, N 15.68; found C 40.27, H 2.32, N 15.91.

*4.1.2.3.3. 4-Nitro-1-(3-nitrophenyl)-1H-imidazole (**11**).* Yield 72%, dark yellow powder, m. p. 229–231 °C (glacial acetic acid); ^{1}H NMR (300 MHz) 7.88 (t, 1H, J = 8.1 Hz, H-5′), 8.28–8.33 (m, 2H, H-4′, H-6′), 8.65 (d, 1H, J = 1.5 Hz, H-2$_{imid.}$), 8.72 (t, 1H, J = 2.4 Hz, H-2′), 9.19 (d, 1H, J = 1.5 Hz, H-5$_{imid.}$); ^{13}C NMR (75.5 MHz) 116.5, 120.0, 123.1, 127.6, 131.4, 135.9, 136.3 (s, 1C, C-1′), 148.24 (s, 1C, C-4$_{imid.}$), 148.51 (s, 1C, C-3′); EA: calcd. for $C_9H_6N_4O_4$ C 46.16, H 2.58, N 23.93; found C 46.32, H 2.65, N 23.91.

*4.1.2.3.4. 4-Nitro-1-{3-(trifluoromethyl)phenyl}-1H-imidazole (**12**).* Yield 79%, yellowish powder, m. p. 114–116 °C (ethyl acetate/hexane); ^{1}H NMR (300 MHz) 7.80–7.87 (m, 2H, Ar–H), 8.14–8.18 (m, 1H, Ar–H), 8.27–8.28 (m, 1H, Ar–H), 8.62 (d, 1H, J = 1.6 Hz, H-2$_{imid.}$), 9.16 (d, 1H, J = 1.6 Hz, H-5$_{imid.}$); ^{13}C NMR (75.5 MHz) 118.4 (q, J = 3.9 Hz, C-4′), 119.9 (s, C-2$_{imid.}$), 123.5 (q, J = 272.8 Hz, –CF_3), 125.1 (q, J = 3.7 Hz, C-2′), 125.3 (s, 1C), 130.7 (q, 1C, J = 32.6 Hz, C-3′), 131.1 (s, 1C), 135.8 (s, 1C), 136.1 (s, 1C), 148.2 (s, C-4$_{imid.}$); UV–vis λ_{max} = 298 nm; MS: m/z: 257 (M^+, 35%), 172 (70%), 145 (100%); EA calcd. for $C_{10}H_6F_3N_3O_2$ C 46.70, H 2.35, N 16.34, found C 46.77, H 2.36, N 16.46.

*4.1.2.3.5. 4-Nitro-1-{4-(trifluoromethyl)phenyl}-1H-imidazole (**13**).* Yield 69%, white powder, m. p. 144–145 °C (methanol/water); ^{1}H NMR (300 MHz) 7.97 (d, 2H, J = 8.4 Hz, Ar–H), 8.08 (d, 2H, J = 8.4 Hz, Ar–H), 8.61 (d, 1H, J = 1.6 Hz, H-2$_{imid}$), 9.13 (d, 1H, J = 1.6 Hz, H-5$_{imid}$); ^{13}C NMR (75.5 MHz) 119.6 (s, C-2$_{imid.}$), 121.9 (s, C-2′, C-6′), 123.7 (q, J = 270.6 Hz –CF_3), 127.1 (q, J = 3.7 Hz, C-3′, C-5′), 128.7 (q, J = 32.3 Hz, C-4′) 135.7 (s, C-5$_{imid.}$), 138.5–138.5 (m, C-1′) 148.3 (s, C-4$_{imid..}$); UV–vis λ_{max} = 307.5 nm (2.4% methanol in water); MS: m/z: 257 (M^+, 37%), 172 (69%), 145 (100%); EA calcd. for $C_{10}H_6F_3N_3O_2$ C 46.70, H 2.35, N 16.34, found C 46.93, H 2.34, N 16.16.

*4.1.2.3.6. 4-Nitro-1-{2-(trifluoromethoxy)phenyl}-1H-imidazole (**14**).* Yield 71%, white plates, m. p. 87–89 °C (ethyl acetate/hexane), white needles m. p. 90.8–92 °C (diethyl ether); ^{1}H NMR (300 MHz) 7.62–7.76 (m, 3H, Ar–H), 7.83–7.86 (m, 1H, Ar–H), 8.23 (d, 1H, J = 1.5 Hz, H-2$_{imid}$), 8.81 (d, 1H, J = 1.5 Hz, H-5$_{imid}$); ^{13}C NMR (75.5 MHz) 119.7 (q, 1C, J = 259.2 Hz, –OCF_3), 122.2, 122.3, 128.1, 128.4, 128.8, 131.4, 137.8, 141.72–141.74 (m, 1C, C-2′), 147.7 (s, 1C, C-4$_{imid}$); UV–vis λ_{max} = 294 nm; MS: m/z: 273 (M^+, 48%), 188 (64%), 95 (100%). EA calcd for $C_{10}H_6F_3N_3O_3$ C 43.97, H 2.21, N 15.38, found C 44.01, H 2.21, N 15.36.

*4.1.2.3.7. 4-Nitro-1-{3-(trifluoromethoxy)phenyl}-1H-imidazole (**15**).* Yield 31%, white powder, m. p. 92–94 °C (ethanol); ^{1}H NMR (600 MHz) 7.49–7.51 (m, 1H, H-4′), 7.73 (t, 1H, J = 8.4 Hz, H-5′), 7.91 (dd, 1H, J = 8.4 Hz, J = 1.2 Hz, H-2′), 7.98 (s$_B$, 1H, H-2′), 8.57 (d, 1H, J = 1.2 Hz, H-2$_{imid}$), 9.10 (d, 1H, J = 1.2 Hz, H-5$_{imid}$); ^{13}C NMR (150 MHz) 114.6, 119.7, 120.0 (q, 1C, J = 257.8 Hz, –OCF_3), 120.2, 120.7, 131.7, 135.7, 136.8, 148.2, 149.0; EA calcd for $C_{10}H_6F_3N_3O_3$ C 43.97, H 2.21, N 15.38, found C 44.18, H 2.19, N 15.34.

*4.1.2.3.8. 4-Nitro-1-{4-(trifluoromethoxy)phenyl}-1H-imidazole (**16**).* Yield 84%, light yellow plates, m. p. 129.5–131 °C (methanol/water); ^{1}H NMR (300 MHz): 7.63 (d, 2H, J = 8.4 Hz, H-2′, H-6′), 7.95–7.99 (m, 2H, H-3′, H-5′), 8.51 (d, 1H, J = 1.2 Hz, H-2$_{imid.}$), 9.05 (d, 1H, J = 1.2 Hz, H-5$_{imid.}$); ^{13}C NMR (75.5 MHz) 120.0 (s, 1C), 120.0 (q, 1C, J = 257.0 Hz, –OCF_3), 122.6 (s, 1C), 123.5 (s, 1C), 134.5 (s, 1C), 135.9 (s, 1C), 147.9 (s, 1C, C-4′), 148.2 (s, 1C, C-4$_{imid.}$); UV–vis λ_{max} = 216, 300 nm; MS: m/z: 273 (M^+, 47%), 188 (100%), 161 (51%), 95 (91%); EA calcd. for $C_{10}H_6F_3N_3O_3$ C 43.97, H 2.21, N 15.38, found C 43.83, H 2.09, N 14.87.

*4.1.2.3.9. 1-(3-Methoxyphenyl)-4-nitro-1H-imidazole (**19**).* Yield 87%, bright yellow needels, m. p. 151–151.5 °C (methanol/water); ^{1}H NMR (300 MHz) 3.86 (s, 3H, –OCH_3), 7.03–7.08 (m, 1H, H-4′), 7.36–7.39 (m, 1H, H-6′), 7.40–7.41 (m, 1H, H-2′), 7.48 (t, 1H, J = 8.1 Hz, H-5′), 8.51 (d, 1H, J = 1.5 Hz, H-2$_{imid.}$), 9.03 (d, 1H, J = 1.5 Hz, H-5$_{imid.}$); ^{13}C NMR (75.5 MHz) 55.6 (s, 1C, –OCH_3), 106.9 (s, C-2′), 113.0 (s, C-4′), 114.4 (s, C-6′), 119.6 (s, C-5$_{imid}$), 130.8 (s, C-5′), 135.6 (s, C-2$_{imid}$), 136.5 (s, C-1′), 148.0 (s, C-4$_{imid}$), 160.3 (s, C-6′); UV–vis λ_{max} = 303 nm; EA calcd. for $C_{10}H_9N_3O_3$ C 54.79, H 4.14; N 19.17, found: C 54.6, H 4.20, N 19.35.

*4.1.2.3.10. Butyl 4-(4-nitro-1H-imidazol-1-yl)benzoate (**21**).* Yield 53%, bright yellow plates, m. p. 134–135.5 °C (methanol); ^{1}H NMR (600 MHz) 0.96 (t, 3H, J = 7.5 Hz, –CH_3), 1.45 (m, 2H, –CH_2–CH_3), 1.70–1.75 (m, 2H, –CH_2–CH_2–CH_3), 4.31 (t, 2H, J = 6.6 Hz, –O–CH_2–CH_2–), 7.97–7.99 (m, 2H, Ar–H), 8.09–8.11 (m, 2H, Ar–H), 8.59 (d, 1H, J = 1.5 Hz, H-2$_{imid}$), 9.09 (d,-1H, J = 1.5 Hz, H-5$_{imid}$); ^{13}C NMR (150 MHz) 13.5, 18.7, 30.2, 64.7, 119.4, 121.1 (s, 2C), 129.52, 130.73 (s, 2C), 135.59, 138.85, 148.32 (s, 1C, C-4$_{imid}$), 164.67 (s, 1C, C=O); EA calcd. for $C_{14}H_{15}N_3O_4$ C 58.13, H 5.23, N 14.53, found C 58.45, H 5.18, N 14.49.

*4.1.2.3.11. 1-(3-Chloro-4-fluorophenyl)-4-nitro-1H-imidazole (**33**).* Yield 64%, bright yellow prisms, m. p. 115–116 °C (methanol); ^{1}H NMR (300 MHz) 7.63–7.69 (m, 1H, Ar–H), 7.86–7.88 (m, 1H, Ar–H), 8.19–8.20 (m, 1H, Ar–H), 8.48 (s$_b$, 1H, H-2$_{imid}$), 9.02 (s$_b$, 1H, H-5$_{imid}$), ^{13}C NMR (75.5 MHz) 117.9 (d, 1C, J = 22.6 Hz, C-5′), 119.9 (s, 1C, C-5$_{imid}$), 120.8 (d, 1C, J = 19.1 Hz, C-3′), 122.1 (d, 1C, J = 7.8 Hz, C-6′), 123.8 (s$_b$, 1C, C–Ar), 132.5 (d, 1C, J = 3.1 Hz, C-1′), 135.8 (s, 1C, C-2$_{imid}$), 148.0 (s, 1C, C-4$_{imid}$), 156.8 (d, 1C, J = 247.7 Hz, C-4′); UV–vis λ_{max} = 299 nm; MS: m/z: 241 (M^+, 38%), 156 (100%), 129 (87%); EA calcd. for $C_9H_5ClFN_3O_2$ C 44.74, H 2.09, N 17.39, found C 44.77, H 2.14, N 18.32.

*4.1.2.3.12. 1-(2-Bromo-4-methylphenyl)-4-nitro-1H-imidazole (**34**).* Yield 56%, yellowish thin plates, m. p. 144–145 °C (methanol); ^{1}H NMR (300 MHz) 2.41 (s, 3H, –CH_3), 7.41 (dd, 8.0 Hz, J = 0.9 Hz, H-5′), 7.58 (d, 1H, J = 8.0 Hz, H-6′), 7.75 (d, 1H, J = 0.9 Hz, H-3′), 8.12 (d, 1H, J = 1.5 Hz, H-2$_{imid}$), 8.73 (d, 1H, J = 1.5 Hz, H-5$_{imid}$); ^{13}C NMR (75.5 MHz) 20.3 (s, 1C, –CH_3), 119.1 (s, 1C, C-2′), 122.8 (s, 1C, C-5$_{imid}$), 128.4 (s, 1C, C-6′), 129.5 (s, 1C, C-5′), 132.3 (s, 1C, C-1′), 133.6 (s, 1C, C-3′), 138.1 (s, 1C, C-4′), 142.2 (s, C-2$_{imid}$), 147.4 (s, 1C, C-4$_{imid}$); EA calcd. for $C_{10}H_8BrN_3O_2$ C 42.58, H 2.86, N 14.90, found C 42.55, H 2.86, N 14.85.

*4.1.2.3.13. 1-(4-Bromo-3-methylphenyl)-4-nitro-1H-imidazole (**35**).* Yield 88%, yellowish powder, m. p. 161–163 °C (methanol/water); ^{1}H NMR (300 MHz) 2.42 (s, 3H, CH_3), 7.60 (dd, 1H, J = 8.7 Hz, J = 2.4 Hz, H-6′), 7.76 (d, 1H, J = 8.7 Hz, H-5′), 7.87 (d, 1H, J = 2.4 Hz, H-2′), 8.48 (d, 1H, J = 1.5 Hz, H-2$_{imid}$), 8.98 (d, 1H, J = 1.5 Hz, H-5$_{imid}$); ^{13}C NMR (75.5 MHz) 22.4, 119.4, 120.3, 123.5, 123.7, 133.3, 135.4, 139.3, 148.1; UV–vis λ_{max} = 229, 303 nm; EA calcd. for $C_{10}H_8BrN_3O_2$ C 42.58, H 2.86, N 14.90, found C 42.59, H 2.91, N 15.24.

*4.1.2.3.14. 1-(3-Bromo-4-methylphenyl)-4-nitro-1H-imidazole (**36**).* Yield 70%, bright yellow powder m. p. 147–148 °C (methanol/water); ^{1}H NMR (300 MHz) 2.40 (s, 3H, CH_3), 7.53 (d, 1H, J = 8.4 Hz, H-5′), 7.74 (dd, 1H, J = 8.4 Hz, J = 2.1 Hz, H-6′), 8.12 (d, 1H, J = 2.1 Hz, H-2′), 8.50 (d, 1H, J = 0.9 Hz, H-2$_{imid}$), 9.02 (d, 1H, J = 0.9 Hz, H-2$_{imid}$); ^{13}C NMR (75.5 MHz) 21.9, 119.6, 120.2, 124.5, 124.7, 131.8, 134.3, 135.5, 137.8, 148.0; UV–vis λ_{max} = 303 nm; EA calcd. for $C_{10}H_8BrN_3O_2$ C 42.58, H 2.86, N 14.90, found C 42.80, H 2.93, N 14.94.

*4.1.2.3.15. 1-(2,4-Dimethylphenyl)-4-nitro-1H-imidazole (**37**).* Yield 78%, yellowish prisms, m. p. 123–124.5 °C (methanol); ^{1}H NMR (300 MHz) 2.16 (s, 3H, –CH_3), 2.36 (s, 3H, –CH_3), 7.20 (d$_b$, 1H, J = 7.8 Hz, H-5′), 7.27 (s$_b$, 1H, H-3′), 7.33 (d, 1H, J = 7.8 Hz, H-6′), 8.06 (d, 1H, J = 1.5 Hz, H-2$_{imid}$), 8.67 (d, 1H, J = 1.5 Hz, H-5$_{imid}$); ^{13}C NMR (75.5 MHz) 17.1 (s, –CH_3), 20.6 (s, –CH_3), 122.5 (s, C-5$_{imid}$), 126.3

(s, 1C), 127.5 (s, 1C), 131.7 (s, 1C), 132.6 (s, C-2$_{imid}$), 133.0 (s, C-1′), 137.9 (s, 1C), 139.4 (s, 1C), 147.5 (s, C-4$_{imid}$); EA calcd. for $C_{11}H_{11}N_3O_2$ C 60.82, H 5.10, N 19.34, found C 60.70, H 5.05, N 19.37.

*4.1.2.3.16. 1-(3,4-Dimethylphenyl)-4-nitro-1H-imidazole (**38**).* Yield 77%, white powder, m. p. 114.5–116 °C (methanol); ^{1}H NMR (300 MHz) 2.27 (s, 3H, −CH$_3$), 2.30 (s, 3H, −CH$_3$), 7.31 (d, 1H, J = 8.1 Hz, H-5′), 7.50 (dd, 1H, J = 8.1 Hz, J = 2.4 Hz, H-6′), 7.60 (d, 1H, J = 2.4 Hz, H-2′), 8.41 (d, 1H, J = 1.4 Hz, H-2$_{imid}$), 8.91 (d, 1H, J = 1.4 Hz, H-5$_{imid}$); ^{13}C NMR (75.5 MHz) 18.8 (s, −CH$_3$), 19.3 (s, −CH$_3$), 118.3 (s, 1C), 119.4 (s, 1C), 122.0 (s, 1C), 130.5 (s, 1C), 133.2 (s, C-1′), 135.3 (s, 1C), 136.9 (s, Ar−CH$_3$), 138.2 (s, Ar−CH$_3$), 147.9 (s, C-4$_{imid}$); UV–vis λ_{max} = 308 nm; EA calcd. for $C_{11}H_{11}N_3O_2$ C 60.82, H 5.10, N 19.34, found C 60.78, H 4.90, N 19.43.

*4.1.2.3.17. 1-(2,4-Dimethoxyphenyl)-4-nitro-1H-imidazole (**39**).* Yield 66.5%, gray powder m. p. 161.5–162 °C (methanol); ^{1}H NMR (300 MHz) 3.85 (s$_b$, 6H, 2×−OCH$_3$), 6.67 (dd, 1H, J = 0.9 Hz, J = 0.3 Hz, H-5′), 6.82 (d, 1H, J = 0.3 Hz, H-3′), 7.46 (d, 1H, J = 0.9 Hz, H-6′), 8.02 (d 1H, J = 0.2 Hz, H-2$_{imid}$), 8.56 (d, 1H, J = 0.15 Hz, H-5$_{imid}$); ^{13}C NMR (75.5 MHz) 55.7 (s, −OCH$_3$), 56.2 (s, −OCH$_3$), 99.6 (s, 1C), 105.2 (s, 1C), 117.6 (s, C-1′), 122.5 (s, C-5$_{imid}$), 127.0 (s, 1C), 137.9 (s, C-2$_{imid}$), 147.1 (s, C-4$_{imid}$), 153.6 (s, Ar−OCH$_3$), 161.1 (s, Ar−OCH$_3$); EA calcd. for $C_{11}H_{11}N_3O_4$ C 53.01, H 4.45, N 16.86, found C 53.01, H 4.39, N 16.61.

*4.1.2.3.18. 1-(3,4-Dimethoxyphenyl)-4-nitro-1H-imidazole (**40**).* Yield 82.5%, yelow powder, m. p. 167.5–168.5 °C (methanol); ^{1}H NMR (300 MHz) 2.14 (s, 3H, −CH$_3$ $_{imid}$), 3.82 (s, 3H, −OCH$_3$), 3.86 (s, 3H, −OCH$_3$), 6.68 (dd, 1H, J = 8.7 Hz, J = 2.4 Hz, H-5′), 6.82 (d, 1H, J = 2.4 Hz, H-3′), 7.58 (d, 1H, J = 8.7 Hz, H-6′), 8.37 (s, 1H, H-5$_{imid}$); ^{13}C NMR (300 MHz) 12.7 (s, −CH$_3$ $_{imid}$), 55.7 (s, −OCH$_3$), 56.1 (s, −OCH$_3$), 99.5 (s, 1C), 105.3 (s, 1C), 117.1 (s, 1C, C-5$_{imid}$), 123.7 (s, 1C), 128.8 (s, 1C), 145.9 (s, 1C, C-4$_{imid}$), 146.0 (s, 1C, C-1′), 154.8 (s, Ar−OCH$_3$), 161.6 (Ar−OCH$_3$); EA calcd for $C_{12}H_{13}N_3O_4$ C 54.75, H 4.98, N 15.96, found C 54.86, H 4.86, N 16.05.

*4.1.2.3.19. 1-(Benzo[d][1,3]dioxol-5-yl)-4-nitro-1H-imidazole (**41**).* Yield 58%, gray powder, m. p. 206–207 °C (acetone); ^{1}H NMR (300 MHz) 6.15 (s, 2H, −CH$_2$−), 7.09 (d, 1H, J = 8.4 Hz, H-5′), 7.26 (dd, 1H, J = 2.4 Hz, H-6′), 7.26 (dd, 1H, J = 2.4 Hz, H-6′), 7.47 (d, 1H, J = 2.4 Hz, H-2′), 8.36 (d, 1H, J = 1.7 Hz, H-2$_{imid}$), 8.89 (d, 1H, J = 1.7 Hz, H-5$_{imid}$); ^{13}C NMR (75.5 MHz) 102.2, 103.2, 108.6, 115.0, 120.0, 129.7, 135.7, 147.4, 147.8, 148.2. MS: m/z: 233 (M^+, 100); EA calcd. for $C_{10}H_7N_3O_4$ C 51.51, H 3.03, N 18.02, found C 51.64, H 3.00, N 17.95.

*4.1.2.3.20. Butyl 2-chloro-5-(4-nitro-1H-imidazol-1-yl)benzoate (**42**).* Yield 37%, yellowish powder, m. p. 88–90 °C (methanol); ^{1}H NMR (600 MHz) 0.94 (t, 3H, J = 7.5 Hz, −CH$_3$), 1.41–1.47 (m, 2H, −CH$_2$−CH$_3$), 1.70–1.75 (m, 2H, −CH$_2$−CH$_2$−CH$_3$), 4.34 (t, 2H, J = 6.6 Hz, −O−CH$_2$−CH$_2$−CH$_2$−CH$_3$), 7.81 (d, 1H, J = 9.0 Hz, H-5′), 8.02 (dd, 1H, J = 9.0 Hz, J = 3.0 Hz, H-6′), 8.20 (d, 1H, J = 3.0 Hz, H-2′), 8.53 (d, 1H, J = 1.2 Hz, H-2$_{imid}$), 9.06 (d, 1H, J = 1.2 Hz, H-5$_{imid}$). ^{13}C NMR (150 MHz) 13.5, 18.6, 30.0, 65.5, 119.9, 123.3, 125.5, 131.0, 132.0, 132.1, 134.4, 135.8, 148.1 (s, 1C, C-4$_{imid}$), 164.39 (s, 1C, C=O). EA calcd. for $C_{14}H_{14}ClN_3O_4$ C 51.94, H 4.36, N 12.98, found C 52.12, H 4.44, N 12.78.

*4.1.2.3.21. 2-Chloro-5-(4-nitro-1H-imidazol-1-yl)benzoic acid (**43**).* Yield 89%, light brown prisms, m. p. 266–268 °C (methanol), ^{1}H NMR (600 MHz) 7.77 (d, 1H, J = 8.4 Hz, H-3), 7.98 (dd, 1H, J = 8.4 Hz, J = 3.0 Hz, H-4), 8.20 (d, 1H, J = 3.0 Hz, H-6), 8.53 (d, 1H, J = 1.2 Hz, H-2$_{imid}$), 9.07 (d, 1H, J = 1.2 Hz, H-5$_{imid}$), 13.77 (s$_b$, 1H, −COOH). ^{13}C NMR (150 MHz) 119.8, 123.2, 124.9, 131.0, 131.9, 133.2, 134.2, 135.7, 148.1 (s, 1C, C-4$_{imid}$), 165.8 (s, 1C, C=O); EA calcd. for $C_{10}H_6ClN_3O_4$ C 44.88, H 2.26, N 15.70, found C 44.96, H 2.31, N 15.92.

*4.1.2.3.22. 2-Methyl-4-nitro-1-{4-(trifluoromethoxy)phenyl}-1H-imidazole (**44**).* Yield 73.5%, bright yellow powder, m. p. 130–132 °C (methanol–chloroform); ^{1}H NMR (300 MHz) 2.32 (s, 3H, −CH$_3$ $_{imid}$), 7.59–7.62 (m, 2H, Ar–H), 7.74–7.79 (m, 2H, Ar–H), 8.61 (s, 1H, C-H$_{imid}$). ^{13}C NMR (75.5 MHz) 13.4 (s 1C, −CH$_3$ $_{imid}$), 120.0 (q, J = 257.3 Hz, −CF$_3$), 122.2 (s, 2C), 122.7 (s, 1C, C-2$_{imid}$), 128.1 (s, 2C), 134.6 (s, 1C, C-1′), 144.8 (s, 1C, C-5$_{imid}$), 146.2 (s, 1C, C-4$_{imid}$), 148.5 (s$_b$, 1C, C-4′); EA calcd. for $C_{11}H_8F_3N_3O_2$ C 46.00, H 2.81, N 14.63, found C 45.66, H 2.70, N 14.35.

*4.1.2.3.23. 2-Methyl-4-nitro-1-{4-(trifluoromethyl)phenyl}-1H-imidazole (**45**).* Yield 66%, yellow powder, m. p. 96–98 °C (ethyl acetate/hexane); ^{1}H NMR (300 MHz) 2.37 (s, 3H, −CH$_3$ $_{imid}$), 7.86 (d, 2H, J = 8.4 Hz, Ar–H), 8.00 (d, 2H, J = 8.4 Hz, Ar–H), 8.67 (s, 1H, H-5$_{imid}$); ^{13}C NMR (75.5 MHz) 13.5 (s, 1C, −CH$_3$ $_{imid}$), 122.5 (s, C-2$_{imid}$), 123.7 (q, J = 272.1 Hz, −CF$_3$), 126.8 (m, C-2′, C-3′, C-5′, C-6′), 129.0 (d, J = 1.3 Hz, C-1′), 144.7 (s, 1C, C-5$_{imid}$), 146.3 (s, 1C, C-4$_{imid}$); EA calcd. for $C_{11}H_8F_3O_2N_3$ C 48.72, H 2.97, N 15.49, found C 48.66, H 2.63, N 14.33.

*4.1.2.3.24. 1-(2-Bromo-4-methylphenyl)-2-methyl-4-nitro-1H-imidazole (**46**).* Yield 40%, yellow powder, m. p. 126–128 °C (ethyl acetate/hexane), ^{1}H NMR (300 MHz) 2.14 (s, 3H, −CH$_3$ $_{imid}$), 2.42 (s, 3H, −CH$_3$), 7.24 (dd, 1H, J = 7.8 Hz, J = 0.9 Hz, H-5′), 7.59 (d, 1H, J = 7.8 Hz, H-6′), 7.76 (d, 1H, J = 0.9 Hz, H-3′), 8.54 (s, 1H, H-5$_{imid}$); ^{13}C NMR (75.5 MHz) 12.8 (s, −CH$_3$ $_{imid}$), 20.3 (s, −CH$_3$), 120.4, 123.1, 129.0, 129.7, 132.1, 133.6, 142.6, 145.3, 146.2 (s, 1C, C-4$_{imid}$); EA calcd. for $C_{11}H_{10}BrN_3O_2$ C 44.62, H 3.40, N 14.19, found C 45.01, H 3.46, N 14.09.

*4.1.2.3.25. 1-(3-Chloro-4-fluorophenyl)-2-methyl-4-nitro-1H-imidazole (**47**).* Yield 72%, white powder, m. p. 177–178 °C (methanol/water); ^{1}H NMR (300 MHz) 2.32 (s, 3H, −CH$_3$), 7.63–7.72 (m, 2H, Ar–H), 7.99–8.02 (m, 1H, Ar–H), 8.59 (s, 1H, H-5$_{imid}$); ^{13}C NMR (75.5 MHz) 13.3 (s, 1C, −CH$_3$ $_{imid}$), 117.7 (d, 1C, J = 22.5 Hz, C-5′), 120.4 (d, 1C, J = 19.0 Hz, C-3′), 122.8 (s, 1C, C-5$_{imid}$), 127.1 (d, 1C, J = 8.2 Hz, C–Ar), 128.6 (s, 1C, C–Ar), 132.6 (d, 1C, J = 3.5 Hz, C-1′), 145.0 (s, 1C, C-2$_{imid}$), 146.0 (s, 1C, C-4$_{imid}$); EA calcd. for $C_{10}H_7ClFN_3O_2$ C 46.99, H 2.76, N 16.44, found C 47.02, H 2.97, N 16.97.

*4.1.2.3.26. 1-(4-Bromo-3-methylphenyl)-2-methyl-4-nitro-1H-imidazole (**48**).* Yield 64%, yellowish powder, m. p. 156–158 °C (methanol/water); ^{1}H NMR (300 MHz) 2.33 (s, 3H, CH$_3$ $_{imid}$), 2.42 (s, 3H, CH$_3$), 7.38 (dd, 1H, J = 8.7 Hz, J = 2.7 Hz, H-6′) 7.62 (d, 1H, J = 2.7 Hz, H-2′), 7.79 (d, 1H, J = 8.7 Hz, H-5′), 8.54 (s, 1H, H-5$_{imid}$); ^{13}C NMR (75.5 MHz) 13.4 (s, 1C, −CH$_3$ $_{imid}$), 22.4 (s, 1C, −CH$_3$), 125.0 (s, 2C), 128.2 (s, 1C), 133.1 (s, 1C), 135.0 (s, 1C), 139.1 (s, 1C), 144.6 (s, 1C), 146.1 (s, 1C); EA calcd. for $C_{11}H_{10}BrN_3O_2$ C 44.62, H 3.40, N 14.19, found C 44.75, H 3.38, N 14.29.

*4.1.2.3.27. 1-(3-Bromo-4-methylphenyl)-2-methyl-4-nitro-1H-imidazole (**49**).* Yield 69%, light brown powder, m. p. 150–152 °C (methanol/water); ^{1}H NMR (300 MHz) 2.31 (s, 3H, CH$_3$ $_{imid}$), 2.43 (s, 3H, CH$_3$), 7.53 (dd, 1H, J = 8.1 Hz, J = 1,8 Hz, H-6′), 7.57 (d, 1H, J = 8.1 Hz, H-5′), 7.90 (d, 1H, J = 1.8 Hz, H-2′), 8.57 (s, 1H, H-5$_{imid}$); ^{13}C NMR (75.5 MHz) 13.4 (s, 1C, CH$_3$ $_{imid}$), 22.1 (s, 1C, CH$_3$), 122.7 (s, 1C), 124.3 (s, 1C), 125.1 (s, 1C), 129.2 (s, 1C), 131.6 (s, 1C), 129.2 (s, 1C), 131.6 (s, 1C), 134.5 (s, 1C), 138.9 (s, 1C), 144.8 (s, 1C, C-5$_{imid}$), 146.0 (s, 1C, C-4$_{imid}$); EA calcd. for $C_{11}H_{10}BrN_3O_2$ C 44.62, H 3.40, N 14.19, found C 44.60, H 3.49, N 14.57.

4.2. Antiprotozoal activity testing

4.2.1. Drug preparation

For the *in vitro* assays, a 10 mg/mL stock solution in dimethyl sulfoxide (DMSO) of the test compounds was prepared. For the *in vivo* studies, the test compounds were dissolved in DMSO, the resulting solution was further diluted with water up to 10% in volume. Drugs were administered to mice by gavage (oral administration) or intraperitoneal injection (i.p. administration).

4.2.2. In vitro trypanocidal and cytotoxicity assays

The compounds were tested in Minimum Essential Medium (50 μl) with Earle's salts, supplemented [41] with the following modifications: 2-mercaptoethanol 0.2 mM, Na-pyruvate 1 mM,

hypoxanthine 0.5 mM and 15% heat-inactivated horse serum. Serial drug dilutions of seven 3-fold dilution steps covering a range from 90 to 0.123 μg/mL were prepared. Then 3×10^4/mL bloodstream forms of *T. b. rhodesiense* STIB900 in 50 μl medium was added to each well and the plate was incubated at 37 °C under a 5% CO_2 atmosphere for 70 h. 10 μl of the viability marker Alamar blue (12.5 mg resazurin [Sigma] dissolved in 100 mL phosphate buffered saline) was then added to each well and the plates incubated for an additional 2–4 h to determine cell viability [42]. The assay was assessed by reading the fluorescence in each well at an excitation wavelength of 536 ηm and at an emission wavelength of 588 ηm. The IC_{50}s were calculated from the sigmoidal inhibition curves using SoftmaxPro software.

For the cytotoxicity assessment, 4×10^4/mL L-6 rat skeletal myoblast cells were seeded in 96 well plates. Test compounds were prepared and added as above. Incubations and assessment of cell viability were carried out as for parasite cultures.

4.2.3. In vivo trypanocidal assays

Acute infection with *Trypanosoma brucei rhodesiense* (STIB900): Groups of 4 mice were infected by intraperitoneal injection with 10^4 bloodstream forms of *T. b. rhodesiense* (STIB900) and treated with the test compounds or reference drugs once or twice daily for 4 consecutive days, starting on day 3 post-infection [33]. A control group was infected but not treated. Parasitemia was monitored using smears of tail-snip blood twice a week after treatment for two weeks followed by once a week until 60 days post-infection. Mice were considered cured if there was no parasitemia relapse detected in tail blood over the 60 days observation period.

Chronic CNS infection with *Trypanosoma brucei brucei* (GVR35): Groups of 5 mice were infected intraperitoneally with 2×10^4 bloodstream forms of *T. b. brucei* (GVR35) and treated with the test compounds or reference drugs once or twice daily from day 21 post-infection for 5 days [33,43]. A control group was treated on day 21 with a single dose of diminazene aceturate at 40 mg/kg i.p., which is subcurative as it clears the trypanosomes only in the hemolymphatic system but not in the CNS leading to a subsequent reappearance of trypanosomes in the blood. Parasitemia was monitored twice in the first week after treatment followed by once a week until 180 days post-infection. Mice were considered cured when there was no parasitemia relapse detected in tail blood over the 180 days observation period [33,43].

All protocols and procedures used in the current study were reviewed and approved by the local veterinary authorities of the Canton Basel-Stadt.

4.3. Genotoxicity assays

4.3.1. Drug preparation

For all tests, stock solutions were prepared by formulating the test compound in DMSO under subdued lighting conditions with the aid of vortex mixing, warming at 37 °C and ultrasonication, immediately prior to assay to give the maximum required treatment solution concentration. Subsequent dilutions were made using DMSO. The test article solutions were protected from light and used within approximately 4 h of initial formulation.

4.3.2. Bacterial mutagenicity tests

Strains: *Salmonella typhimurium* strains TA1535, TA1537, TA100, TA98 and TA102 were obtained from the UK NCTC. TA100NR and TA98NR, which lack the classical *Salmonella typhimurium* nitro-reductase, were obtained from Novartis Pharma AG (Switzerland) and the University of York CRU (UK) respectively. It is known that TA98 and TA98NR are also deficient in a second 'supplementary' nitro-reductase [34]. TA1535NR and derivatives of TA1535, TA1537 and TA102 containing knock-outs of the classical nitro-reductase gene were constructed and kindly supplied by Dr Masami Yamada, National Institute of Health Sciences, Tokyo (Japan).

Ames tests: Standard bacterial plate incorporation assays were carried out, using triplicate plating, essentially as described by [44]. Tests were carried out with and without rat liver post-mitochondrial fraction plus co-factors (S9 mix) to provide a mammalian metabolic activation system. The S9 fraction was obtained from Molecular Toxicology Incorporated USA and was prepared from Sprague–Dawley rats pretreated with the mixed cytochrome P 450 enzyme inducer Aroclor 1254. After incubation at 37 °C for three days, plates were scored for mutant colonies using a Seescan Colony Counter (Seescan plc) plate reader.

In the absence of rat liver S9, the positive controls used were for TA98 and TA98NR 4-nitroquinoline-1-oxide and for TA1535 and TA1535NR, TA100 and TA100NR, sodium azide. For TA1537, 9-aminoacridine was used and for TA102, mitomycin C. In the presence of S9, 2-aminoanthracene was used for all strains. 2-Nitrofluorene and nitrofurantoin were used as additional positive controls to check the effects of loss of the nitro-reductase enzymes in the NR strains on the detection of nitro-containing compounds. In the presence of S9, the positive controls used were benzo(a)pyrene for the TA98 strains and 2-aminoantracene for the TA100 strains.

4.3.3. In vitro micronucleus tests

Lymphocytes were obtained from healthy, non-smoking female donors. Whole blood cultures were established by placing 0.4 mL of pooled heparinised blood into a sufficient volume of HEPES-buffered RPMI medium containing 20% (v/v) heat-inactivated fetal calf serum and 50 μg/mL gentamycin. The mitogen, phytohemagglutinin (PHA), reagent grade, was included in the culture medium at a concentration of approximately 2% of culture to stimulate the lymphocytes to divide. Blood cultures were incubated at 37 ± 1 °C and rocked continuously for 48 h prior to treatment. Quadruple cultures were used for the negative control and duplicate cultures for the test compounds and the positive control. Tests were carried out with and without rat liver S9-mix.

Preliminary tests were carried out to determine the effects of the test compounds to determine concentrations that induced cytotoxicity. Data from concentrations exhibiting high cytotoxicity (>55%) were excluded from the analysis.

*4.3.3.1. Protocol used for compound **16**.* The test compound was added at 48 h following culture initiation (stimulation by PHA). Cells were exposed to the test compound for 3 h followed by a 21h recovery period (+/-S9). In addition (−S9), a further group was exposed for 24 h with no further recovery time, to explore the effects of extended exposure. Cytochalasin-B (6 μg/mL per culture), was added at the time of treatment. This generates binucleate cells by preventing cytokinesis, without preventing nuclear division. Scoring binucleate cells ensures that the cells scored have passed through one cell division. Several drops of cells suspended in fixative were spread onto multiple clean, dry microscope slides. After the slides were dried the cells were stained for 5 min in filtered 4% (v/v) Giemsa in pH 6.8 buffer. The slides were rinsed, dried and mounted with coverslips for scoring. One thousand binucleate cells were scored per replicate for the presence of micronuclei, the scoring of such cells ensures that the micronuclei seen have resulted from the last nuclear division. Slides were also examined for the proportions of mononucleate and binucleate cells per culture.

Measurements were made to check changes in the osmolality of the culture in the presence of the highest concentration of the test compounds and also for any pH changes. Studies have shown that significant changes in osmolality and pH can induce false positive results in these assays.

The positive controls used were mitomycin C (0.08 μg/mL) and vinblastine (0.03 μg/mL) for the cultures without S9 and cyclophosphamide (12.5 μg/mL), which requires metabolism by S9 to generate clastogenic metabolites, for cultures with S9.

*4.3.3.2. Screening protocol used for compounds **14**, **31** and **33**.* As above, but a 20 h treatment was used in the absence of rat liver S9, with a 28 h recovery period. In the presence of S9, a 3 h treatment period was used, with a 45 h recovery period. For compound **33**, quadruple cultures were used for the vehicle control and duplicate cultures for the test compound. For compounds **31** and **14**, duplicate cultures were scored for the vehicle control and single cultures for the test compounds. The positive controls used were 4-Nitroquinoline-*N*-oxide (2.5 or 5.0 μg/mL) in the absence of S9 and cyclophosphamide (6.25 or 12.5 μg/mL) in the presence of S9.

4.4. Redox potential measurement

One-electron reduction potentials were determined by pulse radiolysis following an established procedure [22].

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Contributors

B Bourdin Trunz identified the 1-aryl-4-nitro-1*H*-imidazoles of the present study as potential new drug candidates for HAT, and coordinated the chemistry work, the parasitology and genotoxicity studies with partners as part of DNDi's nitroimidazoles project. J Suwinski and R Jędrysiak designed and synthesized the molecules, the anrorc coupling reaction was developed in the laboratory of J Suwinski; B Bourdin Trunz, J Suwinski and R Jedysiak contributed to the structure–activity relationship (SAR) analysis. D Tweats acted as a toxicology expert consultant for the design and interpretation of the genotoxicity studies. M Kaiser and R Brun designed, conducted and interpreted the parasitology studies. E Torreele was the overall project leader of DND*i*'s nitroimidazoles project. B Bourdin Trunz drafted the manuscript with input from all authors.

Conflict of interest

The authors declare that they have no conflict of interests.

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Appendix. Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.ejmech.2011.01.071.

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