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doi.org/10.1002/open.202100180

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Development of Novel Isoindolone-Based Compounds against *Trypanosoma brucei rhodesiense*

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This study identified the isoindolone ring as a scaffold for novel agents against *Trypanosoma brucei rhodesiense* and explored the structure-activity relationships of various aromatic ring substitutions. The compounds were evaluated in an integrated in vitro screen. Eight compounds exhibited selective activity against *T. b. rhodesiense* (IC$_{50}$ < 2.2 μм) with no detectable side activity against *T. cruzi* and *Leishmania infantum*. Compound **20** showed low nanomolar potency against *T. b. rhodesiense* (IC$_{50}$ = 40 nм) and no toxicity against MRC-5 and PMM cell lines and may be regarded as a new lead template for agents against *T. b. rhodesiense*. The isoindolone-based compounds have the potential to progress into lead optimization in view of their highly selective in vitro potency, absence of cytotoxicity and acceptable metabolic stability. However, the solubility of the compounds represents a limiting factor that should be addressed to improve the physicochemical properties that are required to proceed further in the development of in vivo-active derivatives.

1. Introduction

Neglected tropical diseases (NTDs) affect more than one billion people and continue to cause immense suffering, disability and deaths worldwide. Sleeping sickness (Human African Trypanosomiasis – HAT) remains one of the more burdensome public health problems caused by two subspecies of *Trypanosoma brucei*. While *T. b. gambiense* causes a chronic illness with the onset of symptoms after a prolonged incubation period, *T. b. rhodesiense* elicits a more acute course with more aggressive effects within a few days or weeks after the bite of infected tsetse. Without prompt diagnosis and treatment preventing the parasites from invading the central nervous system, the disease is generally fatal.[1]

Initiatives to support global work against HAT have helped to discover and develop fexinidazole,[2] a new oral drug developed by DND*i* (Drugs for Neglected Diseases *initiative*) and currently approved to treat *gambiense* sleeping sickness. However, the arsenical melarsoprol that is estimated to kill up to 5% of patients is still the only treatment for the advanced stage *T. b. rhodesiense* infection, although it becomes ineffective after some time due to an increase in resistance.[3] As such, there is an urgent need to replace the currently available toxic drugs with orally bioavailable, highly potent, non-toxic alternatives. Recently, new heterocyclic compounds have been discovered through phenotypic screening as potential antitrypanosomal agents, marking renewed hope to treat sleeping sickness.[4–13] This approach has demonstrated to be successful for the discovery of first-in-class drugs providing an advantage in identifying compounds that are active against the whole cell, being especially efficient in infectious disease drug development.[14] We contributed to this by the development of a large number of new heterocyclic compounds having different ring systems[8,13,14] that were assayed against different *Trypanosoma* species. Some examples are presented in Figure 1. The biochemical targets or mechanisms of action of the hit compounds are unknown.

Our previous structure-activity relationship studies (SARs) revealed that the presence of the urea group attached to the 7-position (R^1, hit compound **1**) of the fused ring system proved to be essential for bioactivity; compounds with aromatic groups in region R^2 (hit compound **2**) were found to be more effective than aliphatics in increasing potency; amide, ester, and organic acid functions can be tolerated at the 3-position (hit compound **3**).[8,13]

Applying standard medicinal chemistry principles to design new compounds, we established the following requirements for the antiparasitic activity: a urea moiety linked to bulky amino-

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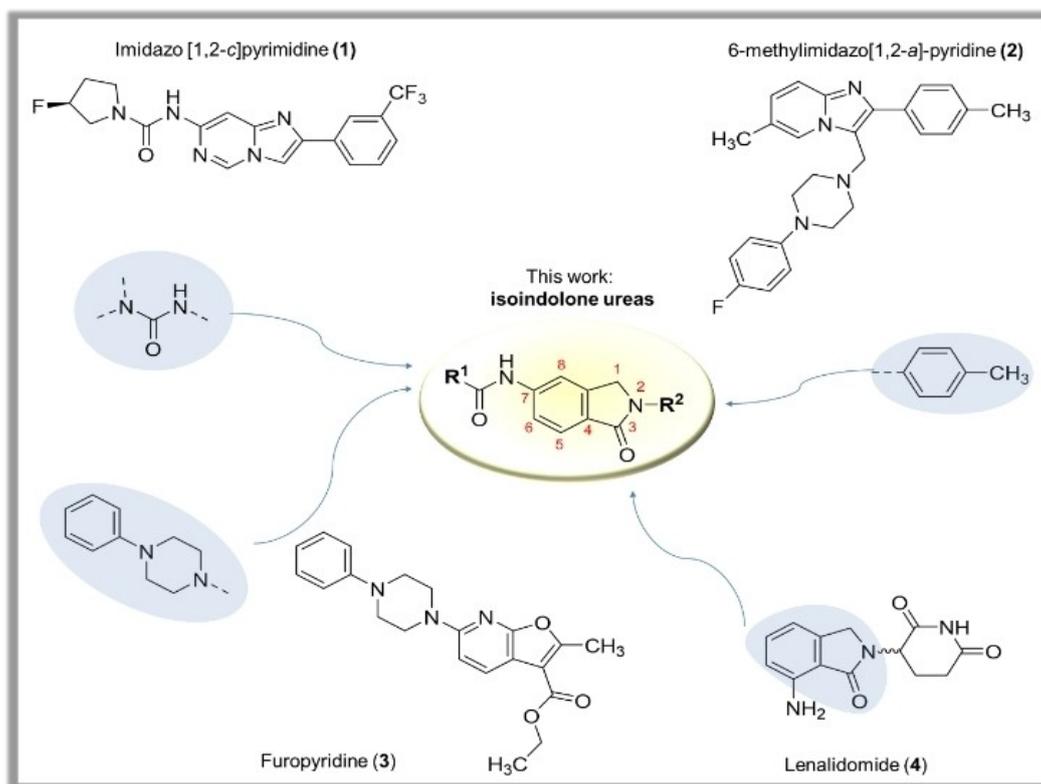European Chemical
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Figure 1. Initial hit compounds **1–4**. In the center: the isoindolone scaffold planned, synthesized, and assayed against a parasitic panel, including *T. b. rhodesiense*, *T. b. brucei*, *T. cruzi* and *Leishmania infantum*; and cytotoxicity for MRC-5 fibroblasts and primary mouse macrophages in the present work.

alkyl groups at the region R^1 (combinations of hit compounds **1** and **3**), an aromatic ring at the region R^2, and a carbonyl group linked to the nitrogen atom (lactam); forming an isoindolone ring. An isoindolone ring is the base scaffold for the commercial drug Lenalidomide (hit compound **4**), used to treat multiple myeloma. Thus, this pharmacophore already has drug-like features associated with the core structure and received our attention to be evaluated as an antitrypanosomal agent due to their similarity with our previously studied scaffolds. Herein, we report the development of a novel class of isoindolone ureas as a promising scaffold that showed selective in vitro potency against *T. b. rhodesiense*. These features can be seen in Figure 1.

Experimental Section

Synthesis of Isoindolone Ureas

We explored modifications of two regions of the central core via the introduction of various substituents at the urea (R^1) and the nitrogen at 2-position of the isoindolone ring (R^2) (Figure 1). The synthesis started with the bromination at the benzylic position through the reaction of *N*-bromosuccinimide with the commercially available 4-nitrobenzoate (**5**), generating the intermediate **6**. The subsequent reaction with the appropriate arylamine yielded the isoindolone core ring. The reduction of the nitro group was done using H_2 over Pd/C or an alternative mild reduction method using tin (II) chloride dihydrate to avoid or limit hydrodehalogenation (for nitroisoindolinone **7i** and **7j**, with phenylbromine or phenylchlorine in the structure, respectively), giving the amines **7f–j**, which reacted with triphosgene and respectivesecondary amines to furnish the corresponding urea derivatives **8–20** (Scheme 1).
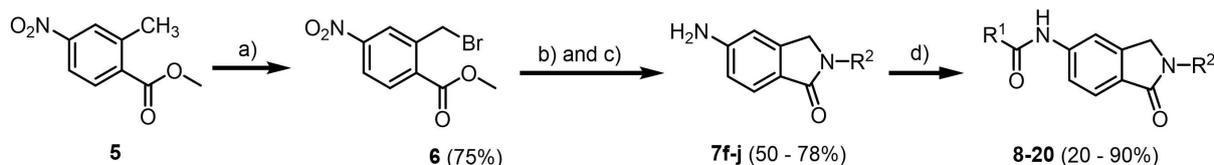
Scheme 1. Synthesis of the isoindolone ureas derivatives **8–20** (see Table 1 for R^1 and R^2). Reagents and conditions: a) NBS, benzoyl peroxide, CCl_4, 9 h, 85 °C; b) appropriate aniline, Et_3N, MeOH, reflux, 24 h, rt; c) 10% Pd/C, H_2, EtOAc, 4 h, rt or $SnCl_2 \cdot 2H_2O$, 48 h; d) triphosgene, Et_3N, DCM, 0 °C and then appropriate secondary amine, 0 °C to 25 °C, 15 h.

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Antiparasitic Activity, Cytotoxicity and Selectivity Index

We evaluated 13 new compounds through phenotypic assay against a parasitic panel, including *T. cruzi*, *T. b. brucei*, *T. b. rhodesiense* and *Leishmania infantum* (Table 1). Cytotoxicity against MRC-5$_{SV2}$ (human lung fibroblast cell line) and PMM (primary mouse macrophages) was evaluated to calculate the selectivity index (SI = ratio of cytotoxic CC$_{50}$ and antiparasitic IC$_{50}$). The β-galactosidase-expressing *T. cruzi* Tulahuen CL2 strain (nifurtimox-sensitive), *T. b. brucei* Squib 427, *T. b. rhodesiense* STIB-900 and *L. infantum* [MHOM/MA(BE)/67] were used. *T. cruzi* was maintained in MRC-5$_{SV2}$ human lung fibroblasts in MEM medium with 5% fetal bovine serum (FBS); *T. b. brucei* and *T. b. rhodesiense* were maintained axenically in HMI-9 medium with 10% FBS. *L. infantum* was maintained in golden hamsters from which spleen-derived amastigotes were collected and used for in vitro infection of peritoneal mouse macrophages (PMM) in RPMI-1640 with 5% FBS. Antiparasitic and cytotoxicity assays (MRC-5 and PMM) were performed as previously described.[15,16] All animal experiments were approved by the ethical committee of the University of Antwerp (UA-ECD-2019-10). Experimental details are provided in the Supporting Information and the cited literature.[17–19]

2. Results and Discussion

2.1. Structure-Activity Relationships (SAR)

The compounds were inactive against *T. cruzi* (IC$_{50}$ ≥ 64 μM) and few showed low antileishmanial activity with compound **13** being the most potent (IC$_{50}$ 12.7 μM). Eight and two of the compounds displayed high potency (IC$_{50}$ values < 2.4 μM) against *T. b. rhodesiense* and *T. b. brucei*, respectively. Two other compounds were also moderately active against *T. b. brucei*. Most compounds exhibited no detectable off-target cytotoxicity, except for compounds **13**, **15** and **16** exhibiting marginal cytotoxicity on PMM (CC$_{50}$. 32.0 μM). Compound **20** displayed no toxicity to MRC-5 and PMM cell lines. It suggests that a more

detailed analysis of the underlying SARs would be of interest for *T. b. brucei* and *T. b. rhodesiense*.

The isoindolone urea scaffold had alterations at either of the two terminal rings (R^1, R^2). To accomplish the SAR analysis in a feasible way, we introduced the substitutions systematically into specific regions to compare the biological activity of molecules that differ only by a single group.

The evaluation of the SAR is shown in Figure 2. The different regions of the molecule (R^1, R^2) were explored with 13 variants. At the left and right of Figure 2 the SAR analysis for the regions R^1 and R^2 is presented, respectively. The IC$_{50}$ (μM) values for analogs **8**–**20** are highlighted in green for *T. b. brucei* and in blue for *T. b. rhodesiense*. In the following, we discuss the most representative SARs established in this work.

The first SAR analysis aimed at the introduction of 3-fluoropyrrolidinyl (**9**) or 3-hydroxypyrrolidinyl (**11**) groups in region R^1. These modifications did not result in any detectable activity against the tested trypanosomatids. Meanwhile, the exchange of pyrrolidine group for a bulkier 4-fluorophenylpiperazine improved the activity (compound **14**, *T. b. brucei* IC$_{50}$ = 3.7 м; *T. b. rhodesiense* IC$_{50}$ = 0.8 м) compared to the inactive compounds **9** and **11** (Figure 2).

Modifications at the phenylpiperazine group also resulted in improvement of the *T. b. rhodesiense* activities (Figure 2). A five-fold increment of potency was achieved by the insertion of a chlorine atom at the 4-position (**17** vs. **18**). However, no significant improvement of activity against *T. b. rhodesiense* was reached by alteration of the chlorine for a fluorine atom (**13** vs. **19**, Table 1). On the other hand, a CF$_3$ group at the 3-position of the phenyl ring attached to piperazine moiety instead of a fluorine atom at the 4-position enhanced the potency 2.5-fold (**15** vs. **20**). Compound **20** is one of the most active and selective analogs against *T. b. rhodesiense* in this work (IC$_{50}$ = 0.04 м, 61 times more potent than against *T. b. brucei*).

The second SAR evaluation aimed at insertions of electron-withdrawing or -donating substituents in the phenyl group

Table 1. Antitrypanosomal/antileishmanial activities and cytotoxicity expressed in IC$_{50}$ (μM). All compounds show IC$_{50}$ > 64 μM towards *T. cruzi*.
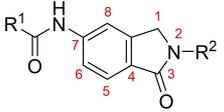
Compounds	R^1	R^2	*T. b. brucei*	*T. b. rhod.*	*L.inf*	MRC-5	PMM
8	(*S*)-3-fluoropyrrolidin-1-yl	phenyl	n.a	n.a	n.a	n.a	n.a
9	(*S*)-3-fluoropyrrolidin-1-yl	4-chlorophenyl	n.a	n.a	n.a	n.a	n.a
10	(*S*)-3-hydroxypyrrolidin-1-yl	phenyl	n.a	n.a	n.a	n.a	n.a
11	(*S*)-3-hydroxypyrrolidin-1-yl	4-chlorophenyl	n.a	n.a	n.a	n.a	n.a
12	4-fluorophenylpiperazin-1-yl	phenyl	n.a	n.a	45.2	n.a	n.a
13	4-fluorophenylpiperazin-1-yl	3,4-difluorophenyl	n.a	2.0	12.7	n.a	32
14	4-fluorophenylpiperazin-1-yl	4-chlorophenyl	3.7	0.8	n.a	n.a	n.a
15	4-fluorophenylpiperazin-1-yl	4-methylphenyl	n.a	0.1	32.5	n.a	32
16	4-fluorophenylpiperazin-1-yl	4-bromophenyl	0.4	0.05	20.3	n.a	32
17	phenylpiperazin-1-yl	3,4-dichlorophenyl	27.6	2.1	n.a	n.a	n.a
18	4-chlorophenylpiperazin-1-yl	3,4-dichlorophenyl	6.9	0.4	n.a	n.a	n.a
19	4-chlorophenylpiperazin-1-yl	3,4-difluorophenyl	44.7	1.8	n.a	n.a	n.a
20	3-(trifluoromethyl)phenylpiperazin-1-yl	4-methylphenyl	2.4	0.04	n.a	n.a	n.a

The values of IC$_{50}$ (μM) activities are averages of n = 3. n.a: not active at a limit concentration of 64 μM. Benznidazole, Suramin, Miltefosine and Tamoxifen were used as reference drugs to measure the activities (more details in Supporting Information Tables S1 and S2).
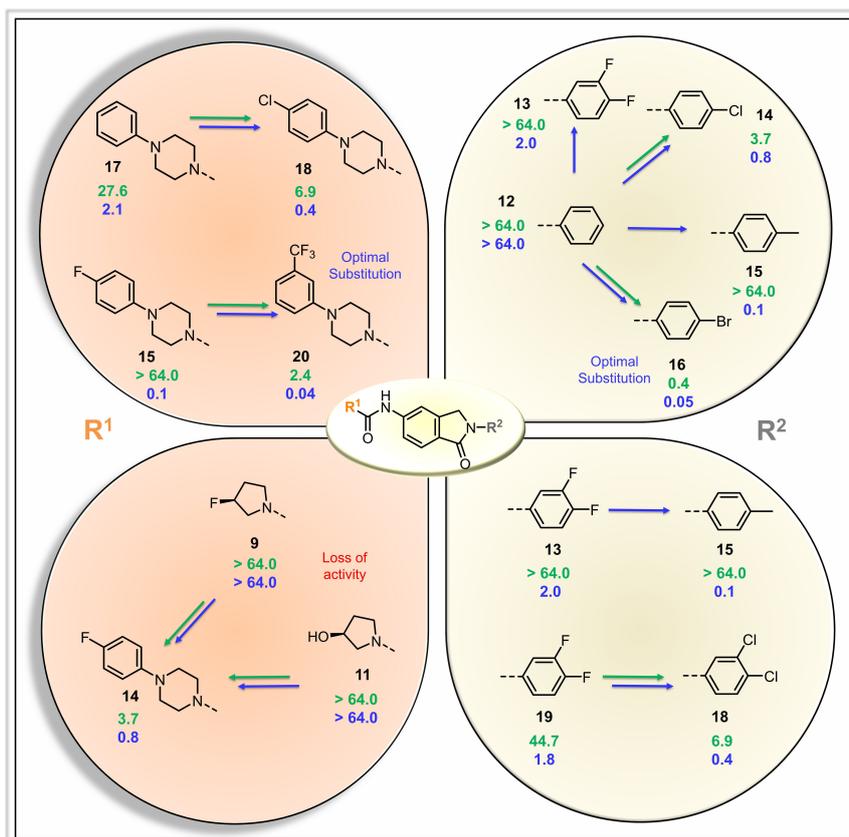
Figure 2. Activity profiles of the isoindolone ureas. Compounds assayed against *T. b. brucei* (highlighted in green)/*T. b. rhodesiense* (highlighted in blue) and results are expressed as IC_{50} (μM). Green and blue arrows represent the improvement of *T. b. brucei* and *T. b. rhodesiense* activities, respectively.

present in region R^2 (Figure 2). The addition of fluorine atoms at the 3,4-positions of the phenyl group in region R^2 enhanced the anti-*T.b. rhodesiense* activity more than 32-fold (**12** vs. **13**); compound **13** showed an IC_{50} value of 2.0 M. The insertion of a single chlorine atom at the 4-position of the phenyl group (compound **14**) resulted in an enhancement against both *T. b. brucei* ($IC_{50} = 3.7$ M) and *T. b. rhodesiense* ($IC_{50} = 0.8$ M).

The methyl group inserted at the 4-position of the aromatic ring (compound **15**, *T. b. rhodesiense* IC_{50} 0.1 M) enhanced the potency more than 640-fold compared to compound **12**. A substantial enhancement of activity for compound **16** was achieved by the introduction of a bromine atom at that same position of the aromatic ring (1280-fold, showing a *T. b. rhodesiense* IC_{50} value of 0.05 M), with an eight times greater activity for *T. b. rhodesiense* than for the *T. b. brucei* ($IC_{50} = 0.4$ M), indicating possible selectivity for the subspecies. The exchange of fluorine for chlorine atoms at the phenyl ring attached at the 2-position of the isoindolone portion enhanced the potency four-fold (**19** vs. **18**). Also, exchanging the 3,4-difluoro to a 4-methyl group enhanced the potency 20-fold (**13** vs. **15**).
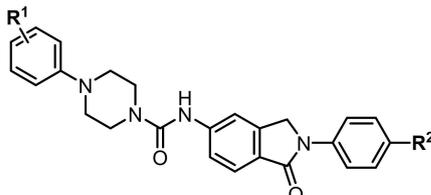
We were intrigued by the difference in the compounds' potency against the subspecies revealed by our studies. A database containing about 30 thousand compounds derived from phenotypic screenings, with available bioactivity against *T. b. rhodesiense*, *T. b. brucei*, *T. cruzi* and *Leishmania infantum*,

were extracted from the ChEMBL (https://www.ebi.ac.uk/chembl/). After applying appropriate filters, the search resulted in compounds based on adenosine (A),[20] pyridyl benzamide (B),[21] melamine nitroheterocycle (C),[22] triazine dimers (D)[23] and tetrazolyl pyrazolone (E, Figure S4 Supporting Information)[18] that were found to be significantly more potent against the human pathogenic subspecies *T. b. rhodesiense* than against *T. b. brucei*. No scaffold displayed similarity to our isoindolone-based compounds. These studies did not provide any rational explanation for the subspecies difference but indicate that finding compounds more potent against *T. brucei rhodesiense* is rare.

2.2. Solubility, in vitro Metabolic Stability and in vivo Efficacy Study

We first investigated the compounds' lipophilicity ($LogD_{7.4}$ values) since it is an important property for drug formulation and oral bioavailability (Table 2). Selected compounds **15**, **16** and **20** showed a $LogD_{7.4}$ value below 5.0. These compounds were furthermore assayed for in vitro metabolic stability using pooled mouse liver microsomes; all exhibited high metabolic stability over a 90 min time period.

Compounds **16** and **20** were selected as candidates for follow-up in vivo efficacy studies in mouse models of HAT. Mice

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Table 2. Solubility (Log$D_{7.4}$) and metabolic stability for selected compounds.

Compd	R^1	R^2	*T. b. brucei* IC$_{50}$ [µM]	*T. b. rhod.* IC$_{50}$ [µM]	logD$_{7.4}$[a]	% parent compound after 90 min[a]
15	4-F	−CH$_3$	>64.0	0.13	4.52 ±0.08	87.30±1.04
16	4-F	−Br	0.4	0.05	4.55 ±0.11	88.60±1.05
20	3-CF$_3$	−CH$_3$	2.4	0.04	4.48 ±0.36	84.80±3.05

[a] Compounds were tested in triplicate (n=3).

were allocated randomly to four groups of three animals each and infected on day 0 with 10^4 *T. b. brucei* trypomastigotes derived from a heavily infected donor mouse. Since this was a primary in vivo evaluation, the projected therapy aimed for maximal drug exposure using a high dose treatment regimen of 50 mg kg^{-1} b.i.d. for five days starting on the day of infection.

The oral bioavailability was checked at 1, 2 and 4 h after the first dose of the first day. Compound 20 showed no detectable blood levels, while compound 16 showed low levels in the range of the in vitro IC$_{50}$. At 4 h, blood levels were still increasing (Table 3). No signs of acute drug intolerance were noted in any of the treated animals.

Compound **16** showed no activity since all animals developed severe clinical trypanosomiasis at day 4 with high parasitemia (mean Log$_{10}$ of 8.9), similar to the vehicle control group (mean Log$_{10}$ of 8.7). At day 7, all animals except one succumbed to the infection. At this point, parasitemia was 1.2× 10^9 mL^{-1} blood (Log$_{10}$ of 9.1) and the animal was euthanized for animal welfare. In the suramin-treated group (10 mg kg^{-1} for 5 consecutive days), all mice survived until day 8 when the experiment was terminated. Nevertheless, the isoindolone-based scaffold may still have the potential to progress into lead-optimization in view of its highly selective in vitro potency, absence of cytotoxicity and acceptable metabolic stability. The

solubility of the compounds represents a limiting factor that should be addressed to improve the physicochemical properties that are required to proceed further in the development of in vivo-active derivatives. We will undertake a medicinal chemistry effort to modify the isoindolone structure with the goal of improving the pharmacokinetic characteristics of the compounds and investigate their ability to cross the blood-brain barrier.

3. Conclusions

A total of thirteen isoindolone ureas were prepared and tested for their antileishmanial and antitrypanosomal effects. The isoindolone ureas have been identified as a new class of compounds that exhibit selective potency against *T. b. rhodesiense*; compounds **16** and **20** were the most potent derivatives in this series with IC$_{50}$ values of 0.05 and 0.04 µM, respectively. Most compounds exhibited no cytotoxicity against MRC-5 and PMM cell lines. A CF$_3$ group at the 3-position of the phenyl ring attached to piperazine moiety in compound **20** resulted in the highest improvement of activity and selectivity, which warrants further exploration. To bring this class of compounds to the next step of drug discovery, we aim to synthesize new candidates with improved bioavailability profiles for further investigation of the SAR and further investigate their putative mode of action.

Author Contributions

The manuscript was written through the contributions of all authors. All authors have given approval to the final version of the manuscript. D.G.S. planned and synthesized the compounds. Biological assays were performed by P.B.F., R.H., A.M. and L.G.

Conflict of Interest

The authors declare no conflict of interest.

Table 3. *In vivo* efficacy studies in mouse model of HAT: blood levels of compound **16** at 1, 2 and 4 h after the first dose on the first day of treatment. Blood levels for compound **20** remain below the limit of detection (not shown).

Concentration in blood		[ng ml^{-1}]			[nM]		
	Time (h)	1	2	4	1	2	4
Compound 16	Mouse 1	50	288	275	98	566	541
50 mg kg^{-1}	Mouse 2	88	163	338	172	320	664
PO b.i.d.	Mouse 3	50	63	138	98	123	271
5 days	*Mean*	**63**	**171**	**250**	**123**	**336**	**492**
	Stdev	22	113	102	43	222	201

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