YALE JOURNAL OF BIOLOGY AND MEDICINE 94 (2021), pp.199-207.

ORIGINAL CONTRIBUTION

YALE JOURNAL OF BIOLOGY AND MEDICINE
YJBM
CELEBRATING 90 YEARS 1928–2018

New Series of Imidazoles Showed Promising Growth Inhibitory and Curative Potential Against *Trypanosoma* Infection

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The *Trypanosoma* spp. cause animal and human trypanosomiasis characterized with appreciable health and economic burden mostly in developing nations. There is currently no effective therapy for this parasitic disease, due to poor drug efficacy, drug resistance, and unwanted toxicity, etc. Therefore, new anti-*Trypanosoma* agents are urgently needed. This study explored new series of imidazoles for anti-*Trypanosoma* properties *in vitro* and *in vivo*. The imidazoles showed moderate to strong and specific action against growth of *T. congolense*. For example, the efficacy of the imidazole compounds to restrict *Trypanosoma* growth *in vitro* was ≥ 12-fold specific towards *T. congolense* relative to the mammalian cells. Additionally, the *in vivo* study revealed that the imidazoles exhibited promising anti-*Trypanosoma* efficacy corroborating the *in vitro* anti-parasite capacity. In particular, three imidazole compounds (C1, C6, and C8) not only cleared the systemic parasite burden but cured infected rats after no death was recorded. On the other hand, the remaining five imidazole compounds (C2, C3, C4, C5, and C7) drastically reduced the systemic parasite load while extending survival time of the infected rats by 14 days as compared with control. Untreated control died 3 days post-infection, while the rats treated with diminazene aceturate were cured comparable to the results obtained for C1, C6, and C8. In conclusion, this is the first study demonstrating the potential of these new series of imidazoles to clear the systemic parasite burden in infected rats. Furthermore, a high selectivity index of imidazoles towards *T. congolense in vitro* and the oral LD_{50} in rats support anti-parasite specific action. Together, findings support the anti-parasitic prospects of the new series of imidazole derivatives.

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Abbreviations: HAT, human African trypanosomiasis; NECT, Nifurtimox Eflornithine Combination Therapy; AAT, African animal trypanosomiasis; BSF, bloodstream form; HFF, human foreskin fibroblast; ROS, reactive oxygen species.

Keywords: Chemotherapy, Drug discovery, Medicinal biochemistry, Medicinal chemistry, Infectious Diseases, Trypanosomiasis

INTRODUCTION

Trypanosoma infection in animals or humans could result in dire outcomes if not treated [1]. At the same time, available treatment options are ineffective due to problems of limited efficacy, unwanted toxicity, remission, and drug resistance [2-4]. In developing nations and particularly in sub-Saharan Africa, tens of millions of people are at risk of *Trypanosoma* infection annually [5,6]. Human trypanosomiasis is among the parasitic diseases with significant health and socio-economic implication, particularly in less developed countries [1-4]. For example, both humans and livestock are at risk of the African trypanosomiasis with dire consequences for the economies of countries in sub-Saharan Africa [7]. The hemoflagellated protozoan parasites (ie, *Trypanosoma* species) are etiological agents for trypanosomiasis, and these pathogenic infections could be lethal in African mammalian hosts. The bovine trypanosomiasis or *nagana* is caused by *Trypanosoma brucei brucei* [8-10], while the *T. brucei rhodesiense* and *T. brucei gambiense* is responsible for the human African trypanosomiasis also referred to as sleeping sickness. Chemotherapy represents a key infection control strategy for this parasitic disease, but the current treatment options have several shortcomings, among which are poor efficacy and undesired toxicity [1,2,11]. There are few chemotherapeutic options available for the treatment of human African trypanosomiasis (HAT), especially the late stage of the disease, and until 2009 the drug of choice for the treatment of HAT was melarsoprol [12]. Among the few treatment options available is melarsoprol; this drug is used for the treatment of the late stage of the trypanosomiasis. However, melarsoprol has been shown to be highly toxic, causing encephalopathy in more than 10% of patients, of which 40% of these cases had fatal outcomes [13,14]. Also, reduced efficacy of melarsoprol has been reported in some areas [15,16]. Considered together, these factors underscore the necessity for new treatments for combating trypanosomiasis, a disease that currently threatens the health of tens of millions of people annually in the sub-Saharan Africa [6]. Accordingly, Nifurtimox Eflornithine Combination Therapy (NECT) was introduced, and recently fexinidazole has been approved (for treating both first and second stage HAT); both are currently being used for the treatment of HAT [17-19]. The NECT proved more advantageous than monotherapy, but there are limitations, like the requirement of intravenous administration for 7 days and all treatment-emergent adverse events (AEs), such as vomiting and the need for repeated administration of drugs. However, fexinidazole, a 5-nitroimidazole drug, emerged as a great promise to overcome the shortcomings of NECT [20,21]. On the other hand, African animal trypanosomiasis (AAT, *nagana*) is currently being treated with isometamidium chloride, diminazene aceturate, homidium bromide, and homidium chloride [22,23]. However, many cases of treatment failures and resistance to isometamidium chloride and diminazene aceturate, the main drugs of choice against AAT, have been documented across several countries in Africa posing significant threat to eradicating AAT [24-26]. Consequently, the absence of effective therapy and/or vaccine for both animal and human trypanosomiasis makes this parasitic disease a huge public health burden for as long as efforts at developing new trypanocides and vaccines are largely neglected. Therefore, new compounds that could become candidates in the drug development against trypanosomiasis are urgently needed.

In this regard, imidazole-derived compounds hold prospects for development as alternative anti-*Trypanosoma* agents. For decades, compounds with imidazole nucleus have been used to treat parasitic diseases [27,28]. The imidazole nucleus, a constituent of many bioactive heterocyclic compounds, is of great importance in medicinal and pharmaceutical chemistry research because a lot of imidazole derivatives have been shown to exhibit antimicrobial and antiparasitic activities [27-29]. In addition, we recently reported that imidazole-based compounds exhibited potent anti-*Trypanosoma* [4] and anti-*Toxoplasma* [30] activities. Furtherance to this, we modified the chemistry of these structures in a bid to enhance the anti-parasite selectivity. Interestingly, our findings (unpublished) revealed that, of the several modified imidazole-based compounds screened for anti-parasite activity, some of them showed specific *in vitro* anti-parasitic activity versus the mammalian cell. In light of this fact, the present study explored the potential of a new series of imidazole compounds for *in vitro* and *in vivo* action against *Trypanosoma* infection.

MATERIALS AND METHODS

Imidazole Compounds

We have previously reported the synthesis and characterization procedures for these series of compounds elsewhere [30,31]. The structural representations of the imidazole derivatives are as depicted in Figure 1.

Parasite Strain

The parasite culture was as described elsewhere [1,11]. For this study, a savannah type strain of *Trypanosoma congolense* IL3000 was used. A bloodstream form (BSF) of the parasite strain was maintained in the HMI-9 medium [32] and propagated in the air at 33°C. The culture medium consisted of Iscove's modified Dulbecco's medium (Sigma-Aldrich, Tokyo, Japan), 20% fetal bovine serum (FBS; Gibco, Invitrogen, Waltham,

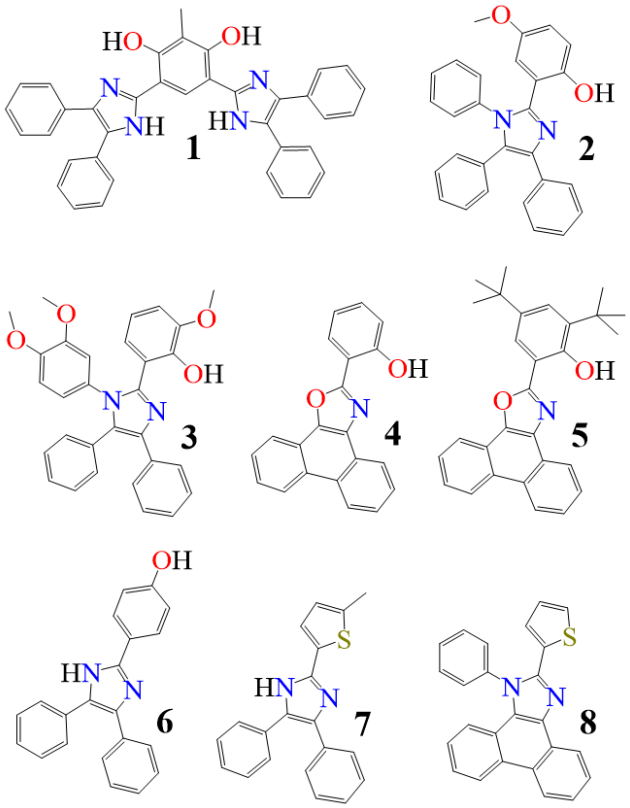

Figure 1. Structural representation of imidazole compounds.

MA, USA), 1 mM pyruvic acid sodium salt, 0.05 mM bathocuproine and 1.5 mM L-cysteine (Sigma-Aldrich, St Louis, MO, USA), 1 mM hypoxanthine and 160 µM thymidine (HT supplement: Thermo Fisher Scientific, Japan), 0.0001% 2-β-mercaptoethanol (Sigma-Aldrich), 10 µg/L insulin, 5.5 µg/L transferrin, and 6.7 ng/L sodium selenite (ITS-X: Thermo Fisher Scientific, Pittsburgh, USA). Every other day, the supernatant is replaced with a fresh medium to maintain the culture medium.

The Assessment of the Imidazole Compounds for In Vitro Anti-Trypanosoma Property

The assay to determine the anti-*Trypanosoma* activity of the imidazole derivatives was performed as described elsewhere [1,11]. Briefly, *T. congolense* were seeded at 1×10^5 cells/mL, in an optical bottom plate (Nunc™ 96-well, Thermo Fisher Scientific, Pittsburgh, USA). The parasites were dosed with imidazole compounds at various concentrations (0 to 25 µg/mL). After 72 h-incubation at 33°C, a luminescence-based protocol (CellTiter-Glo™, Promega Japan, Tokyo, Japan) was used to determine the parasite viability. We followed the manufacturer's instruction for the luminescence assay and the reading was performed on a microplate reader (GloMax, Promega Japan). The biological experiments were done in triplicate and performed independently three times.

The Assessment of Imidazole Compounds for Cytotoxic Property in Mammalian Cells

For this cytotoxicity assay, we used human foreskin fibroblast cells (HFF; ATCC®, Manassas, VA, USA). Initially, cultures of the HFF monolayers were grown to confluence in an atmosphere of 5% CO_2 and 37°C. The culture medium consisted of Dulbecco's Modified Eagle Medium (DMEM; Nissui, Tokyo, Japan), 10% (v/v) FBS, penicillin/streptomycin (10,000 U/mL, Leicestershire, UK) and GlutaMAX™-I (Gibco, Invitrogen, Waltham, MA, USA). For the cytotoxic assay, 1×10^5 cells were seeded per well in optical Nunc™ plates and incubated in an atmosphere of 5% CO_2 and 37°C for 72 h. Thereafter, the imidazole derivatives freshly prepared in a culture medium at various concentrations (0 to 1000 µg/mL) were added and the plates were further incubated for 72 h under the same condition. Control well had only the culture medium while the background correction was achieved with the medium only well. The viability of cells was assessed by using a colorimetric assay reagent (CellTitre-Aqueous, Promega, Madison, USA). We followed the manufacturer's instruction to determine the

cell viability and the absorbance reading was taken at 490 nm on a microplate reader (MTP 500; Corona Electric, Hitachinaka Japan). The biological experiment was carried out in triplicate and performed independently three times.

IN VIVO EXPERIMENTS

Parasite Strain

For the *in vivo* study, we used a *T. brucei brucei* (*Lafia* strain) sourced from the Nigerian Institute for Trypanosomiasis Research (NITR) in Vom, Nigeria. To maintain the *T. b. brucei* in the laboratory for this experimental purpose, parasite was passaged repeatedly in rats. Mostly, the inoculum suspension contained three or four trypanosomes per view at ×100 magnification and the rats received 0.2 mL of the inoculum. The monitoring of parasitemia was performed daily initially to establish infection and every other day afterwards until death of infected rats.

Experimental Rats

For the *in vivo* experimental model of trypanosomiasis, we used apparently healthy male Wistar rats weighing between 120 and 130 g. The rats were obtained from the Department of Biochemistry, Federal University of Technology, Minna, Nigeria and allowed to acclimate for 14 days before the experimental treatment commenced. The rats were kept in hygienic laboratory environment and allowed free access to rat chow and clean water *ad libitum*. Treatment of rats followed approved guidelines for humane handling of experimental animals [33]. The study was duly approved by the local institutional ethics committee on scientific research 19/20-04BCH.

Dose Determination

The LD_{50} in rats was determined to afford dose selection. The acute oral toxicity of the samples was conducted according to the Up and Down method described by OECD [34]. Briefly, healthy male rats whose body weights vary from 120 to 130 g were used for the experiment. The animals were fasted for 3 hours prior to the experiment. Animals were administered with a single dose (2000 mg/kg body weight) of each of the imidazole compounds and observed for mortality during 14-day study period.

Anti-Trypanosoma Evaluation of Imidazole Compounds In Vivo

Blood of a highly infected rat was collected and diluted with 0.9% physiological saline which served as inoculum (0.2 mL, 1 x 10^2 trypanosomes) for serial passaging into 50 healthy rats. The parasitemia was monitored through a 72-h lag phase of parasite growth and 5 days treatment commenced on day 0 of appearance of parasite (post pre-patent period) in the blood smear of rat tail using microscopic examination at ×100 as previously reported [1,35]. Diminazene aceturate was given as a reference drug. Details of the animal grouping were;

Group 1: Uninfected and untreated

Group 2: Infected with *T. b. brucei* but received corn oil (a vehicle for compound administration)

Group 3: Infected with *T. b. brucei* and given diminazene aceturate (3.5 mg/kg bw) in corn oil

Group 4-11: Infected with *T. b. brucei* and treated with imidazole derivatives (C1, C2, C3, C4, C5, C6, C7, C8) in corn oil (40 mg/kg bw).

Statistical Consideration and Presentation of Data

The results were analyzed by using a one-way ANOVA (GraphPad Software Inc., San Diego, CA, USA) and the data are presented as the average value plus or minus standard error of mean (SEM). Comparisons among groups were determined by using Dunnet post-hoc test, while *p*-values at ≤ 0.05 were taken to be statistically significant. For *in vitro* assay, a dose-response plot was used to estimate the imidazole concentration that reduced the viability of mammalian cells and/or parasites by 50% (ie, EC_{50} and/or IC_{50} value), while the curve was fitted using a non-linear regression analysis.

RESULTS AND DISCUSSION

Recently, we demonstrated that some imidazole-based compounds have strong anti-parasitic potentials including against *Trypanosoma* sp. and *Toxoplasma gondii* [4,30]. Furtherance to these findings, in the present study, we investigated the *in vitro* and *in vivo* efficacy of a new series of imidazole derivatives against *Trypanosoma* sp.

To determine the anti-*Trypanosoma* property of the imidazole compounds, we incubated fresh suspension of *T. congolense* with eight imidazole compounds at various concentrations between 0 and 25 µg/mL for 72 h and the parasite viability was assessed by using a luminescence-based method. To validate the *in vitro* anti-parasite assay, a reference drug pentamidine was included. As expected, pentamidine appreciably hindered the *in vitro* growth of *T. congolense*. Interestingly, all the imidazole compounds screened for *in vitro* anti-parasite properties, showed moderate to strong promising action and selectivity against the parasite versus the mammalian cell (Table 1, Figure 2). In particular, C2 and C5 showed strong activity against the growth of *T. congolense* (Figure 2). At the same time these compounds exhibited no detectable cytotoxicity in mammalian cells at the effective anti-*Try-*

Table 1. *In vitro* activity of imidazole derivatives against *Trypanosoma congolense*.

Compound codes	EC_{50} against *T. congolense* (µg/mL)	IC_{50} against HFF cells (µg/mL)	Selectivity index
1	21.17	257	≥12
2	11.29	8182	≥724
3	28.62	5885	≥206
4	44.48	ND	ND
5	6.82	ND	ND
6	19.20	ND	ND
7	25.26	1988	≥79
8	71.79	4526	≥63

ND – Not determined

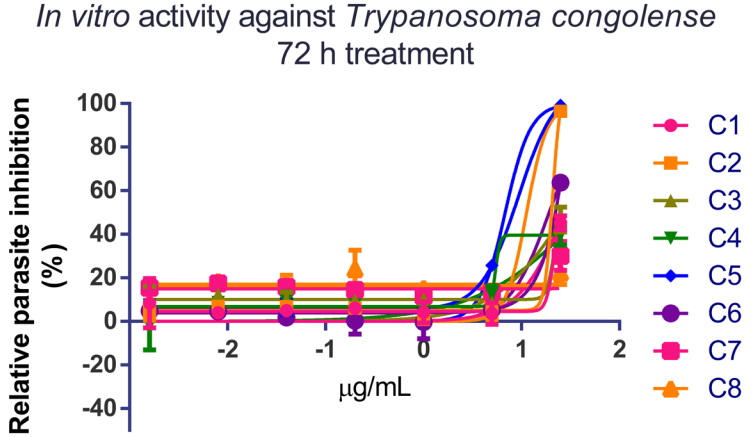

Figure 2. Effect of imidazole derivatives on the *in vitro* growth of *Trypanosoma congolense*. Data are presented as the mean of three replicates plus or minus standard error of mean (SEM). After a 72 h-incubation at 33°C, the viability of parasites was evaluated through a luminescence-based protocol. The biological experiment was done in triplicate and repeatedly and independently performed three times.

panosoma doses. In fact, the selectivity index which is the ratio of the dose that caused 50% toxicity (IC_{50}) in mammalian cells versus that of the parasite revealed good and promising prospective specific action of the imidazole compounds towards the parasite versus mammalian cell. The imidazole compounds demonstrated ≥ 724 to ≥ 12-fold specificity against the parasite versus the mammalian cells. The finding suggests parasite specific toxic action by the imidazole compounds. Considered together, the findings are consistent with the anti-parasitic potential of imidazole derivatives as reported previously [4,30]. Furthermore, these findings affirm the prospects of imidazole compounds as alternative source of anti-parasite therapy. Recently, imidazole-based drug was approved for treatment of human African trypanosomiasis [36]. Although we may not be able to tell how the imidazole derivatives affect the parasite, several investigations have revealed the capacity of these compounds to cause cellular toxicity through production of reactive oxygen species (ROS) [37]. Therefore, it is plausible that ROS and/or oxidative stress likely form part of the anti-parasitic action of these imidazole compounds.

Next, we sought to assess the *in vivo* efficacy of the imidazole compounds on *Trypanosoma* infection using a rat model of experimental infection. Treatment efficacy was evaluated *viz-a-viz* reduction in rat systemic parasite burden. The rat parasite load has been correlated with severity of infection [35]. Firstly, we determined the oral LD_{50} in rats and found that it was >2000 mg/kg body weight (bw). The infected animals were treated daily for 5 days after establishment of infection. Interestingly, the results are promising (Figures 3 and 4); the parasite burden for the imidazole-treated animals reduced appreciably when compared with the untreated control. Even though, all the imidazole derivatives caused drastic reduction in parasite burden compared with the control, only C1, C6, and C8 caused total clearance of the rat systemic parasite and the animals were declared cured since

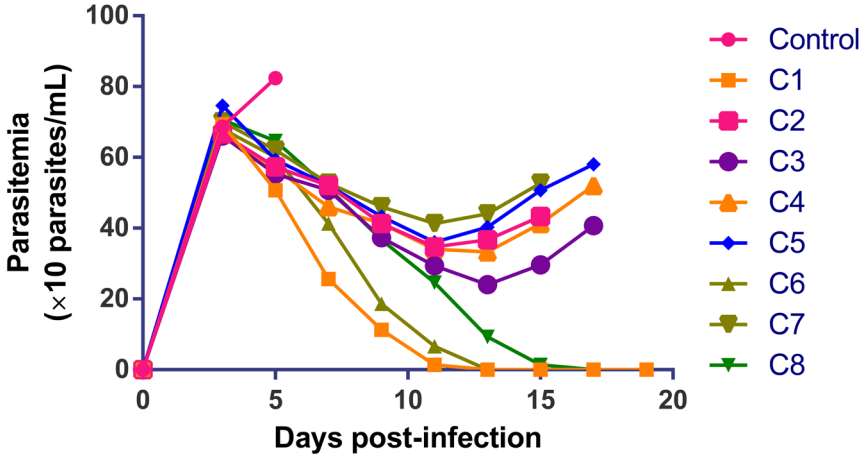

Figure 3. Effect of imidazole derivatives on *Trypanosoma brucei* infection in rats. Data are presented as the mean of five replicates plus or minus standard error of mean (SEM). Treatment commenced 72 h post-infection. Parasitemia was estimated from a fresh blood smear obtained from the tail vein of infected rats. Parasite count was performed under a light microscope (×100).

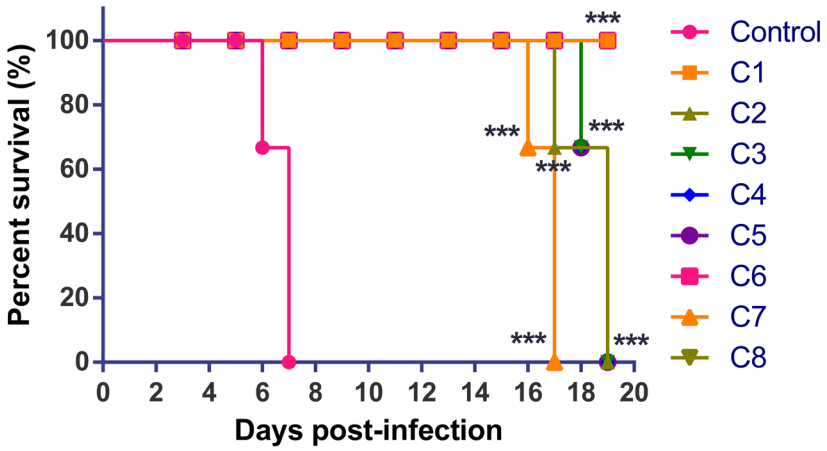

Figure 4. Survival curve for infected animals following treatment with imidazole derivatives. Data are presented as the mean of five replicates plus or minus standard error of mean (SEM). Treatment commenced 72 h post-infection. *** versus control is significant at $p<0.05$.

animals did not die and no remission occurred even after more than 12 weeks of daily observation. The untreated animals died 3 days post-infection. In contrast, C2, C3, C4, C5, and C7 decreased the parasite burden reduced and extended survival time for 14 days, but animals succumbed to death thereafter. Meanwhile, the infected rats that received diminazene aceturate (3.5 mg/kg bw) survived the infection and had their systemic parasite burden cleared (data not shown). That the imidazole derivatives not only decreased parasite burden but extended survival time of infected rats further underscore their prospects for development as likely alternative agents for treatment of *Trypanosoma* infections. In this study, it is worthy of note that compounds 1, 6, and 8 which showed promising curative potential against the *in vivo Trypanosoma* infection also had strong parasite specific activity *in vitro*.

Over the years, research has revealed several imidazole-based compounds as potent antiparasitic agents. Bhandari et al. [38] synthesized a series of aryloxy alkyl/aryl alky imidazole compounds and evaluated their activity against *Leishmania donovani* both *in vitro* and *in vivo*. *In vitro*, the 19 imidazole derivatives inhibited

the promastigotes by 94-100% at concentrations as low as 10 µg/mL while some of the imidazole compounds inhibited the amastigotes at an IC_{50} range of 0.47-4.85 µg/mL. In fact, some of these imidazole compounds have shown promising specific action *in vitro* and *in vivo*. In addition, Papadopoulou et al. [39] reported an *in vitro* characterization of a series of novel 3-nitro-, and 2-nitro-1*H*-imidazole-based amides and sulfonamides. Of the 36 imidazole compounds evaluated, 23 showed significant activity against the intracellular amastigotes of *Trypanosoma cruzi*, with about 58-fold potency more than the standard drug benznidazole and IC_{50} value between 28 nM and 3.72 µM. There was no accompanying toxicity to mammalian L6 host cell lines. The nitrotriazoles which had moderate activity against the trypomastigotes of *Trypanosoma brucei rhodesiense* had 5- to 31-fold activity against the trypomastigotes of *T. b. brucei*. Furthermore, three of the 3-nitro imidazole expressed activity against the axenic form of *L. donovani*. More so, Papadopoulou et al. [40] synthesized and assessed a series of 5-nitro-2-aminothiazole-based compounds for *in vitro* anti-trypanosomastid activity against *T. cruzi, T. brucei rhodesiense,* and *L. donovani.* All compounds showed significant activity against the amastigotes of *T. cruzi* and *T. b. brucei,* while only four of the compounds showed a level of activity against *L. donovani* and none of the compounds had activity against *T. rhodesiense*. However, all the thiazole-based compounds displayed PSA values > 100 $Å^2$, typifying their unlikelihood to cross the blood-brain barrier *in vivo* and are as such less potent anti-chagasic agents than the 3-nitrotriazole counterparts.

Furthermore, on account of the structure-activity relationships, the notable inhibitory activities of the eight compounds (1, 2, 3, 4, 5, 6, 7, and 8) against the *T. congolense* may be connected to the peculiarity of their structures. The structural motif of any class of compound is responsible for the observed bioactivities [30,41,42]. While the imidazole compounds were structurally diverse, some notable features responsible for the activities are observed. For example, the bis-imidazole compound with a notable *T. congolense* inhibitory activity has a central hydroxyl phenol and is quite uniquely symmetric. The central methylresorcinol group seems to enhance the activation of the two linked imidazole derivatives for improved interaction with the *T. congolense.* Although, it has a relatively high molecular weight, the two hetero-nitrogen molecules in each of the imidazole moieties are cheaply available for interactions unlike in scenarios wherein the nitrogen molecules are locked by attachment of bulky phenyl groups. More so, this bis-imidazole may ionize to give two charged entities with identical properties and thus increases the potency.

Compounds 2 and 3 have improved electron donating methoxy groups on the attached aryl forming a C-C bond with the imidazole ring, thereby enhancing the activity of the entire compound. Compounds 4 and 5 are oxazole with related structural similarities. It is likely that the small molecular sizes of the phenyl-substituted oxazoles C4 and C5 and imidazole C6 apparently contributed to their pharmacokinetics and bioactivities. The ortho-phenolic substituents on oxazole C4 and C5 as well as the para-phenolic substituent on the imidazole C6 might enhance the increased electron density on the central heteronuclei, thereby facilitating improved interactions with the proteins of the organism. Compounds that increase binding energies or affinity to protein targets of organisms are known to be better inhibitors.

In thiophene-imidazole compounds, the selective activity of some of the compounds cannot be vividly explained. However, the relatively small size and the addition of heteroatom with lone pairs of electrons increase the potential for interaction as observed in C7. In addition, compound 8 having phenanthrene moiety yielded an increased inhibitory activity against the *T. congolense*. Taken together, the careful consideration of the structural diversity of compounds as indicated could partly explain the observed activities of some of the tested compounds.

Identifying new compounds with potent activity against trypanosome will reduce the heavy burden of the disease on both humans and animals. Currently, the animal trypanosomiasis is responsible for the economic loss which has been estimated to about $1 billion annually [1]. This disease reduces animal production yield and is a contributing factor to protein malnutrition among growing children. Human trypanosomiasis on the other hand affects quality of family life and constitutes a heavy disease burden on the impoverished population. The findings in this study hold prospects for identifying newer anti-parasitic agents and thereby leading to reduced disease burden, better treatment outcomes, as well as improved socio-economic status of the affected population. In conclusion, this is the first report showing that imidazole compounds cleared systemic parasite burden in rats. In addition, our findings support further the *in vitro* anti-parasitic potential of imidazole compounds against *T. congolense*. Taken together, results warrant further studies to explore C1, C6, and C8 as promising candidates for newer anti-*Trypanosoma* therapy. In spite of the promising prospects of the new imidazole derivatives as potential therapeutics for trypanosomiasis, it should be noted that due to available resources, two different strains of the parasite were used for *in vitro* and *in vivo* work. This might represent a limitation to the interpretation of the findings in this study.

Acknowledgments: The authors appreciate the International Foundation for Science (IFS; Grant F/5672-2).

Additional Information: CCDC 850686 contains the supplementary crystallographic data for compound C1. The data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44 01223 336033; Email: deposit@ccdc.cam.ac.uk).

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