Discovery of potent nitrotriazole-based antitrypanosomal agents: *In vitro* and *in vivo* evaluation

Maria V. Papadopoulou[a]*, William D. Bloomer[a], Howard S. Rosenzweig[b], Ivan P. O'Shea[c], Shane R. Wilkinson[c], Marcel Kaiser[d,e] , Eric Chatelain[f], Jean-Robert Ioset[f]

[a]*NorthShore University HealthSystem, Evanston, IL, US;* [b]*Oakton Community College, Des Plaines, IL, US;* [c] *School of Biological & Chemical Sciences, Queen Mary University of London, London, UK;* [d]*Swiss Tropical and Public Health Institute, Parasite Chemotherapy, Basel, Switzerland;* [e]*University of Basel, Basel, Switzerland;* [f]*Drugs for Neglected Diseases initiative (DNDi), Geneva, Switzerland.*

*Correspondence to: Maria V. Papadopoulou, Ph.D., NorthShore University HealthSystem, Department of Radiation Medicine, 2650 Ridge Ave., Evanston IL, 60201, USA.

Tel: (847)570-2262; Fax: (847)570-1878

e-mail: mpapadopoulou@northshore.org

Running Title: *Potent nitrotriazole-based antitrypanosomal agents*

Abstract: 3-Nitro-1*H*-1,2,4-triazole- and 2-nitro-1*H*-imidazole-based amides with an aryloxy-phenyl core were synthesized and evaluated as antitrypanosomal agents. All 3-nitrotriazole-based derivatives were extremely potent anti-*T. cruzi* agents at sub nM concentrations and exhibited a high degree of selectivity for the parasite. The 2-nitroimidazole analogs were only moderately active against *T. cruzi* amastigotes and exhibited low selectivity. Both types of compound were active against *L. donovani axenic* amastigotes with excellent selectivity for the parasite, whereas three 2-nitroimidazole-based analogs were also moderately active against infected macrophages. However, no compound demonstrated selective activity against *T.b. rhodesiense.* The most potent *in vitro* anti-*T. cruzi* compounds were tested in an acute murine model and reduced the parasites to an undetectable level after five days of treatment at 13 mg/kg/day. Such compounds are potential inhibitors of *T. cruz*i CYP51 and, being excellent substrates for the type I nitroreductase (NTR) which is specific to trypanosomatids, work as prodrugs and constitute a new generation of effective and more affordable antitrypanosomal agents.

Key words: *nitrotriazoles; nitroimidazoles; type I nitroreductase; antitrypanosomal agents; Chagas disease; L. donovani*

[1]**Abbreviations:** NTD**,** Neglected tropical diseases; *T. brucei, Trypanosoma brucei;* HAT, human African trypanosomiasis; *T. cruzi, Trypanosoma cruzi;* Bnz, benznidazole (N-benzyl-2-(2-nitro-1H-imidazol-1-yl)acetamide); Nfx, nifurtimox (4-(5-nitrofurfurylindenamino)-3-methylthio-morpholine-1,1-dioxide); NTR, type I nitroreductase; TcNTR, *T. cruzi* NTR; TbNTR, *T. brucei* NTR; CYP51, sterol 14α-demethylase enzyme; TcCYP51, *T. cruzi* CYP51; IC_{50}, concentration for 50% growth inhibition; SI, selectivity index; SAR, structure-activity relationships; TDR, Tropical Diseases Research (http://www.who.int/tdr/en/).

1. Introduction

Parasitic infections caused by trypanosomatids constitute a major health problem in mainly resource poor countries around the world and diseases acquired by such infections are considered 'neglected' because they have received limited funding for discovery, development and delivery of new treatments. Amongst neglected tropical diseases (NTD)[1], human African trypanosomiasis (HAT; caused by *T. b. rhodesiense* and *T. b. gambiense*) is endemic throughout sub-Saharan Africa while American trypanosomiasis (Chagas disease; caused by *T. cruzi*) affects populations in South and Central America. In contrast, leishmaniasis (caused by *Leishmania* species) is prevalent in many sub-tropical and tropical regions of the World [1]. It is estimated that together these three insect transmitted diseases are responsible for more than 110,000 deaths per year [2]. Although the incidence of *T. cruzi* infection has significantly declined recently due to implementation of vector control initiatives, the number of cases in non-endemic sites (United States, Australia, Europe and Japan) is rising, primarily due to international population migration and contaminated blood transfusions [3,4].

The treatment of neglected diseases is based on pharmaceuticals with poor efficacy, significant side effects, high cost, poor oral bioavailability requiring a parenteral administration, long treatment course and emergence of resistance. Thus, severe toxicity and long treatment requirements coupled with limited efficacy are associated with nifurtimox (Nfx) and benznidazole (Bnz), the two currently used medications for Chagas disease [5, 6]. Similarly, drugs used to treat HAT and leishmaniasis are highly toxic (e.g. melarsoprol, antimonials), or require i.v. administration (e.g. melarsoprol, suramin, DFMO, antimonials) resulting in severe side effects, or are of high cost (e.g. DFMO, liposomal amphotericin B, miltefosine, and

paromomycin) [7-9]. Therefore, new effective, safe and affordable drugs are urgently needed for the treatment of these neglected diseases.

Inhibitors of the fungal sterol 14α-demethylase enzyme (CYP51) and currently inhibitors of the orthologous enzyme *T. cruzi* CYP51 (TcCYP51) have demonstrated promising efficacy against Chagas disease in preclinical studies [10-15]. However, in patients with chronic Chagas disease, the antifungal agent posaconazole was shown to be inferior to Bnz based on qPCR detection of *T. cruzi* DNA, measured 10 months after the end of treatment [16]. Moreover, in newly designed *in vitro* assays, nitroheterocyclic compounds were more efficacious trypanocidal agents compared to CYP51 inhibitors [17], and in an acute murine infection model with Bnz and posaconazole administered concomitantly or in sequence, a positive interaction was observed between the two drugs [18]. These data suggest that inhibitors of sterol biosynthesis may not be as efficient as single chemotherapeutic agents against Chagas disease as they are against fungal infections, but they may be effective in combination with known or new nitroheterocyclic antichagasic agents. However, combining agents *in vivo* cannot always be effective due to differential pharmacokinetic behavior of each drug, failure to obtain an optimal required concentration and possible unexpected adverse effects. Combining CYP51 inhibition characteristics with bioreduction properties of a nitro-group in one molecule may offer a better solution in drug development against Chagas disease and other trypanosomatid-induced diseases. This strategy has been successfully used in numerous occasions, including drugs for cancer, asthma and chronic obstructive pulmonary disease as well as neurodegenerative diseases [19-22].

We have demonstrated that a variety of 3-nitro-1*H*-1,2,4-triazole-based compounds exhibit excellent anti-*T. cruzi* activity both *in vitro* and *in vivo*, with several analogs also showing appreciable anti-*T. b. rhodesiense* activity *in vitro* [23-27]. Interestingly, 3-nitrotriazole-based

compounds are significantly more potent and less toxic than their 2-nitroimidazole-based counterparts [23-27], with part of the trypanocidal activity being dependent on the parasite's expression of an oxygen-insensitive type I nitroreductase (NTR), an enzyme absent from most other eukaryotes [23, 24, 26, 27]. Type I NTR, via a series of 2 electron reduction reactions which lead to the production of toxic metabolites, is responsible for the trypanocidal activity of Nfx, Bnz and other nitroheterocyclic prodrugs in general [29-32]. More recently, we have developed 3-nitrotriazole-based amides with a linear, rigid core, as well as 3-nitrotriazole-based carbinols (fluconazole analogs) which, in addition to being substrates for type I NTR, are also inhibitors of TcCYP51 [33]. These bifunctional agents exhibit promising anti-*T. cruzi* activity *in vitro* and *in vivo* and have excellent ADMET characteristics [33].

In the current work we have expanded our search for more potent bifunctional antichagasic agents by investigating the role of an aryloxy-group in the scaffold of 3-nitrotriazole-based linear amides. Imidazole-based inhibitors of TcCYP51 with an aryloxy group in their scaffold have been previously identified [15]. Therefore, we hypothesized that 3-nitrotriazole-based analogs could potentially be inhibitors of this enzyme, in addition to their capacity of being substrates of type I NTRs.

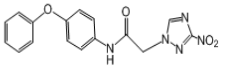
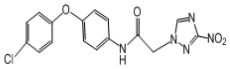
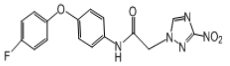
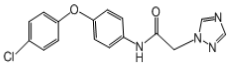
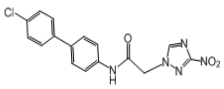
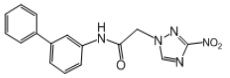
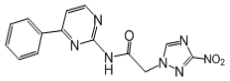
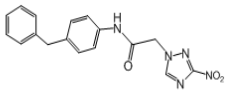
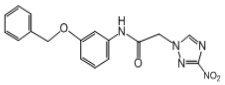
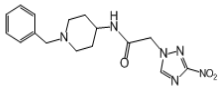
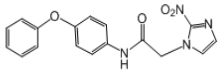
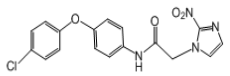
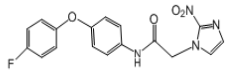
2. Results and Discussion

2.1. Chemistry

The structures of synthesized compounds are shown in Table 1. Their synthesis is straightforward, based on well-established chemistry and outlined in Scheme 1. Thus, amides **2-14** were obtained by nucleophilic substitution of the appropriate chloroacetamides **1a-h** with the potassium salt of 3-nitro-1,2,4-triazole, 2-nitroimidazole or 1,2,4-triazole under refluxing

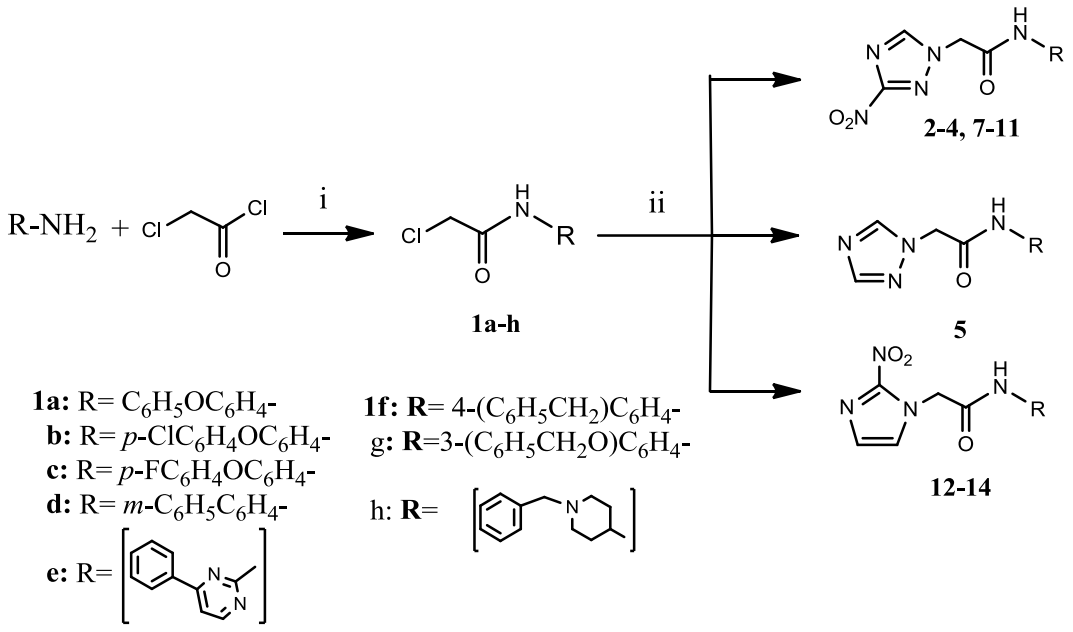
conditions. Chloroacetamides **1a-h** were prepared from appropriate, commercially available aryl/alkylamines and chloroacetyl chloride in the presence of triethylamine at room temperature.

Table 1. *In vitro* antiparasitic activity, host toxicity and physical properties of tested compounds.

ID No	*T.b.rhod.*[a] IC50 µM	SI	*T. cruzi*[b] IC50 µM	SI	*L.don. Axen.*[c] IC50 µM	SI	Cytotox. L6[d] IC50 µM	Chemical Structure	Bnz/comp	clogP	PSA (Å^2)
melar.	0.009 ± 0.0014										
Bnz			2.153 ± 0.176								
Milt.					0.162 ± 0.017						
2	2.7	34	0.065	1416	0.27	339	92		31	2.161	118
3	1.3	22	0.008	3615	0.034	853	29		254	2.874	118
4	2.0	35	0.036	1954	0.18	384	70		56	2.304	118
5	26		18	7.5	29	5	137			3.030	66
6	0.90	140	0.045	2797	0.17	738	126		45	2.716	109
7	58	0.78	0.18	252	1.8	25	45		13	1.951	109
8	139	1.2	2.5	66	>308		169		0.8	2.03	131
9	2.1	17	0.068	528	1.2	31	36		34	2.130	109
10	4.9	14	0.028	2415	3.4	20	68		17	2.81	115
11	12	9.6	8.0	14	133	<1	114		0.28	0.054	112
12	15		4.2	15	0.79	79	62		0.48	2.972	106
13	2.6	50	2.6	49	0.14	937	127		0.78	3.685	106
14	13	9	3.3	37	0.29	418	123		0.61	3.115	106

[a]*T.b. rhodesiense,* strain STIB 900 trypomastigotes; [b] *T. cruzi,* strain Tulahuen C4 amastigotes; [c] *L. donovani axenic,* strain MHOM-ET-67/L82 amastigotes; [d]Cytotoxicity in the host L6 cells. SI is the ratio: IC_{50} in L6 cells/IC_{50} in each parasite. The highest concentration tested in L6 cells was 100 µg/mL and no solubility problems were observed for any compound. Reference drugs: Melarsoprol (Melar.), Benznidazole (Bnz), Miltefosine (Milt.). The IC_{50} value of each reference drug is the mean from multiple measurements in parallel with the compounds of interest. Bnz/comp is the ratio of IC_{50} values against *T. cruzi* obtained by Bnz and each compound. PSA: polar surface area. All physical properties were predicted by using the Marvin Calculator (www.chemaxon.com). IC_{50} values are means of 2 to 3 measurements. The SD was < 5%.

Scheme 1: Synthesis of compounds on Table 1.



i) Et_3N, CH_2Cl_2, RT; ii) 3-Nitro-1,2,4-triazole/2-nitroimidazole/1,2,4-triazole, KOH, CH_3CN, reflux 9h.

2.2. Biological Evaluation

2.2.1. Anti-parasitic activity

Compounds on Table 1 were screened for anti-parasitic activity against three trypanosomatids: *T. cruzi, T. b. rhodesiense* and *Leishmania donovani*. The concentration of compound that inhibits parasite growth by 50 % (IC_{50}) was calculated from dose response curves for each parasite (Table 1). Compounds were also tested for toxicity in L6 rat skeletal myoblasts, the host cells for *T. cruzi* amastigotes, in order to calculate a selectivity index for each parasite (SI = $IC_{50L6}/IC_{50parasite}$) (Table 1). According to the TDR (Special Programme for Research and Training in Tropical Diseases, World Health Organization) criteria for antiparasitic activity and selectivity, an IC_{50} of <4.0 μM, between 4.0-60 μM or >60 μM, designates 'active', 'moderately active' or 'inactive' compounds, respectively, against *T. cruzi* amastigotes, while a SI of ≥50 is required; for blood stream form (BSF) *T. b. rhodesiense,* IC_{50} values of <0.5 μM, between 0.5-6.0 μM or > 6.0 μM identify 'active', 'moderately active' or 'inactive' compounds, respectively, while a SI value of ≥100 is desired; finally, for *L. donovani* amastigotes, IC_{50} of <1 μM, between 1.0-6.0 μM or > 6.0 μM, provides 'active', 'moderately active' or 'inactive' compounds, respectively, whereas a SI value of ≥20 is ideal [34].

According to the criteria described above, all 3-nitrotriazole-based analogs, except **11,** were selectively active against *T. cruzi* amastigotes, whereas the 2-nitroimidazole-based analogs **12-14,** although active against *T. cruzi*, exhibited unacceptable SI values (Table 1). Compound **5**, the des-nitro analog of **3**, was moderately active against *T. cruzi* but was also not selective against the parasite.

Several 3-nitrotriazole-based compounds (**2-4, 6, 9, 10**) and one 2-nitro-imidazole-based analog (**13**) were moderately active against *T. b. rhodesiense*, however, only the chloro-biphenylamide **6** demonstrated an acceptable selectivity for this parasite (Table 1).

Interestingly and, for the first time, both 3-nitrotriazole-based- and 2-nitroimidazole-based analogs demonstrated a remarkable and selective activity against *L. donovani* amastigotes (Table 1). Thus, only the des-nitro analog of **3**, namely compound **5**, the phenylpyrimidinylacetamide **8** and the *N*-benzylpiperidine-amide **11** weren't active against *L. donovani*.

2.2.2. SAR analysis of antichagasic activity

The unsubstituted 3-nitrotriazole-based aryloxy-phenylamide **2** was a very potent anti-*T. cruzi* agent displaying an IC_{50} of 65 nM against this parasite, 31-fold lower than that observed using Bnz, and having a SI of 1416. When a *p*-chloro group was introduced in the terminal phenyl of **2**, the anti-*T. cruzi* potency increased by 8-fold and the SI by 2.6 in analog **3**. Therefore, compound **3** was 254-fold more active than Bnz, and represents the most potent analog in this series. This increase in potency may be related to the increased lipophilicity of **3** compared to **2** (Table 1). However, the presence of the nitro-group was far more important for activity than lipophilicity. Therefore, the des-nitro analog of **3**, compound **5**, albeit more lipophilic than **3**, was only moderately active against *T. cruzi*, while its increased lipophilicity resulted in an unfavorable SI (<50). The fact that compound **5** demonstrated moderate activity against *T. cruzi* also indicates an alternative mechanism of action, independent of nitroreduction.

The fluoro-analog of **3,** compound **4**, was also more potent against *T. cruzi* than the unsubstituted compound **2**, with an IC_{50} value of 36 nM and a SI of 1954. However, **4** was 4.5-

fold less potent than **3**, presumably due to its lower lipophilicity, but still 56-fold more potent than Bnz (Table 1).

To better understand the role that the ether link in **3** plays in mediating anti-*T. cruzi* activity, we included some biphenyl/biaryl compounds in the study. The biphenyl compound **6** has been described before by our group [33]. Compound **6,** which shares a similar lipophilicity with its aryloxy-analog **3,** was also very potent and selective against *T. cruzi*, however its potency was ca. 5.6- fold lower than that of **3**, signifying the importance of the ether link in the core of these compounds for anti-*T. cruzi* activity. Similarly, the biphenyl compound **7** was about 2.8-fold less potent against *T. cruzi* compared to the unsubstituted aryloxy-compound **2** and 4-fold less potent than **6**. In the latter case the drop in activity of **7** may be related to its significantly lower lipophilicity compared to **6** rather than the meta-position of the terminal phenyl group.

When the second phenyl ring in **7** was replaced with a pyrimidine ring in **8**, the anti-*T. cruzi* activity was reduced by a factor of 14, despite the fact that both **7** and **8** share a similar clogP value. However, a greater PSA value in **8** may negatively affect permeability and thus activity [35].

Replacing the phenoxy-group of **2** with a benzyl-group in **9** did not affect anti-*T. cruzi* activity. However, the SI of **9** was reduced >2.5, due to higher toxicity in host cells and was independent of lipophilicity since both compounds share a similar clogP value (Table 1). Inserting an oxygen in **9** to obtain the benzyloxy-analog **10** increased anti-*T. cruzi* activity and selectivity compared to both analog compounds **9** and **2**, consistent with an increase in the lipophilicity of **10**. In addition, **10** was about 2-fold less toxic than **9,** presumably due to its slightly greater PSA value which may decrease permeability [35]. Furthermore, when the anilino-ring of **9** was replaced with a 4-piperidine-amino ring in **11**, disturbance in the planarity

decreased significantly the activity against *T. cruzi*, resulting in an IC_{50} of 8 μM and an unacceptable SI of ca. 14 for compound **11.**

For comparison purposes we have synthesized and evaluated the anti-*T. cruzi* activity of 2-nitroimidazole-based aryloxy-phenyl amides **12-14**, analogs of **2-4**, respectively. Although compounds **12-14** were more lipophilic than their 3-nitrotriazole-based analogs **2-4**, they demonstrated significantly less activity (65- to 324-fold) against *T. cruzi*. In addition, albeit compounds **12-14** were active/moderately active according to the TDR criteria, they demonstrated an unfavorable SI, confirming again the superiority of the 3-nitrotriazole- versus the 2-nitroimidazole ring against *T. cruzi*. From the above discussion it is concluded that a 3-nitrotriazole ring coupled with a aryloxy-phenyl core is important for potent antichagasic activity and superior selectivity. With the exception of compound **8,** there was also a good correlation between anti-*T. cruzi* activity and lipophilicity among the 3-nitrotriazole-based analogs. Thus, antichagasic activity increased with increasing clogP value (Fig.1s in supplementary material). Good correlation also existed between lipophilicity and antichagasic activity in the 2-nitroimidazole-based analogs, although the limited number of analogs does not allow definitive conclusions.

2.2.3. SAR analysis of anti-T. brucei activity

Moderate activity was observed against BSF of *T. b. rhodesiense* with several analogs (**2-4, 6, 9, 10, 13**). Only the 3-nitrotriazole-based biphenylamide **6,** however, demonstrated an acceptable selectivity (>100). Interestingly, there was relatively good correlation between anti-*T. cruzi* and anti-*T. b. rhodesiense* activity ($R^2 = 0.77$) among nitrotriazoles active against both parasites (**Fig. 2s** in supplementary material), something that was not observed before with other 3-nitrotriazole-

based scaffolds [23-25, 33]. Thus, anti-*T. b. rhodesiense* activity (similarly to anti-*T. cruzi* activity) increased as the clogP increased with chloro-substitution enhancing potency in comparison to fluoro- or no-substitution (compare **2, 3** and **4** in the 3-nitrotriazole-based subgroup, and compare **12, 13** and **14** in the 2-nitroimidazole-based subgroup). Although there was relatively good correlation between *T. cruzi* and *T. b. rhodesiense* IC_{50} values for the diaryl derivatives **6-8** (R^2=0.87), the 3-biphenyamide **7** was inactive against *T. b. rhodesiense*, in contrast to its 4-biphenyl analog **6**, which displayed moderate activity and selectivity. Similarly, the 4-phenyl-pyrimidine amide **8** was inactive against *T. b. rhodesiense.* Once again, the des-nitro analog of **3**, compound **5,** was deemed "inactive" against *T. b. rhodesiense* based on the TDR criteria, although the trypanocidal effect it did display made it more potent than the nitro-compounds **7** and **8**, suggesting that the latter two may not be such good substrates for TbNTR.

2.2.4. SAR analysis of anti-L. donovani activity

This is the first report of 3-nitrotriazole- and 2-nitroimidazole-based compounds demonstrating selective anti-*L. donovani* activity. If we consider the active/moderately active 3-nitrotriazole-based analogs with the exception of **10**, there was a relatively good correlation between anti-*T. cruzi* and anti-*L. donovani* activity (R^2 = 0.82), whereas in the case of compounds with better structural similarity we observe an even better correlation, although the number of analogs in these cases is inadequate for correlations. In addition, increasing lipophilicity resulted in an increased anti-*L. donovani* activity (Table 1). Chloro-substitution was more beneficial for activity than fluoro- or no substitution (compare **2, 3** and **4**, as well as **12-14**). The ether link in the core was more beneficial for activity than the methylene- or methylenoxy-link (**2** *vs* **9** and **10**) or no link at all (**3** *vs* **6**). Disturbance in the planarity of the core in **11**, or deletion of the nitro

group in **5**, abolished anti-*L. donovani* activity. Similarly, replacement of the middle phenyl ring in **7** with a pyrimidine ring in **8,** resulted in abolishment of the anti-*L. donovani* activity (Table 1). Two 3-nitrotriazoles (**4** and **6**) and one 2-nitroimidazole (**13**) demonstrated similar activity to the reference compound miltefosine, whereas the 3-nitrotriazole-based *p*-chlorophenyloxyphenyl amide **3** was ca. 4.8-fold more potent than miltefosine, with a SI of 853. Compounds **2, 12** and **14** were selectively active against this parasite at nM concentrations, but slightly less potent than miltefosine. Finally, compounds **7**, **9** and **10** were moderately active and with acceptable selectivity against the parasite (Table 1). If we compare the corresponding 3-nitrotriazole-based and 2-nitroimidazole-based analogs, the former were 1.6 to 4-fold more active than the latter against *L. donovani* parasites (**2** *vs* **12**, **3** *vs* **13** and **4** *vs* **14**).

Table 2. Antileishmanial activity and toxicity in infected macrophages

ID No	*L.don. axen.* IC_{50} µM	SI	*Activity in Macrophages* IC_{50} µM	*Toxicity in Macrophages* IC_{50} µM	Milt./Comp.
Milt.	0.162 ± 0.017		6.894		
2	0.27	339	>30	89	0.60
3	0.034	853	>27	80	4.8
4	0.18	384	>28	84	0.89
6	0.17	738	>84	>84	0.95
9	1.2	31	>30	>89	0.12
12	0.79	79	16	>89	0.21
13	0.14	937	15	>81	1.2
14	0.29	418	14	>84	0.55

Milt./Comp. is the ratio of the coresponding IC_{50} values against *L. don. axen.* amastigotes.

Compounds with substantial selective activity against *L. donovani axenic* amastigotes were also tested for activity and toxicity in infected macrophages, a model more suitable for drug screening (36), since it takes into account host cell-mediated effects (Table 2). Despite activity against *L. donvani axenic* amastigotes, the 3-nitrotriazole-based analogs (**2-4, 6** and **9**) were not particularly active in infected macrophages, whereas the 2-nitroimidazole-based analogs (**12-14**) demonstrated moderate activity (IC_{50} <20 μM) in this assay. Reduced intracellular activity might be associated with metabolic stability of the compounds in the macrophages or permeability issues, although permeability studies through Caco-2 cells with some analogs did not indicate any permeability problems.

2.2.5. Involvement of type I Nitroreductase

Representative analogs from Table 1 were evaluated as substrates of purified, recombinant trypanosomal NTRs and compared to benznidazole (Table 3). Enzyme specific activity was measured as oxidized NADH per min per mg of protein. All tested compounds were excellent substrates of both TcNTR and TbNTR and metabolized by the *T. cruzi* enzyme at rates comparable or better than that of Bnz. However, the same analogs were metabolized by TbNTR at rates 1.6 to 2.2-fold higher than that of Bnz (Table 3). When these studies were extended to investigate the role of NTR within the parasite itself, BSF *T. b. brucei* was used and induced to express elevated levels of TbNTR. *T. b. brucei* is the model organism for all three sub-species (*T. b. rhodesiense, T. brucei gambiense and T. b. brucei*) and can be handled in a level 2 containment lab (with appropriate clearance from Ministry of Agriculture) whereas *T. b. rhodesiense* can only be used in a level 3 containment lab. In addition, the *T. b. brucei* genome has been sequenced and recombinant DNA technology is well advanced (gene replacement,

inducible/constitutive gene expression, RNAi etc) allowing investigation of potential drug mode of action. Such technology cannot be done by using *T. b. rhodesiense*.

Table 3. Activity of recombinant TbNTR and TcNTR towards selected analogs of Table 1.

Compound	Specific activity values (nmol NADH oxidised per min per mg NTR)	
	TbNTR	TcNTR
Bnz	1540 ± 5	680 ± 8
2	2931 ± 23	815 ± 27
3	2699 ± 60	628 ± 54
4	2702 ± 23	516 ± 80
6	2492 ± 31	585 ± 28
13	3337 ± 432	817 ± 157

NTR-overexpressing *T. b. brucei* were ca. 2- to 5-fold more sensitive to most of the tested compounds with **6** being the exception (Table 4). However, there was no good correlation between TbNTR/TcNTR enzymatic activity and specific antitrypanosomal activity. This, coupled with the modest fold difference in susceptibility observed between cells expressing different levels of TbNTR in comparison to the larger changes noted when using other nitroheterocycles such as Nfx, indicates that this oxidoreductase may not be the only activator of the aryloxy-phenylamide prodrug within the parasite or that other factors (*e.g.* permeability into mitochondrion, compound stability and pharmacokinetic factors in general) may play a role in the antiparasitic activities.

Table 4. Antimicrobial activity of selected nitrotriazoles against BSF of *T. b. brucei*

IC_{50} values (μM) T. b. brucei				
Compound	**Wild type**	**TbNTR (-tet)**	**TbNTR (+ tet)**	**Ratio -tet/+tet**
Nfx		4.41± 0.22	0.43 ± 0.03	10.3
2	1.35 ± 0.02	2.34 ± 0.07	1.37 ± 0.02	1.7
3	5.09 ± 0.12	5.90 ± 0.50	1.18 ± 0.03	5.0
4	3.13 ± 0.09	3.67 ± 0.32	0.77 ± 0.04	4.8
6	0.69 ± 0.01	0.69 ± 0.01	0.85 ± 0.04	0.8
13	4.72 ± 0.10	6.48 ± 0.22	1.71 ± 0.09	3.8

2.2.6. TcCYP51 inhibition

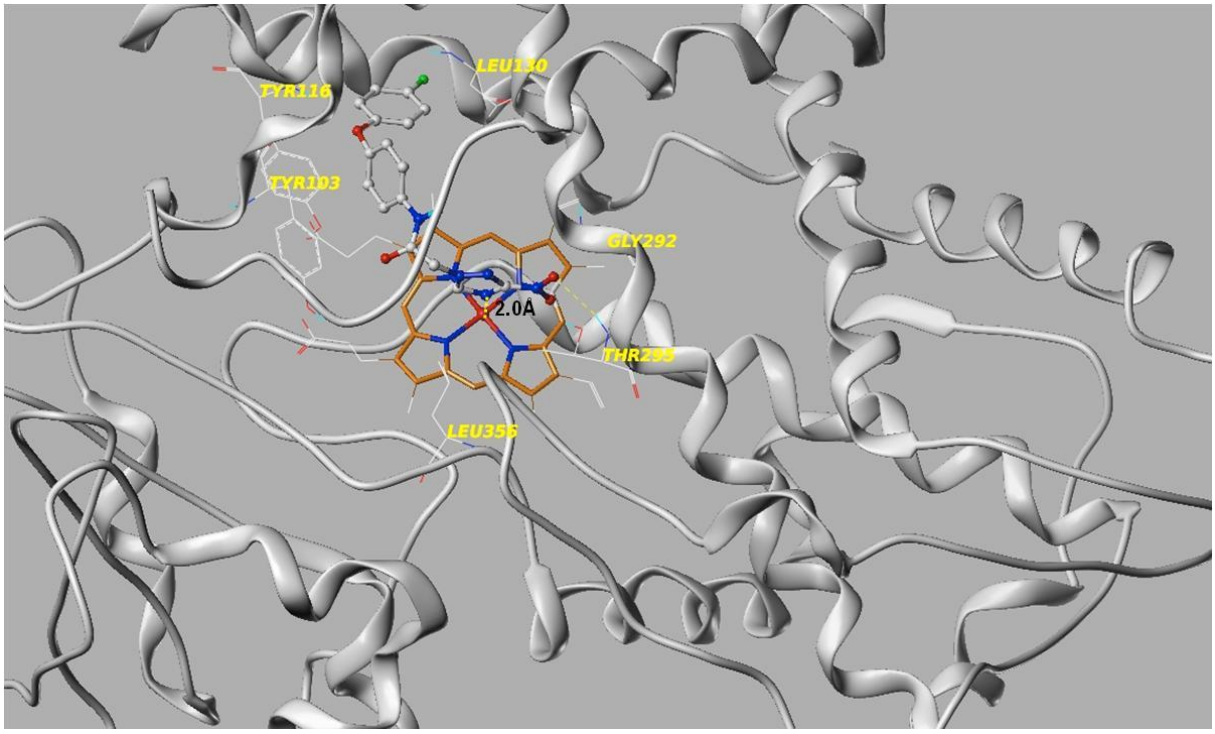
Azole-based scaffolds are effective inhibitors of the *T. cruzi* enzyme sterol 14α-demethylase, TcCYP51 [37]. In addition, aryloxybenzylimidazoles have been described as CYP51 inhibitors [15]. Therefore, *in silico* docking studies were performed by using the ligand binding pocket of the crystal structure of 4H6O sterole-14α demethylase and Glide docking protocols, to investigate whether or not the present compounds bind to the protein as the reference compound **15** (Fig. 1) [15]. The docking scores, which were a function of –logKd, are shown in Table 5.

Table 5. Docking scores of compounds in Table 1 and reference **15**, in the ligand binding pocket of 4H6O sterole-14α demethylase. The score is a function of –logKd.

Compound	Docked Score in the 4H6O Ligand Binding Pocket
2	9.63
3	9.82
4	9.91
5	5.48
6	8.93
7	7.86
8	6.65
9	7.54
10	7.23
11	6.13
12	6.83
13	6.52
14	6.98
15	8.96

The 3-nitrotriazole-based aryloxyphenylamides **2-4** showed a higher docking score than the reference compound **15,** whereas the more rigid 3-nitrotriazole-based biphenylamide **6** showed a similar docking score with **15**. The rest of the compounds demonstrated docking scores lower than compound **15,** with compound **5** (the des-nitro analog of **3**) showing the lowest docking score (Table 5). Examples of docked poses for compounds **3** and **15** are given in Fig. 1**,** which

show coordination between the heme iron of the enzyme and the N4 or N3 of the nitrotriazole and imidazole rings, respectively.



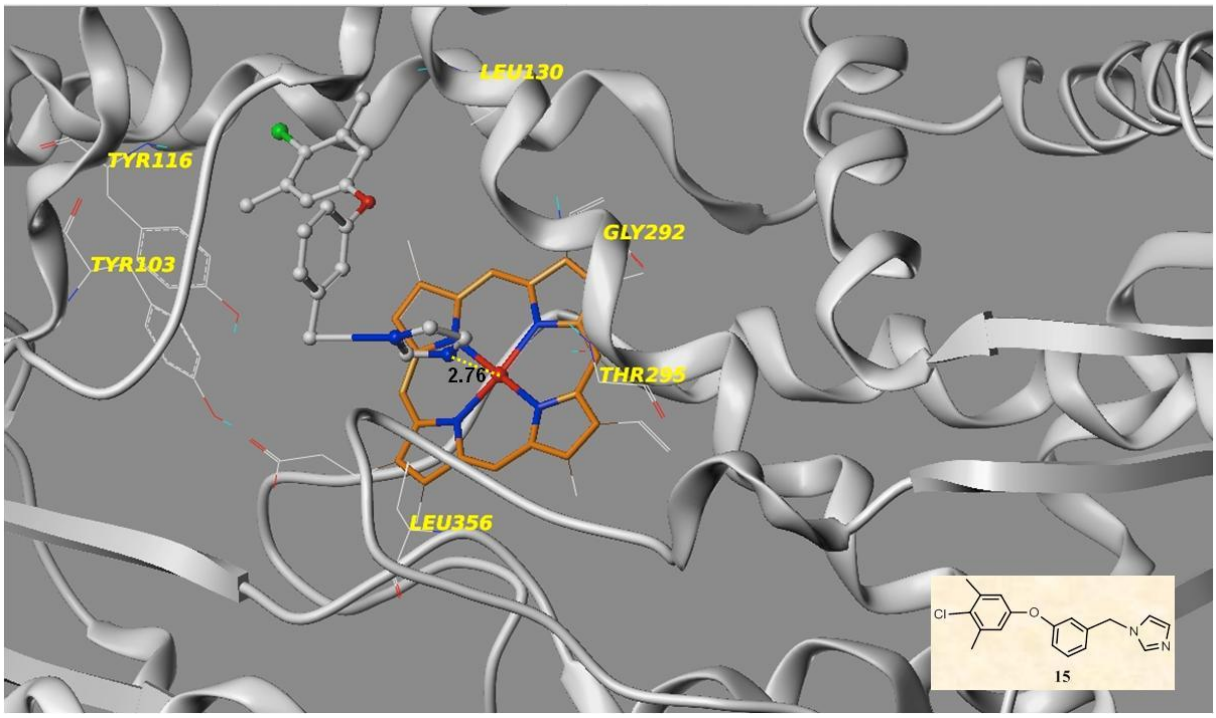


Fig. 1. Docked poses of compound **3** (top) and reference compound **15** (bottom). The distance (in Å) is shown between the iron atom of the heme and the N4 (top) or N3 (bottom) of the nitrotriazole and imidazole rings, respectively.

A distance of 2.0 and 2.76 Å is shown between the iron atom and the corresponding nitrogen atom for compounds **3** and **15**, respectively. The docking score seems to be affected primarily by the distance between the N4 of the nitrotriazole ring and the iron atom of the heme in the active site of the enzyme, whereas the orientation of the core group of each molecule can change significantly, making a SAR correlation much more complicated in this case.

There was good correlation between anti-*T. cruzi* activity (IC_{50} values against *T. cruzi*) and docking score (Fig. 2), suggesting that at least for compounds **2-4** and **6** the antichagasic activity may be also related to *T. cruzi* CYP51 inhibition, an assumption than needs to be verified.

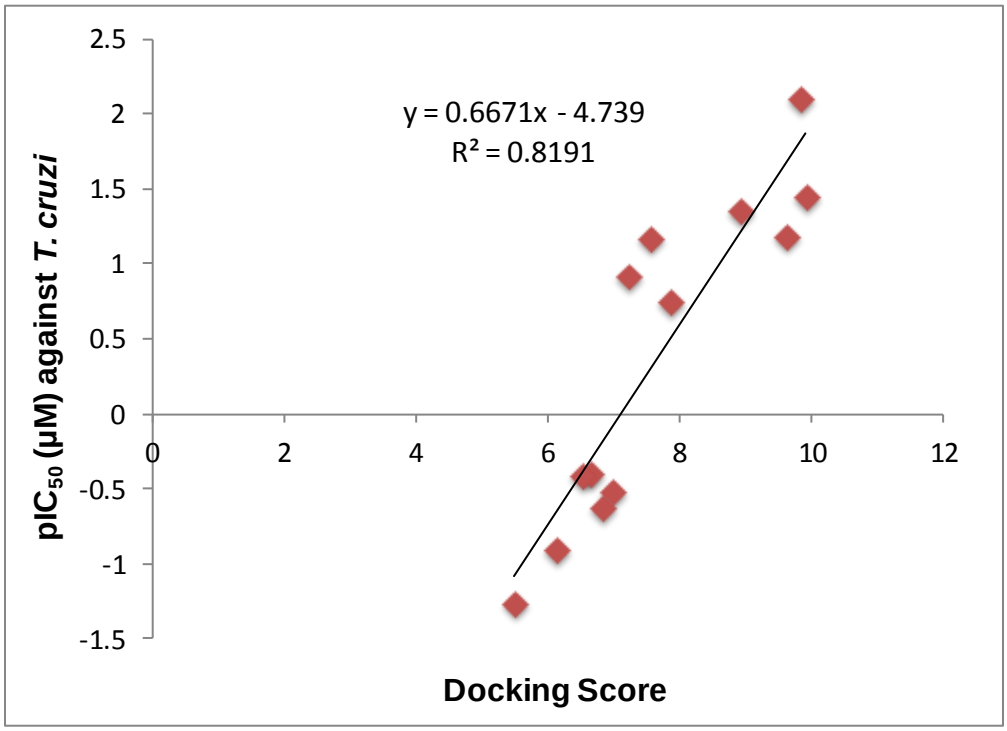


Fig. 2. Correlation between antichagasic activity and docking scores.

2.2.7. In vivo antichagasic activity

The most active compounds (**3, 4** and **6)** against *T. cruzi in vitro* were evaluated for *in vivo* anti-chagasic activity by using an acute *T. cruzi* infected murine model. Bnz was used in parallel as a positive control. Groups of 5 mice/group were treated i.p. for 10 consecutive days with each compound, at 13 mg/kg/day. In our previous work with 3-nitrotriazole-based rigid amides we have seen *in vivo* antichagasic activity at daily doses of 15 mg/kg, therefore we decided to use a slightly lower dose for the present tested analogs which demonstrate *in vitro* anti-*T. cruzi* activity at low nM concentrations (Table 1). The mean ratio of parasite levels was calculated after 5 and 10 days of treatment. The data are summarized in Fig. 3 and images are presented in Fig. 4.

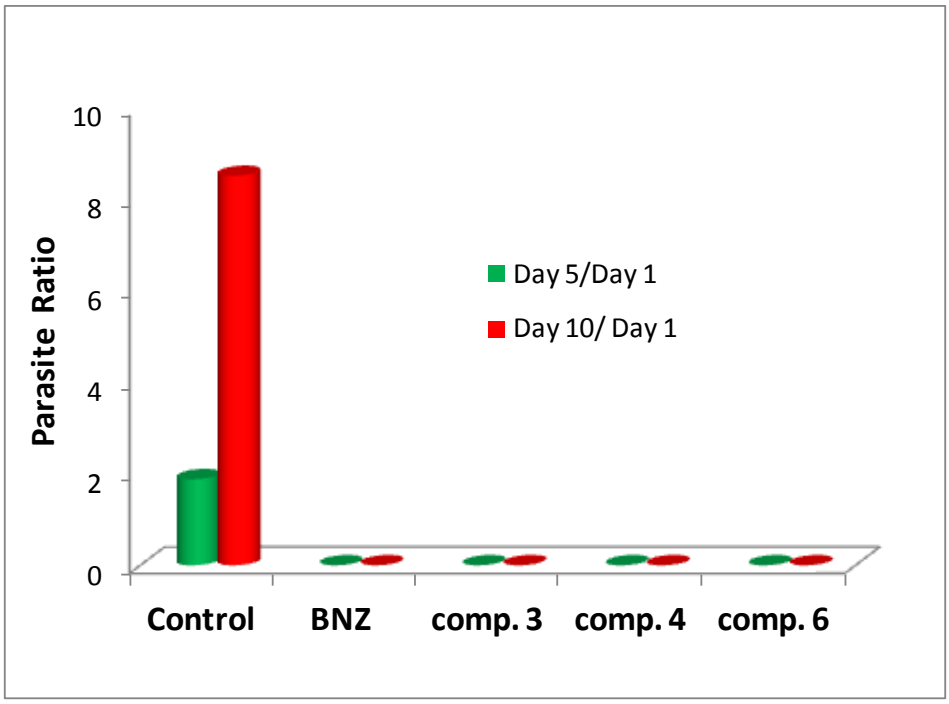


Fig. 3. *In vivo* evaluation of the antichagasic efficacy of compounds **3, 4, 6** and benznidazole in the acute murine model. All compounds (including Bnz) were administered i.p. at 13 mg/kg/day

for 10 consecutive days. Parasite ratios were calculated after 5 and 10 days of treatment. The *P* values between each treated group and control group were < 0.0001. Groups of 5 mice/group were used.

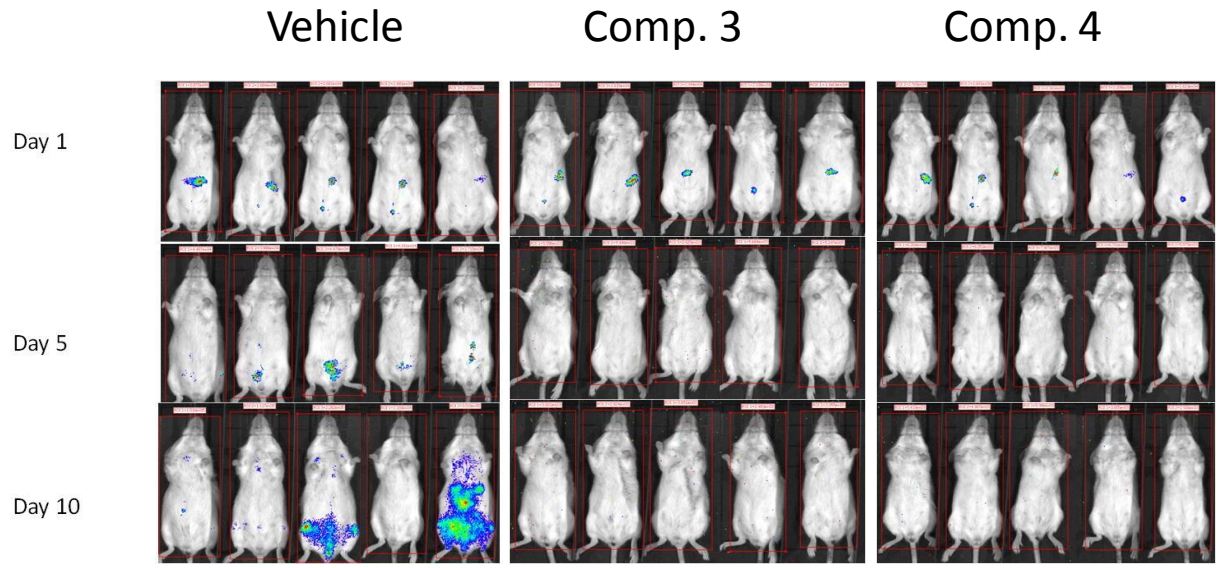


Fig. 4. Images from mice treated with vehicle or compounds **3** and **4** after 1, 5 and 10 days of treatment.

All tested compounds weren't toxic at the given dose and time-frame and all, including Bnz, reduced the parasite load to undetectable levels (Fig. 4) after 5 days of treatment with statistical significance ($p < 0.0001$).

3. Conclusions:

3-Nitrotriazole-based aryloxyphenylamides are very potent and selective anti-*T. cruzi* agents both *in vitro* and *in vivo*, being able to reduce the parasite load to undetectable limits after just 5 days of treatment of infected mice, at the very small i.p. dose of 13 mg/kg/day (Figures 3 and 4).

In addition, limited ADMET studies demonstrated that these compounds are metabolically stable and have a very good Caco-2 permeability, suggesting a favorable profile regarding oral administration (an example is given in the Supplementary material). However, since activity in the acute model is not predictive for efficacy in chronic indeterminate Chagas patients, future experiments in a chronic murine model will be necessary to validate these agents for human use.

Additionally, 2-nitro-imidazole-based aryloxyphenylamides demonstrated promising and selective antileishmanial properties in the macrophage model (which is more appropriate for drug screening) exhibiting IC_{50} values similar or better than pentostam (36). Therefore, this scaffold should be further explored for lead drug optimization.

Both types of compounds act as substrates for trypanosomal type I NTR, whereas the 3-nitrotriazole-based analogs demonstrated slightly better docking scores for 4H6O sterol-14α demethylase than the reference compound **15**, suggesting that the antichagasic properties of these compounds may be partially attributed to *T. cruzi* CYP51 inhibition, but this needs to be confirmed with enzymatic studies.

Finally, although the present compounds should be evaluated for mutagenicity/genotoxicity due to their nitro group, it is worth mentioning that previous studies have shown that 3-nitrotriazole-based amides are not mutagenic compared to their 2-nitroimidazole-based analogs [26], and do not cause developmental toxicity in zebrafish [28]. Therefore, 3-nitrotriazole-based aryloxyphenylamides constitute a new generation of potentially effective and more affordable antichagasic agents in humans.

4. Experimental

4.1. Chemistry

4.1.1. General

All starting materials and solvents were purchased from Sigma-Aldrich (Milwaukee, WI), were of research-grade quality and used without further purification. Solvents used were anhydrous and the reactions were carried out under a nitrogen atmosphere and exclusion of moisture. Melting points were determined by using a Mel-Temp II Laboratory Devices apparatus (Holliston, MA) and are uncorrected. Proton NMR spectra were obtained on a Varian Inova-500 or an Agilent Hg-400 spectrometer at 500 or 400 MHz, respectively, and are referenced to Me_4Si or to the corresponding solvent, if the solvent was not $CDCl_3$. High-resolution electrospray ionization (HRESIMS) mass spectra were obtained on a Agilent 6210 LC-TOF mass spectrometer at 11000 resolution. Thin-layer chromatography was carried out on aluminum oxide N/UV_{254} or polygram silica gel G/UV_{254} coated plates (0.2 mm, Analtech, Newark, DE). Chromatography was carried out on preparative TLC alumina GF (1000 microns) or silica gel GF (1500 microns) plates (Analtech). All compounds were purified by preparative TLC chromatography on silica gel or alumina plates and also checked by HPLC (Agilent 1100) coupled to mass spectrometry (MS) and ultraviolet (UV) absorbance detectors. Purity was determined by comparing the area under the curve of the test compound peak to the combined areas of all peaks in the chromatogram, excluding DMSO and any peaks appearing in blank runs. Priority reporting was given to the UV traces (since mass spectrometer response can vary widely depending on a given compound's ease of ionization). Purity of compounds lacking UV activity was reported using mass spectrometry detection. Purity was ≥ 95% purity for all analogs.

*4.1.2. Synthesis of chlorides **1a-h:***

Some of the chlorides **1a-h** are known in the literature and others are unknown or known through

chemical libraries but without any reference. The synthesis and spectroscopic data of **1a-h** are provided in the supporting material.

4.1.3. General Synthetic Procedure of amides ***2-14:***

The potassium salt of 3-nitro-1,2,4-triazole or 2-nitroimidazole or 1,2,4-triazole (1 eq) was formed in CH_3CN (6-10 mL), by refluxing with KOH (1.2 eq) for 30 min. To this suspension, **1a-h** (1.1 eq) was added and the reaction mixture was refluxed under a nitrogen atmosphere for 9 h. In certain cases, **1a-h** was added in CH_3CN solution. The reaction mixture was checked by TLC for completion of the reaction and the solvent was evaporated. The residue was dissolved in ethyl acetate and the inorganic salts were filtered away. Upon preparative TLC (silica gel or alumina, depending on the mobility of the major band; ethyl acetate-petroleum ether), the desired product was obtained usually as a powder. Purity was checked also by HPLC and it was > 95%.

4.1.3.1. 2-(3-nitro-1H-1,2,4-triazol-1-yl)-N-(4-phenoxyphenyl]acetamide (**2**)**:** Off white microcrystallic powder (89%): mp 156-158 ^oC ; ^1H NMR (500 MHz, (CD_3OD) δ: 8.70 (s, 1H), 7.56 (d, *J* = 9.5 Hz, 2H), 7.34 (t, *J* = 8.0 Hz, 2H), 7.09 (t, *J* = 8.0 Hz, 1H), 6.97 (d, *J* = 9.0 Hz, 4H), 5.28 (s, 2H). HRESIMS calcd for $C_{16}H_{13}N_5NaO_4$ *m/z* $[M+Na]^+$ 362.0860 found 362.0863.

4.1.3.2. N-[4-(4-chlorophenoxy)phenyl]-2-(3-nitro-1H-1,2,4-triazol-1-yl)acetamide (**3**)**:** White microcrystallic powder (52%): mp 167-169 ^oC; ^1H NMR (500 MHz, (CD_3OD) δ: 8.70 (s, 1H), 7.58 (d, *J* = 9.5 Hz, 2H), 7.32 (d, *J* = 9.0 Hz, 2H), 7.00 (d, *J* = 9.0 Hz, 2H), 6.95 (d, *J* = 9.0 Hz, 2H), 5.29 (s, 2H). HRESIMS calcd for $C_{16}H_{12}ClN_5NaO_4$ *m/z* $[M+Na]^+$ 396.0475, 397.0527, 398.0512, 399.0514 found 396.0470, 397.0526, 398.0514, 399.0515.

4.1.3.3. N-[4-(4-fluorophenoxy)phenyl]-2-(3-nitro-1H-1,2,4-triazol-1-yl)acetamide (**4**)**:** Off white powder (80%): mp 179-181 ^{o}C (dec); ^{1}H NMR (500 MHz, (CD_3COCD_3) δ: 9.68 (br s, 1H), 8.73 (s, 1H), 7.63 (d, *J* = 9.5 Hz, 2H), 7.14 (t, *J* = 9.0 Hz, 2H), 7.04 (m, 2H), 6.98 (d, *J* = 9.0 Hz, 2H), 5.40 (s, 2H). HRESIMS calcd for $C_{16}H_{12}FN_5NaO_4$ *m/z* $[M+Na]^+$ 380.0766 , 381.0793, found 380.0767, 381.0791.

4.1.3.4. N-[4-(4-chlorophenoxy)phenyl]-2-(1H-1,2,4-triazol-1-yl)acetamide (**5**)**:** Off white powder (88%): mp 157-159 ^{o}C; ^{1}H NMR (500 MHz, ($CClD_3$) δ: 8.32 (br s, 1H), 8.26 (s, 1H), 8.14 (s, 1H), 7.44 (d, *J* = 8.5 Hz, 2H), 7.28 (d, *J* = 9.5 Hz, 2H), 6.97 (d, *J* = 9.5 Hz, 2H), 6.91 (d, *J* = 9.0 Hz, 2H), 5.02 (s, 2H). HRESIMS calcd for $C_{16}H_{14}ClN_4O_2$ and $C_{16}H_{13}ClN_4NaO_2$ *m/z* $[M+H]^+$ and $[M+Na]^+$ 329.0800, 331.0776 and 351.0619, 353.0595 found 329.0804, 331.0784 and 351.0629, 353.060.

4.1.3.5. N-([1,1'-biphenyl]-3-yl)-2-(3-nitro-1H-1,2,4-triazol-1-yl)acetamide (**7**): White powder (72%): mp 121-123 ^{o}C; ^{1}H NMR (400 MHz, CD_3COCD_3) δ: 9.77 (br s, 1H), 8.75 (s, 1H), 7.99 (s, 1H), 7.64-7.35 (m, 8H), 5.45 (s, 2H). HRESIMS calcd for $C_{16}H_{14}N_5O_3$ and $C_{16}H_{13}N_5NaO_3$ *m/z* $[M+H]^+$ and $[M+Na]^+$ 324.10912 and 346.0911, found 324.1099 and 346.0915.

4.1.3.6. 2-(3-nitro-1H-1,2,4-triazol-1-yl)-N-(4-phenylpyrimidin-2-yl)acetamide **(8):** Off white powder (55 %): mp 175-177 ^{o}C (dec); ^{1}H NMR (400 MHz, (CD_3COCD_3) δ: 10.04 (br s, 1 H), 8.74 (d, *J* = 5.2 Hz, 1H), 8.73 (s, 1H), 8.26 (dd, *J* = 8.4, 1.2 Hz, 2H), 7.77 (d, *J* = 5.2 Hz, 1H), 7.58-7.53 (m, 3H), 6.03 (s, 2H). HRESIMS calcd for $C_{14}H_{12}N_7O_3$ *m/z* $[M+H]^+$ 326.0996, found 326.0995.

4.1.3.7. N-(4-benzylphenyl)-2-(3-nitro-1H-1,2,4-triazol-1-yl)acetamide (**9**)**:** White microcrystallic powder (71%): mp 166-168 ^{o}C; ^{1}H NMR (400 MHz, (CD_3COCD_3) δ: 8.72 (s, 1H), 7.55 (dd, *J* = 8.8, 0.8 Hz, 2H), 7.29-7.16 (m, 7H), 5.39 (s, 2H), 3.95 (s, 2H). HRESIMS calcd for $C_{17}H_{16}N_5O_3$ and $C_{17}H_{15}N_5NaO_3$ *m/z* $[M+H]^+$ and $[M+Na]^+$ 338.1248 and 360.1067, found 338.1250 and 360.1076.

4.1.3.8. N-(3-(benzyloxy)phenyl)-2-(3-nitro-1H-1,2,4-triazol-1-yl)acetamide **(10):** White powder (77 %): mp 147-149 ^{o}C; ^{1}H NMR (400 MHz, (CD_3COCD_3) δ: 9.67 (br s, 1 H), 8.73 (s, 1H), 7.55-7.30 (m, 6H), 7.24 (t, *J* = 8.0 Hz, 1H), 7.14 (dd, *J* = 8.0, 1.6 Hz, 1H), 6.79 (dd, *J* = 8.0, 1.5 Hz, 1H), 5.40 (s, 2H), 5.10 (s, 2H). HRESIMS calcd for $C_{17}H_{16}N_5O_4$ *m/z* $[M+H]^+$ 354.1197, found 354.1195.

4.1.3.9. N-(1-benzylpiperidin-4-yl)-2-(3-nitro-1H-1,2,4-triazol-1-yl)acetamide (**11**): White microcrystallic powder (68%): mp 152-155 ^{o}C (dec); ^{1}H NMR (400 MHz, ($CClD_3$) δ: 8.37 (s, 1H), 7.33-7.24 (m, 5H), 5.83 (br d, *J* = 8.0 Hz, 1H), 4.92 (s, 2H), 3.83 (m, 1H), 3.49 (s, 2H), 2.81 (d, *J* = 11.6 Hz, 2H), 2.12 (t, *J* = 11.2 Hz, 2H), 1.91 (d, *J* = 9.6 Hz, 2H), 1.50 (m, 2H). HRESIMS calcd for $C_{16}H_{21}N_6O_3$ *m/z* $[M+H]^+$ 345.1670 found 345.1677.

4.1.3.10. 2-(2-nitro-1H-imidazol-1-yl)-N-(4-phenoxyphenyl]acetamide (**12**): Off white powder (72%): mp 167-169 ^{o}C; ^{1}H NMR (500 MHz, ($CClD_3$+ 1 drop of acetone -6d) δ: 8.97 (br s, 1H), 7.45 (d, *J* = 9.5 Hz, 2H), 7.27 (t, *J* = 7.5 Hz, 2H), 7.19 (s, 1H), 7.16 (s, 1H), 7.03 (t, *J* = 7.5 Hz, 1H), 6.90 (dd, *J* = 9.0, 7.5 Hz, 4H), 5.22 (s, 2H). HRESIMS calcd for $C_{17}H_{15}N_4O_4$ and $C_{17}H_{14}N_4NaO_4$ *m/z* $[M+H]^+$ and $[M+Na]^+$ 338.1015 and 361.0907, found 338.0974 and 361.0915.

4.1.3.11. N-[4-(4-chlorophenoxy)phenyl]-2-(2-nitro-1H-imidazol-1-yl)acetamide (**13**): White microcrystallic powder (46 %): mp 185-186 ^{o}C; ^{1}H NMR (500 MHz, (CD_3OD) δ: 7.55 (d, *J* = 9.0 Hz, 2H), 7.50 (d, *J* = 1.0 Hz, 1H), 7.32 (d, *J* = 9.5 Hz, 2H), 7.20 (d, *J* = 0.5 Hz, 1H), 6.98 (d, *J* = 9.0 Hz, 2H), 6.94 (d, *J* = 9.0 Hz, 2H), 5.34 (s, 2H). HRESIMS calcd for $C_{17}H_{14}ClN_4O_4$ and $C_{17}H_{13}ClN_4NaO_4$ *m/z* $[M+H]^+$ and $[M+Na]^+$ 373.0698, 375.0675 and 395.0518, found 373.0696, 375.0672 and 395.0519.

4.1.3.12. N-[4-(4-fluorophenoxy)phenyl]-2-(2-nitro-1H-imidazol-1-yl)acetamide (**14**): Off white powder (75 %): mp 194-196 ^{o}C (dec); ^{1}H NMR (500 MHz, (CD_3OD) δ: 7.52 (d, *J* = 8.5 Hz, 2H), 7.50 (d, *J* = 1.0 Hz, 1H), 7.20 (d, *J* = 1.5 Hz, 1H), 7.08 (t, *J* = 8.5 Hz, 2H), 6.99 (dd, *J* = 9.5, 5.0 Hz, 2H), 6.95 (d, *J* = 9.5 Hz, 2H), 5.34 (s, 2H). HRESIMS calcd for $C_{17}H_{13}FN_4NaO_4$ *m/z* $[M+Na]^+$ 379.0813, found 379.0821.

4.2. Biological evaluation.

4.2.1. In vitro screening:

In vitro activity against *T. cruzi*, *T. b. rhodesiense, L. donovani* and cytotoxicity assessment using L6 cells (rat skeletal myoblasts) was determined using a 96-well plate format as previously described [38]. Data were analyzed with the graphic program Softmax Pro (Molecular Devices, Sunnyvale, CA, USA), which calculated IC_{50} values by linear regression from the sigmoidal dose inhibition curves.

4.2.2. Activity against Leishmania donovani intracellular amastigotes; macrophage assay: Mouse peritoneal macrophages (4 x 10^4 in 100 µL RPMI 1640 medium with 10% heat-inactivated FBS) were seeded into wells of Lab-tek 16-chamber slides. After 24 hrs 1.2 x 10^5 amastigote *Leishmania donovani* in 100 µL were added. The amastigotes were taken from an axenic amastigote culture grown at pH 5.4. Four hrs later the medium containing free amastigote forms was removed and replaced by fresh medium. Next day the medium was replaced by medium containing different compound dilutions. Parasite growth in the presence of the drug was compared to control wells. After 96 hours of incubation the medium was removed and the slides fixed with methanol for 10 min followed by a staining with a 10% Giemsa solution. Infected and non-infected macrophages were counted for the control cultures and the ones exposed to the serial drug dilutions. The infection rates were determined. The results were

expressed as % reduction in parasite burden compared to control wells, and the IC_{50} calculated by linear regression analysis [39].

4.2.3. In vitro T. brucei brucei antiproliferating assays and susceptibility studies.

The BSF *of T. brucei brucei* parasites were seeded at 1 x 10^3 ml^{-1} in 200 μL of growth medium containing different concentrations of nitroheterocycle. Where appropriate, induction of the TbNTR was carried out by adding tetracycline (1 μg/mL). After incubation for 3 days at 37 ^oC, 20 μL of Alamar blue was added to each well and the plates incubated for a further 16 h. The cell density of each culture was determined as described before [29] and the IC_{50} established.

4.2.4. Enzymatic activity studies with Type I NTRs.

Recombinant TbNTR and TcNTR were prepared and assayed as previously described [40, 41]. The specific activity of purified his-tagged TbNTR was assessed spectrophotometrically at 340 nm using various nitrotriazole substrates (100 μM) and NADH (100 μM) and expressed as nmol NADH oxidized min^{-1} mg^{-1} of enzyme.

4.2.5. Molecular Modeling studies: Docking into the active site of 4H6O sterole-14 alpha demethylase.

The crystal structure of CYP51 (4H6O.pdb) which is available in protein database was imported for the analysis and found to be suitable for the docking experiments as there are no missing atoms found in the active site pocket of the enzyme. Moreover, the "b" values of the atoms are well within the average value of the crystal structure. 4H6O.pdb was validated with MolProbity [42] and the structure optimized by using Schrodinger software (Schrodinger LLC, Version 9.2 , 2014, New York). A MolProbity score of 1.5 (90^{th} percentile; N=5412, 2.70Å ± 0.25Å) was

obtained. MolProbity score combines the clashscore, rotamer, and Ramachandran evaluations into a single score, normalized to be on the same scale as X-ray resolution.

Next, the Ligand Binding Site (LBS) of the protein was prepared. The 4H6O.pdb is a co-crystal structure, therefore the bound ligand (reference compound) present in the active site of the CYP51 structure was extracted out, to visualize the binding pocket. Using the LigPrep tools, 3-D low energetic conformer of each compound was generated at pH = 7.4 ±1, retaining the absolute chirality of the compounds.

Docking of the reference compound was carried out in the Glide-XP (extra precision) module of the Schrodinger software (Glide Docking Tools, Schrodinger Inc, 2014, NY) and the docked and crystal poses were compared. The RMSD between the two conformers was found to be < 1.5 Å, which validates our docking protocols. Using the same protocols, all other compounds were docked into the active site pocket of CYP51, the binding poses were visualized and the Glide scores were collected. In order to cross check the binding poses, we used another docking engine, the Surflex dock tool implemented in SYBYLx1.3 (Sybylx 1.3, Tripos International, St.Louis, MO, 2013). Both the Glide and Surflex docking engines generated similar binding poses.

4.2.6. In vivo antichagasic activity assessment of selected compounds:

For *in vivo* studies, a Brazilian strain trypomastigotes from transgenic *T. cruzi* parasites expressing firefly luciferase were used as described before [26]. Briefly, parasites were injected in Balb/c mice (10^5 trypomastigotes per mouse) and three days later mice were anesthesized by inhalation of isofluorane, followed by an injection with 150 mg/kg of D-luciferin potassium-salt in PBS. Mice were imaged 5 to 10 min after injection of luciferin with an IVIS 100 (Xenogen,

Alameda, CA) and the data acquisition and analysis were performed with the software LivingImage (Xenogen) as described before [43]. Treatment with test compounds was started 4 days after infection at 13 mg/kg/day x 10 days, given i.p. The vehicle control was 2% methylcellulose + 0.5% Tween 80 and groups of 5 mice/group were used. Mice were imaged after 5 and 10 days of treatment. The ratio of parasite levels was calculated for each animal dividing the luciferase signal after treatment by the luciferase signal on the first imaging (before treatment). Mean values of all animals in each group ± SD were used for plotting.

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Graphical Abstract: