Hybrid furoxanyl N-acylhydrazone derivatives as hits for the development of neglected diseases drug candidates

ARTICLE *in* EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY · NOVEMBER 2012

Impact Factor: 3.45 · DOI: 10.1016/j.ejmech.2012.10.047 · Source: PubMed

8 AUTHORS, INCLUDING:

Michel Sauvain
Institute of Research for Development
117 PUBLICATIONS **1,735** CITATIONS
SEE PROFILE

Eliezer Barreiro
Federal University of Rio de Janeiro
445 PUBLICATIONS **5,342** CITATIONS
SEE PROFILE

Mercedes Gonzalez
University of the Republic, Uruguay
234 PUBLICATIONS **4,104** CITATIONS
SEE PROFILE

Hugo Cerecetto
University of the Republic, Uruguay
235 PUBLICATIONS **4,116** CITATIONS
SEE PROFILE

European Journal of Medicinal Chemistry 59 (2013) 64–74

Contents lists available at SciVerse ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Hybrid furoxanyl *N*-acylhydrazone derivatives as hits for the development of neglected diseases drug candidates

Paola Hernández [a], Rosario Rojas [b], Robert H. Gilman [b], Michel Sauvain [c,d], Lidia M. Lima [e], Eliezer J. Barreiro [e,**], Mercedes González [a,*], Hugo Cerecetto [a,*]

[a] Grupo de Química Medicinal, Laboratorio de Química Orgánica, Facultad de Ciencias-Facultad de Química, Universidad de la República, Igua 4225, 11400 Montevideo, Uruguay
[b] Laboratorios de Investigación y Desarrollo, Facultad de Ciencias y Filosofía, Universidad Peruana Cayetano Heredia, Lima, Peru
[c] Université de Toulouse, UPS, UMR 152 PHARMA DEV, Toulouse, France
[d] Mission IRD, Lima, Peru
[e] LASSBio-Laboratório de Avaliação e Síntese de Substâncias Bioativas, Faculdade de Farmácia, Universidade Federal do Rio de Janeiro, PO Box 68023, ZIP 21941902 Rio de Janeiro, RJ, Brazil

ARTICLE INFO

Article history:
Received 22 August 2012
Received in revised form
24 October 2012
Accepted 26 October 2012
Available online 7 November 2012

Keywords:
N-Acylhydrazone
Furoxane
Anti-*M. tuberculosis*
Anti-*Leishmania*
Anti-*T. cruzi*

ABSTRACT

Neglected diseases represent a major health problem. It is estimated that one third of the world population is infected with tuberculosis and additionally Leishmaniosis and Chagas disease affect approximately 30 million people. *N*-Acylhydrazone moiety is a repeated functional group present in several prototypes and drug candidates for these neglected diseases. On the other hand, furoxan system has been studied as pharmacophore for Leishmaniosis and Chagas diseases. Here we report on the design and preparation of forty hybrid furoxanyl *N*-acylhydrazones and on their activity on *Mycobacterium tuberculosis*, H37Rv and MDR strains, *Trypanosoma cruzi*, and *Leishmania amazonensis*. Among them, four derivatives displayed excellent to good selectivity indexes against the three different microorganisms. Hybrid compound *N*′-(4-phenyl-3-furoxanylmethylidene)isoniazide **9** showed the best antibacterial profile with MIC value 4.5 lesser than the value for the reference isoniazid against MDR strain. Furoxanyl *N*-acylhydrazone (*E*)-2-methyl-*N*′-(4-phenyl-3-furoxanylmethylidene)-4*H*-imidazo[1,2-*a*]pyridine-3-carbohydrazide **15** was ten-fold more potent against *T. cruzi* Amastigotes than the standard drug nifurtimox. On the other hand, derivatives (*E*)-*N*′-(5-benzofuroxanylmethylidene)benzo[*d*][1,3]dioxole-5-carbohydrazide **25** and (*E*)-*N*′-(4-hydroxy-3-methoxyphenylmethylidene)-3-methylfuroxan-4-carbohydrazide **37** emerged as leads for the development of new leishmanicidal agents. The adequate stability, in simulated biological system and plasma, and the lack of mutagenicity of these derivatives allow us to propose them as candidates for further pre-clinical studies.

1. Introduction

Mycobacterium tuberculosis (*M. tuberculosis*), and to a lesser extent *Mycobacterium bovis* and *Mycobacterium africanum*, can cause a chronic and fatal condition in humans known as tuberculosis (TB). With the discovery of several active anti-TB agents the disease was considered to be curable, however, in only a few years, became evident that the use of drugs as single agents led to rapid drug resistance and treatment failures among a substantial number of patients. Of particular concern is the development of multi-drug-resistant forms of the disease (MDR-TB), defined as forms resistant to two or more of the frontline anti-TB agents. It is estimated that one third of the world's population is infected with TB, with about eight million new cases and dying 3.1 million annually [1,2]. The current frontline therapy for TB consists of administering three different drugs (the antibiotic rifampicin, and the azaheterocycles isoniazid and pyrazinamide, Isnz and Pyzd, Fig. 1) over an extended period of time as well as the problems that arise due to MDR-TB. Consequently, it is necessary to develop new, potent, fast-acting anti-tuberculosis drugs with low-toxicity profiles, actives against MDR-TB and able to be given in conjunction with drugs used to treat HIV infections [3,4]. Another pharmacological strategy is the use of thioacetazone (Tzn, Fig. 1) in the cases of resistances by rifampicin and Isnz.

* Corresponding authors. Tel.: +598 2 5258618x216; fax: +598 2 5250749.
** Corresponding author. Tel./fax: +55 21 25626644.
E-mail addresses: ejbarreiro@ccsdecania.ufrj.br (E.J. Barreiro), megonzal@fq.edu.uy (M. González), hcerecet@fq.edu.uy (H. Cerecetto).

0223-5234/$ – see front matter © 2012 Elsevier Masson SAS. All rights reserved.
http://dx.doi.org/10.1016/j.ejmech.2012.10.047

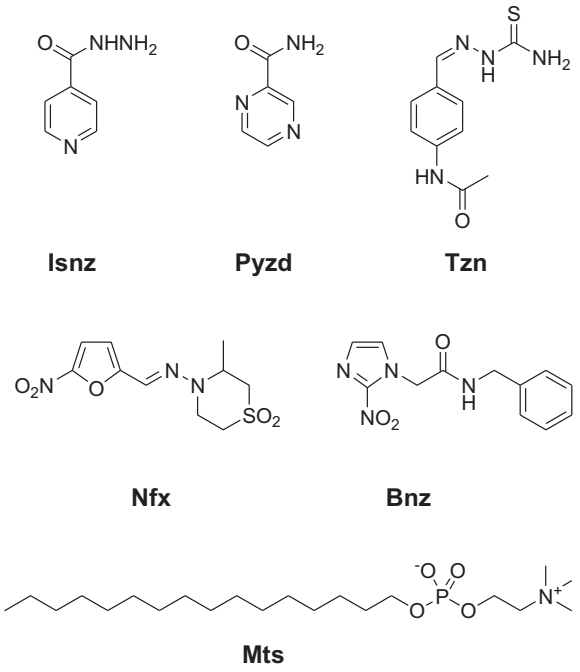


Fig. 1. Some of the therapeutic tools for tuberculosis (**Isnz**, **Pyzd**, and **Tzn**), Chagas disease (**Nfx** and **Bnz**) and leishmaniosis (**Mts**).

Besides TB, the parasitic diseases represent a major health problem in Third World countries. More specifically, Chagas disease, or American trypanosomiasis, caused by the protozoan *Trypanosoma cruzi* (*T. cruzi*), and leishmaniosis, caused by a protozoan belong to the genus *Leishmania*, are the largest parasitic diseases burden in the American continents. Chagas disease affects approximately 20 million people from the southern United States to southern Chile. Even though the enforcement of public health programs towards vector elimination in some Latin American countries has decreased the incidence of new infections, the disease is still endemic in large areas [5]. Currently, there are only two clinically used drugs, nifurtimox (Nfx, Fig. 1) and benznidazole (Bnz, Fig. 1). These drugs are very deficient and there is an urgent need for the development of safe and effective drugs [6]. On the other hand, leishmaniosis is widespread around the world except in South-east Asia. Currently, infections are found in 16 countries in Europe. The current anti-leishmaniosis therapy involves the use of pentavalent antimonials (meglumine antimoniate and sodium stibogluconate), with manifested resistant in the treatment of visceral and mucocutaneous forms [7], the use of Amphotericin B (Amph) and miltefosine (Mts, Fig. 1). Mts is active against visceral and cutaneous leishmaniosis.

Since beginnings of the nineties we have investigated and developed new hits for neglected disease drugs [8–15]. In this sense, we have identified quinoxaline dioxides **1** (Fig. 2), with excellent selectivity for *M. tuberculosis* $H_{37}Rv$ strain, and the lead **2** (Fig. 2), that combined anti-*M. tuberculosis* and anti-*T. cruzi* activities. Moreover, *N*-acylhydrazones (**3** and **4**) and thiosemicarbazones (**5**, Fig. 2) have emerged as potential *T. cruzi*-cysteine protease inhibitors where quinoxaline **4** and benzofuroxan **5** displayed good selectivity index for the parasites over mammalian cells. Further, the furoxans **6** and **7** displayed excellent anti-*T. cruzi* and anti-*Leishmania* activities. These compounds (**1**–**7**) possess some common structural features, i.e. the presence of amide or *N*-acylhydrazone moieties and *N*-oxide containing heterocycles (Fig. 2). On the other hand, the amide moiety together

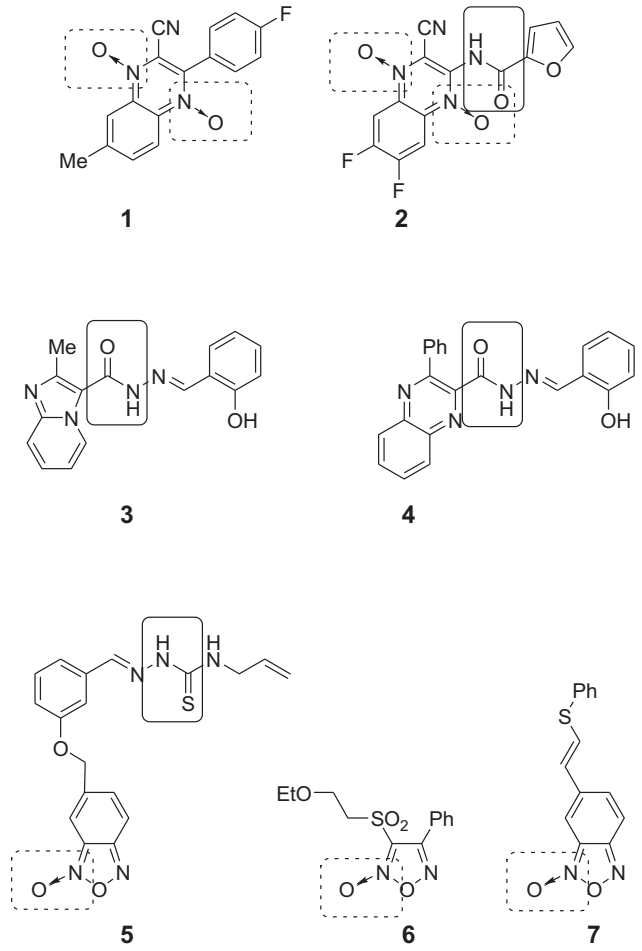
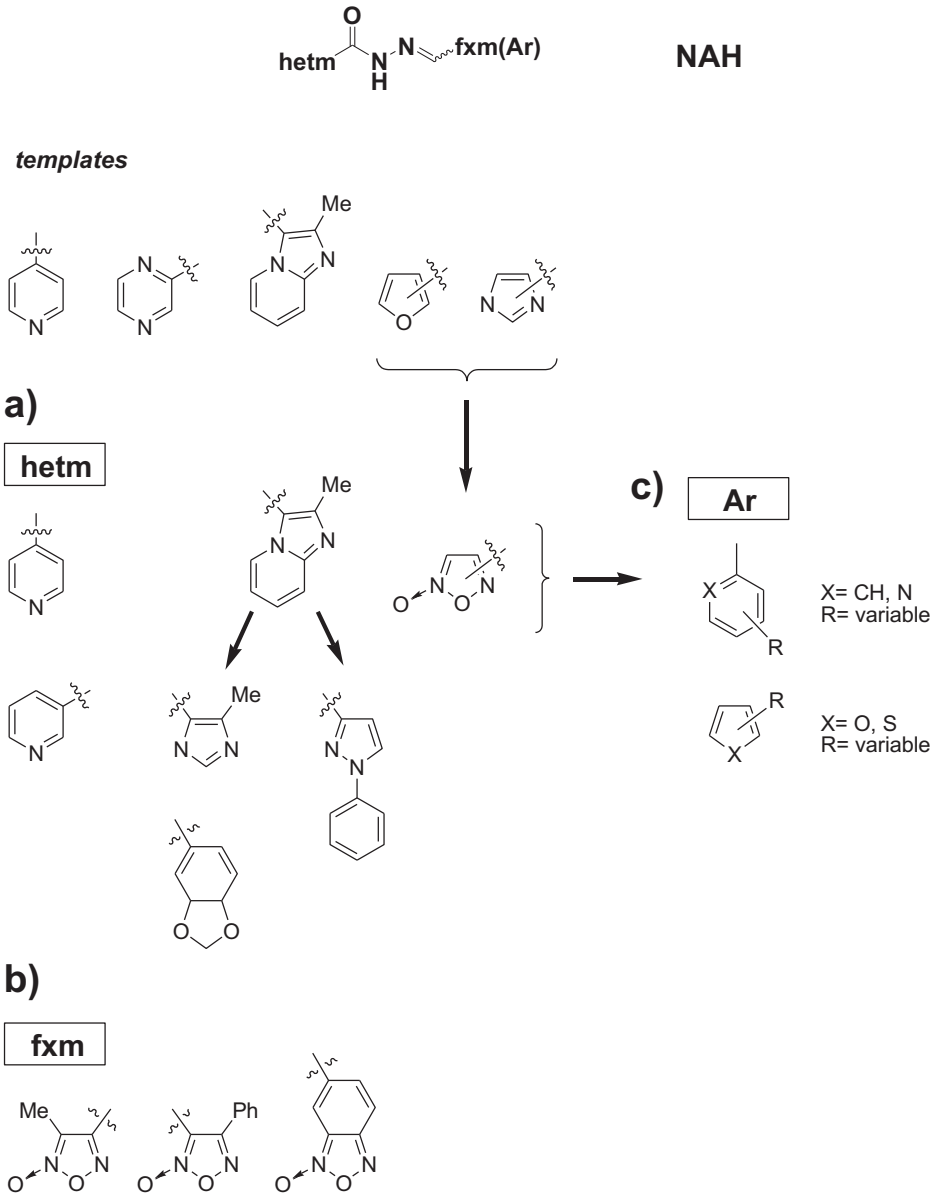


Fig. 2. Previous identified hits with anti-*M. tuberculosis*, anti-*T. cruzi* and anti-*Leishmania* activities. Common moieties are marked (dotted: *N*-oxide; line: amide).

aromatic cycles also constitute the backbone of current drugs for tuberculosis and Chagas diseases (Fig. 1).

This information encourage us to design and develop hybrid agents (Scheme 1), which combine different heterocyclic (hetm) and furoxanyl moieties (fxm) connected by a *N*-acylhydrazone (NAH) linker. As hetm we used the standards Isnz, Pyzd, Nfx, Bnz, and the prototypes **3**, and **4** as templates. Consequently, aza-heterocycle (pyridine-3-, and 4-yl), diaza-heterocycle (1*H*-imidazole-4-yl, 4*H*-imidazo[1,2-*a*]pyridine-3-yl, and 1-phenyl-1*H*-pyrazole-4-yl), and other heterocyles (furoxan-4-yl) as the combination of furan and imidazole heterocycles, and benzo[*d*][1,3]dioxole-5-yl- were proposed as substituent of the acyl-position of the NAH framework (Scheme 1a). The studied fxm, linked to the imine group of the NAH unit, were 3-methyl-4-furoxanyl, 4-phenyl-3-furoxanyl and 5-benzofuroxanyl (Scheme 1b). Additionally, when the acyl-position of the NAH was furoxan-4-yl group, the imine position was substituted by different aromatic groups (Scheme 1c) in order to cover different stereo–electronic parameters.

Forty hybrid furoxanyl *N*-acylhydrazones (fx-NAH), belonging to seven different families of heterocycles (**A**–**G**), have been synthesized and their *in vitro* abilities to decrease the growth of *M. tuberculosis*, *T. cruzi*, and *Leishmania* have been determined. The selectivity indexes were evaluated analysing their *in vitro* cytotoxicity against J774 murine macrophages. Besides, stability, in simulated biological system and plasma, and mutagenicity of some



Scheme 1. Design of hybrid furoxanyl *N*-acylhydrazone (fx-NAH) as neglected disease drugs.

of selected derivatives were studied in order to confirm their potentials as novel antiparasitic drug candidates.

2. Methods and results

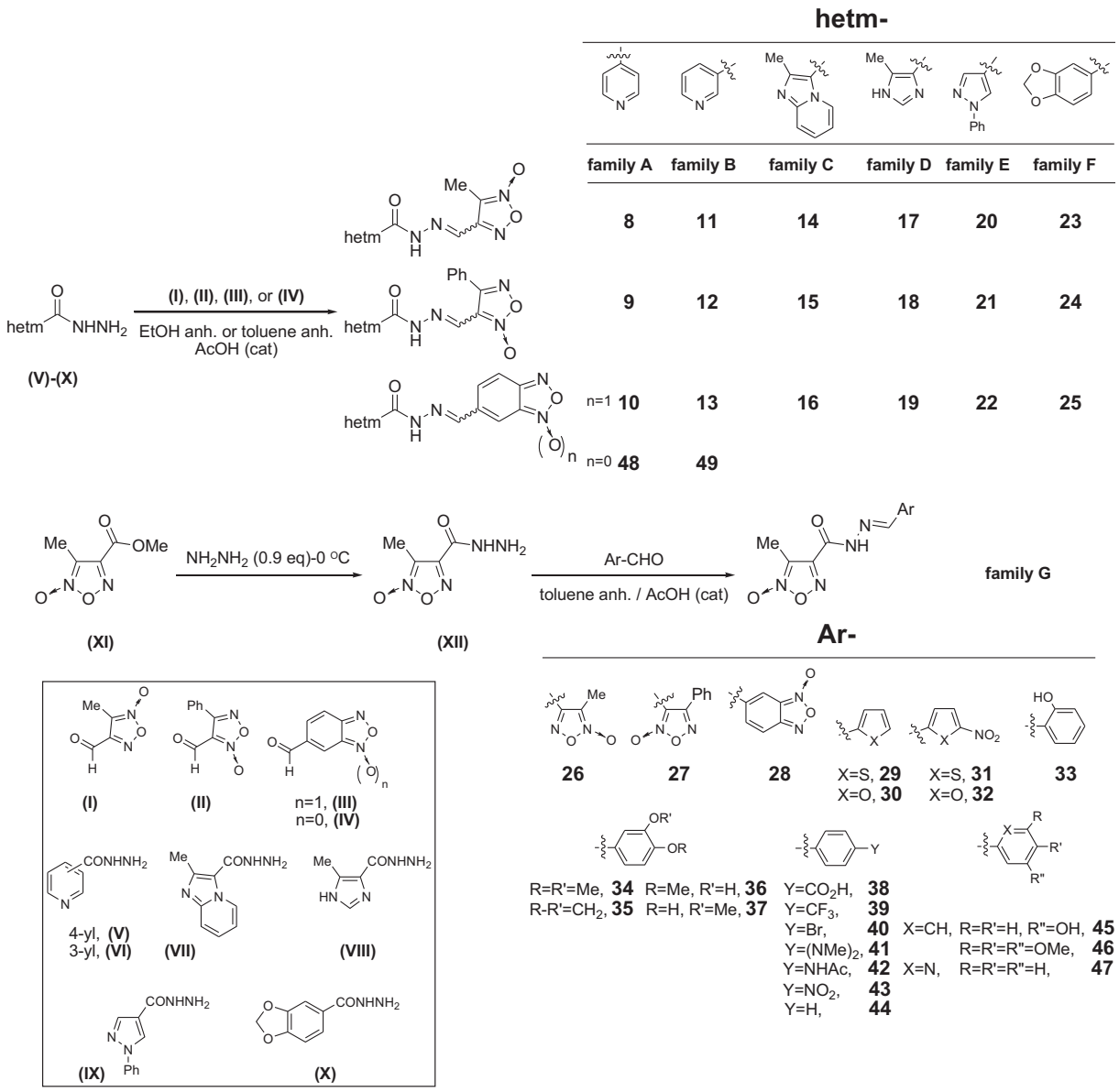
2.1. Chemistry

The first designed family of fx-NAH was the Isnz derivatives (family **A** **8**–**10**, Scheme 2) prepared by the condensation of Isnz with the corresponding furoxanyl aldehydes (**I**, **II** or **III**). The same procedure was used to prepare fx-NAH family **B** (nicotinic acid derivatives, **11**–**13**, Scheme 2), family **C** (imidazo[1,2-*a*]pyridine derivatives, **14**–**16**, Scheme 2), family **D** (imidazole derivatives, **17**–**19**, Scheme 2), family **E** (pyrazol derivatives, **20**–**22**, Scheme 2), family **F** (benzo[*d*][1,3]dioxole derivatives, **23**–**25**, Scheme 2) and family **G** (furoxan derivatives, **26**–**47**, Scheme 2) [16]. In order to probe the relevance of the *N*-oxide moiety in the biological activity the deoxygenated analogues of **10** and **13** (**48** and **49**, Scheme 2) were also prepared [17]. All the new compounds, **8**–**10**, and **42**–**49**, were characterized by ^{1}H NMR, ^{13}C NMR, COSY, HMQC, and HMBC experiments, IR and MS. As previously it was observed for derivatives **11** and **20** [16] in accordance to the NMR experiments (^{1}H NMR and NOE-diff) [16–20] and characteristic fragmentations in MS experiments, the Isnz-derivatives (**8**–**10** and **48**), were obtained as mixture of *E*- and *Z*-isomers around the ylidenic moiety, with ratio of *E*:*Z* near to 91:9. The purity of the synthesized compounds was established by TLC and microanalysis. Only compounds with analytical results for C, H and N, within ±0.4 of the theoretical values were considered pure enough.

2.2. Biological characterization

2.2.1. In vitro anti-M. tuberculosis activity

All the hybrid fx-NAHs (**8**–**47**) and the reduced analogues (**48** and **49**) were tested *in vitro* for their anti-mycobacterial activities against sensitive *M. tuberculosis* H37Rv strain and multidrug-



Scheme 2. Synthetic procedures used to prepare the studied fx-NAH.

resistant (MDR) clinical isolate strain (Table 1). A modified microplate Alamar blue assay [21,22], where Alamar was substituted by tetrazolium bromide, was used to detect viable *M. tuberculosis* [23,24]. This tetrazolium microplate assay allowed us to determine the MIC values in 6–7 days. Isnz (**V**) was included as reference drug. Furthermore, unspecific cytotoxicity of the studied hybrid compounds was studied using J774.1 murine macrophages (Table 1).

Results revealed that fx-NAH **9** was better anti-mycobacterial agent against MDR strain than the reference drug, with a MIC value 4.5 lower than the value for Isnz and a selectivity index (SI, Table 1) higher than the value for Isnz. The behaviour of **9** against H37Rv strain and macrophages is comparable to Isnz. The other two members of the family **A**, fx-NAHs **8** and **10**, also possess very good biological profiles against both Mycobacterium strains. The positional isomers **11**–**13**, belonging to family **B**, displayed similar biological behaviours as fx-NAHs **8**–**10** being phenylfuroxan **12** the most selective derivative with more than two-fold active against MDR strain than Isnz. The most active derivative against MDR strain was phenylfuroxan **15**, belonging to family **C**, however, its high unspecific cytotoxicity produces lower SIs than compounds **9**, **10** or **12**. Similarly, fx-NAH **19**, member of family **D**, had lower SIs with good behaviour against MDR strain. Derivatives **31** and **32** were the exclusive active members of family **G**. The presence of nitro-heterocyclic substituents in the Ar position of these fx-NAHs (Scheme 2) could be the responsible of these activities being the nitrophenyl analogue **43** inactive. In this family of fx-NAH derivative **42** with an acetamidophenyl scaffold like the drug Tzn (Fig. 1) was also inactive. The rest members of this family and members of families **E** and **F** displayed irrelevant biological behaviours in these assays. The absence of *N*-oxide moiety, derivatives **48** and **49**, confirm the relevance of this function in the anti-Mycobacterium activity and macrophages toxicity. These benzofurazan derivatives were less actives and cytotoxic than the *N*-oxide analogues.

2.2.2. *In vitro anti-T. cruzi activity*

The hybrid fx-NAHs **8**–**33**, **35**, **38**–**45**, **47**, and the reduced analogues (**48** and **49**) were tested *in vitro* for their

Table 1
Anti-mycobacterial activity and unspecific cytotoxicity of hybrid fx-NAH derivatives.

Family	Compd.	MIC H37Rv (μM)	MIC MDR (μM)	IC_{50} J774.1 (μM)	SI_{H37Rv}[a] (SI_{MDR})	Family	Compd.	MIC H37Rv (μM)	MIC MDR (μM)	IC_{50} J774.1 (μM)	SI
A	**8**	1.6	>25.4	>400.0	>250	**G**	**26**	>93.6	>93.6	>400.0	–
	9	0.6	10.1	>400.0	>667 (>40)		**27**	>75.8	>75.8	>400.0	–
	10	0.35	11.0	134.9	385 (12)		**28**	82.2	82.2	>400.0	>5
	48	>93.6	>93.6	>400.0	–		**29**	>99.2	>99.2	>400.0	–
B	**11**	>101.2	>101.2	>400.0	–		**30**	>106.0	>106.0	>400.0	–
	12	40.5	20.2	194.9	5 (10)		**31**	42.1	42.1	>400.0	>10
	13	44.2	22.1	31.0	0.7 (1.4)		**32**	44.5	44.5	68.0	1.5
	49	>93.6	46.8	208.9	<2 (5)		**33**	>95.4	>95.4	246.0	<3
C	**14**	>83.3	>83.3	>400.0	–		**34**	>81.6	>81.6	>400.0	–
	15	17.3	8.6	56.2	3.2 (6.5)		**35**	>86.2	>86.2	>400.0	–
	16	77.2	38.6	141.2	1.8 (3.7)		**36**	>85.6	>85.6	>400.0	–
D	**17**	>100.0	>100.0	>400.0	–		**37**	>85.6	>85.6	>400.0	–
	18	>80.1	>80.1	>400.0	–		**38**	>86.2	>86.2	>300.0	–
	19	43.7	21.9	33.9	0.8 (1.5)		**39**	>79.6	>79.6	>400.0	–
E	**20**	>80.1	>80.1	295.0	<4		**40**	>74.0	>74.0	162.0	<2
	21	>66.8	>66.8	41.7	<0.6		**41**	>86.5	>86.5	129.0	<1.5
	22	>17.9	>17.9	37.1	<2		**42**	>82.5	>82.5	235.0	<3
F	**23**	>86.2	>86.2	131.8	<1.5		**43**	>85.9	>85.9	>300.0	–
	24	>71.0	>71.0	162.0	<2		**44**	>101.6	>101.6	>400.0	–
	25	>76.7	>76.7	173.9	<2		**45**	>95.4	>95.4	>400.0	–
							46	>74.4	>74.4	>300.0	–
	Isnz	0.4	45.6	>400.0	>1000 >9		**47**	>101.2	>101.2	>400.0	–

[a] $SI = IC_{50,macrophages}/IC_{50,mycobacteria}$.

trypanosomicidal properties using two different forms of *T. cruzi*. Axenic epimastigotes of Tulahuen 2 strain and amastigotes Tulahuen C4 strain growing in VERO (normal African green monkey epithelial) cells were used in the *in vitro* models [25,26]. The IC_{50} and the SI are showed in Table 2. Nfx was included as reference drug. The furoxan intermediate (**XI**) and the hydrazide reactants, **V**–**VII**, **IX**, **X** and **XII**, were also included to analyse their anti-*T. cruzi* activities.

In general, members of the families **A**–**F** displayed some activity against the epimastigote form of the parasite being, except for derivative **15**, lower than the Nfx activity. Again, the structural motifs 5-nitrothienyl and 5-nitrofuryl conferred activity to members of family **G**, i.e. **31** and **32**. This nitroheterocyclic substituent together *o*-hydroxyphenyl, in fx-NAH **33**, are framework-templates belonging to the parents compounds Nfx, **3** and **4** (Figs. 1 and 2).

Phenylfuroxan derivatives **9**, **15**, **24**, and **27** were the best anti-epimastigote member in each family showing that this structural scaffold could play a role in the studied biological response. Regarding to the anti-amastigote activity, the most relevant form thinking in therapy, a clear relationship between anti-epimastigote and anti-amastigote activities was observed. The most relevant fx-NAHs were the pyrimidin-4-yl derivatives **9** and **10** with similar activities and selectivities to that of Nfx. Compound **9** has excellent SI (>63), when murine macrophages were taken into account as mammal system, and good SI (2.5) when VERO cells were considered. Similarly occurred with family **A** member compound **10**. The best anti-amastigote derivative was fx-NAH **15**, which was

Table 2
Trypanosomicidal activity of hybrid fx-NAHs.

Family	Compd.	$IC_{50,epi}$ (μM)	$IC_{50,ama}$ (μM)	SI[a,b]	Family	Compd.	$IC_{50,epi}$ (μM)	$IC_{50,ama}$ (μM)	SI[a,b]
A	**8**	>300.0	>40.5	–	**G**	**26**	81.3	>37.5	–
	9	11.0	6.4	>63 (2.5)		**27**	25.1	>30.3	–
	10	33.4	5.6	24 (2.5)		**28**	>150.0	>32.9	–
	48	>150.0	>37.5	–		**29**	>150.0	>39.7	–
B	**11**	>150.0	>40.5	–		**30**	>150.0	>42.4	–
	12	58.9	>32.4	<6		**31**	50.0	>33.7	–
	13	35.5	>35.3	<0.9		**32**	50.1	>35.6	<2
	49	>150.0	>37.5	<5.5		**33**	39.8	>38.7	<6
C	**14**	>150.0	>33.3	–		**35**	>150.0	>34.5	–
	15	5.0	0.91	62 (12)		**38**	>150.0	>34.5	–
	16	>150.0	>30.9	<4		**39**	>150.0	>31.9	–
D	**17**	>150.0	>40.0	–		**40**	>150.0	>29.6	<5
	18	>150.0	>32.1	–		**41**	>150.0	>34.6	<3
	19	171.5	>35.0	<1		**42**	>150.0	>33.0	–
E	**20**	>150.0	>32.1	–		**43**	>150.0	>34.4	–
	21	>150.0	>26.7	–		**44**	151.4	>40.7	–
	22	29.5	>30.9	–		**45**	>150.0	>38.2	–
F	**23**	>150.0	>34.5	<1.5		**47**	>150.0	>40.5	–
	24	50.0	<30.7	>5					
	25	50.0	>28.4	<5		**VII**	>150.0	>52.6	–
						IX	ns	>49.5	–
	Nfx	7.7	10.4	27 (7.7)		**X**	>150.0	>55.6	–
	V	>150.0	ns[c]	–		**XI**	>50.0	>62.3	–
	VI	>150.0	>73.0	–		**XII**	>50.0	>62.3	–

[a] $SI = IC_{50,macrophages}$ (Table 1)$/IC_{50,amastigotes}$.
[b] Values in parenthesis are SIs according to $IC_{50,VERO}/IC_{50,amastigotes}$.
[c] ns: not studied.

eleven-fold more active than Nfx with excellent SIs, near to two-fold more selective in both systems than Nfx. The corresponding hydrazide, **VII**, was inactive against both forms of the parasite. The rest of the studied intermediates, **V**, **VI**, **IX**–**XII**, did not display relevant activities. Once again, the absence of *N*-oxide moiety, derivatives **48** and **49**, produced compounds with lower activities.

2.2.3. *In vitro anti-Leishmania activity*

Some selected hybrid fx-NAHs were evaluated against *L. amazonensis* axenic amastigotes. For that, MTT colorimetric assay using MHOM/BR/76/LTB-012 strain was employed [26]. The IC_{50} and the SI are showed in Table 3. Amph was included as reference drug.

All the studied compounds displayed remarkable anti-*Leishmania* activities being the best the fx-NAHs **22**, **25**, and **37**. However, these compounds displayed lower leishmanicidal activities than Amph. Nonetheless, according to the cytotoxicity against murine macrophages (Table 1), they were five- to fifty-fold more selective than the reference drug. Besides the most selective hybrid compounds, derivatives **25** and **37**, derivatives **14**, member of family **A**, **23**, member of family **F**, and **36**, **43**, and **45**, members of family **G**, also displayed very good SIs.

2.3. *Hybrid fx-NAHs as drug candidates*

Considering the great potential therapeutic use of this class of compounds, it is indispensable for pharmaceutical development to assess their bio-safety. Consequently, we evaluated the *in vitro* mutagenic potential of the selected compounds **8**–**10**, **15**, **25**, and **37** using Ames test with *Salmonella typhimurium* TA98 strain [28] in absence and presence of S9 in order to simulate the metabolic process (Table 4).

The negative results of these mutagenicity studies, except for benzofuroxanyl fx-NAHs **10** and **19** in absence of S9, showed the safety of this class of compounds to be further developed.

Moreover, the potential capability of these hybrid compounds to act as pro-drug, where the hydrolysis of acylhydrazone moiety could produce the active drug, was studied. For that, we analysed the stability, of selected derivatives **8**–**10**, **13**, **15**, and **24**, on aqueous solution at different pHs and in the presence of rat plasma (Table 5). All the studied derivatives, and specially **8**–**10**, the potential pro-dugs of **Isnz**, were stable on plasma for 3 h at 37 °C. Additionally, all the compounds were stable during 24 h on aqueous solution of pH 2.0, 5.0, 7.4, and 8.3. On aqueous solution at pH 8.3 derivative **15** showed some degree of instability, lower than 10% of decomposition (no attempts to isolate the decomposition products were done).

3. Discussion and conclusions

We reported the synthesis and biological evaluation of new hybrid furoxanyl *N*-acylhydrazones as antimicrobial and antiparasitic agents to treat neglected diseases. The compounds, designed as hybrid entities combining different aromatic residues and furoxan systems connected by *N*-acylhydrazone moiety, were evaluated against *M. tuberculosis*, *T. cruzi*, and *L. amazonensis*.

Hybrid furoxan **9** (Fig. 3a), belonging to family **A**, was the best *in vitro* anti-*M. tuberculosis* derivative. It displayed good activity against both studied strains of bacteria with excellent selectivity indexes, macrophage/bacteria, especially considering MDR-strain. Additionally, derivative **9** was not mutagenic, against *S. typhimurium* TA98, and the stability at different pH and on plasma showed that it could not act as **Isnz** pro-drug.

On the other hand, one derivative from family **C**, the hybrid compound **15** (Fig. 3a), exhibited excellent profile as anti-*T. cruzi* agent comparing to the reference drug **Nfx** (1.5- and 11.4-fold higher active than **Nfx** against epimastigote and amastigote form, respectively). In addition, it had higher selectivity than **Nfx** and it was not mutagenic in absence and presence of S9. Only, in basic aqueous milieu, pH 8.3, compound **15** showed some degree of instability after 24 h of incubation at 37 °C.

Regarding, *in vitro* anti-*Leishmania* activity, we identified excellent molecular hits with higher selectivity than the reference drug **Amph**. The best hybrid compound was the member of family **G** derivative **37** (Fig. 3a). This compound was 50-fold more selective than the reference drug. Other product with excellent biological behaviour was derivative **25** (Fig. 3a) that possessed a selectivity index 28.5-fold higher than the selectivity for **Amph**. Furthermore, these hybrid derivatives, **25**, and **37**, were not mutagenic.

Lipinski's rule of five [30] serves as a guide to determine if compounds will be orally bioavailable. Analysis of compounds **9**, **15**, **25**, and **37** suggested that it would be orally administered (Fig. 3b). On the other hand, the lipophilicity of these compounds resulted very different, see molecular lipophilicity potential (MLP, Fig. 3c), virtual LogP [31,32], as their biological activities.

In line with these findings, hybrid compounds **9**, **15**, **25**, and **37** are excellent leads for further studies. Currently we are performing mechanism of action and *in vivo* studies.

Table 3
Leishmanicidal activity of hybrid fx-NAHs.

Family	Compd.	IC_{50} (μM)	SI[a]
A	**8**	25.3	>16
C	**14**	12.6	>32
E	**22**	1.4	27
F	**23**	3.3	40
	25	1.3	134
G	**36**	8.3	>48
	37	1.7	>235
	43	7.9	>38
	45	4.5	>89
	Amph	0.11	4.7[b]
	VII	29.8	>13

[a] $SI = IC_{50,macrophages}$ (Table 1)/$IC_{50,amastigotes}$.
[b] Using as $IC_{50,macrophages} = 0.52$ μM (from reference [27]).

4. Experimental

Reagents were purchased from Aldrich and used without further purification. Melting points were performed using an Electrothermal Engineering Ltd melting point apparatus, and the results were uncorrected. Infrared spectra were recorded on a Bomem FTLA2000 spectrophotometer instrument as films on KBr discs. ^1H and ^{13}C NMR spectra were recorded in the indicated solvent on Bruker DX 400 MHz spectrometer. Chemical shifts are quoted in parts per million downfield from TMS and the coupling constants are in Hertz and, in case of E/Z mixture, for the main isomer. Mass spectra were recorded on a Hewlett Packard MSD 5973 (electronic impact, EI) or on a Hewlett Packard LC/MS Series 1100 (electrospray ionization, ESI) instruments. All solvents were dried and distilled prior to use. All the reactions were carried out in a nitrogen atmosphere. Reactions were monitored by TLC using commercially available precoated plates (Merck Kieselgel 60 F254 silica) and developed plates were examined under UV light (254 nm) or as iodine vapour stains. Column chromatography was performed using 200 mesh silica gel. To determine the purity of the compounds, micro-analyses were done on a Fisons EA 1108 CHNS–O instrument from vacuum-dried samples and were within ±0.4 of the values obtained by calculated compositions. Compounds **11**–**41**, **I**–**IV**, and **VI**–**XII** were prepared following synthetic procedures previously reported [16,17].

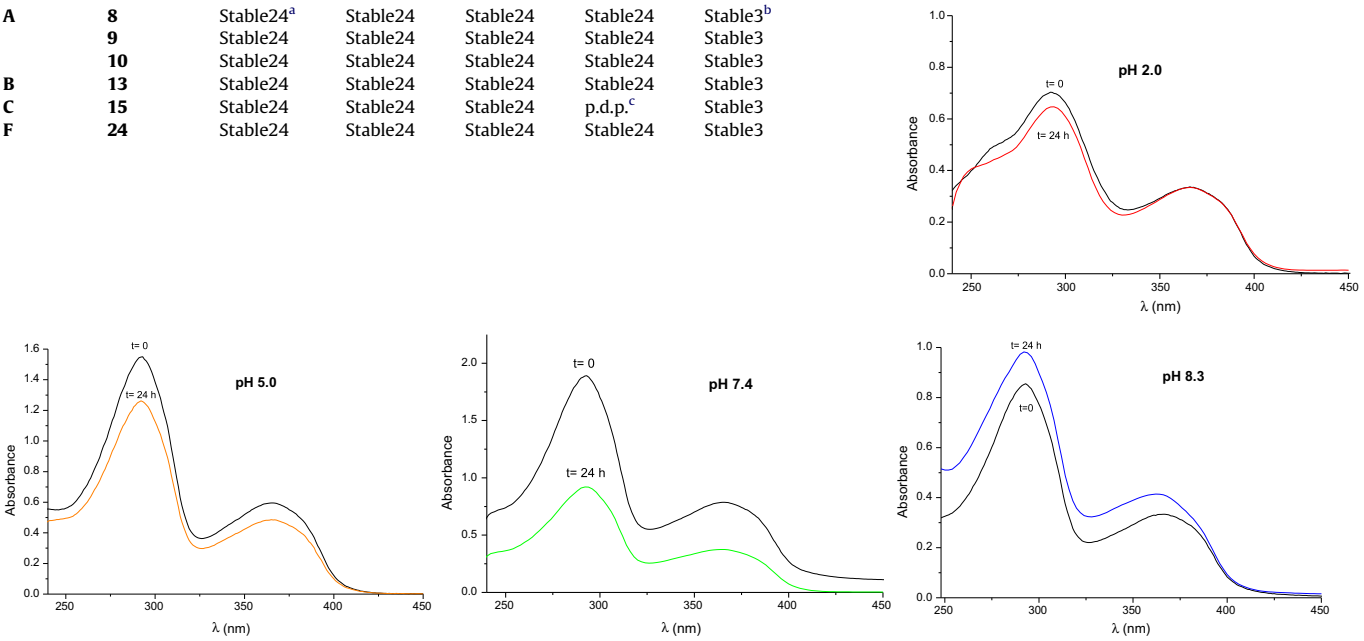
Table 4
Number of revertants of studied derivatives on TA98 *S. typhimurium* strain.

Family	S9	Type of furoxanyl moiety											
		3-Methyl-4-furoxanyl			4-Phenyl-3-furoxanyl			5-Benzofuroxanyl					
		8			9			10				Controls	
		D[a]	NR[b,c]	M[d]	D	NR	M	D	NR	M		D	NR	M
(**A**)	(−)	500.0	12.5 ± 0.7	M−	400.0	14.0 ± 4.2	M-	150.0	76.0 ± 7.1	M+				
		166.7	9.0 ± 5.7		133.3	9.5 ± 2.1		50.0	60.5 ± 19.1					
		55.6	7.0 ± 1.4		44.4	12.0 ± 5.7		16.7	37.5 ± 3.5		**wt**[e]	−	11.7 ± 4.4	−
		18.5	8.5 ± 0.7		14.8	17.0 ± 1.4		5.6	19.0 ± 2.8		**NPD**[f]	20.0	1460 ± 580	M+
		6.2	12.0 ± 2.8		4.9	8.5 ± 0.7		1.9	15.5 ± 0.7					
	(+)	500.0	12.0 ± 1.4	M−	400.0	9.0 ± 5.7	M-	150.0	82.5 ± 6.4	M+/−[g]				
		166.7	11.5 ± 7.8		133.3	8.5 ± 0.7		50.0	21.0 ± 0.5					
		55.6	17.0 ± 5.7		44.4	9.5 ± 3.5		16.7	19.0 ± 5.7		**wt**	−	11.5 ± 5.1	−
		18.5	14.5 ± 6.4		14.8	8.0 ± 5.7		5.6	12.0 ± 2.8		**2-AF**[h]	10.0	900 ± 290	M+
		6.2	11.5 ± 2.1		4.9	6.5 ± 3.5		1.9	12.0 ± 2.8					
(**C**)	(−)				**15**									
					250.0	6.0 ± 2.8	M-							
					83.3	4.5 ± 2.1								
					27.8	9.5 ± 2.1					**wt**	−	11.7 ± 4.4	−
					9.3	7.5 ± 0.7					**NPD**	20.0	1460 ± 580	M+
					3.1	7.5 ± 2.1								
	(+)				250.0	7.0 ± 4.2	M-							
					83.3	9.0 ± 1.4								
					27.8	8.0 ± 2.8					**wt**	−	11.5 ± 5.1	−
					9.3	8.0 ± 1.4					**2-AF**	10.0	900 ± 290	M+
					3.1	6.5 ± 0.7								
(**D**)	(−)							**19**[i]						
								500.0	47.0 ± 8.5	M+				
								166.7	39.5 ± 6.4					
								55.6	30.5 ± 4.9		**wt**	−	10.0 ± 3.0	−
								18.5	23.5 ± 2.1		**NPD**	20.0	1900 ± 200	M+
								6.2	10.5 ± 2.1					
	(+)							500.0	27.0 ± 14.1	M+/−				
								166.7	17.5 ± 0.7					
								55.6	13.0 ± 1.4		**wt**	−	11.0 ± 1.0	−
								18.5	12.0 ± 5.7		**2-AF**	10.0	844 ± 80	M+
								12.0 ± 7.1						
(**F**)	(−)				**24**[i]			**25**						
					250.0	11.0 ± 1.4	M-	500.0	25.0 ± 1.4	M−				
					83.3	7.0 ± 1.4		166.7	9.0 ± 2.8					
					27.8	10.0 ± 3.0		55.6	13.0 ± 4.2		**wt**	−	10.0 ± 2.0[j]	−
					9.3	9.0 ± 4.0		18.5	7.5 ± 3.5		**NPD**	20.0	1900 ± 200[j]	M+
					3.1	8.5 ± 0.7		6.2	8.0 ± 0.5					
	(+)				250.0	13.0 ± 3.0	M-	500.0	9.0 ± 3.5	M−				
					83.3	12.0 ± 3.0		166.7	9.0 ± 1.4					
					27.8	3.5 ± 3.5		55.6	4.5 ± 2.1		**wt**	−	8.0 ± 4.0[j]	−
					9.3	4.0 ± 1.3		18.5	11.0 ± 2.8		**2-AF**	10.0	844 ± 80[j]	M+
					3.1	3.0 ± 1.4		6.2	7.5 ± 2.1					
(**G**)	(−)	**37**												
		500.0	10.5 ± 0.7	M−										
		166.7	7.5 ± 6.4											
		55.6	12.5 ± 2.1								**wt**	−	11.7 ± 4.4	−
		18.5	11.0 ± 4.2								**NPD**	20.0	1460 ± 580	M+
		6.2	5.5 ± 0.7											
	(+)	500.0	22.0 ± 1.4	M−										
		166.7	13.5 ± 7.8											
		55.6	15.0 ± 7.1								**wt**	−	11.5 ± 5.1	−
		18.5	11.0 ± 1.4								**2-AF**	10.0	900 ± 290	M+
		6.2	11.5 ± 0.7											

[a] D: doses in μg/plate.
[b] NR: number of revertants.
[c] The results are the means of two independent experiments ±SD.
[d] M: mutagenicity, according to reference [29] (see Experimental Section for details).
[e] wt: without treatment.
[f] NPD: 4-nitro-*o*-phenylendiamine.
[g] M+/−: Only the highest studied dose was mutagenic. Higher doses did not study due to solubility problems.
[h] AF: 2-aminofluorene.
[i] From reference [16].
[j] Values for derivative **24** [16]. Derivative **25** controls were the same that the other herein studied hybrid compounds.

Table 5
Stability of selected hybrid fx-NAHs in physiological conditions. As example the UV-spectra of selected derivative **13** in the different conditions were showed.

Family	Compd.	Stability in aqueous solution				Stability in plasma	**13**
		pH 2.0	pH 5.0	pH 7.4	pH 8.3		
A	**8**	Stable24[a]	Stable24	Stable24	Stable24	Stable3[b]	
	9	Stable24	Stable24	Stable24	Stable24	Stable3	
	10	Stable24	Stable24	Stable24	Stable24	Stable3	
B	**13**	Stable24	Stable24	Stable24	Stable24	Stable3	
C	**15**	Stable24	Stable24	Stable24	p.d.p.[c]	Stable3	
F	**24**	Stable24	Stable24	Stable24	Stable24	Stable3	



[a] Stable 24 h, @ 37 °C.
[b] Stable 3 h, @ 37 °C.
[c] p.d.p.: presence of decomposition products was observed at the end of the assay.

4.1. General procedure for the preparation of hybrid derivatives *8*–*10*, *48*, and *49*

To a solution of **Isnz** (1 equiv.) or **VI** in dry EtOH (2.5 mL) was added to the corresponding aldehyde (**I**, **II**, **III**, or **IV**, 0.9 equiv.) and a drop of glacial AcOH. The mixture was stirred until the aldehyde was consumed (checked by TLC). The solid precipitated was filtered and washed with cold EtOH.

4.1.1. N′-(2-Methyl-3-furoxanylmethylidene) isoniazide (*8*)

(*E*:*Z* proportion = 91:9). White solid (40%). *E*-isomer: ^{1}H NMR (DMSO-d_6) δ ppm: 2.40 (3H, s), 7.84 (2H, d, J = 4.0), 8.50 (1H, s), 8.84 (2H, d, J = 4.0), 12.61 (1H, s). ^{13}C NMR (DMSO-d_6) δ ppm: 9.6, 112.2, 121.9, 137.9, 140.1, 151.0, 154.4, 164.6. IR (KBr) υ (cm^{-1}): 3200, 1679, 1611, 1552, 1289, 1029, 855, 797. MS (ESI) m/z (%): 248 ($M^{+\bullet}$ + H, 100). Calculated analysis for $C_{10}H_9N_5O_3$: C: 48.6; H: 3.7; N: 28.3. Found: C: 48.4; H: 3.6; N: 28.1.

4.1.2. N′-(4-Phenyl-3-furoxanylmethylidene) isoniazide (*9*)

(*E*:*Z* proportion = 98:2). White solid (42%). *E*-isomer: ^{1}H NMR (DMSO-d_6) δ ppm: 7.62 (3H, m), 7.85 (2H, d, J = 4.0), 7.97 (2H, d, J = 8.0), 8.40 (1H, s), 8.81 (2H, d, J = 4.0), 12.45 (1H, s). ^{13}C NMR (DMSO-d_6) δ ppm: 113.2, 121.5, 129.2, 129.5, 131.0, 131.5, 135.0, 140.1, 151.0, 156.5, 162.0. IR (KBr) υ (cm^{-1}): 3239, 1669, 1584, 1548, 1297, 1277, 1046, 854, 784. MS (ESI) m/z (%): 310 ($M^{+\bullet}$ + H, 100). Calculated analysis for $C_{15}H_{11}N_5O_3$: C: 58.2; H: 3.6; N: 22.6. Found: C: 58.1; H: 3.4; N: 22.5.

4.1.3. N′-(5-Benzofuroxanylmethylidene)isoniazide (*10*)

(*E*:*Z* proportion = 91:1). White solid (83%). ^{1}H NMR (DMSO-d_6) δ ppm: 7.30–8.20 (3H, bm), 7.85 (2H, d, J = 4.0), 8.52 (1H, s), 8.81 (2H, d, J = 4.0), 12.45 (1H, s). ^{13}C NMR (DMSO-d_6) δ ppm: 112.1, 123.4, 130.6, 142.7, 146.6, 150.1, 150.9, 162.5. IR (KBr) υ (cm^{-1}): 3176, 1690, 1617, 1596, 1548, 1267, 1010, 846, 803. MS (ESI) m/z (%): 284 ($M^{+\bullet}$ + H, 100), 268 ($M^{+\bullet}$ + H-16, 5), 224 (40). Calculated analysis for $C_{13}H_9N_5O_3$: C: 55.1; H: 3.2; N: 24.7. Found: C: 54.9; H: 2.8; N: 24.8.

4.1.4. N′-(5-Benzofurazanylmethylidene)isoniazide (48)

(*E*:*Z* proportion = 97:3). White solid (75%). ^{1}H NMR (DMSO-d_6) δ ppm: 7.85 (2H, d, J = 4.0), 8.14 (2H, bs), 8.36 (1H, s), 8.61 (1H, s), 8.81 (2H, d, J = 4.0), 12.45 (1H, s). ^{13}C NMR (DMSO-d_6) δ ppm: 117.2, 117.6, 122.1, 129.8, 138.8, 140.5, 147.1, 149.6, 149.7, 150.9, 162.5. Calculated analysis for $C_{13}H_9N_5O_2$: C: 58.4; H: 3.4; N: 26.2. Found: C: 58.1; H: 3.5; N: 25.9.

4.1.5. (E)-N′-(5-Benzofurazanylmethylidene) nicotinohydrazide (*49*)

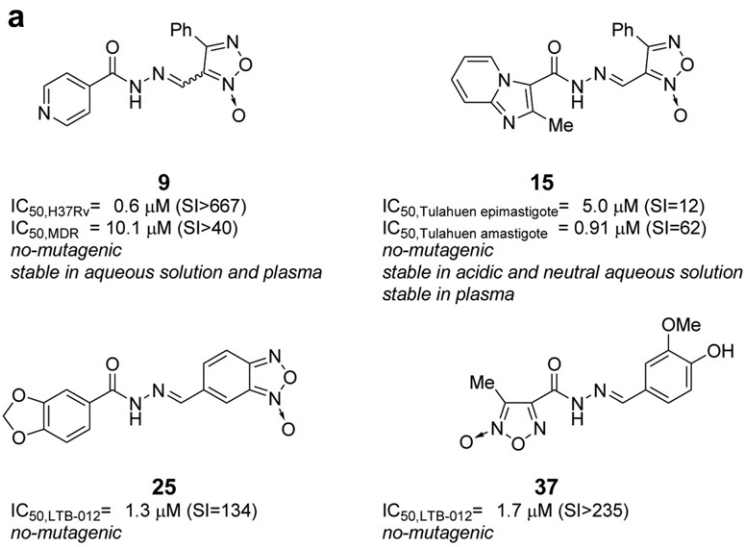
White solid (25%); mp: 181.4–182.9 °C ^{1}H NMR (DMSO-d_6) δ ppm: 7.60 (1H, dd, J_1 = 7.2, J_2 = 8.0), 8.08 (2H, s), 8.29 (1H, dd, J_1 = 8.4, J_2 = 6.4), 8.37 (1H, s), 8.59 (1H, s), 8.80 (1H, d, J = 4.0), 9.10 (1H, s), 12.45 (1H, s). ^{13}C NMR (DMSO-d_6) δ ppm: 117.0, 117.2, 124.2, 129.9, 130.0, 130.1, 146.5, 149.2, 149.5, 153.1, 162.6. Calculated analysis for $C_{13}H_9N_5O_2$: C: 58.4; H: 3.4; N: 26.2. Found: C: 58.5; H: 3.1; N: 26.4.

4.1.6. General procedure for the synthesis of compounds *42*–*47*

To a solution of (**XII**) (1 equiv.) in dry toluene (5 mL/mmol of hydrazide) was added the corresponding aldehyde (0.9 equiv.) and a drop of glacial AcOH. The mixture was stirred until the aldehyde was consumed (checked by TLC). The solid precipitated was filtered and washed with cold toluene.

4.1.7. (E)-N′-(4-Acetamidophenylmethylidene)-3-methylfuroxan-4-carbohydrazide (*42*)

Beige solid (21%); mp: 205.0–207.4 °C ^{1}H NMR (DMSO-d_6) δ ppm: 2.05 (3H, s), 2.31 (3H, s), 7.70 (4H, m), 8.46 (1H, s), 10.14 (1H,



b

	Lipinski descriptors				
compd.	logP	MW	nON	nOHNH	TPSA
9	2.14	309.3	8	1	105.8
15	3.25	362.3	9	1	110.3
25	2.89	326.3	9	1	111.4
37	1.32	292.3	9	2	122.4

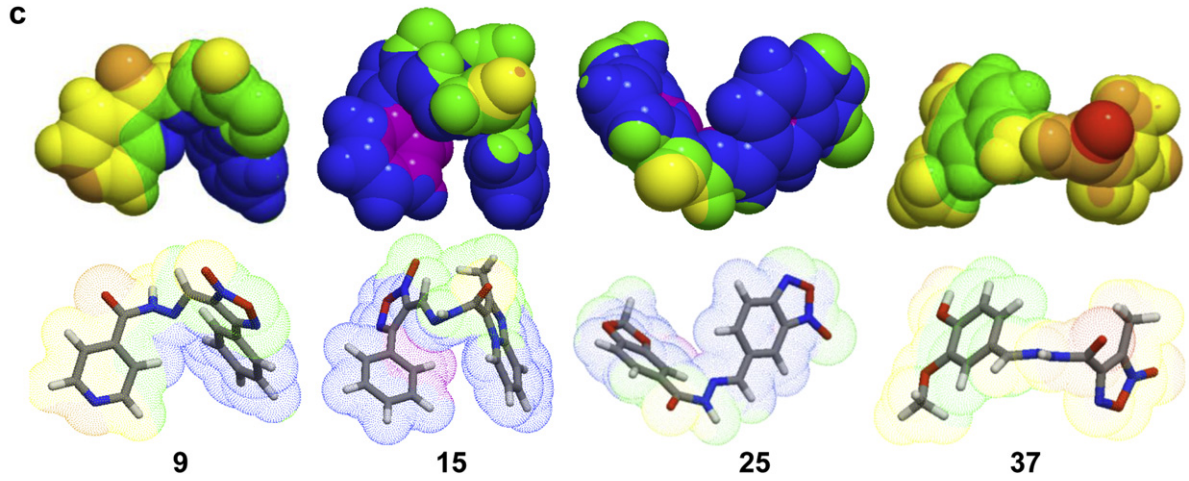


Fig. 3. a) New identified hits with anti-*M. tuberculosis*, anti-*T. cruzi* and anti-*Leishmania* activities from the herein studied hybrid furoxanyl *N*-acylhydrazones. b) Lipinski's physicochemical properties for **9**, **15**, **25**, and **37**. c) Molecular lipophilicity potential (MLP), virtual Log P [22,23], for **9**, **15**, **25**, and **37**. Up: Molecules are represented by CPK and MLP as solid colours are red/orange/yellow for hydrophilic regions; blue/violet for lipophilic regions; green for intermediate regions. Down: Molecules are represented by tubes and MLP as dotted colours. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

s), 12.46 (1H, s). ^{13}C NMR (DMSO-d_6) δ ppm: 9.0, 25.0, 115.0, 119.0, 128.0, 129.0, 142.0, 151.0, 152.0, 154.0, 168.0. IR (KBr) υ (cm^{-1}): 3189, 1676, 1610, 1583, 1520, 1258, 1036, 899, 831. MS (ESI) *m*/*z* (%): 304 ($M^{+\bullet}$ + H, 33). Calculated analysis for $C_{13}H_{13}N_5O_4$: C: 51.5; H: 4.3; N: 23.1. Found: C: 51.2; H: 4.1; N: 23.2.

*4.1.8. (E)-N′-(4-Nitrophenylmethylidene)-3-methylfuroxan-4-carbohydrazide (**43**)*

Yellow solid (52%); mp: 189.8–192.1 °C ^{1}H NMR (DMSO-d_6) δ ppm: 2.31 (3H, s), 8.01 (2H, d, J = 8.0), 8.28 (2H, d, J = 12.0), 8.63 (1H, s), 12.50 (1H, s). ^{13}C NMR (DMSO-d_6) δ ppm: 8.9, 113.7, 124.6, 128.9, 140.4, 148.5, 148.7, 151.9, 154.4. IR (KBr) υ (cm^{-1}): 3216, 1702, 1606, 1553, 1516, 1346, 1306, 1240, 1039, 854, 827. MS (EI) *m*/*z* (%): 291 ($M^{+\bullet}$, 14), 192 (50), 149 (15), 148 (1), 143 (18), 67 (100). Calculated analysis for $C_{11}H_9N_5O_5$: C: 45.4; H: 3.1; N: 24.1. Found: C: 45.1; H: 2.9; N: 23.9.

*4.1.9. (E)-3-Methyl-N′-(4-phenylmethylidene) furoxan-4-carbohydrazide (**44**)*

White solid (42%); mp: 180.4–184.7 °C ^{1}H NMR (DMSO-d_6) δ ppm: 2.39 (3H, s), 7.49 (1H, d, J = 3.0), 7.50 (2H, d, J = 3.0), 7.82 (2H, m), 8.61 (1H, s), 11.37 (1H, s). ^{13}C NMR (DMSO-d_6) δ ppm: 8.1, 112.0, 127.9, 129.2, 131.0, 134.6, 150.7, 152.5, 154.0. IR (KBr) υ (cm^{-1}): 3100, 1682, 1651, 1559, 1539, 1259, 1059, 835, 758. MS (EI) *m*/*z* (%): 246 ($M^{+\bullet}$, 32), 147 (55), 143 (26), 119 (14), 103 (26), 90 (45). Calculated analysis for $C_{11}H_{10}N_4O_3$: C: 53.7; H: 4.1; N: 22.8. Found: C: 53.4; H: 3.9; N: 22.9.

*4.1.10. (E)-N′-(3-Hydroxyphenylmethylidene)-3-methylfuroxan-4-carbohydrazide (**45**)*

Yellow solid (77%); mp: 190.7–193.2 °C ^{1}H NMR (DMSO-d_6) δ ppm: 2.40 (3H, s), 4.54 (1H, s), 6.90 (1H, m), 7.26 (1H, s), 7.28 (1H, m), 7.31 (1H, m), 8.34 (1H, s), 9.91 (1H, s). ^{13}C NMR (DMSO-d_6) δ ppm: 7.0, 112.0, 114.0, 118.0, 119.6, 129.5, 134.9, 151.0, 151.5, 155.0, 157.7. IR (KBr) υ (cm^{-1}): 3372, 3234, 1694, 1614, 1567, 1558, 1252, 1038, 884, 847. MS (EI) *m*/*z* (%): 262 ($M^{+\bullet}$, 100), 163 (66), 143 (10), 135 (20), 119 (89). Calculated analysis for $C_{11}H_{10}N_4O_4$: C: 50.4; H: 3.8; N: 21.4. Found: C: 50.2; H: 3.9; N: 21.5.

*4.1.11. (E)-N′-(3,4,5-Trimethoxyphenylmethylidene)-3-methylfuroxan-4-carbohydrazide (**46**)*

White solid (70%); mp: 179.2–181.9 °C ^{1}H NMR (DMSO-d_6) δ ppm: 2.25 (3H, s), 3.74 (3H, s), 3.77 (6H, s), 7.04 (2H, s), 8.44 (1H,

s), 12.61 (1H, s). ^{13}C NMR (DMSO-d_6) δ ppm: 8.8, 56.5, 60.6, 105.1, 113.7, 129.4, 140.1, 150.9, 152.1, 153.7, 154.0. IR (KBr) υ (cm^{-1}): 3239, 1673, 1610, 1579, 1564, 1241, 1008, 848, 740. MS (EI) *m*/*z* (%): 336 (M^{+}•, 100), 237 (16), 193 (47). Calculated analysis for $C_{14}H_{16}N_4O_6$: C: 50.0; H: 4.8; N: 16.7. Found: C: 49.8; H: 4.9; N: 16.4.

4.1.12. (E)-N′-(2-Pyridinylmethylidene)-3-methyl furoxan-4-carbohydrazide (**47**)

White solid (53%); mp: 174.8 °C (d). ^{1}H NMR (DMSO-d_6) δ ppm: 2.41 (3H, s), 7.45 (1H, dd, $J_1 = 5.0$, $J_2 = 2.0$), 7.91 (1H, dd, $J_1 = 7.8$, $J_2 = 2.0$), 8.13 (1H, d, $J = 8.0$), 8.62 (1H, s), 8.65 (1H, d, $J = 5.0$), 11.63 (1H, s). ^{13}C NMR (DMSO-d_6) δ ppm: 8.2, 113.0, 120.7, 125.1, 136.9, 150.1, 151.0, 151.6, 153.7, 154.3. IR (KBr) υ (cm^{-1}): 3169, 1693, 1620, 1590, 1562, 1242, 1040, 858, 835. MS (EI) *m*/*z* (%): 247 (M^{+}•, 5), 120 (100), 92 (83). Calculated analysis for $C_{10}H_9N_5O_3$: C: 48.6; H: 3.7; N: 28.3. Found: C: 48.5; H: 3.8; N: 28.0.

4.2. Biology

4.2.1. Anti-M. tuberculosis assay

A suspension of MTB was made by mixing growth from slants (20–30 days old) with 100 μL of Tween 80 into 0.2% bovine serum albumin. Turbidity of the suspension was then adjusted to a McFarland standard No. 1 (3×10^7 CFU/mL) by adding Tween 80 and bovine serum albumin. The bacterial suspension (300 μL) was further transferred to 7.2 mL of 7H9GC broth (4.7 g of Middlebrook 7H9 broth base, 20 mL of 10% glycerol, 1 g of Bacto Casitone, 880 mL of distilled water, 100 mL of OADC (oleic acid, albumin, dextrose, catalase)). For the bioassay, the compounds were resuspended in DMSO at a concentration of 4 mg/mL (stock solution). These stock solutions were further diluted with appropriate volumes of 7H9GC broth to yield final concentration of 0.4–25 μg/mL. Final drug concentrations ranges of standard antibiotics used as positive controls were 0.125–32 lg/mL for isoniazid and 0.063–16 μg/mL for rifampin. The drug (100 μL) was mixed in the wells with 100 μL of bacterial inoculums, resulting in a final bacterial concentration of approximately 1.2×10^6 CFU/mL. The wells in column 11 served as inoculums only controls. Solvent (DMSO) was included in every experiment as a negative control. Plants were sealed in plastic bags and then incubated at 37 °C for 5 days. On day 5, 50 μL of tetrazolium-Tween 80 mixture {1.5 mL of tetrazolium[3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide] at a dilution of 1 mg/mL, in absolute ethanol and 1.5 mL of 10% Tween 80} was added to the wells, and the plate was incubated at 37 °C for 24 h. After this period, the growth of the microorganism was visualized by the change in colour of the dye from yellow to purple. The tests were carried out in triplicate. MIC is defined as the lowest drug concentration that prevents the aforementioned change in colour.

4.2.2. Anti-T. cruzi in vitro test

T. cruzi epimastigotes (Tulahuen 2 strain) were grown axenically at 28 °C in BHI-Tryptose complemented with 5% foetal calf serum. Cells were harvested in late log phase, suspended in fresh medium, counted in a Neubauer chamber and placed in 24-well plates (3×10^6/mL). Cell growth was measured as the absorbance of the culture at 590 nm, which was found to be proportional to the number of cells. Before inoculation, the medium was supplemented with the indicated amount of the compound to be analysed from a stock solution in DMSO. The final concentration of DMSO in the culture media never exceeded 1% and a negative control was run with 1% DMSO and the absence of any compound, and a positive control with Nfx was included in each experiment. No effect on epimastigote growth was observed in the presence of up to 1% DMSO in the culture media. The percentage of growth inhibition was calculated as follows $\{1 - [(Ap - A0p)/(Ac - A0c)]\} \times 100$, where $Ap = A_{590}$ of the culture containing the compound to be analysed at day 5; $A0p = A_{590}$ of the culture containing the compound to be analysed just after addition of the inocula (day 0); $Ac = A_{590}$ of the culture in the absence of any compound (control) at day 5; $A0c = A_{590}$ in the absence of the compound at day 0. To determine ID_{50} values, parasite growth was followed in the absence (control) and presence of increasing concentrations of the corresponding compound. The ID_{50} values were determined as the drug concentrations required to reduce the absorbance by half that measured for untreated controls.

The anti-trypanosome activity in amastigote Tulahuen C4 strain transfected with β-D-galactosidase (Lac Z) gene, was evaluated by the colorimetric method based on the reduction of the substrate chlorophenol red β-D-galactopyranoside (CPRG) by β-galactoside resulting from the expression of the gene. A monolayer of VERO cells was seeded in a 96 well plate in complete RPMI 1640 medium without red phenol complemented with 10% of foetal calf serum. The cells were inoculated with 5×10^4 tripomastigotes (Tulahuen C4) per well and then, incubated for 24 h at 37 °C, 5% CO_2. After that, 10 μg/mL of each NAHs were added and the plate was incubated at 37 °C, 5% CO_2 during 5 days. The anti-*T. cruzi* activity was determined by adding 25 μL of 900 μM CPRG substrate (Roche) solution. The plate was incubated at 37 °C during 4–5 h until the colour developed. The compounds that showed anti-parasitic activity (>50% growing inhibition) were re-evaluated to determine the inhibitory concentration for 50% growth of the parasites (IC_{50}). The compounds were evaluated at concentrations 10.0, 2.0, 0.40, 0.80 and 0.16 μg/mL by duplicate. The colour intensity resulting from the cleavage of CPRG by β-galactosidase was measured at 570 nm using a microplate reader. The IC_{50} of compounds were calculated by logarithmic regression of their OD values compared to the untreated control. All active compounds also went through an evaluation of their cytotoxicity in VERO cells.

4.2.3. Anti-Leishmania in vitro test

Experiments were carried out on axenic amastigotes of *L. amazonensis* (strain MHOM/BR/76/LTB-012) maintained by weekly passages in MAA/20 medium at 32 °C with 5% CO_2 in 25 cm^2 tissue culture flasks. Cultures were initiated with 5×10^5 amastigotes with 5 mL of medium. To determine the activity of the NAHs, 100 μL of axenically grown amastigotes were seeded in 96 well plate. The compounds, dissolved in DMSO, were added at final concentrations ranging from 100 to 1 μg/mL. The reference compound, Amphotericin B, was evaluated at 0.1 μg/mL. After 72 h of incubation, 10 μL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma) (10 mg/mL) was added to each well and plates were further incubated for 4 h. The enzymatic reaction was then stopped with 100 μL of 50% isopropanol-10% sodium dodecyl sulphate and incubated for an additional 30 min under agitation at room temperature. Finally, absorbance was measured at 570 nm with a microplate reader. All experiments were performed in triplícate. Percent growth inhibition of the parasite was calculated by the following formula: % of inhibition = ((OD control − OD drugs)/OD control) × 100. The concentration inhibiting 50% of the parasite growth (IC_{50}) was calculated after evaluating percent growth inhibition at different concentrations.

4.2.4. Unspecific mammalian in vitro cytotoxicity

J774.1 murine macrophage cells (ATCC, USA) were grown in DMEM culture medium containing 4 mM L-glutamine and supplemented with 10% heat-inactivated foetal calf serum. The cells were seeded in a 96 well plate (5×10^4 cells in 200 μL culture medium) and allowed to attach for 48 h in a humidified 5% CO_2/95% air atmosphere at 37 °C. Afterwards, cells were exposed for 48 h to

the compounds (12.5–400.0 μM) or vehicle for control and additional controls (cells in medium) were used in each test. Cell viability was then assessed by measuring the mitochondrial-dependent reduction of MTT to formazan. For this propose, MTT in sterile PBS (0.2% glucose) pH 7.4 was added to cells to a final concentration of 0.1 mg/mL and cells were incubated at 37 °C for 3 h. After removing the media, formazan crystals were dissolved in 180 μL of DMSO and 20 μL MTT buffer (0.1 M glycine, 0.1 M NaCl, 0.5 mM EDTA, 10.5 pH) and the absorbance at 560 nm was read using a microplate spectrophotometer. The IC_{50} is determined as the concentration which reduces absorbance by 50% compared to control treated with the solvent of the compounds, and was determined by linear regression analysis.

The cytotoxicity against VERO cells (normal African green monkey kidney epithelial cells) at a maximum concentration of 200 μM was determined for all NAHs active against the amastigote form of *T. cruzi*. The compounds dissolved in DMSO were evaluated at serial dilutions of the maximum concentration in fresh culture medium. The culture was incubated at 37 °C, 5% CO_2 for 48 h. A negative control with DMSO (<0.1%) was included in each experiment. The percentage inhibition of cell growth was determined colorimetrically using Thiozol Blue (MTT; 3-[4.5 dimethylthiazol-2-y1]-2,5-diphenyltetrazolium bromide) (Aldrich, St. Louis MO). and reading of absorbance at 570 nm in a VersaMax Micro microplate reader. Nifurtimox (Bayer) was used as positive control at concentrations of 0.1, 1, and 10 μg/mL.

4.2.5. Mutagenicity assay

The method of direct incubation in plate was performed. Culture of *S. typhimurium* TA98 strain in the agar minimum glucose medium (AMG)—agar solution, Vogel Bonner E(VB) 50×, and 40% glucose solution—was used. First, the direct toxicity of the compounds under study against the bacteria was assayed. DMSO solutions of studied compounds and Nfx at different doses (starting at the highest doses without toxic effects) were assayed in triplicate. Positive controls of 4-nitro-*o*-phenylendiamine (20.0 μg/plate, in the run without S9 activation) and 2-aminofluorene (10.0 μg/plate, in the cases of S9 activation) and negative control of DMSO were run in parallel. The influence of metabolic activation was tested by adding 500 μL of S9 fraction of mouse liver treated with Aroclor, obtained from Moltox, Inc. (Annapolis, MD, USA). The revertant number was counted manually. The sample was considered mutagenic when the number of revertant colonies was at least double that of the negative control for at least two consecutive dose levels.

4.2.6. Stability studies [33]

For pH stability study, we utilized the following aqueous solutions: i) KCl·HCl buffer, pH 2; ii) acetate buffer, pH 5; iii) Tris·HCl buffer, pH 7.4 and iv) Tris·HCl buffer, pH 8.3. The stock solutions of compounds were prepared in DMSO and the final concentration in the aqueous media was 1 mM. The solutions were further homogeneizated and incubated at 37 °C for 24 h. After that, the absorbance spectrum was run between 250 and 450 nm. The spectrum was compared with a solution prepared at the moment of the read-out without incubation. Additionally, thin layer chromatographies were done with the ethyl acetate extracts in order to confirm or discard decomposition products presence.

For the determination of plasma stability, we used a pool of healthy human plasma diluited 1:1 in sterile Tris·HCl buffer, pH 7.4. The stock solutions of compounds were prepared at 10 mM in DMSO, and the final concentration in the biological media was 40 μM (DMSO < 2.5%). The mixture was vortexed, and placed on a 37 °C shaker (gentle) for 3 h. The reaction was quenched with cold acetonitrile followed immediately by mixing and centrifugation at 10,000 rpm, 4 min, 10 °C. The supernatant was used for the determination of stability comparing with a solution of the compound in acetonitrile prepared at the moment of the read-out.

Acknowledgements

We thank PEDECIBA-ANII for scholarship to P.H., and CNPq-PROSUL network for fellowships to P.H. E.J.B., and L.M.L. thanks CNPq (BR) for fellowships. We thank INCT-INOFAR (CNPq CNPq 573.564/2008-6 and FAPERJ E-26/170.020/2008) and RID-IMEDCHAG-CYTED for financial support.

References

[1] TAACF, http://www.taacf.org/about-TB-background.htm, (accessed 05.05.12).
[2] NIAID, http://www3.niaid.nih.gov/topics/tuberculosis/, (accessed 05.05.12).
[3] A. Jaso, B. Zarranz, I. Aldana, A. Monge, J. Med. Chem. 48 (2005) 2019–2025.
[4] R.P. Tangallapally, R. Yendapally, R.E. Lee, K. Hevener, V.C. Jones, A.J. Lenaerts, M.R. McNeil, Y. Wang, S. Franzblau, R.E. Lee, J. Med. Chem. 47 (2004) 5276–5283.
[5] WHO, http://www.who.int/neglected_diseases/diseases/chagas/en/index.html, (accessed 04.06.2012).
[6] H. Cerecetto, M. González, Pharmaceuticals 3 (2010) 810–838.
[7] J. Soto, J.T. Toledo, Lancet Infect. Dis. 7 (2007) 7.
[8] H. Cerecetto, M. González, Mini Rev. Med. Chem. 8 (2008) 1355–1383.
[9] E. Vicente, S. Pérez-Silanes, L.M. Lima, S. Ancizu, A. Burguete, B. Solano, R. Villar, I. Aldana, A. Monge, Bioorg. Med. Chem. 17 (2009) 385–389.
[10] S. Ancizu, E. Moreno, E. Torres, A. Burguete, S. Pérez-Silanes, D. Benítez, R. Villar, B. Solano, A. Marín, I. Aldana, H. Cerecetto, M. González, A. Monge, Molecules 14 (2009) 2256–2272.
[11] N.C. Romeiro, G. Aguirre, P. Hernández, M. González, H. Cerecetto, I. Aldana, S. Pérez-Silanes, A. Monge, E.J. Barreiro, L.M. Lima, Bioorg. Med. Chem. 17 (2009) 641–652.
[12] D.R. Ifa, C.R. Rodrigues, R.B. de Alencastro, C.A.M. Fraga, E.J. Barreiro, J. Mol. Struct. THEOCHEM 505 (2000) 11–17.
[13] A. Merlino, D. Benitez, N.E. Campillo, J.A. Páez, L.W. Tinoco, M. González, H. Cerecetto, Med. Chem. Commun. 3 (2012) 90–101.
[14] L. Boiani, G. Aguirre, M. González, H. Cerecetto, A. Chidichimo, J.J. Cazzulo, M. Bertinaria, S. Guglielmo, Bioorg. Med. Chem. 16 (2008) 7900–7907.
[15] D. Castro, L. Boiani, D. Benitez, P. Hernández, A. Merlino, C. Gil, C. Olea-Azar, M. González, H. Cerecetto, W. Porcal, Eur. J. Med. Chem. 44 (2009) 5055–5065.
[16] P. Hernández, M. Cabrera, M.L. Lavaggi, L. Celano, I. Tiscornia, T. Rodrigues da Costa, L. Thomson, M. Bollati-Fogolín, A.L. Miranda, L.M. Lima, E.J. Barreiro, M. González, H. Cerecetto, Bioorg. Med. Chem. 20 (2012) 2158–2171.
[17] G. Aguirre, L. Boiani, H. Cerecetto, R. Di Maio, M. González, W. Porcal, L. Thomson, V. Tórtora, A. Denicola, M. Möller, Bioorg. Med. Chem. 13 (2005) 6324–6335.
[18] G.J. Karabatsos, J.D. Graham, F.M. Vane, J. Am. Chem. Soc. 84 (1962) 753–755.
[19] G.J. Karabatsos, B.L. Shapiro, F.M. Vane, J.S. Fleming, J.S. Ratka, J. Am. Chem. Soc. 85 (1963) 2784–2788.
[20] G.J. Karabatsos, R.A. Taller, J. Am. Chem. Soc. 85 (1963) 3624–3629.
[21] L.A. Collins, C. Franzblau, Antimicrob. Agents Chemother. 41 (1997) 1004–1009.
[22] S.G. Franzblau, R.S. Witzig, J.C. Mclaughlin, P. Torres, G. Madico, A. Hernandez, M.T. Degnan, M.B. Cook, V.K. Quenzer, R.M. Ferguson, R.H. Gilman, J. Clin. Microbiol. 36 (1988) 362–366.
[23] L. Caviedes, J. Delgado, R.J. Gilman, J. Clin. Microbiol. 40 (2002) 1873–1874.
[24] J. Camacho, A. Barazarte, N. Gamboa, J. Rodrigues, R. Rojas, A. Vaisberg, R. Gilman, J. Charris, Bioorg. Med. Chem. 19 (2011) 2023–2029.
[25] C. Rodrigues, A.A. Batista, J. Ellena, E.E. Castellano, D. Benítez, H. Cerecetto, M. González, L.R. Teixeira, H. Beraldo, Eur. J. Med. Chem. 45 (2010) 2847–2853.
[26] J.C. Aponte, A.J. Vaisberg, D. Castillo, G. Gonzalez, Y. Estevez, J. Arevalo, M. Quiliano, M. Zimic, M. Verástegui, E. Málaga, R.H. Gilman, J.M. Bustamante, R.L. Tarleton, Y. Wang, S.G. Franzblau, G.F. Pauli, M. Sauvain, G.B. Hammond, Bioorg. Med. Chem. 18 (2010) 2880–2886.
[27] V.K. Prajapati, K. Awasthi, S. Gautam, T. Prasad Yadav, M. Rai, O.N. Srivastava, S. Sundar, J. Antimicrob. Chemother. 66 (2011) 874–879.
[28] D.M. Maron, B.N. Ames, Mutat. Res. 113 (1983) 173–215.
[29] C.A. Lipinski, F. Lombardo, B.W. Dominy, P.J. Feeney, Adv. Drug Deliv. Rev. 23 (1997) 4–25.
[30] K.C. Chu, K.M. Patel, A.H. Lin, R.E. Tarone, M.S. Linhart, V.C. Dunkel, Mutat. Res. 85 (1981) 119–132.
[31] P. Gaillard, P.A. Carrupt, B. Testa, A. Boudon, J. Comput. Aided Mol. Des. 8 (1994) 83–96.
[32] A.J.M. Carpy, N. Marchand-Geneste, SAR QSAR Environ. Res. 14 (2003) 329–337.
[33] H.E. Kerns, D. Li, Drug Like Properties: Concepts, Structure, Design and Methods, in: From ADME to Toxicity Optimization, first ed., Academic Press, 2008, pp. 353–358.