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Antitrypanosomal Activities and Cytotoxicity of 5-Nitro-2-furancarbohydrazides

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Abstract—A series of 5-nitro-2-furancarbohydrazides were synthesized. In vitro antitrypanosomal activities of these compounds were determined against the closely related protozoa *Trypanosoma cruzi Trypanosoma brucei* and discussed in relation to potential targets.

Introduction

Trypanosomatids are parasitic hemoflagellate protozoa which are the causative agents of tropical diseases including human African sleeping sickness (*Trypanosoma brucei gambiense*, *T. brucei rhodesiense*), Chagas' disease (South American trypanosomiasis, *T. cruzi*) and the visceral, cutaneous, and mucocutaneous manifestations of leishmaniasis (e.g., *Leishmania donovani, Leishmania tropica, Leishmania braziliensis*). Several millions of new infections by trypanosomatid parasites are detected annually. The current drugs[1] used in the treatment of these diseases have serious side effects, show poor clinical efficacy or are ineffective due to an increase in resistance. For African trypanosomiasis, the trivalent arsenical drug melarsoprol (Fig. 1) is still used against the second stage in which *Trypanosoma brucei rhodesiense* or *gambiense* has invaded the central nervous system. However, its use is limited by the high toxicity of the treatment. Against the *T. cruzi* parasite, nitroheterocyclic compounds[2] such as benznidazole or nifurtimox (Fig. 1) are used for the treatment of Chagas' disease. Benznidazole is the only drug still available since the production of nifurtimox was stopped. The limited available treatment accentuates the need to develop safer and more efficient new therapeutic agents.

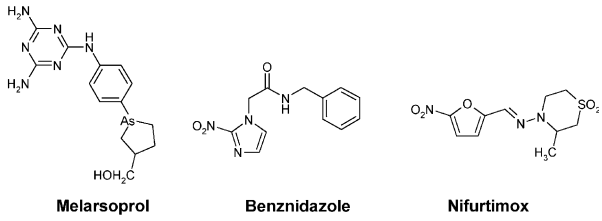

Figure 1. Antitrypanosomal agents.

By random screening, we have identified another nitroheterocyclic derivative (compound **1**, Fig. 2) as a lead structure for a novel class of antitrypanosomal agents. Many 5-nitrofuran derivatives[2b,3] from various series have been synthesized since their structure is associated with antitrypanosomal, antibacterial and antifungal activities. Nevertheless only one example of a 5-nitrothiophenecarbohydrazide has been described so far for its antitrypanosomal activity.[4]

We have therefore undertaken the synthesis and an in vitro antiparasitic activity study against the protozoa

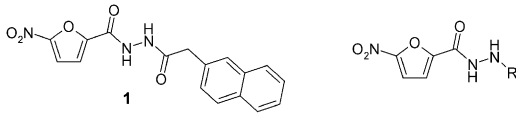

Figure 2. Compound **1** and furancarbohydrazide derivatives.

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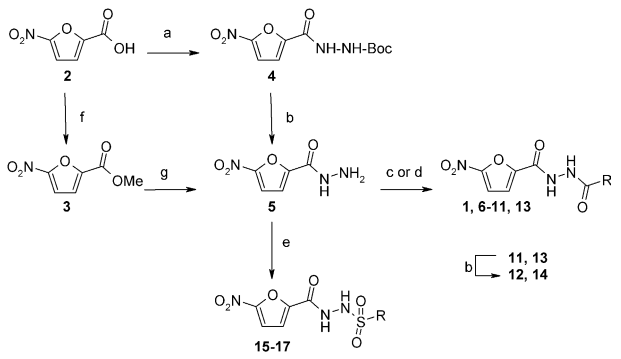


Scheme 1. Reagents and conditions: (a) PyBOP, DIEA, *tert*-butylcarbazate, CH_2Cl_2, rt, 16 h, 71%; (b) MeOH, HCl, rt, 16 h, 95%; (c) PyBOP, DIEA, CH_2Cl_2, appropriate acid, rt, 16 h, 25–72%; (d) appropriate acyl chloride, NEt_3, CH_2Cl_2, rt, 16 h, 50–70%; (e) appropriate sulfonyl chloride, NEt_3, CH_2Cl_2, rt, 16 h, 40–50%; (f) 1. Cs_2CO_3, MeOH, H_2O, 2. CH_3I, DMF, 91%; (g) hydrazine monohydrate, MeOH, reflux, 24 h, 25%.

Trypanosoma cruzi and *Trypanosoma brucei* of 5-nitro-2-furancarbohydrazides in which we have replaced the 2-naphthylmethylcarbonyl of the lead compound by several aromatic acyl or aromatic sulfonyl moieties (Fig. 2). In this study, the 5-nitro-2-furancarbo-hydrazide group presented a good activity/toxicity ratio, allowing to develop a new series of not toxic compounds.

Chemistry

Compounds **1** and **3–17** were obtained as shown in Scheme 1.

The hit **1** was obtained according to standard procedures of peptidic synthesis in solution.[5] Briefly, the commercial 5-nitro-2-furoic acid **2** was reacted with *tert*-butyl carbazate using PyBOP (benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate)[6] as coupling agent and DIEA (*N,N*-diisopropylethylamine) as a base to give compound **4**. Removal of the Boc group by methanolic HCl 1 M solution resulted in 5-nitro-2-furancarbohydrazide hydrochloride **5**.

An alternative synthetic route was also used to prepare this latter compound. It involved the preparation of methyl ester **3** via the alkylation of the cesium salt of 5-nitro-2-furoic acid by methyl iodide in DMF. Then, ester **3** was refluxed with hydrazine monohydrate in MeOH to give the desired 5-nitro-2-furancarbo-hydrazide **5** in 25% yield. However, this method produced a lower yield than the peptidic procedure adopted. Compound **5** was then coupled with 2-naphthyl acetic or another appropriate acid to produce the diacylhydrazines **1** or **9–11**, **13** respectively. For compounds **6–8**, the aromatic moiety was introduced by direct acylation of hydrazide **5** by the commercially available acyl chloride, in CH_2Cl_2 in the presence of NEt_3. The sulfonamide derivatives **15–17** were obtained by reaction of hydrazide **5** with appropriate sulfonyl chloride.

Biological Evaluation

In vitro activity

In vitro activity against intracellular *T. cruzi* amastigotes. Primary mouse peritoneal macrophages were seeded in 96-well microplates at 30,000 cells per well. After 24 h, about 100,000 trypomastigotes of *T. cruzi* were added per well together with 2-fold dilutions of the drug. The cultures were incubated at 37 °C in 5% CO_2–95% air for 4 days. Following fixation in methanol and Giemsa staining, the drug activity was semi-quantitatively scored as % reduction of the total parasite load (free trypomastigotes and intracellular amastigotes) compared with untreated control cultures. Scoring was performed microscopically and ED_{50}-values were then extrapolated.

In vitro activity against *T. brucei* trypomastigotes. Bloodstream forms of *T. brucei* were cultivated in HMI-9 medium. In a 96-well microplate, 10,000 haemoflagellates were incubated at different drug concentrations for 4 days. Parasite multiplication was measured colorimetrically (490 nm) following addition of MTT tetrazolium which converts to an aqueous soluble formazan product. ED_{50}-values were then extrapolated.

In vitro activity against intracellular *Leishmania infantum* amastigotes. Primary mouse peritoneal macrophages were seeded in 16-well LABTEK culture slides at 30,000 cells per well. After 24 h, amastigotes of *L. infantum* (derived from the spleen of an infected donor animal) were added at an infection ratio of 10/1 together with a two-fold dilution of the drug. The cultures were incubated at 37 °C in 5% CO_2–95% air for 7 days. Treatment of uninfected control cultures was also included to determine a selective index. Drug activity was semi-quantitatively scored as % reduction of the total parasite load or the number of infected macrophages in Wright stained preparations. Scoring was performed microscopically and ED_{50}-values were extrapolated.

Cytotoxicity test

A human diploid embryonic lung cell line (MRC-5, Bio-Whittaker 72211D) and mouse primary peritoneal macrophages were used to assess the cytotoxic effects on host cells. The peritoneal macrophages were collected from the peritoneal cavity 48 h after stimulation with potato starch and they were seeded in 96-well microplates at 30,000 cells per well. MRC-5 cells were seeded at 5000 cells per well. After 24 h, the cells were washed and two-fold dilutions of the drug were added in 200 μL standard culture medium (RPMI + 5% FCS). The final DMSO concentration in the culture remained below 0.5%. The cultures were incubated with several concentrations of the compounds (between 32 and 1.6 μM) at 37 °C in 5% CO_2–95% air for 7 days. Untreated cultures were included as controls. For MRC-5 cells, the cytotoxicity was determined using the colorimetric MTT assay and scored as a % reduction of absorption at 540 nm of treated cultures versus untreated control cultures. For macrophages, scoring was performed microscopically.

Enzymology

When tested as potential inhibitors of trypanothione reductase from *T. cruzi* (TcTR), compounds **1** and **4–6** were relatively ineffective with little TcTR inhibition evident at concentrations up to 50 μM (IC_{50} > 50 μM for **4–6**, and > 25 μM for **1**) (data not shown).[7] This was not surprising since many derivatives lack a protonable nitrogen atom in the side-chain, the compounds **1** and **4–6** were not well-recognized by TcTR, which illustrates the essential requirement of a positively charged amino group for TR recognition.[8]

Compounds **1** and **4–6** were also evaluated as subversive substrates of the lipoamide dehydrogenase from *T. cruzi* (TcLipDH).[9] At 133 μM none of the compounds showed any increase in the reaction rate compared to the intrinsic oxidase activity of the enzyme. In comparison, 133 μM nitrofuroxazid[3a] used as positive control yielded an activity of 0.115 U/mL, suggesting another mechanism to explain the observed potent antitrypanosomal action of the synthesized nitrofurans described in this study.

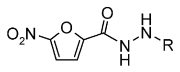
Result and Discussion

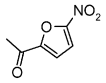
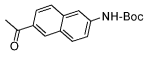
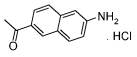
Antitrypanosomal activities of the compounds towards the intracellular amastigote stage of both *T. cruzi* and *L. infantum* and against the *T. brucei* bloodstream trypomastigotes are given in Table 1, as ED_{50} values. The standard drugs melarsoprol, benznidazole and nifurtimox were used as positive controls for *T. brucei* and *T. cruzi* respectively. All compounds were tested also for their cytotoxicity towards human MRC-5 cells (CC_{50} values) and mouse peritoneal macrophages (MPM).

The results show that the 5-nitro-2-furancarbo-hydrazide structure is an excellent scaffold for the design of new antitrypanosomal derivatives. The hydrazides shown in Table 1 present potency profiles that are similar to those seen with standard drugs as melarsoprol, benznidazole and nifurtimox. This was not observed for acid **2** and ester **3** that verified the essential role of hydrazide function. The trypanocidal effects observed in this series suggested to us to study the inhibitory potencies or the redox-cycling properties of several representatives among the most active compounds (**1**,**4–6**,**11**) against *T. cruzi* in cultures, with two flavoenzymes characterized in *T. cruzi,* the trypanothione reductase and the lipoamide dehydrogenase, respectively.[10] Both enzymes have been validated as attractive targets for antiparasitic chemotherapy, by satisfactory correlations between redox-cycling properties and antitrypanosomal action of several nitrofurans and NQ.[3a,11] The data obtained from these experiments showed that obviously the 5-nitro-2-furancarbohydrazides prepared in this study do not act through interaction with TR and LipDH activities.

On the other hand, some acyl hydrazides have already been described as reversible inhibitors of *T. brucei* trypanopain the major cysteine proteinase of *T. b. brucei* and shown to display potent antitrypanosomal action against cultured bloodstream forms of *T. b. brucei*.[4,12] The lack of correlation between the trypanocidal activity and the inhibition of the purified *T. brucei* trypanopain by these products[4] supported the suggestion that the protease was not the major direct target of these compounds. However, the broad antiparasitic—antimalarial,[12] antitrypanosomal[4]—action of the acyl hydrazide series suggests that these compounds could act through a similar mechanism and merit further investigation as potential lead structures for the search of new treatments against trypanosomiasis.

Table 1. In vitro activities towards trypomastigote forms of *T. brucei* and amastigote forms of *T. cruzi* and *L. infantum* and in vitro cytotoxicity upon MRC-5 cells and MPM of compounds **1–17**



Compd[a]	R	*T. cruzi* ED_{50} (μM)	*T. brucei* ED_{50} (μM)	*L. infantum* ED_{50} (μM)[b]	CC_{50} (μM)
1	CO-CH_2-2(Napht)	0.4	0.59	8	4
2	—	> 32	5	> 32	> 32
3	—	> 32	> 32	> 32	> 32
4	Boc	1	2	> 32	12
5	H	0.5	5	> 32	9
6		3	3	8	4
7	CO-Ph	16	32	> 32	17
8	CO-Bzl	1	0.3	8	2
9	CO-2(Napht)	4	0.13	8	2
10	CO-1(Napht)	3	0.4	8	1
11		4	0.25	8	6
12		2	2	> 32	14
13		1	0.5	8	2
14		4	0.64	8	0.77
15	SO_2-Ph	16	2	> 32	4
16	SO_2-2(Napht)	> 32	28	> 32	18
17	SO_2-1(Napht)	0.8	1	8	0.75
Mel	—	—	0.2	—	12.5
Benz	—	3.13	—	—	> 50
Nifur	—	0.4	—	—	> 50

[a]Mel: melarsoprol, Benz: benznidazole, Nifur: nifurtimox.
[b]All the compounds were cytotoxic only at 32 μM against MPM used in *L. infantum* test.

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7. The four compounds **1**, **4–6** were tested in the trypanothione disulfide reduction assay at varying concentrations (2.5–5-12.5–25–50 μM), by following the disappearance of NADPH at 340 nm. The IC_{50} values of compounds **1**, **4–6** were determined in the presence of 50 μM $T(S)_2$ and TR (0.02 U per assay).
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9. The four compounds **1** and **4–6** were tested as substrates of TcLipDH, as well as Nitrofuroxazid[3a] as positive control. 4 mM Stock solutions of the compounds were prepared in DMSO and stored at −20 °C. The LipDH sample used had an activity of 5.3 U/mL in the physiological forward reaction (lipoamide + NAD) and an intrinsic oxidase activity of 0.05 U/mL (the control assay contained only NADH but lacked the nitrofuran derivatives). Nitrofuran reductase assay: assay volume 90 μL in 50 mM potassium phosphate buffer, 1 mM EDTA, pH 7.5, 25 °C, 100 μM NADH, 133 μM nitrofuran derivative. The nitrofuran reductase activity of LipDH was determined by following the disappearance of the NADH absorption at 340 nm.
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