Bioorganic & Medicinal Chemistry 18 (2010) 2880–2886

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Trypanoside, anti-tuberculosis, leishmanicidal, and cytotoxic activities of tetrahydrobenzothienopyrimidines

José C. Aponte [a], Abraham J. Vaisberg [b], Denis Castillo [c], German Gonzalez [c], Yannick Estevez [c,i], Jorge Arevalo [c], Miguel Quiliano [d], Mirko Zimic [d], Manuela Verástegui [b], Edith Málaga [b], Robert H. Gilman [e], Juan M. Bustamante [f], Rick L. Tarleton [f], Yuehong Wang [g], Scott G. Franzblau [g], Guido F. Pauli [g,h], Michel Sauvain [b,i], Gerald B. Hammond [a,*]

[a] Department of Chemistry, University of Louisville, Louisville, KY 40292, USA
[b] Departamento de Microbiología y Laboratorios de Investigación y Desarrollo, Facultad de Ciencias y Filosofía, Universidad Peruana Cayetano Heredia, Lima, Peru
[c] Instituto de Medicina Tropical Alexander von Humboldt, Universidad Peruana Cayetano Heredia, Lima, Peru
[d] Bioinformatics Unit—Drug Design Group, Laboratorios de Investigación y Desarrollo, Facultad de Ciencias y Filosofía, Universidad Peruana Cayetano Heredia, Lima, Peru
[e] Johns Hopkins University School of Public Health, USA
[f] Center for Tropical and Emerging Global Diseases, Coverdell Center for Biomedical Research, University of Georgia, Athens, GA 30602, USA
[g] Institute for Tuberculosis Research, College of Pharmacy, University of Illinois at Chicago, Chicago, IL 60612, USA
[h] Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, Chicago, IL 60612, USA
[i] Université de Toulouse;UPS;UMR 152 (Laboratoire de pharmacochimie des substances naturelles et pharmacophores redox), 118, rte de Narbonne, F-31062 Toulouse cedex 9, France

ARTICLE INFO

Article history:
Received 17 December 2009
Revised 8 March 2010
Accepted 9 March 2010
Available online 12 March 2010

Keywords:
Tetrahydrobenzothienopyrimidine
Trypanosoma cruzi
Mycobacterium tuberculosis
Leishmania amazonensis
Anticancer

ABSTRACT

The synthesis of 2-(5,6,7,8-tetrahydro[1]benzothieno[2,3-*d*]pyrimidin-4-yl)hydrazone-derivatives (BTPs) and their in vitro evaluation against *Trypanosoma cruzi* trypomastigotes, *Mycobacterium tuberculosis*, *Leishmania amazonensis* axenic amastigotes, and six human cancer cell lines is described. The in vivo activity of the most active and least toxic compounds against *T. cruzi* and *L. amazonensis* was also studied. BTPs constitute a new family of drug leads with potential activity against infectious diseases. Due to their drug-like properties, this series of compounds can potentially serve as templates for future drug-optimization and drug-development efforts for use as therapeutic agents in developing countries.

1. Introduction

Chagas' disease, tuberculosis and leishmaniasis are commonly being referred as *Neglected Diseases*. These infectious diseases, prevalent in Third World countries, lack effective, affordable, widely accessible, and/or easy to use drug treatments. A number of factors such as affordability, drugs resistance, poor efficacy and severe adverse effects limit the utility of the current existing drugs. The situation has become more acute as the number of reported cases increased.[1] Because most of the affected population live in developing countries and cannot afford existing drugs, the pharmaceutical industry has traditionally ignored these diseases. Chagas' disease is an endemic tropical disease that infects up to 20 million people in Central and South America and approximately between 50,000 and 100,000 people in the United States.[2] According to the WHO, it is estimated that tuberculosis was responsible for 1.77 million deaths in 2007.[3] In the case of leishmaniasis, it is estimated that over 2 million people are infected, and about 57,000 die annually.[4] Current standard treatments for tuberculosis include antibacterial drugs such as Rifampicin and Isoniazid, which require between six to twelve month-therapies to fully eliminate *mycobacterium* from the body.[5] The antifungal Amphotericin B is currently used to treat leishmaniasis, however its high cost and elevated toxicity limit its use.[6] Furthermore, for the treatment of leishmaniasis, only one available drug, Miltefosine, does not accuse resistance; however, it is contraindicated in pregnancy.[7] Nifurtimox and

Abbreviations: BTP, 2-(5,6,7,8-tetrahydro[1]benzothieno[2,3-*d*]pyrimidin-4-yl)hydrazone-derivatives; *T. cruzi*, *Trypanosoma cruzi*; *M. tuberculosis*, *Mycobacterium tuberculosis*; *L. amazonensis*, *Leishmania amazonensis*; TPSA, topological polar surface area; %ABS, percentage of absorption; *n*-ROTB, number of rotatable bonds; mi Log *P*, logarithm of compound partition coefficient between *n*-octanol and water; *n*-OHNH, number of hydrogen bond donors; *n*-ON, number of hydrogen bond acceptors.

* Corresponding author. Tel.: +1 502 852 5998; fax: +1 502 852 3899.
E-mail address: gb.hammond@louisville.edu (G.B. Hammond).

0968-0896/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2010.03.018

Benznidazole are currently used to treat Chagas' disease, but both are associated with major side effects and need close monitoring.[8]

Clearly, there is a need for a search for new types of drugs with high selectivity, minimum side effects and low manufacturing costs. 2-(5,6,7,8-Tetrahydro[1]benzothieno[2,3-*d*]pyrimidin-4-yl)hydrazone-derivatives (BTPs) are small molecules that have been used as chemical probes in an image-based phenotypic screen for inhibitors of the secretory pathway.[9] Benzothienopyrimidines exhibit a variety of pharmacological activities, among them anticancer,[10] antimicrobial,[11] and anticonvulsant.[14b] We selected tetrahydrobenzothienopyrimidines in our studies because of their structural novelty, diversity of chemical functionalities, straightforward synthesis and affordable starting materials.

In the present study, a series of 38 BTPs were synthesized (Scheme 1) and evaluated in vitro against infectious pathogens *Trypanosoma cruzi*, *Mycobacterium tuberculosis* (Table 1), and three *Leishmania* strains, (Tables 1 and 2). The antiproliferative activity of these BTPs was also studied against panel of six human cancer cell lines and one non-tumorogenic cell line (Table 4). Based on the in vitro study, the in vivo activity of the most active and least toxic BTP against *T. cruzi* and *L. amazonensis* (Table 4) was evaluated. The ADME properties of all molecules were calculated in silico (Table 5).

2. Chemistry

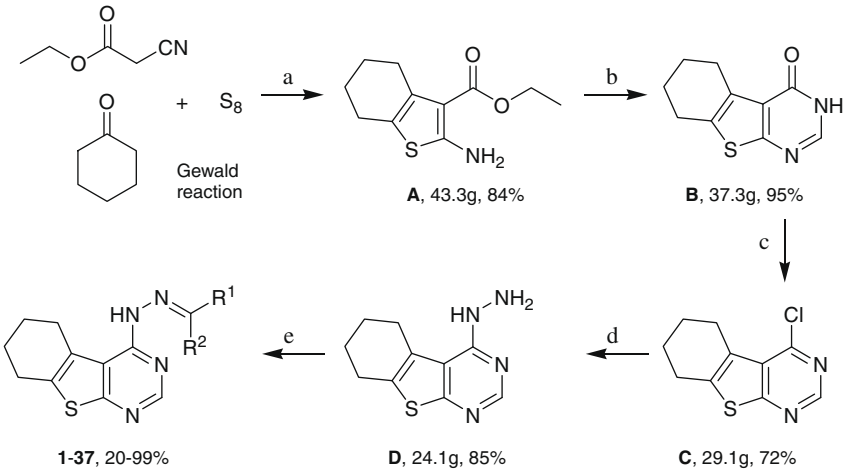
The synthesis of the BTP series is summarized in Scheme 1. It began with the preparation of 2-amino-4,5,6,7-tetrahydrobenzo [*b*]thienophene-3-carboxylic acid ethyl ester (**A**) using a Gewald reaction. **A** reacted with an excess of formamide to obtain the cyclic pyrimidinone **B**,[12a] which underwent chlorination using phosphorus oxychloride ($POCl_3$), yielding **C**. Nucleophilic displacement with aqueous hydrazine formed the corresponding aromatic-hydrazine derivative **D**.[12b] The synthesis of BTPs was concluded with the formation of a Schiff base between **D** and an aldehyde or ketone. All these reactions resulted in high yield and were performed in gram scale; the purification for each step was facilitated because each intermediate in the synthesis could be recrystallized.

3. Results

In our continued search for biologically active compounds that target infective parasites and cancer,[13] a series of 2-(5,6,7,8-tetrahydro[1]benzothieno[2,3-*d*]pyrimidin-4-yl)hydrazone-derivatives (BTPs) was developed and tested for their anti-*T. cruzi*, anti-*M. tuberculosis*, and anti-*L. amazonensis* potential, as well as for their antiproliferative activity against a panel of six human cancer cell lines and one non-tumorogenic cell line. To the best of our knowledge, 13 of a total of 38 compounds (**2**, **3**, **8**, **15**–**17**, **19**, **24**, **27**, **32**–**34**, and **37**) represent new chemical entities. Their structures and in vitro bioactivities are presented in Tables 1, 2 and 4. Compound **4** exhibited the highest selectivity in the in vitro anti-*T. cruzi* assay and was evaluated in a short-term in vivo assay, from which **4** resulted to be inactive. Compound **D** and 37 BTPs were also investigated for their anti-*M. tuberculosis* activity using the H37R*v* virulent strain in a MABA anti-TB assay. In general, this series of compounds showed greater potential against protozoan parasites and tumorogenic cells than against *M. tuberculosis*. However, when compounds **10**, **18**, **24**, **25** and **35** were further tested in a TB non-replicating model, involving exposure under low-oxygen (LORA), compounds **10**, **18** and **35** showed higher anti-TB activity, while compounds **25** and **26**, exhibited the exact same inhibitory activity in both tests (MABA and LORA). Compounds **6**, **21**, **28**, **30** and **36** exhibited moderate-to-high degree of selectivity against *L. amazonensis* axenic amastigotes, and were successively tested in a macrophage-infected assay using the three most prevalent *Leishmania* species in Peru, *L. amazonensis*, *L. brasilensis* and *L. peruviana* (Table 2). With in vitro anti-*Leishmania* bioactivity in hand, we selected compounds **6**, **28** and **36** for their in vivo evaluation (Table 3). It was found that **36** reduced the parasite burden in 65% after four weeks of treatment. Table 4 shows the anti-tumor results for BTPs; in this screening **21** exhibited antiproliferative values ranging from 0.1 to 0.5 μM against the different human cancer cell lines, and had selectivity towards human breast and human colon carcinomas. These results were confirmed by the NCI 60-cancer cell line panel study.

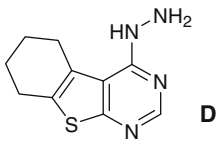
4. Discussion

The benzothienopyrimidine moiety has attracted attention due to their wide variety of biological activities.[9–11,14,15] However the tetrahydrobenzothienopyrimidine system, containing a lipophilic tetrahydrobenzo subunit and two biologically relevant segments—a Schiff base and hydrazone moieties, have not been investigated. The anti-trypanosome activity of BTP was evaluated by a colorimetric method based on the reduction of the substrate chlo-

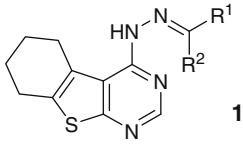


Scheme 1. Synthesis of BTPs. Reagents and conditions: (a) diethyl amine, EtOH, rt, sonication, 30 min; (b) formamide, reflux, 6 h; (c) phosphorus oxychloride, reflux, 3 h; (d) hydrazine 51% in H_2O, MeOH, reflux, 3 h; (e) aldehyde or ketone, EtOH, reflux, 2–72 h.

Table 1
In vitro anti-*T. cruzi*, anti-*M. tuberculosis* and anti-*L. amazonensis* activity of compounds **D** and **1–37**[a]



D



1-37

Compd	R_1, R_2	IC_{50}[b] (μM)	SI[c]	MIC H37Rv (μM) MABA (LORA)	SI[d]	IC_{50} (μM)	SI[e]
		T. cruzi		*M. tuberculosis*		*L. amazonensis*	
D	—	21.3	1.9	374.5	0.3	54.4	0.6
1	R_1 = H, R_2 = C_6H_5	14.4	1.8	75.9	0.5	43.7	0.6
2	R_1 = H, R_2 = 1-Naphthalenyl	13.0	0.2	>250	—	12.7	1.3
3	R_1 = H, R_2 = 2-Naphthalenyl	13.3	1.1	>250	—	39.0	0.4
4	R_1 = H, R_2 = Cinnamyl	1.4	14.6	35.0	0.1	16.6	0.1
5	R_1 = H, R_2 = C_6H_4–4-CH_3	>25	—	>250	—	42.6	0.5
6	R_1 = H, R_2 = C_6H_4–4-CH_2CH_3	>25	—	>250	—	15.7	9.6
7	R_1 = H, R_2 = C_6H_4–4-CH(C_2H_6)	5.2	0.5	>250	—	3.6	2.3
8	R_1 = H, R_2 = C_6H_4–4-CH(C_3H_9)	11.2	1.2	>250	—	1.7	3.8
9	R_1 = H, R_2 = C_6H_4–4-OH	11.3	1.1	72.1	0.6	41.6	4.3
10	R_1 = H, R_2 = C_6H_4–4-OCH_3	16.0	1.1	71.2 (26.9)	3.2 (8.4)	47.5	1.3
11	R_1 = H, R_2 = C_6H_4–3-OCH_3	16.9	0.9	119.4	0.6	36.1	0.5
12	R_1 = H, R_2 = C_6H_3–3,4-O–CH_2–O	22.8	0.7	>250	—	29.4	0.6
13	R_1 = H, R_2 = C_6H_3–2,4-OCH_3	18.5	1.8	>250	—	33.2	4.3
14	R_1 = H, R_2 = C_6H_2–3,4,5-OCH_3	>25	—	>250	—	7.2	1.2
15	R_1 = H, R_2 = C_6H_3–3-OCH_3, 4-OH	17.5	0.6	>250	—	7.2	3.3
16	R_1 = H, R_2 = C_6H_2–3,5-OCH_3, 4-OH	>25	—	>250	—	40.8	3.0
17	R_1 = H, R_2 = C_6H_2-3,5-Allyloxy, 4-Br	15.4	0.4	>200	—	5.3	0.7
18	R_1 = H, R_2 = 2-Furyl	16.1	0.6	39.9 (9.7)	1.0 (4.2)	4.2	0.6
19	R_1 = H, R_2 = 2-Pyrrolyl	22.9	0.1	20.8	0.6	10.1	1.0
20	R_1 = H, R_2 = 4-Pyridinyl	>25	—	>300	—	232.6	0.7
21	R_1 = H, R_2 = 2-Pyridinyl	3.9	—	145.1	0.2	0.5	13.0
22	R_1 = H, R_2 = C_6H_4-4-NO_2	>25	—	>250	—	38.5	5.2
23	R_1 = H, R_2 = C_6H_4–2-NO_2	>25	—	>250	—	107.8	nd
24	R_1 = H, R_2 = C_6H_4–4-CF_3	10.5	0.8	16.2 (15.9)	5.5 (5.6)	2.0	4.1
25	R_1 = H, R_2 = C_6H_4–2-CF_3	13.8	0.6	15.7 (15.4)	1.3 (1.3)	1.2	2.6
26	R_1 = H, R_2 = C_6H_4–4-F	18.4	1.0	71.1	0.6	9.7	1.0
27	R_1 = H, R_2 = C_6H_4–2-F	10.8	1.7	>300	—	3.3	3.1
28	R_1 = H, R_2 = C_6H_4–4-Cl	13.1	1.6	>250	—	1.7	85.2
29	R_1 = H, R_2 = C_6H_4–2-Cl	21.3	0.7	124.3	0.7	2.2	5.0
30	R_1 = H, R_2 = C_6H_4–4-Br	20.3	0.8	>250	—	0.9	8.8
31	R_1 = H, R_2 = C_6H_4–2-Br	9.3	0.8	24.3	0.8	1.3	5.8
32	R_1 = H, R_2 = C_6H_4–4-CN	>25	—	>300	—	32.3	5.0
33	R_1 = H, R_2 = $C(CH_3)_3$	>25	—	299.2	0.3	112.6	1.6
34	R_1, R_2 = CH_3	14.1	2.7	239.7	0.5	40.6	5.9
35	R_1 = CH_3, R_2 = C_6H_5	13.7	0.9	72.6 (8.7)	1.4 (11.5)	28.5	5.6
36	R_1, R_2 = CH_2CH_3	20.7	1.0	160.2	0.7	19.0	8.5
37	R_1, R_2 = C_6H_5	>25	—	>250	—	182.3	0.9
	Nifurtimox	4.5	20.8	—	—	—	—
	Rifampin	—	—	0.1 (>1.9)	1080.0	—	—
	Amphotericin B	—	—	—	—	0.1	54.0

[a] Toxicity assays on VERO cell or macrophages were performed by different research groups using independent methodologies.
[b] IC_{50}: concentration that produces 50% inhibitory effect.
[c] Assay performed at Laboratotios de Investigación y Desarrollo, UPCH; SI = $IC_{50\ VERO\ cell}/IC_{50\ T.\ cruzi}$.
[d] Assay performed at Institute for Tuberculosis Research, UIC; SI = $IC_{50\ VERO\ cell}/IC_{50\ M.\ tuberculosis}$.
[e] Assay performed at Instituto de Medicina Tropical Alexander von Humbolt, UPCH; SI = $IC_{50\ macrophages}/IC_{50\ L.\ amazonensis}$ axenic amastigotes.

rophenol red β-D-galactopyranoside (CPRG) by β-galactosidase resulting from the expression of the gene in *T. cruzi* Tulahuen C4 at the Laboratorios de Investigación y Desarrollo (UPCH). On this study, it was noted that the presence of electron-donating (ED) or electron-withdrawing (EW) groups on the hydrazone moiety has a small impact on the anti-*T. cruzi* activity of BTPs (Table 1). Indeed, only four compounds (**4**, **7**, **21** and **31**) showed anti-trypanosome activity at concentrations below 10 μM, and only **4** was more active, with comparable toxicity to the positive control Nifurtimox, exhibiting a moderate SI value of 15. The Selectivity Index (SI) is the ratio of IC_{50} values between VERO (normal African green monkey epithelial cells) or macrophages, and the corresponding microorganism. Compounds **4** and **31** were further studied in vitro at the Center for Tropical and Emerging Global Diseases (UGA), showing anti-*T. cruzi* activity at 1.4 and 9.3 μM, respectively, the IC_{50} value for **4** being more than four times lower than for the positive control, Benznidazole (6.5 μM). With these in

Table 2
IC_{50} (μM) of BTP against macrophages infected with three *Leishmania* species

Compd	*L. amazonensis* Lma CL1	*L. braziliensis* PER006	*L. peruviana* LCA08
6	8.2	10.5	9.5
21	>2.0	0.6	0.7
28	>30.0	17.3	>30.0
30	>2.0	>2.0	>2.0
36	10.9	11.1	7.0
Amp. B[a]	0.4	0.1	0.1

[a] Amphotericin B.

Table 3
In vivo activity of BTP on *L. amazonensis*-infected BALB/c mice ($n = 10$)[a]

Compd	Lesion diameter[b] (mm)		Reduction of parasite burden (%)	
	After 4 weeks	After 7 weeks	After 4 weeks	After 7 weeks
Control	2.8	4.7		
6	3.0	4.7	30	2
28	3.6	5.2	46	25
36	2.8	4.9	65	17
Gluc.[c]	2.1	2.2	100	100

[a] Effect of treatments after eight intralesional inoculations.
[b] Compounds administrated at 5 mg/kg/day.
[c] *N*-Methylglucamine antimoniate, administrated at 33 mg /kg/day.

Table 4
Cytotoxicity of compounds **D** and **1**–**37** against various cancer cell lines

Compd	GI_{50} (μM) in indicated cell line[a]						
	3T3	**H460**	**DU145**	**MCF-7**	**M-14**	**HT-29**	**K562**
D	<4.5	9.6	13.9	9.0	24.6	20.7	11.1
1	21.7	18.2	20.0	11.2	18.0	12.5	13.2
2	16.7	19.9	24.1	6.3	18.4	6.2	4.7
3	27.9	20.8	24.1	9.3	35.2	19.4	6.2
4	4.4	2.1	5.4	2.1	4.6	8.2	1.6
5	37.5	133.0	>170	12.9	>170	>170	7.1
6	36.1	28.2	58.8	18.5	190.2	32.2	11.1
7	9.4	5.9	10.8	5.2	17.8	5.9	4.1
8	23.2	6.4	21.9	6.3	30.3	15.7	6.4
9	6.6	14.9	20.8	17.0	17.2	19.5	15.8
10	14.9	17.1	23.1	12.5	16.3	17.3	13.9
11	17.6	16.2	17.7	14.9	143.1	7.4	15.5
12	>250	>250	>250	117.4	>250	>250	>250
13	19.9	29.5	20.0	21.1	94.4	20.0	7.9
14	22.5	28.3	22.5	10.5	15.6	11.4	4.7
15	10.2	20.3	9.9	13.6	18.8	18.7	9.0
16	27.3	>170	27.2	>170	>170	>170	>170
17	<2.0	3.1	<2.0	3.8	12.8	3.9	3.9
18	7.8	7.1	15.6	1.0	11.1	8.5	4.0
19	2.2	1.7	4.6	0.4	2.5	3.8	1.9
20	83.2	169.2	83.9	83.9	> 150	152.8	109.3
21	0.04	0.40	0.37	0.09	0.25	0.10	0.16
22	>170	>170	>170	>170	>170	>170	>170
23	>170	52.9	102.9	52.0	>170	>170	>170
24	10.8	15.3	14.8	5.4	16.9	7.0	6.6
25	15.8	5.4	3.5	4.8	6.5	4.5	1.7
26	20.5	9.4	13.9	7.4	17.6	10.4	8.9
27	>250	>250	>250	>250	>250	>250	81.2
28	18.7	12.8	14.6	8.4	>170	15.1	6.7
29	88.1	62.9	77.8	22.0	>170	56.5	19.8
30	17.4	10.5	14.6	5.9	33.6	12.4	5.7
31	11.2	7.7	7.1	4.5	15.4	8.0	3.0
32	150.4	>170	>170	101.7	>170	>170	>170
33	7.3	24.5	25.1	26.0	32.7	33.0	27.5
34	>3.5	9.3	11.6	12.2	23.3	33.0	13.6
35	5.4	15.9	16.1	16.5	19.8	21.9	10.9
36	4.8	15.2	15.0	15.1	20.6	38.9	11.6
37	>170	>170	>170	>170	>170	>170	>170
5FU[b]	<3.0	2.3	5.3	4.5	32.4	14.9	89.9

[a] 3T3, BALB/3T3 clone A31 embryonic mouse fibroblast cells; H460, human large cell lung cancer; DU145, human prostate carcinoma; MCF-7, human breast adenocarcinoma; M-14, human melanoma; HT-29, human colon adenocarcinoma; K562, human chronic myelogenous leukemia cells.
[b] 5FU, 5-Fluoruracil.

vitro information in hand, and aware of the lower cytotoxicity of **4**, we selected it for a short-term in vivo treatment assay. Compound **4** was suspended in 1% DMSO and administered daily to mice through intraperitoneal injection on days 6–11 post-infection at a concentration of 20 mg/kg/day. Treatment with Benznidazole using this protocol resulted in a marked decrease in parasite replication over the 6-day treatment period but administration of **4** had no effect.[16a] There are several possible reasons for the discrepancy between the in vitro and in vivo assays, but we believe these are mostly related to drug bioavailability. Although **4** shows excellent ADME properties in silico (see Table 5), it may be rapidly removed from the body (through the liver perhaps), or it may never make into the circulation because is bound up in the tissue. Further studies are needed to confirm these assumptions.

Table 5
Physico-chemical properties of BTPs[a]

ID	%ABS	TPSA (Å^2)	*n*-ROTB	molecular weight	miLog *P*	*n*-OHNH donors	*n*-ON acceptors	Lipinski's violations
Rule				<500	⩽5	<5	<10	⩽1
D	87.0	63.8	1	220.3	1.84	3	4	0
1	91.7	50.2	3	308.4	3.89	1	4	0
2	91.7	50.2	3	358.5	5.05	1	4	1
3	91.7	50.2	3	358.5	5.07	1	4	1
4	91.7	50.2	4	334.4	4.65	1	4	0
5	91.7	50.2	3	322.4	4.34	1	4	0
6	91.7	50.2	4	336.5	4.80	1	4	0
7	91.7	50.2	4	350.5	5.40	1	4	1
8	91.7	50.2	4	364.5	5.60	1	4	1
9	84.7	70.4	3	324.4	3.41	2	5	0
10	88.5	59.4	4	338.4	3.95	1	5	0
11	88.5	59.4	4	338.4	3.92	1	5	0
12	85.3	68.6	3	352.4	3.78	1	6	0
13	85.3	68.6	5	368.5	3.93	1	6	0
14	82.1	77.9	6	398.5	3.52	1	7	0
15	81.5	79.6	4	354.4	3.23	2	6	0
16	78.3	88.9	5	384.5	3.24	2	7	0
17	85.3	68.6	9	499.4	5.96	1	6	1
18	87.2	63.3	3	298.4	3.15	1	5	0
19	86.2	66.0	3	297.4	3.04	2	5	0
20	87.2	63.1	3	309.4	2.60	1	5	0
21	87.2	63.1	3	309.4	2.72	1	5	0
22	75.9	96.0	4	353.4	3.85	1	7	0
23	75.9	96.0	4	353.4	3.80	1	7	0
24	91.7	50.2	4	376.4	4.78	1	4	0
25	91.7	50.2	4	376.4	4.74	1	4	0
26	91.7	50.2	3	326.4	4.05	1	4	0
27	91.7	50.2	3	326.4	4.00	1	4	0
28	91.7	50.2	3	342.9	4.57	1	4	0
29	91.7	50.2	3	342.9	4.52	1	4	0
30	91.7	50.2	3	387.3	4.70	1	4	0
31	91.7	50.2	3	387.3	4.65	1	4	0
32	83.5	74.0	3	333.4	3.64	1	5	0
33	91.7	50.2	3	288.4	4.00	1	4	0
34	91.7	50.2	2	260.4	3.12	1	4	0
35	91.7	50.2	3	322.4	4.34	1	4	0
36	91.7	50.2	4	288.4	4.12	1	4	0
37	91.7	50.2	4	384.5	5.55	1	4	1

[a] %ABS, percentage of absorption; TPSA, topological polar surface area; *n*-ROTB, number of rotatable bonds; miLog *P*, logarithm of compound partition coefficient between *n*-octanol and water; *n*-OHNH, number of hydrogen bond donors; *n*-ON, number of hydrogen bond acceptors.

The anti-TB activity is presented in Table 1. There is no direct correlation between the presence of ED or EW groups on the hydrazone moiety; however, BTPs containing EW groups gave a slightly higher number of active compounds. Initially, BTPs were tested using the Microwell Alamar Blue Assay (MABA)[19a,b] against H37R*v*, a virulent strain of *M. tuberculosis*. With the exception of **24** and **25**—which were the only compounds exhibiting minimum inhibitory concentration (MIC) values below 20 μM (16.2 and 15.7 μM, respectively)—BTPs exhibited only very moderate anti-TB potential under MABA conditions. A significant number of BTPs had MICs below 100 μM (**1**, **4**, **9**, **10**, **18**, **19**, **26**, **31** and **35**), but only **10**, **18**, **24**, **25** and **35** were relatively non-toxic in a VERO cell assay. Only those compounds with anti-*M. tuberculosis* selectivity indices (SI values) ranging from 1 to 6-fold were further tested in the Low Oxygen Recovery Assay (LORA),[19c] also using the H37R*v* strain of *M. tuberculosis*. Interestingly, compounds **10**, **18** and **35** were 2–8

times more active in LORA than in MABA, whereby **18** and **35** exhibited the highest MICs in LORA (MIC = 9.7 and 8.7 μM, respectively). The same three compounds showed a greater SI in the LORA system as well. On the other hand, compounds **24** and **25** exhibited identical MICs and SI values in both bioassay panels.

The in vitro activity of BTPs against axenic amastigotes of *L. amazonensis* is presented on Table 1. From these results, a direct correlation between BTPs bearing ED or EW groups on the hydrazone moiety can be found. With exception of the moieties containing a nitrile or a nitro group on the aromatic moiety, it appears that EW groups increase the in vitro anti-*L. amazonensis* activity and selectivity of BTPs. With the exception of **26**, aromatic systems containing halogens (**24**, **25**, **27**–**31**) exhibited low IC_{50} values (ranging from 0.9 to 2.0 μM) and high SIs (ranging from 3 to 85). More interestingly, on compounds **28**–**29** and **30**–**31**, the isomer containing a halide substituent on the *para*-position of the phenyl ring is more selective than the *ortho*-isomer. Compounds **6**, **21**, **28**, **30** and **36** exhibited SIs greater than 8; they were selected for an in vitro screening under a macrophage-infected model using three of the most prevalent *Leishmania* strains in Peru, namely *L. amazonensis*, *L. brasilensis* and *L. peruviana*. The results of this in vitro screening are summarized in Table 2. Although there is no direct correlation between the activity of the compounds on the axenic amastigotes model and the macrophage-infected model, it can be observed from this second screening that compounds **6** and **36** exhibited better and more congruent IC_{50} values on the macrophage-infected model than in the values obtained on the axenic amastigote model, while compound **21**, was only congruent in the case of macrophages infected with *L. brasilensis* and *L. peruviana*. Aware of the toxicity of compounds **21** and **30**, and considering their structural characteristics, we choose compounds **6**, **28** and **36** for an in vivo assay; the results are presented in Table 3. From this study, we can conclude that none of the tested compounds was as effective as the positive control (*N*-methylglucamine antimoniate, Gluc.) in reducing the lesion diameter. Because the lesion diameter depends on various factors, such as immune response, the inflammation process and parasite virulence—which are not proportional to the parasite load and the parasite burden—the measure of percentage of reduction of the parasite burden was completed by counting the *L. amazonensis* amastigotes in the foot tissue using a fluorescent probe. From this count, **36** (administered to the infected mice in a concentration almost seven times lower than the positive control) showed a 65% reduction of the parasite burden, after 4 weeks; and a reduction of its antiparasitic potential after completion of the in vivo assay. None of the in vivo-assayed compounds exhibited any cutaneous toxicity at the tested doses and the fact that they did not exhibit a reduction of the lesion diameter is probably due to their lack of anti-inflammatory activity.

The anti-tumor activity of BTPs is presented on Table 4. It can be observed that the activity of this series of compounds is independent of the presence of ED or EW substituents on the phenyl ring of the hydrazone moiety. Compound **5** was found selective against MCF-7 and K562 cell lines (human breast adenocarcinoma and human chronic myelogenous leukemia cell, respectively), while **12** and **18** were selective against MCF-7. Also, **17** was selective against DU145, human prostate carcinoma. Three of the screened compounds (**4**, **19** and **21**) exhibited cytotoxicity below 5 μM to all cell lines; indeed, compound **21** was found to be not only the most cytotoxic compound of the entire series, (GI_{50} values ranged from 0.04 to 0.37 μM) but it also showed selectivity towards MCF-7 and HT-29 cell lines. In isomers **26**–**27** and **28**–**29**, compounds containing the halogen on the *para*-position were more active than their *ortho* counterparts. Compound **21** was at least a threefold more cytotoxic than its structural isomer **20**. Because of its high cytotoxicity, **21** was submitted to the National Cancer Institute (NCI) to be further tested in a 60 cell line panel; the results are shown on Table S1 (in the Supplementary data). The NCI screening confirmed the activity against human breast and human colon cancer, with GI_{50} values of 0.33 and 0.18 μM against MCF-7 and HT-29, respectively. Furthermore, **21** was much more selective towards HL-60 (TB) leukemia cell, HCT-116 colon cancer cell and OVCAR-3 ovarian cancer cell lines than to the rest of cancer cell lines, its GI_{50} values ranging from 0.11 to 0.17 μM. Recently a series of thieno[2,3-*d*]pyrimidin-4-yl] hydrazones were found to be cyclin-dependent kinase 4 (CDK4) inhibitors.[10a] Inhibition of CDK4 induces G1 cell cycle arrest.

A computational study designed to predict the ADME properties of our BTPs was performed, and the results are presented in Table 5. Topological polar surface area (TPSA) is a good indicator of drug absorbance in the intestines, Caco-2 monolayers penetration, and blood–brain barrier crossing.[17a] TPSA was used to calculate the percentage of absorption (%ABS) according to the equation: $\%ABS = 109 - 0.345 \times TPSA$, as reported by Zhao et al.[17b] In addition, the number of rotatable bonds (*n*-ROTB), and Lipinski's rule of five,[17c] were also calculated. All of the most active and selective compounds found for each specific bioassay (compounds **4**, **18**, **21**, **35** and **36)** showed high percentages of intestinal absorption, with values above 87%, and an excellent *n*-ROTB, ranging only from 3 to 4.[17d] None of these compounds violated any of the Lipinski's parameters, an important characteristic for future drug-development.

5. Conclusion

The collaborative effort of a multidisciplinary and international network of scientist led to the synthesis and biological evaluation of 38 tetrahydrobenzothienopyrimidines for their potential activity against three neglected diseases and several tumorogenic cell lines. Compound **4** was effective in vitro against *T. cruzi* parasites; compounds **18** and **35** were mildly selective towards the LORA for *M. tuberculosis*; compound **28** was highly selective in vitro against *L. amazonensis*, while compound **36** showed a 65% reduction of the same parasitic burden on an in vivo assay; finally, compound **21** was the most cytotoxic against human cancer cell lines and has been further screened at the NCI. These compounds contained a variety of chemical functionalities and exhibited moderate to good selectivity in the tested disease models. BTPs are easy to prepare and exhibited promising drug-like properties. These are important characteristics for future drug-optimization studies and possible use in developing countries.

6. Experimental procedures

6.1. General methods

^1H and ^{13}C NMR spectra were recorded at 500 and 125 MHz, respectively, using DMSO-d_6 as a solvent on a Varian Inova 500. The chemical shifts are reported in ppm values relative to DMSO-d_6 (2.62 ppm for ^1H NMR and 40.76 ppm for ^{13}C NMR). Coupling constants (*J*) are reported in hertz (Hz). Melting points were measured on a Thomas Hoover capillary melting point apparatus and are uncorrected. Elemental analysis was performed at Atlantic Microlab, Inc., Norcross, GA. Reactions were monitored on Merck silica gel 60 F254 aluminum sheets. TLC spots were visualized by inspection of plates under UV light (254 and 365 nm) and after submersion in 4% phosphomolybdic acid and heating (110 °C). All commercial reagents were obtained either from Aldrich, Acros or Alfa Aesar and used without any further purification. 3,5-Bisallyloxy-4-bromobenzaldehyde was available from our laboratory.[18]

6.2. Synthesis of BTPs

Cyanoacetic acid ethyl ester (0.23 mol), diethyl amine (1.0 equiv) and cyclohexanone (1.5 equiv) were dissolved in EtOH (75 mL) and placed in an ice bath; after 5 min, elemental sulfur (0.23 mol) was added and the mixture was sonicated at room temperature for 30 min. After cooling inside a fridge for 1 h, the pale yellow solid (**A**, 2-amino-4,5,6,7-tetrahydro-3-ethoxycarbonylbenzothiophene, 43.3 g, 84%) was filtered and washed with a cold solution of 60% EtOH in H_2O. After drying under vacuum overnight, **A** was suspended in formamide (400 mL) and refluxed for 6 h. After cooling to room temperature, the dark solution was placed inside a fridge overnight, and the dark brown needles (**B**, 4-hydroxy-5,6-tetramethylenethieno[2,3-*d*]pyrimidine, 37.3 g, 95%) were filtered and washed with a cold solution of 40% EtOH in H_2O. After drying under vacuum overnight, **B** was suspended in $POCl_3$ (400 mL) and refluxed for 3 h. After cooling to room temperature, the excess of $POCl_3$ was removed under reduced pressure, and then 300 mL of $CHCl_3$ were added and the homogeneous solution was poured over iced deionized water (500 mL). The resulting bilayer mixture was separated and the aqueous layer was adjusted to pH 8 using NH_4OH (concd) and then extracted with $CHCl_3$. The organic fractions were pooled and dried over anhydrous $MgSO_4$. The $CHCl_3$ was removed under reduced pressure and the remaining yellow solid was re-dissolved in hot MeOH. Afterwards, the solution was allowed to cool to room temperature and placed inside a fridge overnight. The precipitated white crystals (**C**, 4-chloro-5,6,7,8-tetrahydro-[1]benzothieno[2,3-*d*]pyrimidine, 29.1 g, 72%) were filtered, washed with a cold solution of 80% EtOH in H_2O and dried overnight under vacuum. **C** was suspended in MeOH (400 mL) and hydrazine hydrate 80% (hydrazine 51%) in H_2O (16 mL) and refluxed for 2 h; immediately after, hydrazine (8 mL) was added to complete the reaction and the mixture was allowed to reflux for 1 h. After cooling to room temperature, it was placed in a fridge overnight. The resulting yellow precipitate (**D**, 4-hydrazinyl-5,6,7,8-tetrahydro-[1]benzothieno[2,3-*d*]pyrimidine, 24.1 g, 85%) was filtered and washed with a cold solution of 40% EtOH in H_2O. An aliquot of **D** (1 mmol) was mixed with 1.2 equiv of the corresponding aldehyde or ketone and dissolved in EtOH (10 to 15 mL), and the mixture was then refluxed from 2 to 48 h. After cooling to room temperature, it was placed overnight in a fridge and the colored-formed solid (**1**–**38**, 2-(5,6,7,8-tetrahydro[1]benzothieno[2,3-*d*]pyrimidin-4-yl)hydrazone-derivative, 20–99%) was filtered, washed with a solution of cold 80% EtOH in H_2O, and recrystallized from EtOH.

6.3. In vitro, in vivo bioassays and calculation of physicochemical parameters

BTPs were evaluated against *T. cruzi* trypomastigotes in vitro,[13a] and in vivo;[16] against *L. amazonensis* axenic amastigotes in vitro and in vivo;[20] against tumorogenic cell lines in vitro,[13b] and against *M. tuberculosis* in vitro under (MABA)[19a,b] and (LORA)[19c] assays, using previously reported methodologies. BTPs physicochemical properties were calculated using literature methods.[13a,17]

Acknowledgments

G.B.H. and J.C.A. wish to acknowledge the early contribution of Mr. Devin Pantess (University of Louisville) and Dr. Rosario Rojas (Universidad Peruana Cayetano Heredia) in the synthesis of BTPs and their anti-tuberculosis screening. The authors are grateful to the USA Department of Defense Prostate Cancer Research Program (PCRP) of the Office of the Congressionally Directed Medical Research Programs (CDMRP) (Grant #W81XWH-07-1-0299 to G.B.H.), to the FINCYT-BID (Banco Interamericano para el Desarrollo) Program in Peru (grant #PIBAB03-084 to M.S.), to the Infectious Diseases Training Program in Peru # 5D43 TW006581 (R.H. G) and to the NIH grant P01-044979 (to R.L.T.) for their financial support. J.M.B and R.L.T. thank Adriana M. C. Canavaci at the CTEGD-University of Georgia for her technical assistance with the in vitro testing of drugs for *T. cruzi*. The skillful technical assistance of Mrs. Baojie Wan and Yuehong Wang, ITR, UIC College of Pharmacy, Chicago (IL), is gratefully acknowledged.

Supplementary data

Supplementary data (^1H and ^{13}C NMR), elemental analysis, individual tables for each test with cell/microorganism control, NCI results for **21**, different Log *P* calculations of all tested compounds and a brief description of the anti-*M. tuberculosis* and anti-*L. amazonensis* bioassays) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.03.018.

References and notes

1. (a) Danzon, P. M. *Nature* **2007**, *449*, 176; (b) Nwaka, S.; Hudson, A. *Nat. Rev. Drug Disc.* **2006**, *5*, 941; (c) Ioset, J.-R. *Curr. Org. Chem.* **2008**, *12*, 643; (d) Chung, M. C.; Ferreira, E. I.; Santos, J. L.; Giarolla, J.; Rando, D. G.; Almeida, A. E.; Bosquesi, P. L.; Menegon, R. F.; Blau, L. *Molecules* **2008**, *13*, 616.
2. (a) Buckner, F. S.; Wilson, A. J.; White, T. C.; Van Voorhis, W. C. *Antimicrob. Agents Chemother.* **1998**, *40*, 3245; (b) El-Sayed, N.; Myler, P. J.; Bartholomeu, D. C.; Nilsson, D.; Aggarwal, G.; Tran, A.-N.; Ghedin, E.; Worthey, E. A.; Delcher, A.; Blandin, G.; Westenberger, S.; Caler, E.; Cerqueira, G. C. *Science* **2005**, *309*, 409.
3. (a) World Health Organization, 2007 Tuberculosis Facts. http://www.who.int/tb/publications/2009/tbfactsheet_2009_one_page.pdf (Accessed 11/12/09).; (b) Houben, E.; Nguyen, L.; Pieters, J. *Curr. Opin. Microbiol.* **2006**, *9*, 76.
4. (a) Herwaldt, B. L. *Lancet* **1999**, *354*, 1191; (b) Dujardin, J.-C. *Trends Parasitol.* **2006**, *22*, 4.
5. (a) Rivers, E. C.; Mancera, R. L. *Curr. Med. Chem.* **2008**, *15*, 1956; (b) Ormerod, L. P. *Arch. Dis. Child.* **1998**, *78*, 169.
6. (a) Larabi, M.; Yardley, V.; Loiseau, P. M.; Appel, M.; Legrand, P.; Gulik, A.; Bories, C.; Croft, S. L.; Barratt, G. *Antimicrob. Agents Chemother.* **2003**, *47*, 3774; (b) Deray, G. *J. Antimicrob. Chemother.* **2002**, *49*, 37.
7. Dorlo, T. P. C.; van Thiel, P. P. A. M.; Huitema, A. D. R.; Keizer, R. J.; de Vries, H. J. C.; Beijnen, J. H.; de Vries, P. J. *Antimicrob. Agents Chemother.* **2008**, *52*, 2855.
8. (a) Filardi, L. S.; Brener, Z. *Trans. R. Soc. Trop. Med. Hyg.* **1987**, *81*, 755; (b) Nozaki, T.; Dvorak, J. A. *Parasitol. Res.* **1993**, *79*, 451.
9. (a) Feng, Y.; Jadhav, A. P.; Rodighiero, C.; Fujinaga, Y.; Kirchhausen, T.; Lencer, W. I. *EMBO Rep.* **2004**, *5*, 596; (b) Yarrow, J. C.; Feng, Y.; Perlman, Z. E.; Kirchhausen, T.; Mitchison, T. J. *Comb. Chem. High Throughput Screening* **2003**, *6*, 279.
10. (a) Horiuchi, T.; Chiba, J.; Uoto, K.; Soga, T. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 305; (b) Jennings, L. D.; Kincaid, S. L.; Wang, Y. D.; Krishnamurthy, G.; Beyer, C. F.; Mginnis, J. P.; Miranda, M.; Discafani, C. M.; Rabindran, S. K. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 4731.
11. (a) Patil, C. D.; Sadana, G. S. *J. Indian Chem. Soc.* **1991**, *68*, 169; (b) Chambhare, R. V.; Khadse, B. G.; Bobde, A. S.; Bahekar, R. H. *Eur. J. Med. Chem.* **2003**, *38*, 89.
12. (a) Tranberg, C. E.; Zickgraf, A.; Giunta, B. N.; Luetjens, H.; Figler, H.; Murphree, L. J.; Falke, R.; Fleischer, H.; Linden, J.; Scammells, P. J.; Olsson, R. A. *J. Med. Chem.* **2002**, *45*, 382; (b) El-Baih, F. E. M.; Al-Blowy, H. A. S.; Al-Hazimi, H. M. *Molecules* **2006**, *11*, 498; (c) Bogolubsky, A. V.; Ryabukhin, S. V.; Stetsenko, S. V.; Chupryna, A. A.; Volochnyuk, D. M.; Tolmachev, A. A. *J. Comb. Chem.* **2007**, *9*, 661.
13. (a) Aponte, J. C.; Verástegui, M.; Málaga, E.; Zimic, M.; Quiliano, M.; Vaisberg, A. J.; Gilman, R. H.; Hammond, G. B. *J. Med. Chem.* **2008**, *51*, 6230; (b) Aponte, J. C.; Vaisberg, A. J.; Rojas, R.; Sauvain, M.; Lewis, W. L.; Lamas, G.; Sarasara, C.; Gilman, R. H.; Hammond, G. B. *J. Nat. Prod.* **2009**, *72*, 524; (c) Aponte, J. C.; Vaisberg, A. J.; Rojas, R.; Caviedes, L.; Lewis, W. L.; Lamas, G.; Sarasara, C.; Gilman, R. H.; Hammond, G. B. *J. Nat. Prod.* **2008**, *71*, 102.
14. (a) Briel, D.; Rybak, A.; Kronbach, C.; Unverferth, K. *Pharmazie* **2008**, *63*, 823; (b) Bhaskar, V. H.; Kumar, M.; Sangameswaran, B.; Balakrishnan, B. R. *Inter. J. Chem. Sci.* **2007**, *5*, 2076; (c) Bhaskar, V. H.; Gokulan, P. D. *Oriental. J. Chem.* **2007**, *23*, 999.
15. (a) Santagati, N. A.; Caruso, A.; Cutuli, V. M.; Caccamo, F. *IL Farmaco* **1995**, *50*, 689; (b) Gundla, R.; Kazemi, R.; Sanam, R.; Muttineni, R.; Sarma, J. A. R. P.; Dayam, R.; Neamati, N. *J. Med. Chem.* **2008**, *51*, 3367.
16. (a) Canavaci, A. M. C.; Bustamante, J. M.; Padilla, A. M.; Perez-Brandan, C. M.; Simpson, L. J.; Xu, D.; Boehlke, C. L.; Tarleton, R. L. *PLoS Negl. Trop. Dis.* Submitted for publication; (b) Bustamante, J. M.; Bixby, L. M.; Tarleton, R. L. *Nat. Med.* **2008**, *14*, 542.
17. (a) Ertl, P.; Rohde, B.; Selzer, P. *J. Med. Chem.* **2000**, *43*, 3714; (b) Zhao, Y.; Abraham, M. H.; Lee, J.; Hersey, A.; Luscombe, N. C.; Beck, G.; Sherborne, B.; Cooper, I. *Pharm. Res.* **2002**, *19*, 1446; (c) Lipinski, C. A.; Lombardo, F.; Dominy,

B. W.; Feeney, P. J. *Adv. Drug Delivery Rev.* **1997**, *23*, 3; (d) Veber, D. F.; Johnson, S. R.; Cheng, H.-Y.; Smith, B. R.; Ward, K. W.; Kopple, K. D. *J. Med. Chem.* **2002**, *45*, 2615.
18. Xu, B.; Pelish, H.; Kirchhausen, T.; Hammond, G. B. *Org. Biomol. Chem.* **2006**, *4*, 4149.
19. (a) Collins, L.; Franzblau, S. G. *Antimicrob. Agents Chemother.* **1997**, *41*, 1004; (b) Falzari, K.; Zhu, Z.; Pan, D.; Liu, H.; Hongmanee, P.; Franzblau, S. G. *Antimicrob. Agents Chemother.* **2005**, *49*, 1447; (c) Cho, S. H.; Warit, S.; Wan, B.; Hwang, C. H.; Pauli, G. F.; Franzblau, S. G. *Antimicrob. Agents Chemother.* **2007**, *51*, 1380.
20. (a) Aponte, J. C.; Castillo, D.; Estevez, Y.; Gonzalez, G.; Arevalo, J.; Hammond, G. B.; Sauvain, M. *Bioorg. Med. Chem. Lett.*, **2010**, *20*, 100.; (b) Aponte, J. C.; Estevez, Y.; Gilman, R. H.; Lewis, W. H.; Rojas, R.; Sauvain, M.; Vaisberg, A. J.; Hammond, G. B. *Planta Med.* **2008**, *74*, 407; (c) Sauvain, M.; Dedet, J. P.; Kunesch, N.; Poisson, J.; Gayral, P.; Gantier, J. C.; Kunesch, G. *Phytother. Res.* **1993**, *7*, 167.