Chem Biol Drug Des 2013

Research Article

Discovery of Synthetic *Leishmania* Inhibitors by Screening of a 2-Arylbenzothiophene Library

Vivian I. Bonano[1], Jenicer K. U. Yokoyama-Yasunaka[1], Danilo C. Miguel[1,†], Scott A. Jones[2], Jeffrey A. Dodge[2] and Silvia R. B. Uliana[1,*]

[1]Departamento de Parasitologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, SP 05508-900, Brazil
[2]Lilly Research Laboratories, Eli Lilly and Company, Lilly Corporate Center, Indianapolis, IN 46285, USA
**Corresponding author: Silvia R. B. Uliana, srbulian@icb.usp.br*
[†]Present address: Departamento de Biologia Animal, Instituto de Biologia, Universidade Estadual de Campinas, SP, Brazil

Tamoxifen has been shown to be active *in vitro* against *Leishmania* and effective in the treatment for leishmaniasis in murine models. Through the screening of a compound library of estrogen receptor modulator analogs, we identified the major characteristics required for antileishmanial activity. To overcome the difficulties presented by tamoxifen's propensity for E/Z isomerization, we used the 2-arylbenzothiophene compound BTP as a more stable alternative. Directed screening of a small compound library based on BTP led to active compounds against *Leishmania*. Subsequent structure–activity data for the synthetic 2-arylbenzothiophenes evaluated in this study indicate that optimal antileishmanial potency is dependent on the presence of two basic side chains. In addition, the primary structural features required for estrogen receptor binding, the phenols, are not required for inhibiting parasitic growth. Significantly, the most active antileishmanial benzothiophenes lack the pharmacophore for estrogen receptor activity and therefore address potential concerns about the undesirable effects of using selective estrogen receptor modulators in women and children with leishmaniasis. Three compounds selected from the screening have shown consistent activity against all species and stages of *Leishmania in vitro* although improvements in selectivity are needed. These compounds represent viable starting points for further optimization as antileishmanial agents.

Key words: leishmaniasis, selective estrogen receptor modulator, tamoxifen

Received 3 July 2013, revised 18 September 2013 and accepted for publication 27 September 2013

Leishmaniasis is a neglected tropical disease caused by protozoan parasites endemic to 98 countries globally, with 367 million people at risk and about 2 million new cases yearly (1,2). Over 20 *Leishmania* species are known to infect humans. Clinical manifestations range from the disfiguring skin lesions of cutaneous leishmaniasis (CL) to the often fatal visceral leishmaniasis (VL) (3). CL presentation varies from self-healing cutaneous ulcers, through very aggressive and disfiguring cases of disseminated or mucocutaneous lesions to the rare but life-threatening diffuse leishmaniasis. VL is characterized by prolonged fever, enlarged spleen and liver, substantial weight loss and progressive anemia and invariably leads to death if untreated. The disease is even more severe in children and in the course of immuno-depression as is the case for patients with concomitant HIV infections (4).

Human leishmaniasis treatment is based on parenteral administration of highly toxic drugs, including pentavalent antimonials, amphotericin B (deoxycholate or as a liposomal formulation), and pentamidine. Resistance to antimonials is widespread in India, and oral administration of miltefosine has emerged as an alternative approach, having been approved for management of VL. However, the efficacy of miltefosine outside India is still a matter of investigation. Furthermore, there is reason for concern about the rapid emergence of resistance to miltefosine and its teratogenicity (5). Clinical failure rates of CL treatment with antimonials are also rising in Latin America (6,7), suggesting that the emergence of resistant strains may be occurring (8,9).

The need for the development of new treatment strategies for leishmaniasis is therefore evident. New drugs or therapeutic schemes would ideally be effective against all species of the parasite, allowing their use in all forms of the disease.

Tamoxifen (Figure 1) is a selective estrogen receptor modulator (SERM) that has been in clinical use for the treat-

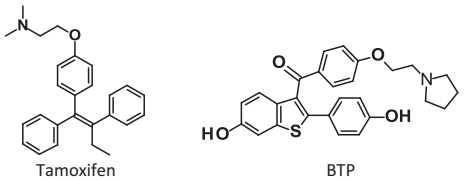
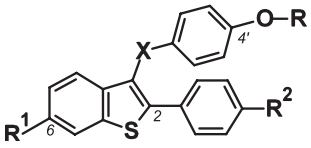
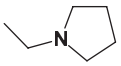
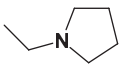
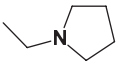
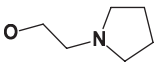
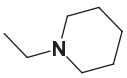
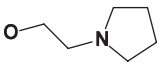
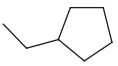
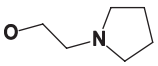
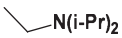
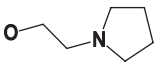
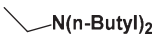
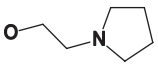
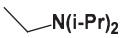


Figure 1: Structures of SERMs tamoxifen and BTP.

 doi: 10.1111/cbdd.12239

Table 1: Structures and biological activity of BTP and benzothiophene analogs against promastigotes of *L. chagasi*

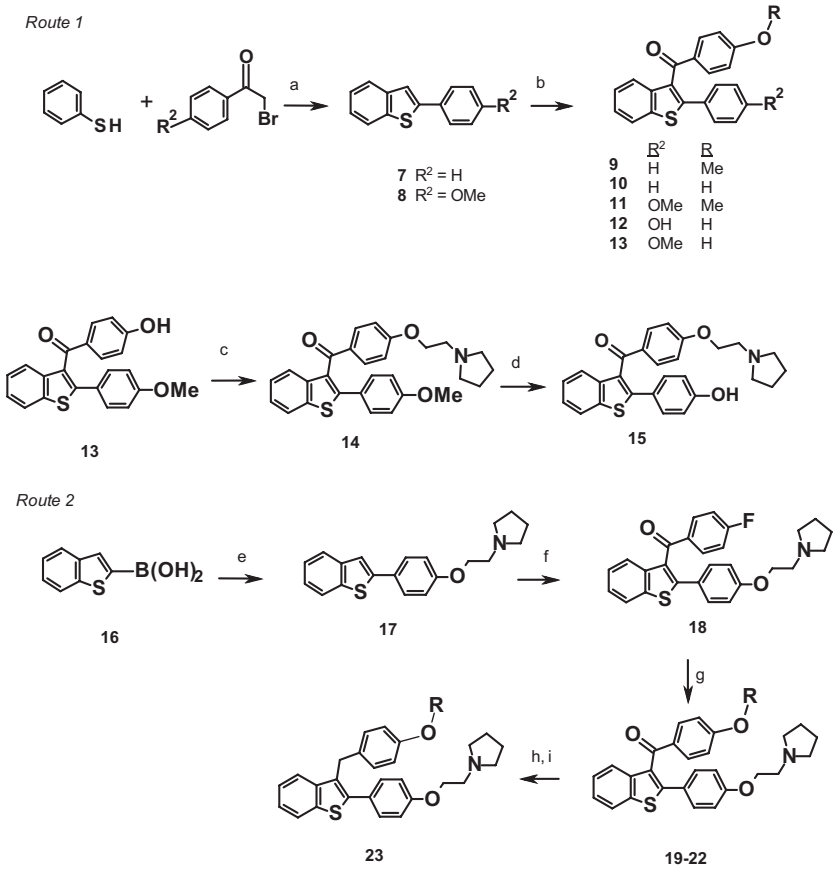


Compound	R^1	R^2	R	X	IC_{50}[a] (μM ± SD)	cLogP	cLogD
BTP[b]	OH	OH		C=O	52.2 ± 18.2	5.18	4.79
15[b]	H	OH		C=O	34.6 ± 4.1	5.55	5.09
14[c]	H	OMe		C=O	36.5 ± 5.7	6.06	5.25
12	H	OH	OH	C=O	88.7 ± 0.2	5.35	5.10
13	H	OMe	OH	C=O	79.0 ± 0.1	5.49	5.35
10	H	H	OH	C=O	65.8 ± 0.1	5.65	5.51
19[d]	H			C=O	3.7 ± 0.3	6.93	4.72
20[c]	H			C=O	28.7 ± 1.8	8.10	6.59
21[d]	H			C=O	4.3 ± 0.2	7.63	4.37
22[d]	H			C=O	5.4 ± 0.4	8.73	5.49
23[d]	H			CH_2	3.6 ± 0.2	8.26	4.28

[a]Tamoxifen was used as a positive control in all experiments and the overall mean IC_{50} for *L. chagasi* promastigotes was 12.8 ± 6.0 μM.
[b]Free base.
[c]Oxalate salt.
[d]Dioxalate salt.

ment and prevention of breast cancer for over 30 years. However, in an estrogen receptor (ER) independent way, tamoxifen also demonstrates several other effects in tumor cells, for example, alkalinization of intracellular organelles (10), protein kinase C modulation (11), apoptosis induction (12) and modulation of sphingolipid metabolism (13).

Our group has recently described the antileishmanial activity of tamoxifen *in vitro* (14,15) as well as *in vivo* in both cutaneous and visceral experimental models of leishmaniasis (15,16). The sensitivity of different species of *Leishmania*, agents of cutaneous (e.g. *Leishmania braziliensis*, *L. amazonensis*, *L. major*) as well as of visceral disease (*L. donovani* and *L. chagasi*) is uniform with *in vitro* IC_{50} in the low micromolar range. While in the treatment for breast cancer tamoxifen is usually given continuously for 5 years, in the leishmaniasis murine models, the drug was effective when administered for 2–3 weeks. We have previously



Scheme 1: Synthesis of 2-Arylbenzothiophenes. Reagents: (a) KOH, PPA; (b) anisoyl chloride, AlCl3; (c) *N*-(2-chloroethyl) pyrrolidine hydrochloride, NaH; (d) 2N NaOH, EtSH; (e) $Pd(PPh_3)_4$, 2 N aqueous Na_2CO_3, 1-[2-(4-bromophenoxy)ethyl]pyrrolidine; (f) p-fluorobenzoyl chloride, $TiCl_4$; (g) RH, NaH; (h) $LiAlH_4$; (i) Et_3SiH, TFA.

shown that tamoxifen's antileishmanial activity cannot be competed out by estradiol, suggesting that the effect against the parasite is unrelated to ER interaction (14).

While there are several advantages in repurposing a drug so widely used and with an established safety profile, the use of tamoxifen in leishmaniasis chemotherapy raises some safety concerns, including its use in children in which bone development could be impaired (17) or in childbearing age women. In addition, treatment for women with tamoxifen has been associated with an increased risk of endometrial cancer (18,19). To address these concerns, we sought to identify alternative chemotypes that have antileishmanial activity yet are devoid of ER activity.

With the aim of identifying hits for further optimization, we assessed the structural requirements of the tamoxifen scaffold and evaluated two SERM scaffolds, triphenylethylenes and benzothiophenes, to compare their antiparasitic activity. We also determined the structural requirements for antileishmanial activity and whether these determinants are distinct from those known to be responsible for ER activity.

Methods and Materials

Leishmania strains used in this work were as follows: *Leishmania* (*Leishmania*) *infantum chagasi* (MHOM/BR/1974/M2682), *Leishmania* (*Leishmania*) *amazonensis* (MHOM/BR/1973/M2269) and *Leishmania* (*Viannia*) *braziliensis* (MHOM/BR/1975/M2903). Culture media and fetal bovine serum were acquired from Invitrogen. Chemicals used in biological tests were from Sigma-Aldrich (St. Louis, MO, USA).

Compound **1** (Tamoxifen [(*Z*)-1-(p-Dimethylaminoethoxyphenyl)-1,2-diphenyl-1-butene, *trans*-2-[4-(1,2-Diphenyl-1-butenyl)phenoxy]-*N*,*N*-dimethylethylamine]) and compound **2** (2-[4-[(*E*)-1,2-diphenylbut-1-enyl]phenoxy]-*N*,*N*-dimethylethanamine) were purchased from commercial sources (Sigma-Aldrich).

Compound synthesis

The syntheses of tamoxifen analogs have been reported, that is, for analogs **3** and **4** see Shiina *et al.* (20); for analogs **5** and **6** see (21). The compound library screened for antileishmanial activity consisted of 147 structurally divergent benzothiophenes from the Lilly compound collection. These compounds were >95% pure as determined by LCMS analysis. The synthesis and pharmacology of the benzothiophene BTP (Figure 1) has been previously described (22,23). The general synthetic routes for the preparation of the 2-arylbenzothiophene compounds shown in Table 1 are depicted in Scheme 1. Two complementary routes into the benzothiophene scaffold were developed. In the first route, Friedel–Crafts acylation (for a

review of Friedel-Crafts acylation, see (24) with known 2-arylbenzothiophenes **7** and **8** with anisoyl chloride provided analogs **9** and **11** substituted at the C-3 position of the benzothiophene. *O*-Demethylation of **9** and **11** was accomplished by complementary methods, that is, pyridine hydrochloride was used to make **10** and **12**, while selective removal of the 4′-OMe group was accomplished using EtSNa (25) to provide **13**. Subsequent alkylation with *N*-(2-chloroethyl)pyrrolidine hydrochloride followed *O*-demethylation with EtSNa gave compound **15**. The second general synthetic approach that was employed utilized benzo[b]thiophene-2-boronic acid (**16**). Palladium catalyzed coupling with 1-(2-(4-bromophenoxy)ethyl)pyrrolidine gave compound **17** which was acylated at the C-3 position with *p*-fluorobenzoyl chloride affording the common intermediate **6**. Employing the methods of Schmid *et al.* (26), a diverse set of basic side chains was installed at the 4′-position through the displacement of the fluoride with a variety of oxygen nucleophiles to yield derivatives **19**–**22**. Reductive deoxygenation to the C-3 methylene derivative (**23**) was accomplished by a two-step procedure involving initial hydride reduction to the secondary benzylic alcohols ($LiAlH_4$), followed by a second reductive deoxygenation (TFA/Et_3SiH) to give the desired methylene product **23**. Attempts to affect both the reduction and deoxygenation steps in a single transformation using $NaBH_4$/TFA were unsuccessful. Experimental procedures and characterization for all compounds in Table 1 are included in the Supporting Information. Further information on the preparation of these and other analogs has been described (27,28).

Biological evaluation

Promastigotes were cultured at 25 °C in M199 supplemented with 40 mM HEPES pH 7.4, 0.1 mM adenine, 0.005% hemine, 10% fetal bovine serum, and 100 μg/mL penicillin/streptomycin. Additionally, cultures of *L. braziliensis* and *L. chagasi* were supplemented with 2% human male sterile urine.

In vitro assays against *Leishmania* promastigotes were performed by incubating 5×10^6 parasites in media containing increasing concentrations (0.3–250 μM) of the compounds dissolved in DMSO at 25 °C for 24 h, in 96-well plates. The maximal concentration of DMSO used to add the compounds was 3%, which was used as a negative control, while tamoxifen and amphotericin B were used as positive controls. The effective antileishmanial concentration was assessed by colorimetry using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) as described previously (29). MTT reduction was quantified by measuring the absorbance at 595 nm using 690 nm as a reference, in a Multiscan EX spectrophotometer (Lab Systems, Inc., Vantaa, Finland). Assays were performed in triplicate, and results are expressed as the mean percentage reduction in parasite numbers compared with untreated control wells calculated for at least two independent experiments. Percent survival was used to determine the 50% and 90% inhibitory concentration (IC_{50} and IC_{90}) of each compound with ORIGIN PRO 7.5 analysis software (OriginLab Corporation, Northampton, MA, USA).

Cytotoxicity against mammalian cells *in vitro* was evaluated by testing each compound against Vero cells. Cells were seeded in 24-well plates at a density of $4–5 \times 10^5$ cells/well and allowed to adhere overnight at 37 °C, in a 5% CO_2 atmosphere. Cells were then treated for 48 h with various concentrations (0.3–81 μM) of the compounds of interest, replacing the media with drug after the initial 24 h. Cell viability after treatment was assayed by MTT as described above.

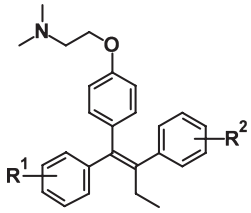
In vitro activity testing against *Leishmania* intracellular amastigotes was performed using BALB/c bone marrow-derived macrophages (BMDM) which were seeded at a density of 4×10^5 cells/well in 24-well plates containing round coverslips. Cells were allowed to adhere overnight at 37 °C with 5% CO_2 and then infected for 3 h with *L. chagasi* promastigotes at a ratio of 10 parasites/macrophage. Infected macrophages were then treated with various concentrations of each compound (0.3–8 μM) for 48 h, replacing the media with drug at 24 h. Cells were then washed, fixed, and stained using Instant ProV (Newprov, Pinhais, PR, Brazil). Coverslips containing stained cells were fixed onto slides using Entellan (Merck KGaA, Darmstadt, Germany), and cells were evaluated by light microscopy (Nikon Eclipse E200; Nikon Corporation, Tokyo, Japan) to determine the number of infected cells and the number of amastigotes/cell. Percent survival was calculated for each treatment performed intriplicate.

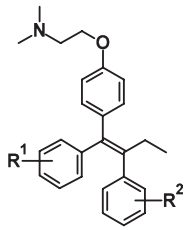
Results and Discussion

The E and Z isomers of tamoxifen analogs are known to have distinct estrogen-dependent activities (30). While the (*Z*)-isomer of tamoxifen (**1**) is a SERM, the cis isomer (E) (**2**) is a pure estrogen agonist with no estrogen-antagonistic actions (31). We wanted to test whether these isomers demonstrated different antileishmanial activity. (*Z*)-Tamoxifen (**1**) kills *L. chagasi* promastigotes in a concentration dependent way with a calculated 50% inhibitory concentration (IC_{50}) of 17.7 ± 0.8 μM (14). When (E)-tamoxifen (**2**) (Table 2) was used, a calculated IC_{50} of 12.6 ± 1.1 μM was obtained indicating that both isomers are active against *Leishmania*. We also tested other (Z) and (E) analogs of tamoxifen, as shown in the comparison between compounds **3**–**4** and **5**–**6**, respectively, in Table 2. In each case, the isomeric pairs were similar in potency. Because interconversion between (Z) and (E) isomers is known to occur among tamoxifen analogs (32), we could not rule out that the similarities in potency seen between each pair are due to E/Z conversion under the assay conditions. Rather than explore the propensity for isomerization in promastigotes, we elected to look for other scaffolds with fewer structural liabilities.

The 2-arylbenzothiophene scaffold has been shown to mimic the stilbene core of tamoxifen (**1**) based on molecu-

Table 2: Structures and biological activity of (Z) and (E) geometrical isomers of tamoxifen and diarylethylene analogs against promastigotes of *Leishmania chagasi*





Isomer (Z)	R^1	R^2	IC_{50} (μM ± SD)	IC_{90} (μM)	Isomer (E)	R^1	R^2	IC_{50} (μM ± SD)	IC_{90} (μM)	cLogP	cLogD
1	**H**	**H**	17.7 ± 0.8	46.6	**2**	**H**	**H**	12.6 ± 1.1	26.1	6.35	4.97
3	**3-OH**	**H**	13.4 ± 4.5	27.6	**4**	**3-OH**	**H**	9.2 ± 0.5	22.6	5.68	4.66
5	**4-OMe**	**4-OMe**	7.0 ± 0.8	15.2	**6**	**4-OMe**	**4-OMe**	6.6 ± 0.9	20.8	6.19	4.81

lar modeling overlays and crystal structures (33,34). In addition, the thiophene ring prevents potential isomerization issues by locking the three aryl groups in a topological display similar to **1** but not **2**. Because of our experience with SERMS, we selected the 2-arylbenzothiophene BTP (Figure 1) to test for antileishmanial activity. Like tamoxifen, BTP is a SERM and possesses pharmacological properties similar to second generation SERMs in rodents (22,23). The synthesis and pharmacology of the benzothiophene BTP has been previously described (22,23).

When tested against *L. chagasi,* BTP inhibits parasitic growth with an IC_{50} of 52.2 ± 18.2 μM (see Table 1). Apart from being active against a viscerotropic species, BTP was also shown to be effective against the cutaneous species *L. amazonensis* with an IC_{50} of 50.6 ± 21.3 μM. While BTP is approximately fivefold less potent than tamoxifen, it is configurationally stable, and thus provided a seed for further hit expansion. Thus, holding the core 2-arylbenzothiophene constant, we conducted a similarity search of the Lilly compound collection that resulted in the identification of 147 divergent 2-arylbenzothiophenes which were collected for screening purposes and subsequently tested against *L. chagasi*. An initial screen was performed against *L. chagasi* promastigotes in a dose-response test with 0.5, 5, 50, and 250 μM of each compound. Compounds with IC_{50} lower than 10 μM were subsequently subjected to more detailed dose-response tests.

In general, most molecules tested exhibited activity (123 compounds) with an IC_{50} that ranged from 3.6 ± 0.2 to 121.9 ± 0.2 μM. Among these, 101 compounds exhibited an $IC_{50} \leq$ BTP (IC_{50} 52.6 ± 18.4 μM) and 59 had an $IC_{50} \leq$ tamoxifen (IC_{50} 17.7 ± 0.8 μM), respectively. Twenty-four compounds with an IC_{50} over 250 μM were deemed unsuitable for our purposes, and their exact activity was not determined.

Structure–activity relationships in the benzothiophene scaffold initially examined two of its structural subunits, the phenols (R^1 and R^2, Table 1) and the basic side chain (R, Table 1). We were particularly interested in determining whether the structural features responsible for antiparasitic activity were related to those responsible for ER activity. A phenol at R^1 is known to be important for binding to ER for BTP (27).

To determine whether the phenol is important for antileishmanial activity, we analyzed benzothiophene **15** (Table 1) in which the phenol at the 6-position of the benzothiophene is not present. This compound inhibits parasitic growth with a half-maximal effective concentration of 34.6 ± 4.1 μM, marginally more potent than BTP (52.2 μM), indicating that this phenol is not a requirement for antileishmanial activity. As the 4′-phenol is also known to play a role in ER binding, we tested benzothiophene **14** in which the OH has been masked as a methyl ether, a group which is known to diminish ER binding (33). These two compounds, **14** and **15**, are equipotent, that is, 36.5 ± 5.7 μM versus 34.6 ± 4.1 μM, respectively. Taken together, this indicates that neither phenol is required for antileishmanial activity, validating our previous findings that indicated that tamoxifen's antileishmanial activity and ER binding are not related (14).

To determine the significance of the basic side chain R, the inhibition potencies for compounds **15** and **14** were compared to analogs that lack the side chain, compounds **10**, **12**, and **13** (Table 1). An approximate twofold drop-off in potency is observed for these compounds indicating that the presence of the side chain is important for antileishmanial potency. However, one of the more potent compounds in the original screening cassette was compound **19** (Table 1), which contains a pyrrolidine and a piperidine base and is 10-fold more potent than **15** which contains only a single basic pyrrolidine, that is, compare 3.7 μM for **19** to 34.6 μM for **15**. Thus, we expanded the SAR in these regions. Replacement of either of the basic nitrogen's with a non-basic carbon atom results in a loss of activity, that is, cyclopentyl analog **20** has an IC_{50} of

28.7 μM, respectively, compared with 3.7 μM for **19**. These data further demonstrate the significance of the nitrogen for biological activity.

Variation in the base is well tolerated. For example, the potencies of *N*,*N*-di-isopropylamine in compound **21** (4.3 μM) and *N*,*N*-di-*n*-butylamine in **22** (5.4 μM) are similar to pyrrolidine in **19** (3.7 μM). Changing the carbonyl group to the corresponding methylene (compare **22**–**23** in Table 1) does not alter the potency. In summary, the structure–activity data for the synthetic 2-arylbenzothiophenes evaluated in this study indicate that optimal antileishmanial potency is dependent on the presence of two basic side chains. In addition, the primary structural features required for ER binding, the phenols, are not required for inhibiting parasitic growth.

In terms of the physical properties of these compounds, the calculated logP's, which are a measure of drug-likeness, are shown in Tables 1 and 2. Historically, launched drugs have cLogP's of approximately 2.5, while tamoxifen and raloxifene, the most closely structurally related launched drugs to the chemical entities in our study, have clogP's of 6.35 and 6.34, respectively. The most active of the benzothiophene analogs against *Leishmania*, **19**, **21**, **22** and **23**, has clogP's in the 6.93–8.26 range. Likewise, the molecular masses are somewhat high relative to marketed drugs. As these physicochemical parameters are somewhat higher than both the average drug and even structurally related ones, optimization of these properties may be required in future SAR.

Based on these findings, we selected the compounds with the most potent activity to be tested further. Tamoxifen was previously shown to be active against all *Leishmania* species tested. Furthermore, it was demonstrated to be equally active against the promastigote stage as well as against the intracellular amastigote stage, present in the mammalian host (14,15). The activity of compounds **19**, **21** and **23** was also homogeneous across the different *Leishmania* species tested, with values of IC_{50} in the sub-10 μM range (see Table 3), a desirable characteristic for a future drug development.

Cytotoxicity against mammalian cells *in vitro* was evaluated in Vero cell cultures. Selectivity indexes were around 3.0 (Table 3). We also evaluated **19** for cell-based activity against the ER and found it to be inactive (>25 μM) in a GAL4 luciferase cotransfected with ERα or ERβ (data not shown).

Concentrations in the range of the IC_{50} against promastigotes (4 μM) were used to test the activity against intracellular amastigotes in *L. chagasi* infected macrophages. At these concentrations, the treatment with compounds **19**, **23** and **21** reduced the number of infected cells by 70.1 ± 8.1, 64.8 ± 11.8, and 34.4 ± 6.3%, respectively, demonstrating the compounds' activity against intracellular amastigotes. While it is desirable that selectivity indexes *in vitro* are as high as possible, for some drugs, *in vitro* data do not correlate well with *in vivo* drug safety. For example, an *in vitro* selective index of only 1.3 was found for tamoxifen (14), while *in vivo* safety data allow very wide safety margins for this drug.

The antileishmanial mechanism of action of tamoxifen is presently under study. Dibasic benzothiophenes such as **19** have been shown to inhibit the serine protease thrombin, but there appears to be little correlation between the reported thrombin activity and the observed antileishmanial activity (28). For example, the thrombin inhibitory activity of the compound **19** is 30 times more potent than of compound **23** (28), while they are equipotent in their leishmanicidal effect.

Some SERMs (clomiphene, toremifene, tamoxifen, and raloxifene) were recently shown to possess *in vitro* and *in vivo* potent inhibitory activity against Ebola virus infection (35). This activity was also unrelated to ER expression and response. Mechanistic studies suggested that this inhibitory activity (for toremifene and clomiphene) is related to a late step in viral entry, possibly through interference in endosome membrane composition (35).

Studies in progress in our laboratories indicate that disturbances of membrane fluidity and interference with sphin-

Table 3: IC_{50} of selected compounds against promastigotes of different *Leishmania* species[a] and toxicity to mammalian cells

	Leishmania chagasi		*Leishmania amazonensis*		*Leishmania braziliensis*		*VERO*		
Compound	IC_{50} (μM ± SD)	IC_{90} (μM)	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	SI[b]
19	3.7 ± 0.3	8.2	9.9 ± 0.5	20.4	5.2 ± 0.8	10.0	11.7 ± 3.6	15.2	3.16
21	4.3 ± 0.2	7.9	8.0 ± 1.2	13	3.5 ± 0.9	6.3	13.6 ± 1.6	16.7	3.16
23	3.6 ± 0.2	9.2	7.3 ± 1.8	11.4	4.2 ± 0.1	5.4	13.0 ± 2.9	16	3.6
Amphotericin B	0.111 ± 0.013	ND	0.091 ± 0.006	ND	0.070 ± 0.013	ND	ND	ND	ND

ND, not determined.
[a]*Leishmania* strains used in this work were as follows: *L.* (*L.*) *infantum chagasi* (MHOM/BR/1974/M2682), *L.* (*L.*) *amazonensis* (MHOM/BR/1973/M2269), and *L.* (*V.*) *braziliensis* (MHOM/BR/1975/M2903).
[b]Selectivity index calculated for *L. chagasi*: IC_{50} mammalian cell/IC_{50} *L. chagasi.*

golipid biosynthesis seem to be responsible for tamoxifen's leishmanicidal activity (unpublished data). Future experiments using the compounds identified in this work will be directed at investigating whether they induce the same biochemical modifications as tamoxifen.

Conclusions and Future Directions

The directed screening of a 2-arylbenzothiophene library based on the SERM BTP resulted in the discovery of a novel class of potent antileishmanial agents. Structure–activity studies indicate that the critical determinant for antileishmanial potency is the presence of two basic side chains. Representative compounds within this structural class have shown consistent activity against all species and stages of *Leishmania in vitro* although improvements in selectivity are needed. As such, these compounds represent viable starting points for further optimization as antileishmanial agents. Significantly, the most active antileishmanial benzothiophenes lack the pharmacophore for ER activity, and therefore address potential concerns about the undesirable effects of using SERMs in women and children with leishmaniasis.

Acknowledgments

We thank Andrew S. Bell for the critical reading of the manuscript. This work was supported by Grants #2011/20484-7, São Paulo Research Foundation (FAPESP) and 473343/2012-6, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). V.I.B. was supported by a PNPD/CAPES fellowship (2289/2009).

Conflict of Interest

None.

References

1. den Boer M., Argaw D., Jannin J., Alvar J. (2011) Leishmaniasis impact and treatment access. Clin Microbiol Infect;17:1471–1477.
2. Alvar J., Vélez I.D., Bern C., Herrero M., Desjeux P., Cano J., Jannin J., den Boer M., WHO Leishmaniasis Control Team. (2012) Leishmaniasis worldwide and global estimates of its incidence. PLoS ONE;7:e35671.
3. Murray H.W., Berman J.D., Davies C.R., Saravia N.G. (2005) Advances in leishmaniasis. Lancet;366:1561–1577.
4. Bern C., Maguire J.H., Alvar J. (2008) Complexities of assessing the disease burden attributable to leishmaniasis. PLoS Negl Trop Dis;2:e313.
5. Seifert K., Perez-Victoria F.J., Stettler M., Sanchez-Canete M.P., Castanys S., Gamarro F., Croft S.L. (2007) Inactivation of the miltefosine transporter, LdMT, causes miltefosine resistance that is conferred to the amastigote stage of *Leishmania donovani* and persists *in vivo*. Int J Antimicrob Agents;30:229–235.
6. Machado P.R., Ampuero J., Guimaraes L.H., Villasboas L., Rocha A.T., Schriefer A., Sousa R.S., Talhari A., Penna G., Carvalho E.M. (2010) Miltefosine in the treatment of cutaneous leishmaniasis caused by *Leishmania braziliensis* in Brazil: a randomized and controlled trial. PLoS Negl Trop Dis;4:e912.
7. Neves L.O., Talhari A.C., Gadelha E.P., Silva Junior R.M., Guerra J.A., Ferreira L.C., Talhari S. (2011) A randomized clinical trial comparing meglumine antimoniate, pentamidine and amphotericin B for the treatment of cutaneous leishmaniasis by *Leishmania guyanensis*. An Bras Dermatol;86:1092–1101.
8. Inocencio da Luz R., Romero G.A., Dorval M.E., Cruz I., Canavate C., Dujardin J.C., Van Assche T., Cos P., Maes L. (2011) Drug susceptibility of *Leishmania infantum* (syn. *Leishmania chagasi*) isolates from Brazilian HIV-positive and HIV-negative patients. J Antimicrob Chemother;66:677–679.
9. Rojas R., Valderrama L., Valderrama M., Varona M.X., Ouellette M., Saravia N.G. (2006) Resistance to antimony and treatment failure in human *Leishmania (Viannia) infection*. J Infect Dis;193:1375–1383.
10. Altan N., Chen Y., Schindler M., Simon S.M. (1999) Tamoxifen inhibits acidification in cells independent of the estrogen receptor. Proc Natl Acad Sci USA;96:4432–4437.
11. O'Brian C.A., Liskamp R.M., Solomon D.H., Weinstein I.B. (1985) Inhibition of protein kinase C by tamoxifen. Cancer Res;45:2462–2465.
12. Mandlekar S., Kong A.N. (2001) Mechanisms of tamoxifen-induced apoptosis. Apoptosis;6:469–477.
13. Cabot M.C., Giuliano A.E., Volner A., Han T.Y. (1996) Tamoxifen retards glycosphingolipid metabolism in human cancer cells. FEBS Lett;394:129–131.
14. Miguel D.C., Yokoyama-Yasunaka J.K., Andreoli W.K., Mortara R.A., Uliana S.R. (2007) Tamoxifen is effective against *Leishmania* and induces a rapid alkalinization of parasitophorous vacuoles harbouring *Leishmania (Leishmania) amazonensis* amastigotes. J Antimicrob Chemother;60:526–534.
15. Miguel D.C., Zauli-Nascimento R.C., Yokoyama-Yasunaka J.K.U., Katz S., Barbieri C.L., Uliana S.R.B. (2009) Tamoxifen as a potential antileishmanial agent: efficacy in the treatment of *Leishmania braziliensis* and *Leishmania chagasi* infections. J Antimicrob Chemother;63:365–368.
16. Miguel D.C., Yokoyama-Yasunaka J.K., Uliana S.R. (2008) Tamoxifen is effective in the treatment of *Leishmania amazonensis* infections in mice. PLoS Negl Trop Dis;2:e249.
17. Karimian E., Chagin A.S., Gjerde J., Heino T., Lien E.A., Ohlsson C., Sävendahl L. (2008) Tamoxifen impairs both longitudinal and cortical bone growth in young male rats. J Bone Miner Res;23:1267–1277.

18. Kedar R.P., Bourne T.H., Powles T.J., Collins W.P., Ashley S.E., Cosgrove D.O., Campbell S. (1994) Effects of tamoxifen on uterus and ovaries of postmenopausal women in a randomised breast cancer prevention trial. Lancet;343:1318–1321.
19. Iqbal J., Ginsburg O.M., Wijeratne T.D., Howell A., Evans G., Sestak I., Narod S.A. (2012) Endometrial cancer and venous thromboembolism in women under age 50 who take tamoxifen for prevention of breast cancer: a systematic review. Cancer Treat Rev;38:318–328.
20. Shiina I., Sano Y., Nakata K., Kikuchi T., Sasaki A., Ikekita M., Hasome Y. (2007) Synthesis of the new pseudo-symmetrical tamoxifen derivatives and their anti-tumor activity. Bioorg Med Chem Lett;17:2421–2424.
21. Collins D.J., Hobbs J.J., Emmens C.W. (1971) Antiestrogenic and antifertility compounds 4. 1,1,2-Triarylalkan-1-ols and 1,1,2-Triarylalk-1-enes containing basic ether groups. J Med Chem;14:952–957.
22. Black L.J., Goode R.L. (1980) Uterine bioassay of tamoxifen, trioxifene and a new estrogen antagonist (LY117018) in rats and mice. Life Sci;26:1453–1458.
23. Jones C.D., Jevnikar M.G., Pike A.J., Peters M.K., Black L.J., Thompson A.R., Falcone J.F., Clemens J.A. (1984) Antiestrogens. 2. Structure-activity studies in a series of 3-aroyl-2-arylbenzo[b]thiophene derivatives leading to [6-hydroxy-2-(4-hydroxyphenyl)benzo[b] thien-3-yl] [4-[2-(1-piperidinyl)ethoxy]-phenyl]methanone hydrochloride (LY156758), a remarkably effective estrogen antagonist with only minimal intrinsic estrogenicity. J Med Chem;27:1057–1066.
24. Olah G.H. (1963) Friedel-Crafts and Related Reactions, Vol. 1. General Aspects. New York: Wiley; p. 91–115.
25. Dodge J.A., Stocksdale M.G., Fahey K.J., Jones C.D. (1995) Regioselectivity in the alkaline thiolate deprotection of aryl methyl ethers. J Org Chem;60:739–741.
26. Schmid C.R., Sluka J.P., Duke K.M. (1999) Nucleophilic aromatic substitution on 3-aroyl-2-arylbenzothiophenes. Rapid access to raloxifene and other selective estrogen receptor modulators. Tetrahedron Lett;40:675–678.
27. Sall D.J., Bastian J.A., Briggs S.L., Buben J.A., Chirgadze N.Y., Clawson D.K., Denney M.L. *et al.* (1997) Dibasic benzo[b]thiophene derivatives as a novel class of active site-directed thrombin inhibitors. 1. Determination of the serine protease selectivity, structure-activity relationships, and binding orientation. J Med Chem;40:3489–3493.
28. Sall D.J., Bailey D.L., Bastian J.A., Buben J.A., Chirgadze N.Y., Clemens-Smith A.C., Denney M.L. *et al.* (2000) Diamino benzo[b]thiophene derivatives as a novel class of active site directed thrombin inhibitors. 5. Potency, efficacy, and pharmacokinetic properties of modified C-3 side chain derivatives. J Med Chem;43:649–663.
29. Zauli-Nascimento R.C., Miguel D.C., Yokoyama-Yasunaka J.K., Pereira L.I., Pelli de Oliveira M.A., Ribeiro-Dias F., Dorta M.L., Uliana S.R. (2010) *In vitro* sensitivity of *Leishmania (Viannia) braziliensis* and *Leishmania (Leishmania) amazonensis* Brazilian isolates to meglumine antimoniate and amphotericin B. Trop Med Int Health;15:68–76.
30. Harper M.J., Walpole A.L. (1966) Contrasting endocrine activities of cis and trans isomers in a series of substituted triphenylethylenes. Nature;212:87.
31. Jordan V.C., Haldemann B., Allen K.E. (1981) Geometric isomers of substituted triphenylethylenes and antiestrogen action. Endocrinology;108:1353–1361.
32. Katzenellenbogen B.S., Norman M.J., Eckert R.L., Peltz S.W., Mangel W.F. (1984) Bioactivities, estrogen receptor interactions, and plasminogen activator-inducing activities of tamoxifen and hydroxy-tamoxifen isomers in MCF-7 human breast cancer cells. Cancer Res;44:112–119.
33. Grese T.A., Cho S., Finley D.R., Godfrey A.G., Jones C.D., Lugar C.W. III, Martin J. *et al.* (1997) Structure-activity relationships of selective estrogen receptor modulators: modifications to the 2-arylbenzothiophene core of raloxifene. J Med Chem;40:146–167.
34. Grese T.A., Pennington L.D., Sluka J.P., Adrian M.D., Cole H.W., Fuson T.R., Magee D.E. *et al.* (1998) Synthesis and pharmacology of conformationally restricted raloxifene analogues: highly potent selective estrogen receptor modulators. J Med Chem;41:1272–1283.
35. Johansen L.M., Brannan J.M., Delos S.E., Shoemaker C.J., Stossel A., Lear C., Hoffstrom B.G., Dewald L.E., Schornberg K.L., Scully C., Lehár J., Hensley L.E., White J.M., Olinger G.G. (2013) FDA-approved selective estrogen receptor modulators inhibit ebola virus infection. Sci Transl Med;5:190ra79.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Complementary data with experimental procedures and characterization of arylbenzothiophenes.