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Antiparasitic lethality of sulfonamidebenzamides in kinetoplastids

Amber Hackler^{a,e}, Stephen L. Patrick^{a,f}, Elizabeth W. Kahney^{a,g}, Daniel P. Flaherty^{b,h}, Elizabeth R. Sharlow^{c,d}, James C. Morris^a, and Jennifer E. Golden^{b,i}

^aEukaryotic Pathogens Innovation Center, Department of Genetics and Biochemistry, Clemson University, Clemson, SC 29634, USA

^bKU Specialized Chemistry Center, University of Kansas, Lawrence, KS 66047, USA

^cDepartment of Pharmacology, University of Virginia, Charlottesville, VA 22908, USA

^dFiske Drug Discovery Laboratory, University of Virginia, Charlottesville, VA 22908, USA

Abstract

A sulfonamidebenzamide series was assessed for anti-kinetoplastid parasite activity based on structural similarity to the antiparasitic drug, nifurtimox. Through structure-activity optimization, derivatives with limited mammalian cell toxicity and increased potency toward African trypanosomes and *Leishmania* promastigotes were developed. Compound **22** had the best potency against the trypanosome ($EC_{50} = 0.010 \mu M$) while several compounds showed ~ 10-fold less potency against *Leishmania* promastigotes without impacting mammalian cells ($EC_{50} > 25 \mu M$). While the chemotype originated from an unrelated optimization program aimed at selectively activating an apoptotic pathway in mammalian cancer cells, our preliminary results suggest that a distinct mechanism of action from that observed in mammalian cells is responsible for the promising activity observed in parasites.

Graphical abstract

^eCurrent: Department of Chemistry, Scripps Research Institute, Jupiter, FL 33458, USA

^fCurrent: College of Medicine, Medical University of South Carolina, Charleston, SC 29425, USA

^gCurrent: Department of Biology, Johns Hopkins University, Baltimore, MD 21218, USA

^hCurrent: Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, IN 47907, USA

ⁱCurrent: Department of Pharmaceutical Sciences, School of Pharmacy, University of Wisconsin-Madison, Madison, WI 53705, USA

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Keywords

Antiparasitic; African Sleeping; Sickness; Leishmaniasis; Sulfonamide

The Trypanosomatida order of flagellated protists, including the African trypanosome *Trypanosoma brucei* and several *Leishmania spp.*, are responsible for widespread human diseases that include African sleeping sickness¹ and leishmaniasis,² respectively. Together, these diseases infect over 2 million people and place a tremendous burden on the local medical infrastructure where they occur. Therapeutic options for these parasitic diseases are limited by toxicity and efficacy, with the rising specter of emerging resistance increasing the possibility that the suboptimal therapeutics currently in use will soon be obsolete. Consequently, finding novel, safe and effective antiparasitic agents is critical to bridge the chasm between available options and none at all. The recent disclosure of GNF6702, a selective inhibitor of the kinetoplastid proteasome that imparts broad spectrum activity,³ shows progress on this front. For our own efforts, we investigated the possibility of targeting multiple parasites to reveal a distinct, sulfonamide-based compound series that potently impacts both *T. brucei* and *Leishmania* trypanosomatid parasites.

Our sulfonamidebenzamide-based compound library originated from an orthogonal medicinal chemistry program aimed at detecting agents that selectively upregulated the expression of the C/EBP-homologous protein (CHOP) in cancerous mammalian cells.⁴ Increased expression of *CHOP*, which occurs in normal cells in response to unresolved, misfolded protein accumulation, ultimately leads to programmed cell death (PCD) in mammals if cellular homeostasis is not otherwise achieved.^{5–6} While there is evidence that singled-celled protists can respond to stimuli and initiate a programmed cellular response similar to metazoan apoptosis, the noted absence of clear homologs to the caspases involved suggest alternative mechanisms may be engaged or that the phenotypes that are similar to the PCD-based program are due to “incidental death”.^{7–12} Our interest in these compounds as potential antiparasitic agents was piqued by the realization that the series, represented by compound **1**, shared some common skeletal features with anti-trypanosomal drug, nifurtimox (Lampit®, **2**, Fig. 1). Both compounds bear a nitrofuryl group that is conjugated to an adjacent *pi*-system (blue shaded region) which is tethered to a sulfone or sulfonamide (green shaded space) through a nitrogen-carbon framework (beige shaded area).

The exact mechanism of action of nifurtimox has not been elucidated, though studies propose at least two alternative pathways involving the induction of oxidative stress leading to apoptosis^{13–16} or reductive activation by a eukaryotic type I nitroreductase^{17–19} – both of which implicate the involvement of the nitrofuryl moiety as a warhead. Given the structural congruency between these templates and the ambiguity of mechanisms involved with each, we decided to investigate if these compounds harbored antiparasitic activity.

Sulfonamidebenzamides were synthesized as previously described.⁴ Structural integrity was confirmed by ¹H and ¹³C NMR, and purity was validated to be > 95% by UV/LCMS. Compounds were assessed for their impact on parasite viability in 96- or 384-well plate format against *T. brucei* bloodstream form (BSF)^{20–22} and *L. amazonensis* promastigote form parasites,²³ respectively (Table 1). Immortalized cell lines which are commonly used to

assess general mammalian cytotoxicity are hypersensitive to apoptotic pathway modulators.^{4,24} As many of these compounds were originally developed as inducers of the mammalian cell apoptotic CHOP pathway, immortalized cell lines were not useful indicators of general cytotoxicity. As a result, we utilized a non-immortalized, human foetal lung myofibroblast cell line, IMR90, to detect cytotoxic effects on non-parasite cells.²⁵ All compounds were determined to have EC₅₀ values > 25 μ M on IMR90 cells.

Generally, *T. brucei* BSF parasites were more susceptible to this class of compounds than *L. amazonensis* promastigotes with distinguishable structure-activity relationships (SAR) apparent for each parasite. Analogs bearing structural variants in the 4-position of the *N*-morpholine sulfonamide of **1** showed potent cidal activity on *T. brucei* BSF parasites with EC₅₀ values ranging from 0.03 – 0.73 μ M (compounds **1–10**, Table 1). A broader and comparatively weaker activity profile was observed for the same compounds when evaluated against *L. amazonensis*, though compound **4** was highlighted as one of the more potent members with EC₅₀ = 0.28 μ M. Sulfone derivatives **11–13** were also effective in the submicromolar range against *T. brucei*, and sulfone **12** demonstrated nearly equal potency against both parasites. Analogs **14–18** were surveyed with a modified nitrofuryl moiety. Augmentation, in all cases, resulted in loss of potency against both parasites. Notably, compounds **19** and **20** showed no mammalian CHOP activity,⁴ though moderate micromolar potency against parasites was observed with a 3-nitrophenyl group of **19** and interestingly, with the non-nitro group containing 5-trifluoromethylated acylfuran unit in **20**. Acylnitrothiophenes **21–25** were very potent against *T. brucei*, with little potency fluctuation observed for structural changes in the *N*-morpholine sulfonamide portion or when the 1,4-substitution pattern of the central phenyl ring was exchanged for a 1,3-arrangement, as shown in **25**. Analogously for these derivatives, the previously observed preference for a nonpolar alkyl group in the 4-position of the sulfonamide moiety was underscored against *T. brucei*. The best anti-*Leishmania* activity was also observed in this group with several compounds showing potencies of 100 nM. Ultimately, the effort revealed that, for *T. brucei*, the 1,4-substitution of the phenyl ring was preferred in combination with a 5-nitrofuryl- or 5-nitrothiophenyl amide group and a cyclic sulfonamide bearing a lipophilic 4-positioned substituent. Against *T. brucei* parasites, many analogs were generated that demonstrated low nanomolar potency. For *L. amazonensis* promastigotes, acylnitrothiophenes were generally the most potent representatives with an analogous preference for alkyl substitution on the distal end of the sulfonamide moiety.

Given that the activity profile against each parasite could be dramatically improved in a tractable manner, we turned our attention to finding evidence of a similar or disparate mechanism of action between the parasites and mammalian cells. Previous researchers have described a PCD-like response in kinetoplastid parasites, with stimuli such as the lectin concanavalin A,^{26–27} prostaglandin D²⁸, or unresolved ER stress initiating phenotypes consistent with PCD, including alteration in cellular DNA content due to disruption of replication, DNA degradation, and surface membrane blebbing^{12, 29}.

To explore the mode of action of subject compounds, parasites were scored for loss of membrane phospholipid asymmetry, a hallmark of early apoptosis in mammalian cells. Phosphatidylserine (PS) translocation was detected using an Annexin V-FITC Apoptosis

Detection Kit (Enzo Life Sciences, Inc.) Parasites were incubated with test compounds for 6–24 hr (with time points established to limit necrotic/dying cells to less than 0.5% of the population), equilibrated with manufacturer-provided binding buffer, and labeled with Annexin V-FITC (at a 1:200 dilution of the provided stock) along with propidium iodide (PI, 2 µg/mL) to detect necrosis and eliminate dead cells from consideration. Using flow cytometry to analyze parasite staining, 10,000 BSF trypanosomes (a number sufficient to establish statistical significance in observed differences) were scored for changes in Annexin-V labeling after exposure to compound **5** for 6 h at its EC₅₀ value. Compound-treated parasites showed an increase in Annexin-V labeling after exposure (Fig. 2), with a relative fluorescence median signal of 1225 compared to that of the control line, 463. A second structurally related, though less potent, trypanotoxic mammalian apoptotic activator, compound **7**, yielded similar changes in Annexin-V labeling (data not shown). Procyclic form (PF) parasites were similarly responsive, with Annexin-V treatment yielding a mean relative fluorescence of 658 and 849 for the untreated and treated lines, respectively (data not shown). These experiments show that treated parasites demonstrate similar cell membrane rearrangements as observed during early apoptosis events in mammalian cells, though the studies do not give an indication of what pathway may be engaged. Deconvoluting the specific pathway by which the phenotype is induced will require a substantially more in depth effort.

The activators of the mammalian apoptotic branch of the UPR were found to be potent anti-parasitic compounds with selectivity for parasites sufficiently high to suggest that these compounds could prove useful in future therapeutic development. Because the agents trigger apoptotic-like responses in the African trypanosome, it is tempting to speculate that the mode of action is similar in both mammals and trypanosomes. However, the structure-activity assessment identified compounds that did not retain activity against the mammalian CHOP pathway, thereby failing to induce mammalian apoptosis – though the compounds retained activity in killing parasites. This result, combined with the possibility of “incidental death”,¹⁰ suggests that the small molecules yielded PCD-like behavior as a consequence of targeting pathways that yield similar lethal phenotypes, an issue we look forward to resolving in the future as a more robust SAR effort and mechanistic study is undertaken.

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22. Bloodstream form (BSF) parasites 90-13 and procyclic form (PF) parasites 29-13 *T. brucei*, both 427 strains, were grown in HMI-9 or SDM-79 and viability assessed in 96-well microtiter plate format. Parasites (1×10^5 /mL PF or 1×10^4 /mL BSF) were plated in triplicate with varying concentrations of test compound or compound carrier (DMSO), incubated for 72 hr (RT for PFs or 37 °C for BSF parasites), and CellTiter Blue (Promega) was added followed by fluorescence measurement at 560_{Ex}/590_{Em}. *L. amazonensis* promastigote viability assays were performed with cells (5,000 parasites) seeded into 384-well microtiter plates with test and control compounds included for 44 h at 28 °C with 5% CO₂ prior to CellTiter Blue addition.
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Figure 1.
Similarity between scaffold 1 and nifurtimox 2

Figure 2.

Analysis of *T. brucei* BSF 90-13 parasites labeled by Annexin-V after treatment with compound 5. The mean peak fluorescence of the control population is indicated (vertical line).

Table 1

Sulfonamidebenzamide structure-activity data against *T. brucei* and *L. amazonensis*^{1,2}

compound	central ring substitution	R ¹	R ²	cytotoxicity EC ₅₀ (μM) ³	<i>T. brucei</i> EC ₅₀ (μM) ⁴	<i>T. brucei</i> selectivity index, SI ⁵	<i>L. amazonensis</i> EC ₅₀ (μM) ⁶	<i>L. amazonensis</i> selectivity index, SI ⁵
1	1,4	<i>N</i> -morpholine	5-NO ₂ -furyl	> 25	0.46 ± 0.05	> 54	3.0 ± 0.105	> 8
2	1,4	<i>N</i> -piperidine	5-NO ₂ -furyl	> 25	0.22 ± 0.06	> 114	13.7 ± 7.0	> 2
3	1,4	4-CH ₃ - <i>N</i> -piperidine	5-NO ₂ -furyl	> 25	0.12 ± 0.02	> 208	7.7 ± 5.0	> 3
4	1,4	4,4-dimethyl- <i>N</i> -piperidine	5-NO ₂ -furyl	> 25	0.09 ± 0.01	> 278	0.28 ± 0.1	> 89
5	1,4	4- <i>t</i> -butyl piperidine	5-NO ₂ -furyl	> 25	0.03 ± 0.02	> 833	1.4 ± 0.3	> 18
6	1,4	<i>N</i> -3-azaspiro[5.5]undecane	5-NO ₂ -furyl	> 25	0.03 ± 0.00	> 833	1.5 ± 0.9	> 17
7	1,4	4-OH- <i>N</i> -piperidine	5-NO ₂ -furyl	> 25	0.31 ± 0.07	> 81	4.4 ± 1.8	> 6
8	1,4	4-Cl- <i>N</i> -piperidine	5-NO ₂ -furyl	> 25	0.08 ± 0.04	> 313	7.9 ± 2.8	> 3
9	1,4	4-F- <i>N</i> -piperidine	5-NO ₂ -furyl	> 25	0.42 ± 0.03	> 60	7.4 ± 2.5	> 3
10	1,4	4- <i>NH</i> -piperazine	5-NO ₂ -furyl	> 25	0.73 ± 0.03	> 34	10.5 ± 4.3	> 2
11	1,4	cyclohexyl	5-NO ₂ -furyl	> 25	0.49 ± 0.05	> 51	1.6 ± 0.7	> 16
12	1,4	4-pyran	5-NO ₂ -furyl	> 25	0.53 ± 0.04	> 47	0.3 ± 0.05	> 83
13	1,4	phenyl	5-NO ₂ -furyl	> 25	0.11 ± 0.02	> 227	4.3 ± 1.8	> 6
14	1,4	<i>N</i> -morpholine	2-furyl	> 25	> 10	NA	> 25	NA
15	1,4	<i>N</i> -morpholine	2-thiophene	> 25	> 10	NA	> 25	NA
16	1,4	<i>N</i> -morpholine	phenyl	> 25	> 10	NA	> 25	NA
17	1,4	<i>N</i> -morpholine	5-CH ₃ -furyl	> 25	> 10	NA	> 25	NA
18	1,4	<i>N</i> -morpholine	4-NO ₂ -phenyl	> 25	> 10	NA	> 25	NA

compound	central ring substitution	R ¹	R ²	cytotoxicity EC ₅₀ (μM) ³	<i>T. brucei</i> EC ₅₀ (μM) ⁴	<i>T. brucei</i> selectivity index, SI ⁵	<i>L. amazonensis</i> EC ₅₀ (μM) ⁶	<i>L. amazonensis</i> selectivity index, SI ⁵
19	1,4	4,4-dimethyl- <i>N</i> -piperidine	3-NO ₂ -phenyl	> 25	6.6 ± 0.15	> 4	13.3 ± 6.2	> 2
20	1,4	4,4-dimethyl- <i>N</i> -piperidine	5-CF ₃ -furyl	> 25	7.55 ± 1.46	> 3	10.7 ± 1.0	> 2
21	1,4	<i>N</i> -morpholine	5-NO ₂ -thiophene	> 25	0.060 ± 0.00	> 417	1.3 ± 0.03	> 19
22	1,4	4,4-dimethyl- <i>N</i> -piperidine	5-NO ₂ -thiophene	> 25	0.010 ± 0.001	> 2500	0.1 ± 0.01	> 250
23	1,4	4- <i>t</i> -butyl piperidine	5-NO ₂ -thiophene	> 25	0.030 ± 0.001	> 833	0.1 ± 0.01	> 250
24	1,4	<i>N</i> -3-azaspiro[5.5]undecane	5-NO ₂ -thiophene	> 25	0.020 ± 0.000	> 1250	0.1 ± 0.01	> 250
25	1,3	4,4-dimethyl- <i>N</i> -piperidine	5-NO ₂ -thiophene	> 25	0.045 ± 0.001	> 556	1.3 ± 0.1	> 19
26	1,3	4,4-dimethyl- <i>N</i> -piperidine	5-NO ₂ -furyl	> 25	0.32 ± 0.001	> 78	0.1 ± 0.02	> 250
27	1,3	4,4-dimethyl- <i>N</i> -piperidine	2-furyl	> 25	5.11 ± 0.86	> 5	1.2 ± 0.1	> 21
28	1,3	4,4-dimethyl- <i>N</i> -piperidine	3-NO ₂ -phenyl	> 25	10.39 ± 1.06	> 2	3.0 ± 0.03	> 8
29	1,3	<i>N</i> -morpholine	5-NO ₂ -furyl	> 25	1.47 ± 0.19	> 17	0.9 ± 0.1	> 28

¹ Experimental data is an average of at least 3 runs (n = 3);

² Nifurtimox as a control afforded *T. brucei* EC₅₀ = 7.5 ± 0.05 μM, *L. amazonensis* EC₅₀ = 0.8 ± 0.1 μM, IMR90 EC₅₀ > 25 μM;

³ Mammalian cytotoxicity was determined using IMR90 cells;

⁴ Bloodstream form parasites;

⁵ SI = IMR90 EC₅₀/parasite EC₅₀;

⁶ Promastigote stage parasites.

NA = not applicable.