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Identification and Characterization of FTY720 for the Treatment of Human African Trypanosomiasis

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The screening of a focused library identified FTY720 (Fingolimod; Gilenya) as a potent selective antitrypanosomal compound active against *Trypanosoma brucei gambiense* and *T. brucei rhodesiense*, the causative agents of human African trypanosomiasis (HAT). This is the first report of trypanocidal activity for FTY720, an oral drug registered for the treatment of relapsing multiple sclerosis, and the characterization of sphingolipids as a potential new class of compounds for HAT.

Human African trypanosomiasis (HAT), caused by the protozoan parasites *Trypanosoma brucei gambiense* and *T. brucei rhodesiense*, is prevalent in 36 sub-Saharan countries (1). The disease is extremely debilitating, progressing through two stages: an early or hemolymphatic stage in which the parasites are confined to the blood and lymphatic systems, and a second or central nervous system (CNS) stage in which the parasites penetrate the choroid barrier and invade the CNS (2). Without treatment, the disease is nearly always fatal. The currently available drugs are stage and species specific, have poor safety profiles, and require complex protracted parenteral administration, which is impractical in the rural resource-poor regions where the disease occurs (3). New compounds are desperately needed for the treatment of HAT. Despite two new compounds currently in clinical development (4–6), the extremely high attrition rates in drug discovery mean it is paramount that novel molecules continue to be identified and developed for the treatment of HAT (7).

The screening of a library composed of 741 compounds with either FDA approval or that had reached the late stages of clinical development, was undertaken against bloodstream-form *T. brucei brucei* isolates, according to previously published protocols (8, 9). Active hits, i.e., those meeting the predefined selection criterion of >50% activity at both 20 and 2 μM, were simultaneously rescreened in a dose-response study against *T. brucei brucei* and the mammalian cell line HEK293, as previously described (9), to determine 50% inhibitory concentrations (IC_{50}s) and selectivity indices (SI), respectively. Of the 31 compounds identified as active in the primary screening campaign, 4 compounds (Table 1) had IC_{50}s of <10 μM and an SI of >10, criteria defined by the Drugs for Neglected Diseases *initiative* (DND*i*) for the selection of hit compounds for HAT (10).

FTY720, a drug registered by the U.S. FDA in 2010 as an oral treatment for relapsing multiple sclerosis (MS), exhibited promising selective trypanocidal activity against *T. brucei brucei* (Table 1) (11). In addition, FTY720 is trypanocidal and not trypanostatic, as exposure to the MIC_{90} for 32 h resulted in >80% of the trypanosomes being killed and eliminated, with viability determined by microscopic counting of the parasites, according to previously published protocols (9).

This is the first report of the trypanocidal activity of FTY720, a structural analogue of sphingosine that is phosphorylated *in vivo* by sphingosine kinase (SPHK) to FTY720P(S) and FTY720P(R) (11). To further characterize FTY720 and determine if it is the only sphingolipid compound with trypanocidal activity, a panel of commercially available sphingolipid compounds and SPHK inhibitors were evaluated against *T. brucei brucei*, *T. brucei rhodesiense*, and *T. brucei gambiense* (Table 2), according to published methodologies (6, 8).

FTY720 and its phosphorylated enantiomers FTY720P(S) and FTY20P(R) exhibited activity against all 3 *T. brucei* spp., with IC_{50}s in the range of 0.072 to 1.98 μM (Table 2). This is in contrast to what is observed in MS, in which only FTY720P(S) exerts a therapeutic effect through its agonistic activity at sphingosine 1 phosphate receptors (S1P), and FTY720P(R) and FTY720 display no or weak activity (12). The trypanocidal activities observed for FTY720 and both FTY720P enantiomers suggest that S1P receptors may not be the target of FTY720 in trypanosomes, a hypothesis that is further supported by the absence of any receptors bearing sequence homology to S1P receptors in trypanosomes. However, additional experiments are required to confirm this hypothesis.

FTY720 was not the only sphingolipid derivative with trypanocidal activity. D-erythro-sphinganine, *N*-hexanoyl-D-sphingosine, and sphingosine all displayed trypanocidal activity against *T. brucei*, with IC_{50}s ranging from 0.02 to 4.25 μM (Table 2). As in eukaryotic cells, sphingolipids form an essential component of *T. brucei* membranes, and the *de novo* synthesis of sphingolipids is essential for parasite viability (13). Sphingolipid biosynthesis is also unique in *T. brucei*, in contrast to the kinetoplastid parasites *Leishmania* (14) and *Trypanosoma cruzi* (15), which synthesize the higher-order sphingolipid, inositol phosphorylceramide (IPC); IPC is not detected in bloodstream *T. brucei* parasites (13, 16). In bloodstream *T. brucei*, the predominant higher-order sphingolipids are sphingomyelin, which is normally found in mammals, and ethanolamine phosphorylceramide (EPC) (17). The unique sph-

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TABLE 1 IC_{50}s of active compounds from the compound library against *T. brucei brucei* and HEK293 that met the hit selection criteria of IC_{50} of <10 μM and SI of >10

Compound	IC_{50} (mean ± SD) (μM) for[a]:		SI[b]	Indication	Mechanism of action[c]
	T. brucei brucei	HEK293			
Nitidine chloride	0.55 ± 0.05	6.32 ± 0.76	11.39	Antimicrobial/antiviral/anti-inflammatory/analgesic	Inhibits topoisomerase I/II
Ethacridine lactate	0.73 ± 0.14	ND	10.68	Topical antiseptic/abortifacient	Intercalates DNA/induces myometrial contraction
FTY720	0.59 ± 0.00	ND	16.78	Multiple sclerosis	S1P receptors
Geldanamycin	0.02 ± 0.00	0.44 ± 0.19	100.85	Anticancer	Inhibits HSP-90
Pentamidine	0.0017 ± 0.0008	ND	>394	Antibacterial/antiparasitic	DNA, RNA, phospholipids, and protein synthesis inhibitor
Diminazene aceturate	0.046 ± 0.02	ND	>837	Antiparasitic	Binds to DNA
Puromycin	0.042 ± 0.01	0.71 ± 0.04	17	Antibacterial	Inhibits protein synthesis

[a] Results from two independent experiments. ND, no IC_{50} could be determined due to the compound not reaching a plateau of activity in the HEK293 assay.
[b] SI, selectivity index.
[c] S1P, sphingosine 1 phosphate; HSP, heat shock protein.

ingolipid biosynthesis pathway in *T. brucei* has led to the pathway being proposed as a potential drug target in the parasite, and sphingosine has been shown to inhibit *T. brucei brucei*, with an IC_{50} of 0.82 μM, which is similar to the value obtained in the present study (18). In addition to sphingolipid derivatives, a number of SPHK inhibitors also displayed activity against *T. brucei*, with IC_{50}s of 0.02 to 10.79 μM (Table 2). CAY10621, PF543, and SKI 2 are selective inhibitors of SPHK 1, with IC_{50}s of 3.3, 0.002, and 0.5 μM, respectively (19–21). However, there was no correlation between SPHK and antitrypanosomal activity. The SPHK inhibitor di-methyl sphingosine inhibited *T. brucei brucei*, with an IC_{50} of 1.63 μM, which is in agreement with the value previously reported by Pasternack et al. (22). Although further studies are required to confirm if the SPHK inhibitors identified in this study are targeting TbSPHK, the enzyme was previously validated as a drug target in procyclic *T. brucei brucei* parasites (22). Unlike mammals that possess two SPHK isoforms, designated SPHK 1 and SPHK 2, only one orthologue, TbSPHK, has been identified to date in *T. brucei brucei* (23, 24). TbSPHK is constitutively expressed in both procyclic and bloodstream *T. brucei brucei* parasites, and its depletion in procyclics results in impaired growth and abnormal organelle positioning (22).

The potent selective antitrypanosomal activity exhibited by the sphingolipids in this study led to FTY720 being evaluated in an *in vivo* acute *T. brucei rhodesiense* murine model, according to previously published protocols (6). Disappointingly, FTY720 exhibited no *in vivo* trypanocidal activity following oral or intravenous administration (data not shown). The possible reasons for a lack of translation from *in vitro* to *in vivo* activity are complex and multifold. Potential reasons include the *in vivo* phosphorylation of FTY720 to FTY720P(S), sequestration of the drug in tissues, or significant protein binding resulting in insufficient free drug at trypanocidal concentrations (25). However, extensive studies will be required to determine the exact reasons that *in vivo* trypanocidal activity was not observed for FTY720.

This study is the first report of the antitrypanosomal activity of FTY720. Despite a lack of *in vivo* activity, the results presented in this paper further highlight the potential of sphingolipid derivatives and SPHK inhibitors as therapeutics for HAT; thus, further investigation into these chemical classes is warranted.

TABLE 2 *In vitro* trypanocidal activities of FTY720, sphingolipids, and SPHKI against *T. brucei* subspecies

Compound	IC_{50} (mean ± SD) (μM) for[a]:				SI HEK293[b]
	T. brucei brucei	*T. brucei rhodesiense*	*T. brucei gambiense* STIB930	*T. brucei gambiense* K048	
FTY720	0.59 ± 0.01	0.20 ± 0.01	0.01 ± 0.01	0.072 ± 0.08	16.8
FTY720P(S)	1.21 ± 0.07	0.71 ± 0.09	0.03 ± 0.01	0.40 ± 0.13	14.8
FTY720P(R)	1.98 ± 0.26	1.06 ± 0.21	0.11 ± 0.05	0.67 ± 0.05	9
Sphingosine	1.26 ± 0.35	0.63 ± 0.09	0.54 ± 0.02	0.53 ± 0.08	31.4
D-Erythro-sphinganine	1.71 ± 0.05	0.99 ± 0.04	0.86 ± 0.04	0.72 ± 0.08	6
D-Methyl-sphingosine	1.63 ± 0.12	0.58 ± 0.11	0.02 ± 0.02	0.18 ± 0.01	12.2
N-hexanoyl-D-sphingosine	2.89 ± 0.83	3.03 ± 0.17	6.17 ± 0.91	4.25 ± 0.43	1.4
CAY10621	0.48 ± 0.36	0.19 ± 0.03	0.06 ± 0.06	0.09 ± 0.14	20.8
PF543	10.79 ± 3.07	3.10 ± 1.25	0.51 ± 0.14	2.13 ± 0.16	1.8
SKI2	1.95 ± 0.92	3.73 ± 0.09	0.20 ± 0.03	0.34 ± 0.27	20.3
Pentamidine	0.0027 ± 0.0007		0.0007 ± 0.0002	0.051 ± 0.016	260.8
Diminazene aceturate	0.027 ± 0.001				957
Puromycin	0.038 ± 0.002				12
Melarsoprol		0.007 ± 0.003	0.0006 ± 0.002	0.014 ± 0.002	

[a] Results from three independent experiments.
[b] Selectivity index (SI) compared to HEK293 cells.

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