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Enantiomers of Nifurtimox Do Not Exhibit Stereoselective Anti-*Trypanosoma cruzi* Activity, Toxicity, or Pharmacokinetic Properties

Carolina B. Moraes,[a*] Karen L. White,[b] Stéphanie Braillard,[c] Catherine Perez,[d] Junghyun Goo,[a] Luis Gaspar,[a,e] David M. Shackleford,[b] Anabela Cordeiro-da-Silva,[e] R. C. Andrew Thompson,[d] Lucio Freitas-Junior,[a*] Susan A. Charman,[b] Eric Chatelain[c]

Center for Neglected Diseases Drug Discovery, Institut Pasteur Korea, Bundang-gu, Seongnam-Si, Gyeonggi-do, South Korea[a]; Centre for Drug Candidate Optimisation, Monash University, Parkville, Victoria, Australia[b]; Drugs for Neglected Diseases *initiative* (DNDi), Geneva, Switzerland[c]; Murdoch University, Murdoch, WA, Australia[d]; Faculdade de Farmácia e Instituto de Biologia Molecular Celular da Universidade do Porto, Porto, Portugal[e]

With the aim of improving the available drugs for the treatment of Chagas disease, individual enantiomers of nifurtimox were characterized. The results indicate that the enantiomers are equivalent in their *in vitro* activity against a panel of *Trypanosoma cruzi* strains; *in vivo* efficacy in a murine model of Chagas disease; *in vitro* toxicity and absorption, distribution, metabolism, and excretion characteristics; and *in vivo* pharmacokinetic properties. There is unlikely to be any therapeutic benefit of an individual nifurtimox enantiomer over the racemic mixture.

Nifurtimox is one of only two drugs currently registered for the treatment of Chagas disease caused by the parasite *Trypanosoma cruzi*. While nifurtimox is effective when treatment is initiated during the acute stage of infection, its effectiveness in treating the chronic stage of infection is highly variable (1). Treatment with nifurtimox is associated with a high incidence of severe side effects, and as a result, the related nitroheterocycle benznidazole is currently the drug of choice for the treatment of Chagas disease. *In vivo*, different strains of *T. cruzi* have been reported to exhibit differing susceptibilities to nifurtimox, which may contribute to variations in its effectiveness (2, 3).

Nifurtimox, a 5-nitrofuran that undergoes nitroreduction to form the active species, is marketed as a racemate (Lampit); however, information regarding the biological activity and toxicological and pharmacokinetic properties of the individual nifurtimox enantiomers is not available in the scientific literature. With the aim of improving the currently available chemotherapeutic options for treating Chagas disease, we characterized the individual nifurtimox enantiomers for their antitrypanosomal activity against a panel of *T. cruzi* strains and clones *in vitro* and assessed their efficacy in a murine model of acute Chagas disease. We also determined the *in vitro* toxicity profile and *in vitro* and *in vivo* pharmacokinetic properties of each enantiomer relative to those of the racemic mixture.

Nifurtimox racemate was synthesized by Epichem Pty. Ltd., Perth, Australia. Following separation of the enantiomers by supercritical fluid chromatography and recrystallization, their absolute structure was determined by X-ray diffraction (WuXi AppTec Co., Ltd., Shanghai, China). The *R* and *S* enantiomers were assessed against a panel of seven *T. cruzi* strains and clones, each a member of a distinct lineage or discrete typing unit (Table 1.). Mammalian and *T. cruzi* cultures and antiparasitic activity assays were conducted as described previously (4). Briefly, *T. cruzi*-infected U2OS monolayers were treated with benznidazole (control), a nifurtimox racemic mixture, or the individual enantiomers for 72 h (DM28c) or 96 h (remaining clones and strains), and the concentration resulting in 50% inhibition of parasite growth (50% effective concentration [EC_{50}]) relative to nontreated, infected controls was determined. The EC_{50}s of the racemate and each of the enantiomers were in the low micromolar (benznidazole) to submicromolar (nifurtimox) range, with no significant difference in potency between the enantiomers against the strains and clones tested. A first estimation of cytotoxicity was done by looking at the compound's selectivity toward the parasite; it was measured against the host cells, the human cell line U2OS, by calculating the CC_{50}, the ratio of (i) the average number of cells in compound-treated, infected wells to (ii) the average number of cells in nontreated (mock), infected negative controls. We found that differences between the CC_{50}s of the enantiomers were not significant when the same strain or clone was considered (Table 1). Cytotoxicity assessment in the noninfected rat skeletal myoblast L6 cell line did not show any difference between the enantiomers either.

In vivo efficacy was assessed as previously described (5), by using a murine model of acute Chagas disease with approval from the Animal Ethics Committee of Murdoch University. Female Swiss mice (8 weeks old, $n = 5$ per group) were infected intraperitoneally with 50,000 trypomastigotes (Tulahuen strain), and drug treatment commenced on day 8 postinfection. Nifurtimox racemate or each individual enantiomer was administered by oral gavage (100 mg/kg) once a day for 20 days. At the end of the 20-day dosing period, 5 out of 5 mice in the nifurtimox racemate group had undetectable levels of parasites in their blood as determined

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Address correspondence to Eric Chatelain, echatelain@dndi.org.

* Present address: Carolina B. Moraes and Lucio Freitas-Junior, Laboratório Nacional de Biociências, Centro Nacional de Pesquisa em Energia e Materiais, Campinas, Brazil.

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TABLE 1 EC_{50}s of nifurtimox racemate and its purified enantiomers against a *T. cruzi* strain and clone panel

Strain or clone	DTU	Nifurtimox racemate			*R* enantiomer			*S* enantiomer		
		EC_{50} (μM)	CC_{50} (μM)[a]	SI[b]	EC_{50} (μM)	CC_{50} (μM)	SI	EC_{50} (μM)	CC_{50} (μM)	SI
Dm28c	I	3.41	111.6	32.7	4.00	104.0	26.0	3.32	101.0	30.4
Y	II	1.00	161.6	161.6	0.65	167.4	257.5	0.69	143.2	207.5
ARMA13 cl1	III	0.91	68.5	75.3	0.78	54.8	70.3	0.71	67.2	94.6
ERA cl2	IV	0.58	124.4	214.4	0.75	136.8	182.4	0.42	108.3	257.9
92.80 cl2	V	0.33	79.5	240.9	0.35	79.7	227.7	0.28	75.7	270.4
Tulahuen	VI	0.32	117.7	367.8	0.32	120.0	375.8	0.25	107.1	428.4
CL Brener	VI	0.76	63.8	83.9	0.65	58.3	89.7	0.92	74.9	81.4

[a] CC_{50} was measured against the U2OS cell line as described in Materials and Methods.
[b] SI, selectivity index, defined as the CC_{50}/EC_{50} ratio.

microscopically, compared to 4 out of 5 and 5 out of 5 in the *R* and *S* enantiomer groups, respectively. To assess potential cures, the parasitemia-negative animals in each group underwent up to three rounds of immunosuppression with cyclophosphamide (50 mg/kg administered intraperitoneally once daily for 4 days, followed by 3 days of rest); at the end of the immunosuppression phase, a rebound of parasitemia was observed in all of the animals, indicating that there were no complete cures when using this model.

In vitro toxicity tests included inhibition of the human ether-à-go-go-related gene (hERG) potassium channel and mutagenic potential. For the hERG inhibition assay, an automated patch clamp method (QPatch^{HTX}; WuXi AppTec Co., Ltd., Shanghai, China) was used and indicated that neither of the enantiomers exhibited detectable inhibition of the channel (50% inhibitory concentration [IC_{50}] > 30 μM). A mini-Ames assay (WuXi AppTec Ltd., Suzhou, China) using *Salmonella enterica* serovar Typhimurium strains TA98 and TA100 in the presence or absence of exogenous metabolic activation (Aroclor 1254-induced rat liver S9 mix) turned out positive for each enantiomer (>2-fold increase in the mean number of revertant colonies); a similar increase in the number of revertants per plate (reverse mutations at the selected loci of the two tester strains) in the absence or presence of S9 activation was exhibited by each enantiomer.

Physicochemical and metabolic stability properties and CYP inhibition were assessed as described previously (5). Both enantiomers exhibited good aqueous solubility (50 to 100 μg/ml) and moderate lipophilicity (a distribution constant [log $D_{pH\ 7.4}$] of 2.1) and were metabolically stable in the presence of human hepatic microsomes (*in vitro* intrinsic clearance of <7 μl/min/mg of protein). The enantiomers both showed minimal inhibition of cytochrome P450 3A4, with an IC_{50} of >20 μM (data not shown).

The *in vivo* pharmacokinetic properties of (*R*)- and (*S*)-nifurtimox were investigated in male Sprague-Dawley rats using protocols approved by the Monash Institute of Pharmaceutical Sciences Animal Ethics Committee and in accordance with procedures outlined in the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Each enantiomer was administered intravenously (i.v.) by constant-rate infusion (3 mg/kg) over 10 min into a catheter surgically inserted into the jugular vein under isoflurane anesthesia. Oral doses (10 mg/kg) were administered as an aqueous suspension by oral gavage. Blood samples were collected for up to 24 h postdose and analyzed by liquid chromatography-mass spectrometry. Following i.v. administration, plasma (*R*)- and (*S*)-nifurtimox concentrations rapidly declined to levels below the analytical lower limit of quantitation; however, within the first 6 h, there was no discernible difference in i.v. exposure between the two enantiomers. Similarly, after oral administration, there was no substantial difference between the profiles of (*R*)- and (*S*)-nifurtimox during the initial 5-h period, and while concentrations of the *R* enantiomer at later sample times were greater than those of (*S*)-nifurtimox, there was no major difference in the overall exposure of the two enantiomers (Fig. 1).

In summary, these studies reveal no evidence of nifurtimox enantiomer stereoselectivity in relation to *in vitro* anti-*T. cruzi* activity against multiple strains and clones or *in vivo* in a murine model of acute Chagas disease. For selected toxicity parameters,

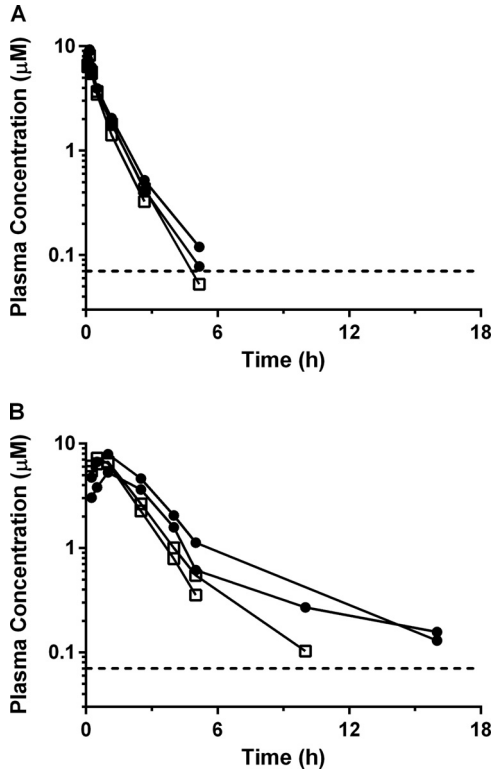

FIG 1 Plasma concentration-versus-time profiles of (*R*)- and (*S*)-nifurtimox in male Sprague-Dawley rats following i.v. (3 mg/kg, panel A) or oral (10 mg/kg, panel B) administration. Data are shown for two individual animals per compound per dose and route. (*R*)-Nifurtimox is represented by filled symbols, and (*S*)-nifurtimox is represented by open symbols. The dashed line in each panel represents the analytical lower limit of quantitation.

both enantiomers exhibited no detectable inhibition of the hERG potassium channel, and both were similarly positive in a mini-Ames mutagenicity test. There was no detectable difference between the physicochemical, metabolic, or pharmacokinetic properties of the two enantiomers. The results suggest that there is unlikely to be any therapeutic benefit in relation to safety or efficacy of an individual nifurtimox enantiomer over the racemic mixture.

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