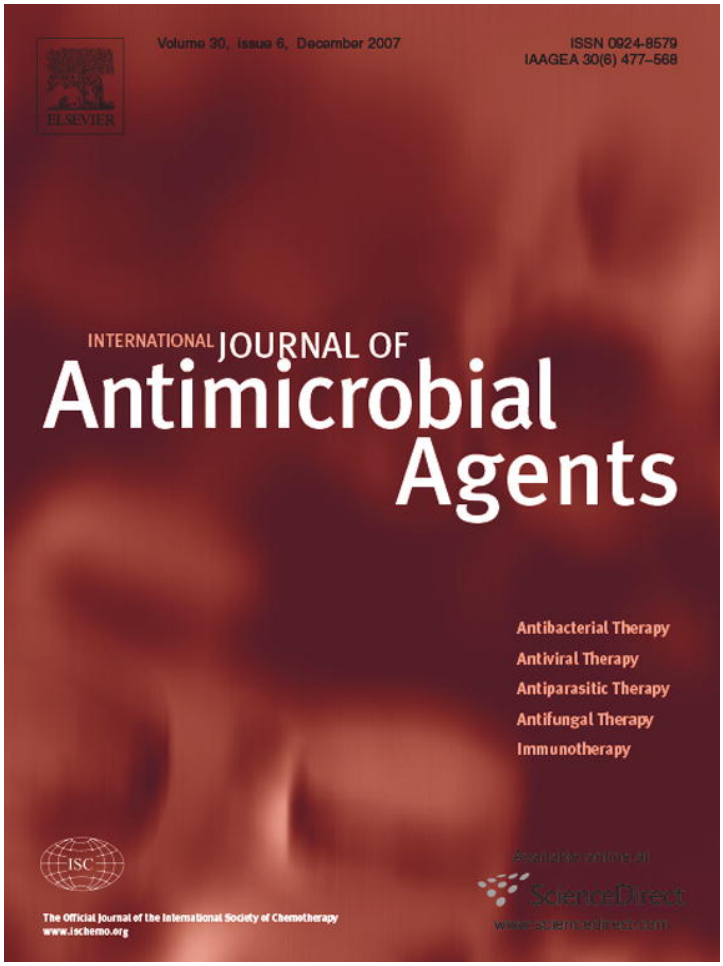

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Short communication

Trypanocidal activity of piperazine-linked bisbenzamidines and bisbenzamidoxime, an orally active prodrug

Tien L. Huang [a,*], Cyrus J. Bacchi [b], Nageswara R. Kode [a], Qiang Zhang [c], Guangdi Wang [c], Nigel Yartlet [d], Donna Rattendi [b], Indira Londono [b], Lakshman Mazumder [b], Jean Jacques Vanden Eynde [a], Annie Mayence [a], Isaac O. Donkor [e]

[a] *College of Pharmacy, Xavier University of Louisiana, 1 Drexel Drive, New Orleans, LA 70125, USA*
[b] *Department of Biological and Health Sciences, Pace University, Haskins Laboratories, 1 Pace Plaza, New York, NY 10038, USA*
[c] *Department of Chemistry, Xavier University of Louisiana, 1 Drexel Drive, New Orleans, LA 70125, USA*
[d] *Department of Chemistry and Physical Sciences, Pace University, Haskins Laboratories, 1 Pace Plaza, New York, NY 10038, USA*
[e] *Department of Pharmaceutical Sciences, University of Tennessee Health Science Center, 847 Monroe Avenue, Memphis, TN 38163, USA*

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Abstract

A series of 32 piperazine-linked bisbenzamidines (and related analogues) were analysed for their in vitro and in vivo trypanocidal activity against a drug-sensitive strain of *Trypanosoma brucei brucei* and a drug-resistant strain of *Trypanosoma brucei rhodesiense*. The compounds showed similar potencies against both strains. The most potent compounds were bisbenzamidines substituted at the amidinium nitrogens with a linear pentyl group (**8**, inhibitory concentration for 50% (IC_{50}) = 1.7–3.0 nM) or cyclic octyl group (**17**, IC_{50} = 2.3–4.6 nM). Replacement of the diamidine groups with diamidoxime groups resulted in a prodrug (**22**) that was effective orally against *T. b. brucei*-infected mice. Three compounds (**7**, **11** and **15**) provided 100% cure when administered parenterally. The results indicate that the nature of the substituents at the amidinium nitrogens of bisbenzamidines strongly influence their trypanocidal activity.

Keywords: Diamidine; Diamidoxime; Prodrug; Trypanocidal agents; Human African trypanosomiasis; *Trypanosoma brucei*

1. Introduction

Trypanosoma brucei gambiense and *Trypanosoma brucei rhodesiense* are parasitic protozoans that cause debilitating sleeping sickness in humans (human African trypanosomiasis (HAT)) and nagana in cattle, diseases that are endemic in sub-Saharan Africa. It has been estimated that there are 300 000–500 000 cases of HAT with 50 000 deaths annually [1]. The parasites are transferred to the host through the bite of an infected tsetse fly. If left untreated, the disease progresses with increasing breakdown of neurological function, to coma and eventually death [1,2]. *Trypanosoma b. gambiense* causes the chronic form of the disease, which proliferates more slowly and can take several years before spreading into the central nervous system (CNS). *Trypanosoma b. rhodesiense* causes the acute form of the disease in which the parasites rapidly invade the CNS, causing death within weeks if untreated.

There are four drugs (suramin, pentamidine, melarsoprol and eflornithine) approved for the chemotherapy of HAT [1]. However, these drugs are plagued with problems such as unacceptable toxicity, increasing incidence of drug failure due to resistance by the parasites, undesirable parenteral route of administration and/or poor efficacy [1–3]. Based on the limitations of current therapy, it is widely accepted that new drugs with improved biological properties are urgently required for the chemotherapy of HAT. The only compound that is in clinical development for the treatment of HAT is an orally bioavailable prodrug DB289 [3], which is the dimethoxy derivative of the diamidine DB75. DB289 recently

* Corresponding author. Tel.: +1 504 520 7603; fax: +1 504 520 7954.
E-mail address: thuang@xula.edu (T.L. Huang).

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entered phase III clinical trials for the treatment of the early stage of HAT [4].

We previously reported on the trypanocidal activity of a series of conformationally restricted pentamidine congeners [2]. From this study, *N*,*N*′-bis(4-amidinophenyl) piperazine (**1**, TH103; Table 1) emerged as an excellent lead compound against trypanosomiasis. Using compound **1** as the lead, we have synthesised 31 analogues in which the 1,4-piperazinyl ring linking the two benzamidine groups was retained while various modifications were made to the terminal amidinium groups. These analogues were then evaluated against a drug-sensitive strain of *Trypanosoma brucei brucei* Lab 110 EATRO and a drug-resistant strain of *T. b. rhodesiense* KETRI 243. An amidoxime prodrug of compound **1** was synthesised and tested in vivo in mice infected with *T. b. brucei* Lab 110 EATRO. A metabolic study of the prodrug (**22**) was conducted using high performance liquid chromatography (HPLC) and mass spectrometry (MS) to characterise the metabolites.

2. Materials and methods

2.1. Synthesis of trypanocidal agents

The compounds used in this study were synthesised in the laboratory of Prof. Tien L. Huang at Xavier University of Louisiana (New Orleans, LA). ^{1}H-nuclear magnetic resonance (NMR) and infrared (IR) spectra were recorded using a Varian Inova instrument (500 MHz; Varian Inc., Palo Alto, CA) and a Perkin-Elmer Spectrum One instrument (Perkin-Elmer Life and Analytical Sciences, Shelton, CT), respectively. Syntheses of compounds **1–21** and **23–32** have been described previously [2,5–7]. Elemental analyses were performed by M-H-W Laboratories (Phoenix, AZ).

4,4′-(1,4-Piperazinediyl)bis(*N*-hydroxybenzenecarboximidamide) (dihydrochloride salt **22**) was synthesised as follows. Hydroxylamine 50% solution in water (11.7 mL, 174 mmol) was diluted with dimethyl sulfoxide (DMSO) (11.7 mL) and then added drop-wise to a stirred solution of 4,4′-(1,4-piperazinediyl)bisbenzonitrile (5.0 g, 17 mmol) [7] in DMSO (100 mL) at 110 °C. Subsequently, the reaction mixture was heated at 100 °C for 2 h until the nitrile peak at 2214 cm^{-1} was no longer detected in IR. The reaction mixture was cooled to room temperature and poured onto crushed ice (ca. 1 kg). The resulting snow white solid was filtered, washed thoroughly with water followed by ethanol (100 mL), acetone (100 mL) and hexane (50 mL), and dried under vacuum at room temperature. The crude bisamidoxime (free base) obtained was re-crystallised from a mixture of dimethylformamide and acetone (7:1) to give creamy white feathery needles. The product was filtered under vacuum, washed with water, ethanol and acetone (100 mL each) followed by hexane (50 mL) and dried under vacuum to afford 5.6 g of **22** free base in 90% yield, melting point (mp) > 300 °C; ^{1}H-NMR (DMSO-d6): δ 9.36 (s, 2H), 7.56 (d, 4H, J = 10 Hz), 6.99 (d, 4H, J = 10 Hz), 5.65 (s, 4H), 3.32 (s, 8H).

Then, 10% methanolic hydrochloric acid (5.0 mL, 14 mmol) was added to a suspension of **22** free base (1.0 g, 2.8 mmol) in 25 mL of anhydrous DMSO and stirred at room temperature for 4 h. The resulting clear solution was filtered directly into 300 mL of anhydrous methylene chloride when a snowy white precipitate resulted. The product was filtered and washed thoroughly with anhydrous methylene chloride followed by hexane (50 mL) and dried under high vacuum at room temperature for 24 h to afford 1.1 g, 91% yield, mp > 265 °C. ^{1}H-NMR (DMSO-d6): δ 12.66 (s, 2H), 11.05 (Br s, 2H), 9.12 (s, 2H), 8.65 (s, 2H), 7.7 (d, 2H, J = 8.5 Hz), 7.12 (d, 2H, J = 8.5 Hz), 3.53 (s, 8H).

Anal. calc for $C_{18}H_{22}N_6O_2$ 2.0 HCl 1.2 H_2O (447.74), C, 48.29; H, 5.67; N, 18.77. Found: C, 48.07; H, 5.73; N, 18.53.

2.2. Trypanosome strains

The two clinical isolates of *T. brucei* used in this study were *T. b. brucei* Lab 110 EATRO (pentamidine-sensitive) and *T. b. rhodesiense* KETRI 243 (melarsoprol-, pentamidine- and berenil-resistant) [8].

2.3. In vitro and in vivo studies

In vitro and in vivo studies were conducted according to previously described procedures [2,8].

2.4. Metabolism studies with prodrug 22

The in vitro metabolism of compound **22** was investigated by incubation of the prodrug in 0.5 mL solutions containing 2 mg/mL rat liver microsomal proteins, 20 mM prodrug, 75 mM potassium phosphate (pH 7.4), 17 mM magnesium chloride, 7 mM NADP$^+$, 17 mM glucose 6-phosphate and 1.2 units/mL of glucose-6-phosphate dehydrogenase. Incubations were halted at different time points by placing the vials in an ice-bath, followed by addition of an equal volume of methanol (0.5 mL). The incubation products were centrifuged (10 000 × *g*, 15 min) to remove proteins and the supernatant was concentrated with a stream of nitrogen at 37 °C to 0.2 mL. Solid phase extraction was used to purify further the incubation solution before HPLC–MS analysis.

Control incubations were performed under identical conditions with heat-inactivated microsomes (heated to 100 °C for 10 min) and in the absence of NADPH or microsomes. In addition, blank incubations were performed in which all elements were present except the drug. A Shimadzu LC-MS 2010 (Shimadzu Scientific Instruments, Inc., Columbia, MD) was used for screening of possible metabolic products generated from the microsomal incubations by obtaining the mass spectra of all chromatographic peaks. An Agilent ZORBAX Rx-C8 column (2.1 × 150 mm; 5 μm pore size) (Agilent Technologies, Inc., Santa Clara, CA) coupled to a C18 guard column (2 × 18 mm, 5 μm) (Sigma-Aldrich Co.,

Table 1
Structure and in vitro trypanocidal activity of piperazine-linked bisbenzamidines and analogues **1–32**

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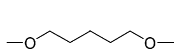
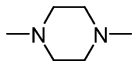
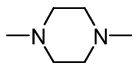
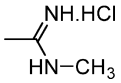
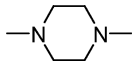
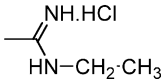
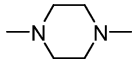
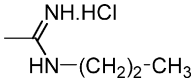
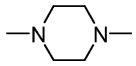
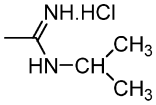
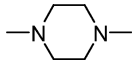
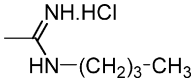
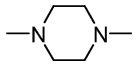
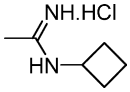
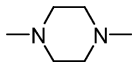
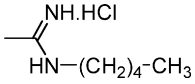
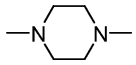
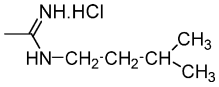
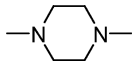
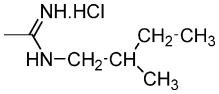
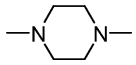
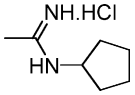
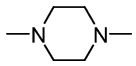
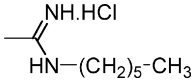
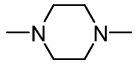
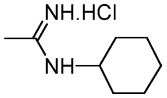
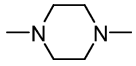
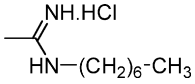
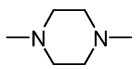
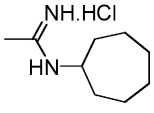
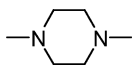
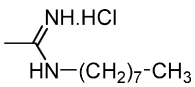
			2.12	2.32
	Pentamidine			
1			16.7	8.90
2			205	33.0
3			13.0	33.0
4			35.0	32.5
5			82.0	70.0
6			13.5	18.9
7			6.00	51.0
8			3.00	1.7
9			72.0	61.0
10			13.0	39.0
11			24.9	37.5
12			13.0	23.9
13			56.5	26.5
14			100	57.0
15			18.0	22.0
16			3.65	19.0

Table 1 (*Continued*)

Compound no.	Linker	R	IC_{50} (nM)[a]	
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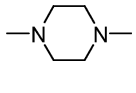
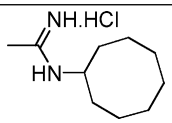
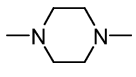
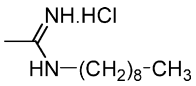
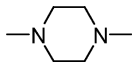
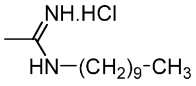
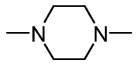
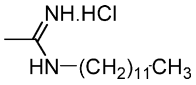
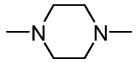
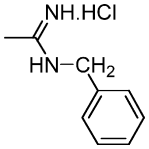
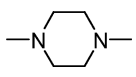
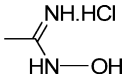
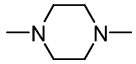
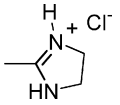
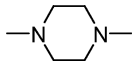
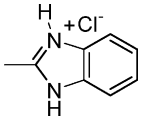
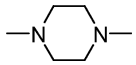
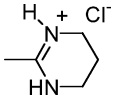
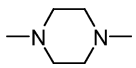
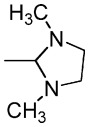
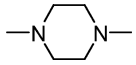
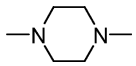
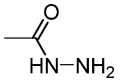
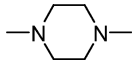
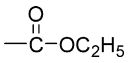
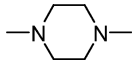
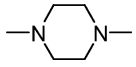
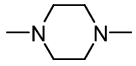
17			2.30	4.6
18			100	210
19			180	220
20			660	125
21			51.0	52.5
22			>1000	>1000
23			16.0	6.50
24			>1000	>1000
25			19.5	20.5
26			>1000	>1000
27			>1000	>1000
28			240	48.0
29			340	350
30			>1000	>1000
31		—CN	>1000	>1000
32		$-NO_2$	137	250

[a] IC_{50} = inhibitory concentration for 50%. Each value is the average of duplicate determinations.
[b] *Trypanosoma brucei brucei* strain Lab 110 EATRO.
[c] *Trypanosoma brucei rhodesiense* strain KETRI 243 (see [8]).

Milwaukee, WI) was used for separation. The HPLC mobile phase flow rate was set at 0.30 mL/min, with gradient elution starting at 100% mobile phase A (aqueous 15 mM triethylamine (TEA) and 35 mM acetic acid) for 5 min, followed by a linear increase of mobile phase B (75% aqueous acetonitrile containing 15 mM TEA and 35 mM acetic acid) to 25% in 22 min, then to 40% B in 5 min, finally to 100% B in 15 min.

3. Results and discussion

Compound **1** was identified as a promising lead compound against *Pneumocystis carinii* [7] and *T. brucei* [2]. Based on this observation, 31 analogues of **1** were synthesised to investigate the structure–activity relationships of this series of compounds. In this study, the trypanocidal activity of this series of compounds was investigated in vitro using a pentamidine-sensitive strain of *T. b. brucei* and a drug-resistant strain of *T. b. rhodesiense* (Table 1).

In general, the compounds exhibited similar potency against both strains of *T. brucei*. Bisbenzamidines substituted with straight alkyl chains were more potent than those with branched alkyl chains (e.g. **8** is more potent than **9** or **10**). Optimum activity was observed with the analogue substituted with a pentyl chain (**8**, inhibitory concentration for 50% (IC_{50}) = 1.7–3.0 nM). Interestingly, the analogue with an *n*-octyl substituent (**16**) was also very potent (IC_{50} = 3.65–19.0), despite its weak binding (ΔT_m = 4.8) to poly (dA-dT). Although most of the active compounds bind to DNA (data not shown), poor correlation was observed between DNA affinity and trypanocidal activity. Trypanocidal activity was significantly reduced with longer chain substitutions (**18–20**). Among the bisbenzamidines

Table 2
In vivo trypanocidal activity of piperazine-linked bisbenzamidines and bisbenzamidoxime[a]

Compound no.	Dosage (mg/kg/day)	Days of treatment	Mean survival (days)	No. of mice cured/total (%)
None	–	–	5.0	0/3
Pentamidine	1.0, 2.5, 5, 10	3	>30	5/5 (100)[b]
3	1.0	3	5.0	0/3
	2.5	3	5.0	0/3
	5	3	18	2/3 (67)
	10	3	17	2/3 (67)
6	1.0	3	7.0	0/3
	2.5	3	9.0	0/3
	5	3	18	5/6 (83)
	10	3	6.3 (toxic)	3/6 (50)
7	1.0	3	5.0	0/3
	2.5	3	5.0	0/3
	5	3	11	2/3 (67)
	10	3	>30	3/3 (100)
11	1.0	3	5.0	0/3
	2.5	3	6.6	0/3
	5	3	16	2/3 (67)
	10	3	>30	3/3 (100)
12	1.0, 2.5, 5, 10	3	5.3–10.6	0/3
15	1.0	3	7.5	0/3
	2.5	3	8.3	0/3
	5	3	9.0	4/6 (67)
	7.5	3	>30	3/3 (100)
	10	3	>30	6/6 (100)
	15	3	>30	3/3 (100)
16	1.0, 2.5, 5, 10	3	5.0	0/3
19	1.0, 2.5, 5, 10	3	5.0	0/3
21	1.0, 2.5, 5, 10	3	5.0	0/3
22	50 (p.o.)	3	5.0	0/3
	100 (p.o.)	3	5.0	0/3
	100 (p.o. bid)	3	>30	3/3 (100)

p.o., orally; bid, twice a day.

[a] In vivo efficacy of compounds given via intraperitoneal (i.p.) route against *T. b. brucei* LAB 110 EATRO. Mice were infected with 250 000 parasites and dosing commenced 24 h post infection. Mice were separated into groups of three and given single i.p. doses for 3 days unless otherwise noted. Infected untreated controls were used for each experiment. Mice were considered cured if surviving more than 30 days beyond death of controls without parasites in tail vein blood smears. Mean survival (in days) is exclusive of cured animals.

[b] All doses cured. Groups of five animals used for all doses.

substituted with saturated cyclic rings, the most potent analogue was **17** (IC_{50} = 2.3–4.6 nM), which has a cyclooctyl substituent. Potent trypanocidal activity was generally associated with analogues containing cycloalkyl substituents (IC_{50} values of **7**, **11**, **13**, **15** and **17** ranged from 2.30 nM to 56.5 nM). Cyclisation of the amidinium groups into a five-membered imidazolinium ring (e.g. **23**) or a six-membered tetrahydropyrimidinium ring (e.g. **25**) resulted in potent analogues with IC_{50} values of 6.5–20.5 nM. Interestingly, the benzimidazole analogue **24** was inactive. This analogue was among the most potent antileishmanial agents in a series of conformationally restricted pentamidine congeners [9]. Replacement of the amidinium groups with non-basic groups (e.g. **27** and **29–32**) resulted in weakly active or inactive compounds. As expected, the diamidoxime prodrug **22** was inactive in the in vitro assay.

Based on the encouraging in vitro data, ten compounds were selected for in vivo efficacy studies using mice infected with the *T. b. brucei* LAB 110 EATRO strain (Table 2). Compounds **3**, **6**, **7**, **11**, **12**, **15**, **16**, **19** and **21** were administered via the intraperitoneal route at doses ranging from 1.0 mg/kg/day to 15.0 mg/kg/day for 3 days unless otherwise noted. The prodrug **22** was administered orally at 50 mg/kg/day and 100 mg/kg/day for 3 days. Although several of the bisbenzamidines substituted with straight alkyl chains (**3**, **6**, **12**, **16** and **19**) showed potent in vitro activity, they were relatively inactive (**12**, **16** and **19**) or moderately active (**3** and **6**) in this animal model. None of these analogues cured the mice, although compound **3** provided 67% cure at doses of 5 mg/kg/day or 10 mg/kg/day; compound **6** at 5 mg/kg/day provided 83% cure of the animals, but it was toxic at 10 mg/kg/day. On the other hand, bisbenzamidines with cycloalkyl substituents were the most active trypanocides in vivo. Compounds with the cyclobutyl (**7**), cyclopentyl (**11**) or cycloheptyl (**15**) substituents when administered at doses ranging from 7.5 mg/kg/day to 15 mg/kg/day for 3 days afforded 100% cure of the animals. Lower doses of 5 mg/kg of these compounds cured 67% of the animals from the infection. Compound **15** appears to be the best trypanocide in vivo since it cured the animals at several doses. Besides good efficacy, these compounds (**7**, **11** and **15**) also demonstrated low cytotoxicity in several human cell lines [6], indicating that the compounds were more selective in inhibiting the parasites compared with human cell lines. The benzyl analogue **21** was inactive in this animal model. A prodrug strategy was used to develop an orally active drug. Drugs with good oral bioavailability are urgently needed to overcome the limitations of the current drugs, which are administered by the parenteral route. The prodrug **22** was not effective in single doses of 50 mg/kg or 100 mg/kg orally. However, administering the drug orally twice a day at 100 mg/kg for 3 days provided 100% cures of the animals. This suggests that prodrug **22** may not be

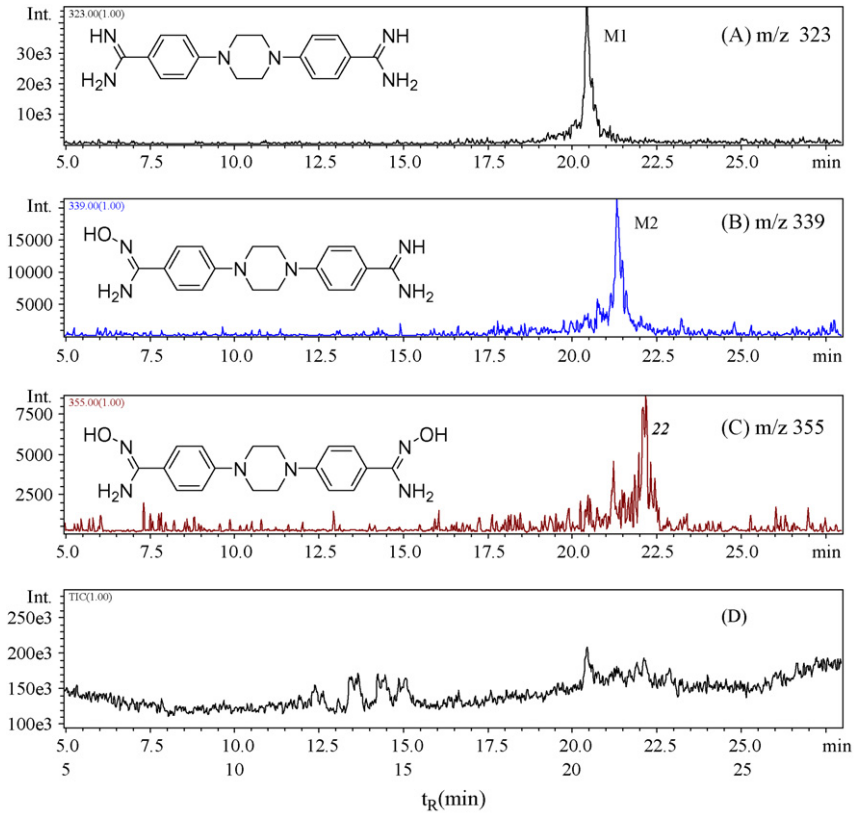


Fig. 1. (A–C) Reconstructed ion chromatograms and (D) total ion chromatogram of a rat microsomal incubation solution of the prodrug **22** (TH125). The identified metabolites are labelled as M1 (**1**, TH103) and M2, and the parent compound is labelled **22**.

sufficiently absorbed from the gastrointestinal tract or that it is rapidly cleared from the bloodstream of the tested animals. Further pharmacokinetic experiments would be needed to confirm this hypothesis.

The in vivo efficacy of the prodrug **22** suggested that the amidoxime group of the prodrug was bioactivated to the active amidinium group by host enzymes. This proposed metabolic pathway was confirmed by incubating the prodrug with rat liver microsomes. Two major metabolites corresponding to the monoamidine (M2) and diamidine (M1) were identified (Fig. 1). The percentages of M1 formed at 30, 60 and 120 min were 51%, 56% and 67%, respectively, whereas the percentages of M2 were 46%, 35% and 27% at the same time intervals. Therefore, most of the parent diamidoxime was metabolised to either M2 or M1 within 30 min. Transformation of the amidoxime to the amidine is probably catalysed by the cytochrome b_5 and NADH cytochrome b_5 reductase enzymes reported recently for an amidoxime prodrug [10].

In conclusion, our data suggest that piperazine-linked bisbenzamidines and a bisbenzamidoxime prodrug constitute a new series of promising trypanocidal agents. Compounds with cycloalkyl substituents (e.g. cyclobutyl, cyclopentyl or cycloheptyl) on the amidinium nitrogens were the most potent trypanocides in vivo. A diamidoxime prodrug (**22**) was effective when administered orally.

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Ethical approval: Not required.

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