Brief Articles

O-Alkoxyamidine Prodrugs of Furamidine: In Vitro Transport and Microsomal Metabolism as Indicators of in Vivo Efficacy in a Mouse Model of *Trypanosoma brucei rhodesiense* Infection

John H. Ansede,*,† Mariappan Anbazhagan,‡ Reto Brun,§ Judy D. Easterbrook,§ James Edwin Hall,† and David W. Boykin‡

Division of Drug Delivery and Disposition, School of Pharmacy, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7360, Department of Chemistry, Georgia State University, Atlanta, Georgia 30303, and Swiss Tropical Institute, Basel, CH4002, Switzerland

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Five *O*-alkoxyamidine analogues of the prodrug 2,5-bis[4-methoxyamidinophenyl]furan were synthesized and evaluated against *Trypanosoma brucei rhodesiense* in the STIB900 mouse model by oral administration. The observed in vivo activity of these prodrugs demonstrates that compounds with an *O*-methoxyamidine or *O*-ethoxyamidine group effectively cured all trypanosome-infected mice, whereas prodrugs with larger side-chains did not completely cure the mice. Permeability across Caco-2 cell monolayers and microsomal metabolism were used to identify the underlying mechanisms of prodrug efficacy.

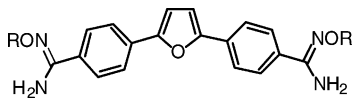
Introduction

Aryl diamidines exhibit broad spectrum antimicrobial activity; however, their potential as chemotherapeutic agents has not been realized, in part due to their low oral bioavailability.[1] Several reports have appeared which have demonstrated that prodrug strategies which lower the p*K* of the functional group can provide orally effective molecules.[2−5] We have reported that a prodrug of furamidine (**1**, Table 1), 2,5-bis[4-methoxyamidinophenyl]furan (**3**), was effective in an immunospressed rat model for *Pneumocystis carinii* pneumonia (PCP).[6] The prodrug, **3**, is currently in Phase II clinical trials against PCP, human African trypanosomiasis and malaria.[1] In our earlier studies, we noted that 2,5-bis[4-ethoxyamidinophenyl]furan (**4**) was not effective in the PCP rat model and speculated that the bis-ethoxy-bearing molecule was either poorly metabolized to furamidine or was transported across the intestinal epithelial at an insufficient rate.[6] More recently, in a study of aza-analogues of furamidine we found that a bis-ethoxyamidino prodrug was quite effective in a mouse model for *Trypanosoma brucei rhodesiense* infection.[7] Albeit in two different animal models and with two different molecules, these contradictory results have led us to study the effect of variation of the alkyl group on the prodrug efficacy of *O*-alkoxyamidines.

Chemistry

Earlier we synthesized **3** from the corresponding bis-nitrile using Pinner methodology.[6] More recently, we have reported the synthesis of **3** and related compounds by first preparing the requisite *O*-alkyl-4-bromobenzamidoxime by reaction of 4-bromobenzonitrile with hydroxylamine to form 4-bromobenzamidoxime and subsequent *O*-alkylation.[8] Reaction of *O*-alkyl-4-bromobenzamidoximes with 2,5-bis(tri-*n*-butylstannyl)furan under Stille coupling conditions readily produced the desired *O*-alkoxyamidines.[8] We have used this latter approach to prepare the compounds used in this study as outlined in Scheme 1.

Table 1. Activity of Amidoxime Prodrugs of Furamidine in an Acute Mouse Model of Trypanosomiasis



		in vitro[a]	in vivo[b]		
code	R	IC_{50} (nM)	dose (mg/kg)[c]	cures[d]	survival (days)[e]
1, furamidine	na	4.5	4 × 10	0/8	>46
2, DB290	H	66.4×10^3	4 × 100	0/4	50
3, DB289	Me	14.6×10^3	4 × 100	4/4	>60
			4 × 50	2/4	>60
			4 × 25	1/4	>60
4, DB377	Et	645	4 × 100	4/4	>60
			4 × 25	0/4	32
5, DB1005	*n*-Pr	472	4 × 100	1/4	>47.5
6, DB1009	*i*-Pr	12.6×10^3	4 × 100	2/4	48
7, DB1010	*n*-Bu	1.9×10^3	4 × 100	0/4	9
8, DB1011	*n*-Hex	5.7×10^3	4 × 100	0/4	6

[a] Average of duplicate determinations; see ref 7 for details. [b] See ref 7 for experimental details of STIB900 mouse model. [c] Dosage for furamidine (**1**) is intraperitoneal, all others dosage by oral administration. [d] Number of mice that survive and are parasite free for 60 days. [e] Average days of survival. Untreated controls expire between day 7 and 8 postinfection.

* Current address and to whom correspondence should be addressed: Qualyst Inc., 7030 Kit Creek Road, P.O. Box 12199, Research Triangle Park, NC, 27709. Tel: (919) 313-0162. Fax: (919) 313-6504. E-mail: JohnAnsede@Qualyst.com.
† The University of North Carolina at Chapel Hill.
‡ Georgia State University.
§ Swiss Tropical Institute.

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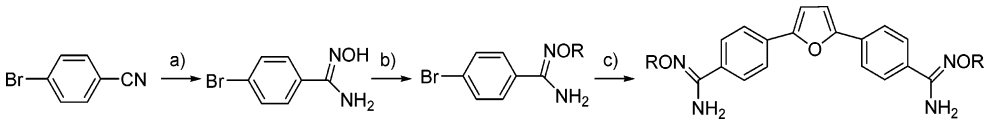
Scheme 1[a]



[a] Reagents and conditions: (a) $NH_2OH \cdot HCl$/KO-*t*-Bu, DMSO; (b)R_2SO_4 or RBr/NaOH, dioxane, 0 °C; (c) 2,5-bis(tri-*n*-butylstannyl)furan, $Pd(P\ Ph_3)_4$.

Biological Results

Table 1 contains the results of both in vitro and in vivo evaluation of these *O*-alkoxyamidine compounds, furamidine (**1**) and 2,5-bis[4-hydroxyamidinophenyl]-furan (**2**), against *T. b. rhodesiense*. As expected, the prodrugs do not exhibit significant in vitro activity due to the absence of the cytochrome P450 and cytochrome b_5 enzymes from trypanosomes needed for bioconversion of the prodrug to the active furamidine.[9−11] The results from the in vivo studies show that significant absorption and bioconversion to furamidine occurs on oral administration of the amidoxime and the *O*-alkoxyamidines when the alkyl group contains three carbons or less (Table 1). It is quite clear that the *O*-*n*-butyl and *O*-*n*-hexyl analogues are not effective, as the average survival time of the treated animals is not significantly different from untreated controls. The *O*-*n*-propyl and *O*-*i*-propyl prodrugs had intermediate activity in vivo, failing to cure all mice at the highest dose tested, but greatly prolonging mean survival time compared to untreated control mice. The *O*-methyl analogue was most active, curing all animals treated orally with 4 × 100 mg/kg/day and greatly prolonging mean survival time of animals treated with 4 × 50 and 4 × 25 mg/kg/day. The *O*-ethyl analogue was nearly as active, curing all animals at the highest dose. However, no animals were cured at 4 × 25 mg/kg/day, and the mean survival time was shortened considerably compared to the *O*-methyl analogue.

In an effort to gain a better understanding behind the in vivo efficacy of these prodrugs, the uptake and metabolism of the *O*-alkoxyamidines were studied in Caco-2 cell monolayers and human liver microsomes, respectively. The transport of a compound across Caco-2 cell monolayers represents an in vitro model for the intestinal absorption of orally administered drugs and thus may predict whether intestinal absorption may be the cause for poor oral activity. Furthermore, the metabolism of the prodrug to its active metabolite must occur at a sufficient rate in order for the prodrug to exert its therapeutic effect. The metabolism of the *O*-alkoxyamidine prodrugs to furamidine was measured in human liver microsomes to gain a better understanding on how the length of the alkyl side-chains may affect the dealkylation of the compounds and thus the in vivo efficacy of the prodrugs.

Tranport across Caco-2 Cell Monolayers. The apparent permeability (P_{app}) of furamidine and its prodrugs was determined across Caco-2 cell monolayers in both the apical (AP) to basolateral (BL) and BL to AP directions. In agreement with previous results, furamidine transport in both directions increased with time and resulted in a low P_{app} value across the cell monolayers ($3.8 \pm 0.2 \times 10^{-7}$ and $8.5 \pm 0.2 \times 10^{-7}$ for AP to BL and BL to AP, respectively).[9,10] At physiological pH, the cationic charges of the diamidine groups impose a barrier for the transcellular diffusion of this compound across the monolayers. A low P_{app} value and high sensitivity to extracellular Ca^{2+} suggests furamidine may be transported via a paracellular route;[9,10] however, the roles of organic cation and other transporters in furamidine transport have not been determined.

Strategies for increasing the oral bioavailability for amidines have focused on masking the positive charges. **3** is an *O*-methoxyamidine prodrug derivative of furamidine which exhibits enhanced oral activity and reduced acute toxicity in animal models for pneumocystosis and trypanosomiasis.[6] The transport in Caco-2 cell monolayers of **3** increased over 100-fold in comparison to furamidine [6.04×10^{-5} ($\pm 8.68 \times 10^{-7}$) and 3.75×10^{-5} ($\pm 1.53 \times 10^{-6}$) for AP to BL and BL to AP, respectively]. These results were consistent with previously reported values and support the hypothesis that **3** is translocated via transcellular passive diffusion.[9,10] P-glycoprotein (P-gp) does not seem to have an effect on the transport of this compound across the monolayers as indicated by no significant difference in the rate of transport for either direction (i.e. AP to BL and BL to AP). The *O*-ethoxyamidine prodrug, **4**, exhibited a slightly lower P_{app} value than did **3** [0.59×10^{-5} ($\pm 1.73 \times 10^{-7}$) and 0.37×10^{-5} ($\pm 1.11 \times 10^{-7}$), AP to BL and BL to AP, respectively.] Again, the transport of **4** showed no significant difference in either the AP to BL or BL to AP directions. The *n*-propyl (**5**), *i*-propyl (**6**), *n*-butyl (**7**) and *n*-hexyl (**8**) *O*-alkoxyamidine prodrugs of furamidine were not transported across the cell monolayers as indicated by no detectable levels of compound in the receiver compartments. These results may help to explain why some of the higher alkyl analogues of these furamidine prodrugs were unable to cure trypanosomal infections in the animal model.

Metabolism of *O*-Alkoxyamidine Prodrugs in Human Liver Microsomes. Thus far, **3** is a promising candidate for treating primary stage trypanosomal infections most likely due to both its increased intestinal absorption in comparison to furamidine and its sufficient rate of metabolism to furamidine.[1,9,10] The metabolism of the *O*-alkoxyamidine analogues of **3** was investigated in human liver microsomes by measuring the loss of the parent compound over time. Quantifying the disappearance of the parent compound measures the first step in the biotransformation of the prodrugs to furamidine and provides some indication of the metabolic stability of these compounds toward dealkylation (an essential and presumably rate-limiting step in the production of furamidine). However, hydroxylation of the *O*-alkyl side-chains (i.e. analogues with side-chains two carbons or more) would also cause a decrease in prodrug concentration and therefore LC-MS analyses was used to identify and quantify the major metabolites formed in the microsomal incubations.

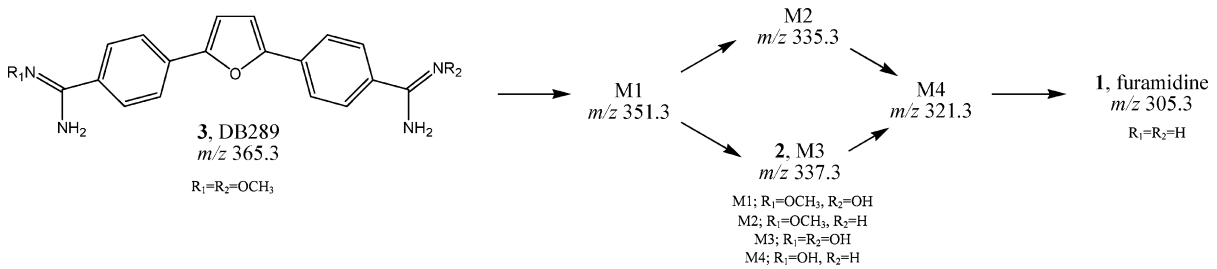


Figure 1. Scheme depicting the metabolism of **3** to furamidine (**1**). Metabolites were identified by LC-MS. The *m*/*z* for each metabolite is provided.

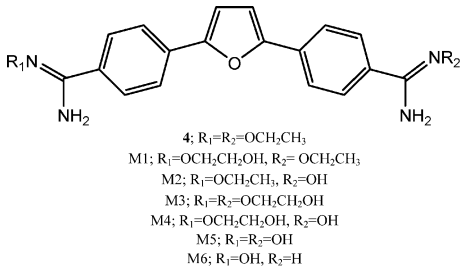


Figure 2. The structure of the most abundant metabolites formed during the metabolism of **4** in human liver microsomes.

The *O*-methoxyamidine prodrug, **3**, was rapidly metabolized in human liver microsomes (HLM) with a microsomal half-life of ca. 4.16 min and was completely metabolized within 40 min. Five metabolites were formed during the metabolism of **3** (Figure 1). LC-MS was used to verify the structure of each metabolite. The methoxyamidine/amidoxime metabolite (M1) reached the highest concentration of all metabolites and was subsequently metabolized to either the diamidoxime (M3) or the methoxyamidine/amidine (M2) metabolites. The concentration of M3 also increased to significant levels and reached a steady-state concentration after 60 min. The amidine metabolites were also identified; however, at much lower concentrations. A detailed description of the metabolism of **3** in both human and rat liver microsomes has been previously noted.[9,11]

The *O*-ethoxyamidine prodrug, **4**, was metabolized less efficiently than its *O*-methoxyamidine analogue with a microsomal half-life of ca. 35 min. More than twelve metabolites were detected during the metabolism of **4** to furamidine in HLM (Figure 2). The metabolites were identified by LC-MS based on their *m*/*z* and their MS^n fragmentation patterns. The three reactions that resulted in the production of these metabolites included (i) hydroxylation of the β-carbon of the ethoxy group, (ii) dealkylation of the ethoxy (or ethoxy-OH) group(s) and (iii) amidoxime reduction to form an amidine group. The *O*-ethoxy side-chain of **4** was rapidly hydroxylated to form M1, reaching a maximum concentration at 20 min and only slightly decreasing thereafter. The dihydroxylated metabolite, M3, was also formed in significant quantities and steadily increased in concentration over the 90-min incubation. The nonhydroxylated and hydroxylated ethoxy side-chains were susceptible to cleavage as indicated by the production of both mono- and diamidoxime metabolites. Low concentrations of the several monoamidine metabolites were also observed; however, their concentrations were much lower than some of the *O*-ethoxyamidine metabolites.

The *O*-*n*-propyl analogue, **5**, was more stable in comparison to both **3** and **4** with a microsomal half-life of ca. 60 min. More than 10 metabolites were detected by LC-MS analysis after 30, 60 and 90-min of incubation. The metabolic transformation of **5** was similar to that of **4** with one additional methylene group of the *O*-*n*-propyl side-chain susceptible to hydroxylation. The *O*-*i*-propyl analogue, **6**, was less susceptible to hydroxylation and dealkylation in comparison to **3**, **4** and **5**. No metabolites were detected after 90 min of incubation with **6**, suggesting that the isopropyl side-chain hindered the direct attack on this molecule by oxidizing enzymes such as cytochrome P450. The *O*-*n*-butyl analogue, **7**, underwent minor metabolism with a greater than 3 h microsomal half-life. Hydroxylation(s) on the butyl side-chain produced the only minor metabolites identified by LC-MS. Metabolic stability of **8** based on microsomal half-life suggested that this *O*-alkoxyamidine analogue was not metabolized at a detectable rate.

In summary, the size of the *O*-alkyl side-chain determined the metabolic stability of the prodrug with the *O*-methyl analogue (**3**) being most susceptible to metabolism and the larger *O*-*n*-butyl and *O*-*n*-hexyl groups least susceptible to metabolism. The production of furamidine requires a cytochrome P450-catalyzed dealkylation of the *O*-alkoxyamidine prodrug with the subsequent reduction of the amidoxime intermediate by a cytochrome b_5/cytochrome b_5 reductase-mediated reaction.[12] However, human liver microsomes are not a favorable environment for reductive reactions, and therefore quantifying furamidine production would most likely not be the best indication for prodrug metabolism to furamidine.

The in vivo studies in the STIB900 mouse model for *T. b. rhodesiense* have shown that *O*-alkoxyamidine prodrugs, where the alkyl chain is less than three carbons, can be effectively used as prodrugs for amidines. As judged from the Caco-2 cell monolayer studies, the *O*-methoxy and *O*-ethoxy analogues are transported at a high rate in comparison to the higher alkyl analogues. The human liver microsome studies demonstrate, at least to a first approximation, that dealkylation of *O*-methoxy, and to a lesser extent, *O*-ethoxy analogues occurs relatively rapidly and is the likely rate-limiting step leading to the bioactive amidines. Taken together, the in vitro transport and metabolism studies are consistent with the in vivo results from the STIB900 mouse model for *T. b. rhodesiense*.

Experimental Section

In Vivo and in Vitro Assays. Antitrypanosomal assays were performed as previously described.[7] Transport of furamidine prodrugs across Caco-2 cell monolayers were performed as previously described.[9,10]

Prodrug Metabolism in Human Liver Microsomes. Furamidine prodrugs were prepared as 5 mM stocks in

methanol prior to each experiment. Reactions consisted of the following: 100 mM potassium phosphate buffer (pH 7.4), 10 μM substrate, 0.2 mg/mL pooled human liver microsomes (GenTest Corp., Woburn, MA) and 1 mM NADPH. Reactions were preincubated for 5 min prior to the addition of NADPH to initiate the reaction and aliquots (250 μL) removed at 0, 5, 10, 15, 20, 30, 45, 60 and 90 min. The reactions were terminated by the addition of 125 μL of acetonitrile and placed on ice. Precipitated protein was removed by centrifugation (15 000 rpm for 4 min) and the supernatant analyzed by HPLC−UV or LC-MS as described previously.[10]

Chemistry. Melting points were recorded using a Thomas-Hoover (Uni-Melt) capillary melting point apparatus and are uncorrected. TLC analysis was carried out on silica gel 60 F_{254} precoated aluminum sheets and detected under UV light. ^{1}H and ^{13}C NMR spectra were recorded employing a Varian GX400 or Varian Unity Plus 300 spectrometer, and chemical shifts (δ) are in ppm relative to TMS as internal standard. Mass spectra were recorded on a VG analytical 70-SE spectrometer. Elemental analyses were obtained from Atlantic Microlab Inc. (Norcross, GA) and are within ±0.4 of the theoretical values. The compounds reported as salts frequently analyzed correctly for fractional moles by water of solvation. In each case proton NMR showed the presence of water. All chemicals and solvents were purchased from Aldrich Chemical Co. or Fisher Scientific. The synthesis of **2** and **4** have been previously reported.[6,8]

Synthesis of *O*-Alkyloxy-4-bromobenzamidoximes Illustrated by the Preparation of *O*-*n*-Propyl-4-bromobenzamidoxime. Hydroxylamine hydrochloride (6.9 g, 100 mmol) was suspended in anhydrous DMSO (50 mL), and the mixture was cooled in an ice bath. KO-*t*-Bu (11.2 g, 100 mmol) was added portionwise under nitrogen atmosphere, and the solution was stirred at room temperature for 1 h. 4-Bromobenzonitrile (1.82 g, 10 mmol) was added to the solution, the reaction mixture was stirred overnight at room temperature and poured into ice−water, and the product was filtered. The 4-bromobenzamidoxime was recrytallized from ethanol. Yield: 1.95 g, 91%. Mp: 144−45 °C; lit. mp 144−145 °C. The 4-bromobenzamidoxime (1.07 g, 5 mmol) was dissolved in dioxane (15 mL) and cooled to 0 °C. A solution of 2 N NaOH (50 mL) was added slowly, followed by dropwise addition of *n*-propyl bromide (923 mg, 7.5 mmol) in dioxane (5 mL). After addition, the ice-bath was removed, and the mixture was stirred at room temperature of 1 h. TLC showed the disappearance of the amidoxime. The mixture was extracted with EtOAc (3 × 50 mL), and the combined organic layers were washed with water, brine and dried over Na_2SO_4. The solvent was removed under reduced pressure. The crude product was purified by passing through a short silica gel column (eluent 5% EA in Hexanes) to yield a pale yellow solid which was recrystallized (hexane).

2,5-Bis (4-*O*-*n*-propyloxyamidinophenyl)furan (5). An oven-dried, 25 mL, round-bottomed flask was charged with 678 mg (1.05 mmol) of 2,5-bis(tri-*n*-butylstannyl)furan and 456 mg (2 mmol) of *O*-*n*-propyl-*p*-bromobenzamidoxime under nitrogen. To that was added 10 mL of anhydrous dioxane and 115 mg of tetrakis(triphenylphosphene)palladium(0), and the mixture was heated at reflux for 16 h. After complete consumption of the amidoxime (determined by TLC), the reaction mixture was cooled, and the solvent was removed using a rotary evaporator. The residue was diluted with EtOAc and filtered through Celite, and the Celite layer was washed with EtOAc. The combined EtOAc layers were washed with water and brine and dried (Na_2SO_4). The solvent was removed under reduced pressure. The crude product was purified by flash chromatography using 35−40% ethyl acetate in hexane to yield 294 mg (70%) of the free base. The hydrochloride salt was obtained by passing hydrochloride gas into an ethanol solution of the free base.

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Supporting Information Available: Elemental analysis, yields, melting point, mass spectral and ^{1}H NMR data for all compounds. Figure 1 metabolism of *O*-alkoxyamidine prodrugs in human liver microsomes. Figures 2 and 3 formation of metabolites from **3** and **4** in human liver microsomes, respectively. This material is available free of charge via the Internet at http://pubs.acs.org.

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