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N-*n*-Alkyl-3,4-Dihydroxybenzamides as Inhibitors of the Trypanosome Alternative Oxidase: Activity In Vitro and In Vivo

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On the basis of our previous demonstration of the high inhibitory activity of a series of *p*-*n*-alkyloxybenzhydroxamic acids and *n*-alkyl esters of 3,4-dihydroxybenzoic acid against the trypanosome alternative oxidase in a cell-free mitochondrial preparation of *Trypanosoma brucei brucei*, we synthesized a series of N-*n*-alkyl-3,4-dihydroxybenzamides for evaluation as inhibitors of this enzyme. This class of compounds was selected with the expectation of their having similar inhibitory activity to but greater solubility than the esters and hydroxamic acids noted above and greater resistance to serum hydrolases in vivo. We predicted that such properties would allow an inhibitor of the trypanosome alternative oxidase to be coadministered with glycerol as a means of providing treatment for infections by African trypanosomes. As expected, such benzamides were both more soluble and more stable, some being more active against the target enzyme than the corresponding ester. One, N-*n*-butyl-3,4-dihydroxybenzamide, was selected for evaluation in vivo against *T. brucei brucei*. When combined with glycerol, this benzamide was found to be curative. A regimen wherein 450 mg of N-*n*-butyl-3,4-dihydroxybenzamide per kg and 15 g of glycerol per kg were given hourly in three divided doses cured 17 of 19 mice with established *T. brucei brucei* infections. This combination is more active in vivo than any other designed to block simultaneously both the unique respiratory electron transport system and the anaerobic glycolytic pathways of these pathogenic protozoa.

The *Trypanosoma brucei* complex, the causative agent of human and animal trypanosomiasis in Africa, has several unusual biochemical pathways, including a mitochondrial terminal oxidase known as the trypanosome alternative oxidase (TAO) (1). This enzyme is the sole terminal oxidase functioning in the life cycle stages found in the mammalian host and, by the following criteria, is similar to the alternative oxidase found in some plants. Both are ubiquinol-oxygen oxidoreductases (1). With a monoclonal antibody against the plant enzyme, Western blots (immunoblots) of *Trypanosoma brucei brucei* show a single band of the same size (35 kDa) as the plant alternative oxidase (3). Furthermore, the two enzymes are sensitive to the same range of specific inhibitors (2).

Since the TAO is an enzyme with no homolog in the mammalian host, it has been the target of a rational approach to the design of drugs for the treatment of African trypanosomiasis. Early work showed that the TAO is sensitive to substituted hydroxamic acids (6). In combination with glycerol, which blocks a glycerol-producing pathway by mass action, salicylhydroxamic acid (SHAM) is trypanocidal (4), although the concentrations required for a cure are toxic to the host (9). In a search for alternatives to SHAM, we designed, synthesized, and tested compounds against TAO activity as measured in a crude mitochondrial preparation of *T. brucei brucei* (5, 7, 8). The most active compounds were *p*-*n*-alkyloxybenzhydroxamic acids and *n*-alkyl 3,4-dihydroxybenzoates, the latter being the more active. In vivo, however, the activity of these compounds was less than that of SHAM, the lead compound (4, 6). This was true despite the fact that SHAM exhibited significantly less activity against the TAO in mitochondrial preparations (5). It was suggested that low solubility and/or hydrolysis, in the case of the esters, contributed to the low in vivo activity since both factors lead to lower bioavailability.

To circumvent these difficulties, we synthesized a series of N-*n*-alkyl-3,4-dihydroxybenzamides. The amide linkage was expected to increase solubility, to provide better stability against serum esterase activity, and to have little or no adverse effect on TAO activity. Here, we present a comparison of these amides and the corresponding esters as well as data showing that at least one of these compounds, N-*n*-butyl-3,4-dihydroxybenzamide, is an effective trypanocide in vivo and results in cures of infected mice when combined with glycerol administration.

MATERIALS AND METHODS

Trypanosomes. *T. brucei brucei* Lab 110 was the same strain as previously used. Growth of the parasites in rats, harvesting by ion-exchange chromatography, and preparation of assay material by Carborundum grinding and differential centrifugation were carried out under the same conditions as previously described (7, 8).

Inhibitor synthesis. The *n*-alkyl 3,4-dihydroxybenzoates were synthesized as previously described (8). The N-*n*-alkyl-3,4-dihydroxybenzamides were synthesized via condensation of the appropriate amines with succinimido 3,4-dihydroxybenzoate or 3,4-dihydroxybenzoyl chloride.

Determination of I_{50} and I_{90}. The concentrations observed to inhibit oxygen uptake by 50 and 90% (I_{50} and I_{90},

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respectively) were taken from curves obtained by measuring alternative oxidase activity in the presence of an inhibitor with glycerol-3-phosphate as the substrate with a Clark-type oxygen electrode. The details of this polarographic assay have been described previously (6).

Solubility measurements. Compounds were dissolved or suspended in 20 mM Tris (pH 7.4) containing 150 mM sodium chloride. After the mixture was stirred at room temperature for 3 h, more compound was added or the suspension was diluted appropriately. The solubilities reported are within a factor of two of the true solubilities. Once the solubility in aqueous buffer was determined, the solubility in buffer containing 1% bovine serum albumin was determined, the latter serving as a better estimate of what might be expected in vivo.

Toxicity determinations. The compounds were placed in wide-mouth glass jars containing several Burundum cylinders (Fisher Scientific, Springfield, N.J.). Normal saline containing 0.25% methylcellulose as a suspending aid was added, after which the jars were placed on a roller mill for 30 min. The resulting solution/suspension was injected intraperitoneally into groups of four female Swiss-Webster mice (20 to 25 g). Step-fold dilutions of each compound were tested until an approximate 50% lethal dose (LD_{50}) was determined, the true single-dose LD_{50} being within ± 25 mg/kg of the value reported.

Stability assessment. Solutions (2 mM) of *n*-butyl 3,4-dihydroxybenzoate and N-*n*-butyl-3,4-dihydroxybenzamide were prepared in 20 mM Tris (pH 7.4) containing 150 mM NaCl. Equal volumes of either the amide or the ester were added to 3-ml aliquots of normal human serum and incubated at 37°C for 0, 0.25, 0.5, 1, 3, 6, or 24 h. At the end of the incubation, 5 drops of 6 N HCl was added and the mixtures were extracted three times with diethyl ether. The combined organic extracts were dried over anhydrous $MgSO_4$, and the solvent was evaporated under reduced pressure. The residues were taken up into 2 ml of methanol (high-performance liquid chromatography [HPLC] grade) and then analyzed via thin-layer chromatography and/or HPLC, the amount of hydrolysis being quantitated by the intensity of the spot or the relative peak height. In the case of thin-layer chromatography, the spots were visualized by spraying with 1% aqueous $FeCl_3$, 1% aqueous $KMnO_4$, or 25% aqueous sulfuric acid (charred).

In vivo assays. Female Swiss-Webster mice averaging 25 g were inoculated intraperitoneally with 0.1 ml of a suspension containing 10^5 trypanosomes. Mice were randomly assigned to treatment groups and monitored daily for parasitemia by observing tail blood smears. Treatment was initiated 3 to 5 days postinoculation, at which time the parasitemia was at least 10^7 parasites per ml of blood. Glycerol was administered by gavage as a 30% solution in water. The mice were not allowed access to water from 1 h prior to the first dose of glycerol until 3 h after the last dose. Three, four, or five doses of glycerol were administered at hourly intervals at the doses indicated. N-*n*-Butyl-3,4-dihydroxybenzamide was administered intraperitoneally in a 1:1 solution of dimethyl sulfoxide-normal saline. Initial doses were administered simultaneously with the initial glycerol dose, with subsequent doses being administered at hourly intervals. Parasitemias were estimated daily. In the initial experiments, animals were considered cured if they remained negative for at least 6 weeks posttreatment. In subsequent experiments involving the optimal dosage, animals were monitored for 16 weeks. Relapsing mice were sacrificed.

TABLE 1. Inhibition of the TAO by *n*-alkyl esters and amides derived from 3,4-dihydroxybenzoic acid[a]

Alkyl chain length	Ester		Amide	
	I_{50}	I_{90}	I_{50}	I_{90}
2	15	54	42	368
4	5.5	22	4	9.4
6	0.5	5.6	2.5	11
8	0.6	4.3	0.6	4.5
10	0.3	3.3	0.32	0.62

[a] Values are millimolar concentrations which inhibit the oxidase by 50 and 90%, respectively.

RESULTS

Table 1 presents the concentrations of the 3,4-dihydroxybenzoates and corresponding N-*n*-alkyl benzamides required for 50 and 90% inhibition of the TAO. As expected, the results show an increase in the activity of the amides as the length of the *n*-alkyl substituent increases, a similar effect having been observed previously in the case of the corresponding esters. However, there does not appear to be a consistent relationship between the activity of the amides and that of the esters. For example, on the basis of I_{90}s, the *n*-butyl amide was more than twice as active as the corresponding ester. The activity of the *n*-decyl amide was characterized by a sharply curved Dixon plot such that the I_{90} was reached at approximately twice the I_{50}. Finally, the *n*-hexyl amide was somewhat less active than both the corresponding ester and the *n*-butyl amide. These differences may result from multiple factors, including aqueous solubility, susceptibility to hydrolysis, and conformational changes.

The aqueous solubilities of the amides and esters are shown in Table 2. Increasing the length of the *n*-alkyl chain decreased the solubilities of both series of compounds, with the amides being at least twice as soluble as the corresponding esters. The addition of 1% albumin to selected solutions increased the solubility by approximately a factor of two (data not shown).

The esters and amides would not be expected to have the same stability when incubated in serum. In three separate experiments, there was no hydrolysis of N-*n*-butyl-3,4-dihydroxybenzamide (1 mM) after incubation for 24 h at 37°C in 50% buffered serum. Incubation of the corresponding ester at the same concentration resulted in significant hydrolysis in as little as 1 h, with 70% hydrolysis being observed after 24 h (data not shown). Although a number of compounds were present in the serum extracts as evidenced by both thin-layer chromatography (chromatograms were charred with sulfuric acid) and HPLC analysis, only two

TABLE 2. Solubility in 0.9% NaCl–20 mM Tris (pH 7.4) of *n*-alkyl esters and amides derived from 3,4-dihydroxybenzoic acid

Alkyl chain length	Solubility (mM) of:	
	Ester	Amide
2	19.0	ND[a]
4	2.0	5.0
6	0.050	0.5
8	0.025	0.125
10	0.0125	0.025

[a] ND, not determined.

TABLE 3. Toxicity of *n*-alkyl esters and amides derived from 3,4-dihydroxybenzoic acid

Alkyl chain length	LD_{50} (mg/kg) of:	
	Ester	Amide
2	162	ND[a]
4	200	300
6	500	250
8	800	500
10	700	600

[a] ND, not determined.

spots were visualized with 1% ferric chloride, the ester and the parent acid, 3,4-dihydroxybenzoic acid (data not shown).

The approximate LD_{50}s of the two groups of compounds are shown in Table 3. No attempt was made to determine LD_{50}s when they exceeded 800 mg/kg, this being arbitrarily considered the upper limit for toxicity. As might be expected from the solubility data, the toxicity of both groups of compounds decreased with increasing alkyl chain length. Nonetheless, some striking differences between the two groups were noted. N-*n*-Butyl-3,4-dihydroxybenzamide was less toxic than the corresponding ester, the LD_{50} being approximately one and a half times that of the *n*-butyl 3,4-dihydroxybenzoate. The N-*n*-hexyl derivative, which has relatively low activity against the alternative oxidase, was twice as toxic as the *n*-hexyl ester. When the length of the alkyl chain was increased to 8 or 10 carbon atoms, toxicity decreased over that of the hexyl compound, with the amides being somewhat more toxic than the corresponding esters.

Taking into consideration all of the activity, solubility, stability, and toxicity data, we chose to test N-*n*-butyl-3,4-dihydroxybenzamide for trypanocidal activity in vivo. The results are shown in Table 4. A matrix was set up for multiple-dose regimens of both glycerol and the butyl amide. In the three trials with single doses of the amide in combination with glycerol at a total dose of 10 g/kg, the only amide dose effecting any cures was the highest, 450 mg/kg. In the absence of glycerol, this dose of amide was ineffective even in temporarily reducing the parasitemia. When combined with glycerol, all doses of the amide tested markedly reduced parasitemia. When the total glycerol concentration was increased and butyl amide at 450 mg/kg was given in multiple doses, the cure rate increased without increased toxicity. The best cure rate (17 of 19 mice) was obtained with a total of 450 mg of amide per kg and 15 g of glycerol per kg given in three equal hourly doses. In general, efficacy decreased as the total dose or dose per administration increased beyond these values. Increasing the glycerol dose to 20 g/kg or spreading out the total dose of glycerol over 5 h to maintain higher glycerol levels in blood did not increase effectiveness, even when the total dose of butyl amide was raised to 500 mg/kg administered in divided doses over the same period.

DISCUSSION

We synthesized and examined a series of N-*n*-alkyl-3,4-dihydroxybenzamides as potential chemotherapeutic agents for the treatment of infections caused by *T. brucei brucei* and, by extension, other African trypanosomes. As was the case for the *p*-alkyloxybenzhydroxamic acids and *n*-alkyl 3,4-dihydroxybenzoates, increasing the alkyl chain length increased activity against the target enzyme when measured in a cell-free mitochondrial preparation. A number of factors undoubtedly contribute to relative effectiveness both within and among the different classes of compounds. As predicted, the benzamides proved to be more soluble than the corresponding hydroxamic acids and esters and more stable than the esters when incubated in serum. Two compounds, N-*n*-butyl-3,4-dihydroxybenzamide and N-*n*-decyl-3,4-dihydroxybenzamide, proved to be more active than the corresponding esters against the TAO in a cell-free preparation; compounds of intermediate chain length behaved in the opposite manner. Compared with N-*n*-decyl-3,4-dihydroxybenzamide, N-*n*-butyl-3,4-dihydroxybenzamide was 15 times less active against the target enzyme and its LD_{50} was approximately 50% lower but the compound was 200 times more soluble. Considering all of the data, we chose N-*n*-butyl-3,4-dihydroxybenzamide for in vivo evaluation as a trypanocidal agent.

Trials with mice infected with *T. brucei brucei* proved that N-*n*-butyl-3,4-dihydroxybenzamide is an effective trypanocidal agent when administered intraperitoneally together with oral administration of glycerol. The best trypanocidal activity was obtained with 450 mg of amide per kg and 15 g of glycerol per kg administered in three divided doses at

TABLE 4. In vivo trypanocidal activity of N-*n*-butyl-3,4-dihydroxybenzamide in combination with glycerol

Glycerol dose (g/kg) at:					Total glycerol dose	Butyl amide (mg/kg)[a]	No. of mice:		No. of deaths	Total no. of mice
0 h	1 h	2 h	3 h	4 h			Cured	Relapsed		
0	0	0	0	0	0	1 × 450	0	2[b]	3	5
5	2.5	2.5	0	0	10	1 × 150	0	3	2	5
5	2.5	2.5	0	0	10	1 × 300	0	3	2	5
5	2.5	2.5	0	0	10	1 × 450	1	0	4	5
5	2.5	2.5	0	0	10	4 × 150	1	3	2	6
5	5	5	0	0	15	3 × 150	17	2	0	19
5	5	5	5	0	20	3 × 150	10	3	2	15
5	5	5	0	0	15	4 × 150	12	0	3[c]	15
5	5	5	0	0	15	3 × 200	7	1	8	16
5	2.5	2.5	2.5	2.5	15	5 × 100	0	0	5	5
5	2.5	2.5	2.5	2.5	15	3 × 150	0	1	5	6
5	5	5	5	5	25	3 × 200	0	0	6	6

[a] The butyl amide was given at hourly intervals beginning with the first dose of glycerol.
[b] Parasitemia never decreased in this group.
[c] One death occurred on the third day after treatment.

hourly intervals. Under these conditions, 17 of 19 treated mice were cured. No increase in the cure rate was obtained upon increasing the amount of amide either by increasing the size (3 × 200 mg/kg) or number (4 × 150 mg/kg) of doses. Such increases in dosage actually reduced the efficacy, most likely because of toxicity. The dose and timing of glycerol administration were also important; neither administering the same dose over a longer period of time nor increasing or decreasing the total dose improved the cure rate.

Our research group has previously synthesized and tested many hundreds of compounds, several hundred of which were active against the TAO. Some had exquisite sensitivity for the target enzyme when measured in a cell-free mitochondrial preparation. Representative examples of these have been reported (5–7). In vivo, however, very few were active and none were more active than SHAM. In fact, until now no inhibitor of the TAO with greater activity against African trypanosome infections than SHAM has been reported. While the therapeutic ratio for a 1-day treatment with the combination of N-*n*-butyl-3,4-dihydroxybenzamide and glycerol is less than optimal, it is significantly better than that obtained with SHAM and glycerol when administered in the same manner. This has important implications for the eventual development of a practical therapy. The efficacy of this combination no doubt could be improved by fine-tuning the dosage regimen, as was done by others with SHAM and glycerol (9). Furthermore, repeated treatments with lower doses over an extended period of time would undoubtedly improve the therapeutic index. For example, in previously unpublished work we used a relatively low intravenous dose of SHAM (319 mg/kg) and glycerol (0.92 g/kg) twice weekly for 7 weeks to treat five rabbits with established late-stage or central nervous system infections of *T. brucei brucei*. Two of the five were cured despite the fact that we were not able to devise a regimen that produced cures in a single day. Although it may be possible to improve the success rate with N-*n*-butyl-3,4-dihydroxybenzamide by careful modulation of the dosage regimen, we do not consider this compound to be a likely clinical candidate. The present results will be used only to guide future efforts to identify compounds more likely to be of clinical utility.

Our current success in using energy inhibition as a means of treating *T. brucei brucei* infections was due in part to consideration of predicted pharmacokinetic and xenobiotic properties in the design of these new inhibitors of the TAO. If a well-tolerated and more active inhibitor of the TAO is identified, combination of that inhibitor with glycerol may provide a total cure of African trypanosomiasis. Furthermore, a short course of treatment may be possible since the mechanism of action involves a complete block of energy production by the parasite, leading to rapid and total parasite lysis (4). This would be a marked improvement over current therapies that usually require multiple treatments over several weeks.

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