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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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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