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Letters

Specific Inhibition of a Family 1A Dihydroorotate Dehydrogenase by Benzoate Pyrimidine Analogues

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Abstract: Dihydroorotate dehydrogenases (DHODs) catalyze the conversion of dihydroorotate to orotate in de novo pyrimidine biosynthesis. We have found that 3,4-dihydroxybenzoate and 3,5-dihydroxybenzoate are competitive inhibitors vs dihydroorotate with the prototypical family 1A DHOD from *Lactococcus lactis*. The dissociation constants of these compounds, determined by spectral titrations, were similar to the dissociation constant of orotate, the enzymatic reaction product, suggesting that hydroxybenzoates could be developed into useful drugs for treating infections by certain protozoan parasites.

Introduction. Pyrimidines are vital precursors in biochemistry. Consequently, the pyrimidine biosynthetic pathway offers attractive targets for drug design. Dihydroorotate dehydrogenase (DHOD; E.C. 1.3.3.1) catalyzes the only redox reaction in pyrimidine biosynthesis, the oxidation of dihydroorotate (DHO) to orotate.[1] DHO is oxidized by an enzyme-bound flavin mononucleotide prosthetic group, which is subsequently reoxidized by a substrate whose identity depends on the type of DHOD. DHODs have been classified into two families, designated family 1 and family 2.[2] Two groups exist within the family 1 DHODs: the family 1A enzymes appear to utilize fumarate as their physiological oxidant,[3] while the family 1B enzymes utilize NAD through the intermediacy of a second protein subunit containing an Fe_2S_2 cluster and a FAD.[4] The family 1 DHODs are cytoplasmic enzymes. The type-B enzyme appears to be prevalent within Gram-positive bacteria, while some express both the A and B enzymes. In contrast, the 1A form of the enzyme appears to be the sole form in the few eukaryotes, such as *Leishmania mexicana*, that express a family 1 DHOD. Most Gram-negative bacteria and most eukaryotes have family 2 DHODs, which are membrane-bound enzymes that use respiratory quinones as their physiological oxidants.[5]

DHOD has been the target in a number of drug development efforts aimed at treating cancer, malaria, gastric ulcers, and rheumatoid arthritis.[6−9] The most successful inhibitors of DHOD have been directed against the hydrophobic tunnel that serves as the quinone binding site in the family 2 enzymes. These inhibitors bind in a site that is distinct from the pyrimidine binding site.[10] Enough diversity between species exists in the quinone binding site that species-specific family 2 DHOD inhibitors have been designed.[9] Perhaps the most prominent example is Leflunomide (Arava), a pro-drug that decomposes into a tight-binding inhibitor of human DHOD,[8] which has been approved for the treatment of rheumatoid arthritis.

While it is feasible to develop specific inhibitors of the family 2 DHODs by targeting the quinone binding site, an analogous quinone binding site does not exist in the family 1 DHODs. In this report we describe the inhibition of the family 1A DHOD from *Lactococcus lactis* (designated DHOD A) by hydroxybenzoates that act as analogues of orotate, the pyrimidine product of the enzymatic reaction. We found that these inhibitors had little effect on the family 2 DHODs from *Escherichia coli* or *Homo sapiens*, or the family 1B enzyme from *L. lactis*, suggesting that it is possible to develop inhibitors acting at the pyrimidine binding site that are selective for the family 1A DHODs. Such an approach should prove valuable in the therapy of diseases caused by protozoan parasites such as *Leishmania* and *Trypanosoma*.

Results. (a) Inhibition. The effects of a series of hydroxyaromatics at 1 mM concentration on catalysis

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Table 1. Survey of Possible Inhibitors

compound	% activity[a]
salicylate	81 ± 3
p-hydroxybenzoate	92 ± 5
6-hydroxynicotinate	94 ± 6
2,4-dihydroxybenzoate	91 ± 6
3,4-dihydroxybenzoate	7 ± 1
3,5-dihydroxybenzoate	6 ± 0.3

[a] The activity of the enzyme was determined at 25 °C by observing the reduction of DCIP at 600 nm, using 32 μM DHO, 33 nM in DHOD A active sites, and a 1 mM concentration of the compound in 0.1 M Tris-HCl, 0.1 mM EDTA, pH 8.0. Percent activities were calculated for the average of at least three assays. The error range represents the standard deviation. The uninhibited reaction rate, defined as 100% activity, was 0.17 μM s^{-1}.

Table 2. Inhibition and Binding Constants

compound	K_d (μM)[a]	K_i (μM)[b]
3,4-dihydroxybenzoate	19 ± 2	12 ± 2
3,5-dihydroxybenzoate	18.4 ± 0.2	21 ± 4

[a] Dissociation constants were determined in static spectral titrations from the change in flavin absorbance at 25 °C, pH 8.0. [b] Inhibition constants were determined by globally fitting initial reaction velocities to a competitive inhibition model. The concentration of DHO was varied from 2 μM to 1 mM, and the concentration of inhibitor was varied from 0 to 100 μM.

by the family 1A DHOD from *L. lactis* were assessed in steady-state assays (Table 1). This survey identified 3,4-dihydroxybenzoate (3,4-diOHB) and 3,5-dihydroxybenzoate (3,5-diOHB) as inhibitors of DHOD A, while other hydroxybenzoate compounds were not effective. In contrast, 3,4-diOHB inhibited *E. coli* and human DHODs only slightly, to 81% and 74% of their uninhibited levels, respectively, and 3,5-diOHB did not inhibit at the 1 mM level. Neither 3,4-diOHB or 3,5-diOHB inhibited the family 1B enzyme from *L. lactis*. 3,4-DiOHB and 3,5-diOHB were studied in further detail by measuring the rate as the concentrations of both DHO and the inhibitor were varied. An intersecting double-reciprocal pattern was obtained for different concentrations of each inhibitor, indicating that the inhibitors were competitive for the DHO binding site (see Supporting Information). The data were fit globally to a competitive model in order to obtain the inhibition constants listed in Table 2.

(b) Ligand Binding. Aliquots of concentrated solutions of monohydroxybenzoates (salicylate and *p*-hydroxybenzoate), dihydroxybenzoates (2,4-dihydroxybenzoate, 3,4-diOHB, and 3,5-diOHB), 3,4,5-trihydroxybenzoate, and 6-hydroxynicotinate were added to solutions of the family 1A DHOD from *L. lactis*, giving a final concentration of 2 mM. Large changes in the absorbance spectrum of the enzyme-bound flavin were observed upon adding either 3,4-diOHB or 3,5-diOHB (Figure 1), indicating that these compounds were binding at the active site of the enzyme. 3,5-DiOHB is an isosteric analogue of orotate, the pyrimidine product of the enzymatic reaction, and caused the flavin absorbance peak to shift from 454 nm in the free enzyme to 469 nm in the complex. The binding of 3,4-dihydroxybenzoate caused a substantial decrease in absorbance in the 450 nm region and also produced a charge-transfer absorbance band with a peak at 635 nm. Charge-transfer absorbance bands are frequently observed in flavoprotein complexes when an electron-rich donor ligand makes contact with the electron-deficient oxidized flavin. 3,4,5-Trihydroxybenzoate (gallate) also

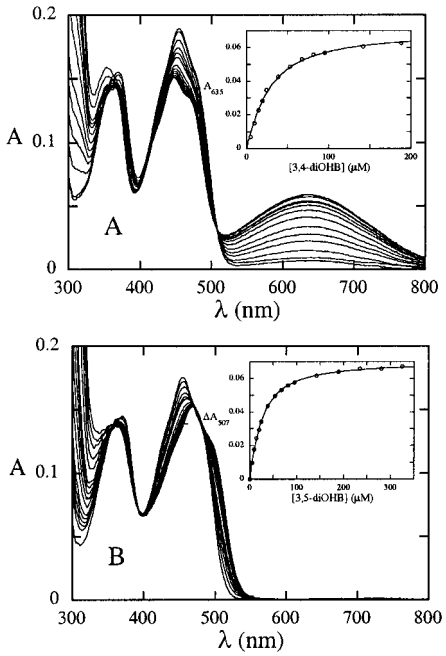

Figure 1. Determination of ligand dissociation constants. Solutions of DHOD A in 0.1 M Tris-HCl, 0.1 mM EDTA, pH 8.0, were titrated at 25 °C with concentrated ligand solutions, and the absorbance spectra were recorded. (A) The titration of 16.5 μM DHOD with 3,4-diOHB caused the development of a charge-transfer band centered at 635 nm. The charge-transfer absorbance was used to calculate a dissociation constant of 19 ± 2 μM (inset). (B) Titration with 3,5-diOHB caused a large bathochromic shift, similar to that reported for pyrimidine ligands. A dissociation constant of 18.4 ± 0.2 μM was obtained (inset).

caused large spectral changes, including a charge-transfer band centered at 650 nm. However, owing to the instability of gallate in aerobic solution, the behavior of this compound was not studied further. The large spectral changes accompanying the formation of the 3,4-diOHB and 3,5-diOHB complexes provided a convenient means for monitoring ligand binding and determining the dissociation constants of each ligand (Figure 1 and Table 2). Both ligands had affinities (18−19 μM) for the family 1A enzyme that were similar to that of orotate (13 μM) determined previously.[2]

In contrast, the addition of these hydroxybenzoates to concentrations up to 2 mM had no effect on the absorbance spectra of the *E. coli* or human DHODs, suggesting that these compounds did not bind to the family 2 enzymes. Alternatively, it is conceivable that these compounds bind to family 2 enzymes without causing any spectral change. This possibility was addressed in competitive binding experiments, in which the family 2 enzyme−orotate complex was titrated with 3,4-diOHB. The orotate complexes of the family 2 DHODs have a red-shifted flavin absorbance spectrum. If 3,4-diOHB were to displace orotate, a large change in the absorbance spectrum would be observed. After correcting for dilution, there was no detectable change in the absorbance spectrum of the orotate complexes of the family 2 enzymes after the addition of 3,4-diOHB, indicating that this ligand did not compete for the pyrimidine binding site of the family 2 enzyme.

Discussion. Currently there are no drugs that specifically inhibit family 1 DHODs. Some eukaryotic mi-

crobes, such as *Trypanosoma cruzi* and *Leishmania mexicana*, express a family 1A DHOD to synthesize pyrimidines and lack a pyrimidine salvage pathway, making them entirely dependent on de novo synthesis. New therapeutic agents that act specifically on family 1A DHODs could, therefore, be useful in treating diseases that are prevalent in tropical climates such as African sleeping sickness (estimated 400 000 cases each year) and leishmaniasis (estimated 2 million cases each year). The binding of the dihydroxybenzoates studied here suggests that these compounds could serve as lead compounds for agents that block pyrimidine synthesis. The minimal inhibition of the family 1B and family 2 DHODs suggests that a high degree of specificity for the family 1A DHODs is possible, so that deleterious side effects caused by the inhibition of pyrimidine synthesis in humans would be avoided.

The likely mode of dihydroxybenzoate binding to DHOD A from *L. lactis* can be inferred from the structure of the orotate complex (Figure 2A).[11] The carboxylate of 3,5-diOHB is expected to make the same hydrogen bonding interactions with the protein backbone (not indicated in the figure) and the side chain of Lys 43 that orotate makes (Figure 2 B). In this orientation, the two phenolic oxygens of 3,5-diOHB would occupy the positions of the two carbonyl oxygens of orotate in the crystal structure, forming hydrogen bonds with the side chains of Asn 127, Ser 194, and possibly Asn 67. Hydrogen bonds analogous to those between the enzyme and the pyrimidine nitrogens of orotate are not possible with the inhibitor. 3,4-DiOHB would also bind in the same position as orotate but would interact somewhat differently with the protein. Asn 193 could form a hydrogen bond to the 4-hydroxyl of the 3,4-diOHB if the side chain moves a short distance further from the ligand binding site in order to accommodate the substituent. The position of the 3-hydroxyl of 3,4-diOHB in the complex cannot be predicted with certainty, because hydrogen bonds are possible with either Asn 127, or, by rotation around the C1−C4 axis, with Ser 194 and Asn 67, and perhaps both conformations are populated significantly. X-ray diffraction will be used to verify the structures of both enzyme−inhibitor complexes.

The origin for the selective inhibition by 3,4-diOHB and 3,5-diOHB is not evident from the crystal structures of the enzymes used in these studies. Very similar enzyme−orotate interactions were observed in the structures of the two *L. lactis* enzymes and the human and *E. coli* enzymes.[10−13] The large red-shift in the flavin absorbance caused by orotate[2,14] or DHO[15] is a common response of all DHODs and indicates a ligand−flavin interaction; its absence in the family 1B and family 2 enzymes indicates that neither 3,4-diOHB nor 3,5-diOHB bind to the pyrimidine binding site. Given the sensitivity of our absorbance measurements, we can set a lower limit of 4 kcal mol^{-1} for the difference in binding energies of the two inhibitors between the family 1A enzyme and the other enzymes, and the actual value is likely to be greater. The weak inhibition of the family 2 enzymes by 3,4-diOHB could be due to binding at the quinone binding site. In the presence of natural quinone oxidizing substrates, we expect 3,4-diOHB to be an ineffective inhibitor.

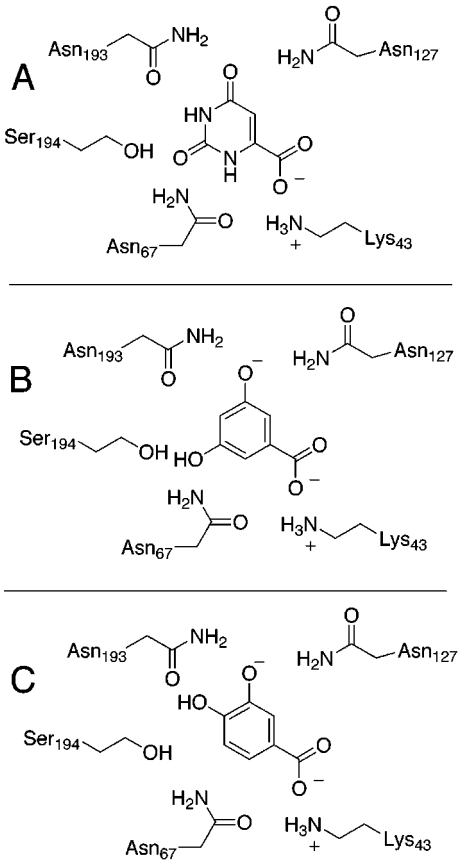

Figure 2. Enzyme−ligand interactions. (A) The interactions between orotate and DHOD A are shown in schematic fashion based on the 2.0 Å resolution crystal structure.[11] The flavin of the enzyme is not represented in the diagram for the sake of clarity, but would lie beneath the ligand, with the isoalloxazine moiety in van der Waals contact and roughly parallel to the pyrimidine ring of orotate. In the orotate complex, the amides of Asn 67 and Asn 193 act both as hydrogen bond donors and acceptors. (B) The likely mode of binding for 3,5-diOHB is shown, in which orotate has been replaced by the inhibitor. (C) A possible 3,4-diOHB-DHOD charge transfer complex is shown. When 3,4-diOHB is bound (C), the 4-hydroxyl would be in steric conflict with Asn 193, so that we expect this residue to move slightly in this complex. In both B and C, hypothesized phenolate forms of the inhibitors are oriented to place a negative charge in the same position that the negative charge of an enolate reaction intermediate would occupy during the oxidation of DHO.

We hypothesize that the differential binding of hydroxybenzoates originates from differences in interactions that the enzyme uses to stabilize the transition states for the reduction of the flavin by DHO in the enzyme families. Two mechanisms for DHO oxidation—concerted deprotonation of C5 and hydride transfer from C6, or the stepwise deprotonation of C5 followed by oxidation of the enolate intermediate—have been proposed. It was suggested that an enolate intermediate was formed in a family 1A enzyme[16] but that a family 1B enzyme and a family 2 enzyme oxidized DHO in a concerted mechanism.[17,18] An enolate intermediate, or a transition state with significant enolate character, would develop considerable negative charge on the 4-carbonyl oxygen of the pyrimidine, and it is reasonable to expect that the enzyme accelerates the reaction by stabilizing this negative charge.[19] In contrast, the charge development in a concerted mechanism would

be lower. Interestingly, 3,4-diOHB and 3,5-diOHB inhibit only the family 1A enzyme, which presumably oxidizes DHO in a stepwise fashion. The phenolic oxygens of the dihydroxybenzoate inhibitors could mimic the charge distribution of an intermediate in DHO oxidation by ionizing to the phenolate. We are currently investigating whether these ligands are bound in their phenolate form by conducting extensive binding studies, but presumably 3,4-dihydroxybenzoate does bind as the phenolate, because it forms a charge-transfer complex upon binding, and the phenolate has been shown to be required in other enzymes for flavin-phenol charge-transfer interactions.[20] Thus, the possibility that hydroxybenzoates act as transition state inhibitors of the family 1A enzymes offers the potential for developing novel therapeutic agents.

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Supporting Information Available: Experimental details and double-reciprocal plots. This material is available free of charge via the Internet at http://pubs.acs.org.

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