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Selective Inhibition of Bacterial Dihydroorotate Dehydrogenases by Thiadiazolidinediones

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ABSTRACT. Dihydroorotate dehydrogenase is a critical enzyme of *de novo* pyrimidine biosynthesis in prokaryotic and eukaryotic cells. Differences in the primary structure of the enzymes from Gram-positive and -negative bacteria and from mammals indicate significant structural divergence among these enzymes. We have identified a class of small molecules, the thiadiazolidinediones, that inhibit prototypical enzymes from Gram-positive and -negative bacteria, but are inactive against the human enzyme. The most potent compound in our collection functioned as a time-dependent irreversible inactivator of the bacterial enzymes with k_{inact}/K_i values of 48 and 500 M^{-1} sec^{-1} for the enzymes from *Escherichia coli* and *Enterococcus faecalis*, respectively. The data presented here indicate that it is possible to inhibit prokaryotic dihydroorotate dehydrogenases selectively while sparing the mammalian enzyme. Thus, this enzyme may represent a valuable target for the development of novel antibiotic compounds. BIOCHEM PHARMACOL **60**;3:339–342, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. thiadiazolidinedione; dihydroorotate dehydrogenase; *Enterococcus faecalis*; *Escherichia coli*; inhibition; time-dependent inactivation

The recent emergence of pathogenic bacteria that are resistant to current antibiotic therapies has prompted renewed interest in antibiotic research with the aim of identifying novel molecular targets for new antibacterials. New agents might help ameliorate the resistance problem by expanding the range of molecular mechanisms of cell killing available to the clinician. Antimetabolites have long been known to be effective mechanisms for halting cell growth. In some cases, the resulting buildup of metabolic intermediates causes not only cell growth arrest but also cell killing. This has been shown to be the case when *de novo* pyrimidine biosynthesis is eliminated by genetic disruption of key enzymes in this metabolic pathway [1].

The enzyme DHODase¶ (EC 1.3.3.1) is a critical enzyme in the *de novo* pathway of pyrimidine synthesis in both prokaryotic and eukaryotic cells. The proteins responsible for this enzymatic activity are, however, quite distinct among different species [2]. Thus, at the level of amino acid sequence, mammalian DHODase is distinct from prokaryotic DHODases, and the prokaryotic enzymes generally are distinguishable between Gram-positive and -negative bacteria. The antiproliferative compounds brequinar sodium [3] and leflunomide [4] function by potent inhibition of mammalian DHODase and have shown utility in treating hyper-proliferative diseases in humans. A distinguishing feature of these compounds is that they inhibit mammalian DHODase selectively, while showing no effects on Gram-positive or -negative bacterial enzymes ([5], and unpublished results). We reasoned that this inhibitor selectivity was based on the structural distinctions among these enzymes and, hence, wondered if reversed selectivity (i.e. inhibitors of the bacterial enzymes that do not inhibit the human enzyme) could be realized with small molecule inhibitors. We report here the discovery of a class of compounds, the thiadiazolidinediones (Fig. 1), that are potent inhibitors of representative Gram-positive and -negative bacterial DHODases but show no inhibition of the human enzyme. This general class of compounds has been reported previously to be effective for killing bacterial cells in industrial applications [6, 7].

MATERIALS AND METHODS

Recombinant DHODases from humans [8], *Escherichia coli* [9], and *Enterococcus faecalis* [10] were expressed and purified essentially as described previously. In the case of the human and *E. coli* enzymes, the recombinant construct was appended with an N-terminal $(His)_6$-tag, and purification was achieved in two chromatographic steps (Ni-affinity and

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¶ *Abbreviations*: DCIP, 2,6-dichloroindophenol; and DHODase, dihydroorotate dehydrogenase.

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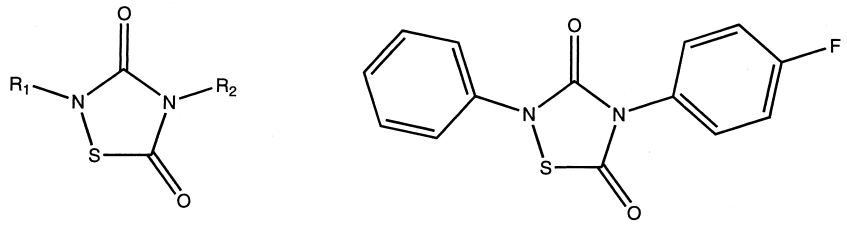

Generic Thiadiazolidinedione **1**

FIG. 1. (Left) Generic chemical structure of thiadiazolidinedione inhibitors of bacterial DHODases. (Right) Chemical structure of compound 1.

S-200 gel filtration). All chromatographic elution buffers included 0.1% Triton X-100. In all cases, the purity of the final enzyme samples was >90% as assessed by SDS–PAGE with Coomassie Blue staining. The specific activities of the final enzyme samples in all cases were comparable to those previously reported for these enzymes.

Initial screening of our chemical library was performed with a nitroblue tetrazolium-based colorimetric assay in 96-well microtiter plates, as described previously [8]. The assay buffer for screening of all the enzymes contained 0.1% Triton X-100 to help solubilize the formazan product of nitroblue tetrazolium, and to minimize artifactual differences in inhibition between the enzymes due to compound partitioning into detergent micelles. A DCIP-based colorimetric assay [8, 10], following reduction of the dye at 600 nm (ϵ = 20,000 M^{-1} cm^{-1}), was used to confirm the inhibitory effects of compounds identified in the primary screening assay. The DCIP-based assays for human and *E. coli* DHODases included 0.1 mM L-dihydroorotate, 0.1 mM coenzyme Q_6, and 0.05 mM DCIP, whereas coenzyme Q_6 was omitted from the *E. faecalis* activity assay, since the latter enzyme is able to reduce the dye directly.

Time-dependent inactivation assays were performed by preincubating 0.5 μM enzyme solution (a 1:4 dilution of the initial enzyme stock solution, resulting in a Triton X-100 concentration in the preincubation mixture of 0.025% for the human and *E. coli* enzymes) with a 0.5 to 10 μM concentration of the compound. Aliquots of 10 μL were taken at different time points and diluted into a 0.2-mL activity assay, containing 0.1 M Tris (pH 7.5), 0.1 mM coenzyme Q_6 with 0.1% Triton X-100 (for human and *E. coli* enzymes), 0.05 mM DCIP. The reaction was initiated by adding dihydroorotate to a final concentration of 0.1 mM. The resulting steady-state velocity was plotted as a function of preincubation time and fit to a first-order equation to obtain estimates of k_{obs} at several inhibitor concentrations.

For initial screening, compounds (at a nominal concentration of 10 μM) were preincubated with the enzyme for *ca.* 5 min before initiating the enzymatic reaction by addition of substrates. A library in excess of 150,000 compounds from the DuPont Pharmaceuticals screening collection was used for initial screening. Compounds were used as available in the library without further purification or confirmation of composition. A compound was scored as an inhibitor if it reduced the activity of the enzyme by more than 3 standard deviations from the assay mean. The inhibitory activity of such compounds then was confirmed at least twice more in independent assays, as described above.

From the screening hits, compound **1** [2-phenyl-4-(4-fluorophenyl)-1,2,4-thiadiazolidine-3,5-dione; see Fig. 1] was chosen for detailed kinetic studies. This compound was prepared from *p*-fluoroisothiocyanate and phenylisothiocyanate using the method of Ottmann and Hooks [11]. An existing sample was purified by preparative HPLC or recrystallized from ethanol (m.p. 140–141°). High resolution mass spectrometry (NH_3-Cl) yielded a mass (M+H) of 289.0447 Da, consistent with the calculated mass for $C_{14}H_{10}N_2O_2FS$ of 289.0444 Da. Buffers, cofactors, detergents, and all other reagents were of the highest grades commercially available.

RESULTS AND DISCUSSION

Screening Results

Screening of a library in excess of 150,000 compounds revealed several examples of thiadiazolidinediones (Fig. 1) that inhibited the Gram-positive, the Gram-negative, or both bacterial enzymes at 10 μM. These compounds generally showed weak (<20%) or no inhibition of the human enzyme at concentrations as high as 100 μM. A structure–activity relationship was apparent among these compounds in that the most effective inhibitors of both the Gram-positive and -negative bacterial enzymes contained at least one aromatic substituent at R_1 or R_2 (data not shown). Compound **1** was one of the two most potent inhibitors found in this structural class, and was available in large quantities; therefore, this compound was selected for further study. After HPLC purification, **1** displayed initial IC_{50} values (i.e. with a 15-min preincubation of the inhibitor with enzyme) of 0.84 ± 0.11 and 0.075 ± 0.005 μM for the *E. coli* and *E. faecalis* enzymes, respectively. In contrast, compound **1** showed zero inhibition of the human enzyme at 100 μM.

Kinetic Analysis

Kinetic analysis [12] revealed that compound **1** behaved as a slow-binding inhibitor of the bacterial enzymes (Fig. 2).

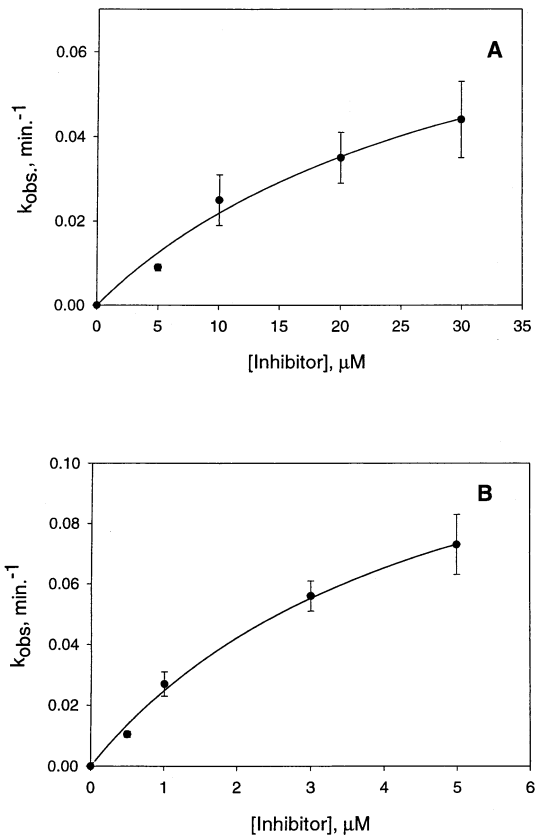

FIG. 2. Observed rate constant for enzyme inactivation as a function of inhibitor concentration for compound 1. (A) Against *E. coli* DHODase. (B) Against *E. faecalis* DHODase. The observed rate constant at each inhibitor concentration was determined by preincubating the enzyme (0.5 μM) with inhibitor for various lengths of time before initiating the enzymatic reaction with substrate, and fitting a plot of the residual activity as a function of preincubation time to a first-order decay of activity equation [12]. The error bars represent the iteratively calculated standard error values from the first-order decay equation using least squares methods (SigmaPlot 5.0).

Analysis of the rate of inactivation as a function of inhibitor concentration yielded values of the second-order inactivation rate constant, k_{inact}/K_i, of 48 ± 23 and 500 ± 121 M^{-1} sec^{-1} for the *E. coli* and *E. faecalis* enzymes, respectively. Prolonged preincubation of **1** with the human enzyme did not lead to inhibition at concentrations as high as 100 μM. Hence, the lack of inhibition of the human enzyme by **1** was not due to a slower onset of inhibition for this enzyme.

Incubation of **1** with the bacterial enzymes for 30 min followed by removal of free inhibitor by size exclusion chromatography failed to reverse the inhibition. Likewise, extensive dialysis of the inhibited enzyme against inhibitor-free buffer showed no recovery of enzymatic activity. These results suggest that either **1** acts as an irreversible inactivator of the enzymes or binding of the inhibitor leads to an enzyme isomerization resulting in extremely tight binding that is dominated by a very slow off rate.

The apparent inactivation of the enzymes by **1** does not

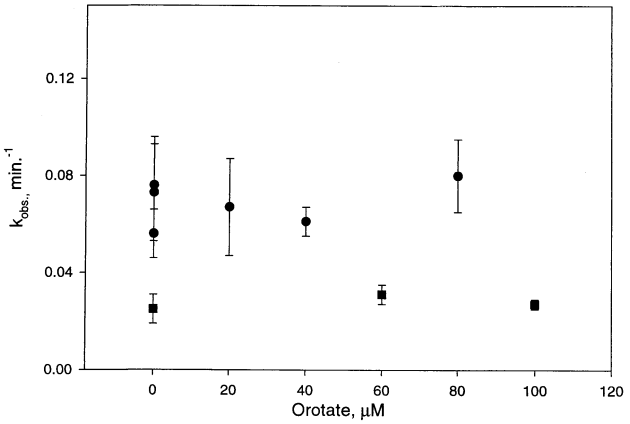

FIG. 3. Inactivation rate constant (k_{obs}) of 1 as a function of orotate concentration in the preincubation mixture for *E. faecalis* (circles) and *E. coli* (squares) DHODases. Error bars are as in Fig. 2.

appear to be the result of modification of residues within the dihydroorotate binding pocket. Classical Lineweaver–Burk analysis gave the characteristic pattern for noncompetitive (mixed-type) inhibition for both bacterial enzymes in the absence of a preincubation of the enzyme with inhibitor (data not shown). These data must be viewed with some caution, however, as the time-dependent nature of inhibition by **1** could lead to a misinterpretation of the double-reciprocal plot pattern [12]. More revealing, the rate of inactivation by **1** for both bacterial enzymes was unaffected by the product inhibitor orotic acid (K_i = 50 μM) over a range of orotic acid concentrations from 0 to 100 μM (Fig. 3). Product inhibition by orotic acid is competitive with dihydroorotate, and the crystal structure of the DHODase from *Lactococcus lactis* confirms that orotic acid binds to the enzyme at the active site for dihydroorotate oxidation [13]. Thus, the inactivation induced by **1** appears to result from compound binding to a site other than the dihydroorotate binding pocket on the enzymes.

The molecular mechanism of this slow-binding inhibition is yet to be worked out completely. The structure of the thiadiazolidinediones suggests that they may undergo nucleophilic attack to covalently inactivate the enzymes. Cysteine residues would be a likely candidate for covalent modification by the thiadiazolidinediones. Compound **1**, however, does not appear to be a general inhibitor of sulfhydryl-dependent enzymes. For example, **1** did not inhibit the activity of human ubiquitin activating enzyme (E1),* or reduced glutathione reductase [14] at concentrations as high as 100 μM. The Gram-positive enzymes contain an active site cysteine that functions as the general base for catalytic hydrogen abstraction from the substrate dihydroorotate [2]. In the Gram-negative and mammalian enzymes this cysteine is replaced by a serine residue [2], but the *E. coli* enzyme contains other cysteine residues within

* Wee KE, Lai Z, Auger K, Horiuchi K, Dowling RL, Dougherty C, Wynn R and Copeland RA, Manuscript submitted for publication.

its primary structure [9]. None of these cysteine residues, however, appears to be critical for the enzymatic activity of this enzyme, based on chemical modification studies with iodoacetamide (unpublished results). We have also found that **1** is an effective inactivator of other Gram-negative bacterial DHODases that contain no cysteine residues within their primary structure (data not shown). Thus, covalent modification of cysteine residues is not likely to be the general mechanism of DHODase inhibition by these compounds. Attempts to elucidate the binding site for the thiadiazolidinediones through peptide mapping of the enzymes are being explored currently.

The results presented here demonstrate that the thiadiazolidinediones, a class of known bactericidal agents [6, 7], are potent and selective inhibitors of bacterial dihydroorotate dehydrogenase. Indeed, preliminary cellular* assays suggest that compound **1** is able to inhibit cell growth of some bacteria in culture; however, compound optimization for cell permeability and other factors would be required before attempts could be made to correlate cellular and enzymatic inhibition effects for this compound class.

The fact that the thiadiazolidinediones inhibited both Gram-positive and -negative bacterial DHODases while sparing the human enzyme is surprising given the structural diversity among these enzymes. Nevertheless, the current results demonstrated clearly that broad spectrum inhibition of prokaryotic DHODases can be achieved without associated effects on the human enzyme. Other inhibitors of selected bacterial DHODases have been reported. For example, hydantoins and spirocyclopropanobarbiturates have been reported to be modest (mM) inhibitors of the enzyme from *Clostridium oroticum* [15]. The thiadiazolidinediones, however, represent the first examples of potent and selective inhibitors of bacterial DHODases. The use of these compounds as antibiotic agents *in vivo* may be limited by chemical liabilities of the thiadiazolidinedione class (for example: structural instability in blood, potential chemical reactivity with other proteins, and the possibility of non-mechanism-based toxicity). Nevertheless, these compounds demonstrate the possibility of selectively targeting prokaryotic DHODases for the development of novel antibacterial agents, and may serve as useful research tools for scientists studying prokaryotic DHODase structure and function.

* Sizemore C, Amsler KM, Marcinkeviciene J, Rogers MJ, Hobbs FW, Haque T and Copeland RA, unpublished data.

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