Comparative Inhibition of Bacterial and Microsomal 3-Ketodihydrosphingosine Synthetases by L-Cycloserine and Other Inhibitors

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Eleven compounds were examined for their capacity to inhibit the first enzyme of the sphingolipid pathway, 3-ketodihydrosphingosine synthetase. Of these, L-cycloserine was the most potent, affecting both bacterial and brain microsomal enzymes to a significant degree at 0.04 mM. D- and L-cycloserine irreversibly inactivated the enzyme, indicating a suicide substrate mode of action. L-Cycloserine was a more potent inhibitor of the growth of *Bacteroides levii* than was D-cycloserine, indicating that inhibition of sphingolipid synthesis could be a factor in the growth inhibition.

The mode of action of D-cycloserine as an antibiotic has been established as an inhibitor of D-alanine racemase (10). Further studies have indicated that its mode of action is that of a suicide substrate, a mechanism-based enzyme inactivator (11). Besides D-alanine racemase, other enzymes could be inhibited by D- (and also by L-) cycloserine and so contribute to its antibacterial properties. The description of sphingolipids in the membranes of *Bacteroides* sp. (12) and other microorganisms (3) has opened up the possibility of the sphingolipid pathway as a potential focus for antimicrobial attack. Our investigations of sphingolipid synthesis in *Bacteroides levii*, formerly a subspecies of *Bacteroides melaninogenicus* (4), and the enzymology of the first enzyme of the sphingolipid pathway, 3-ketodihydrosphingosine (3KDS) synthetase, suggest that this enzyme could be susceptible to cycloserine. 3KDS synthetase catalyzes the condensation between palmitoyl coenzyme A and serine to form 3KDS, with pyridoxal phosphate as the cofactor. Inhibitors of this enzyme previously described have been compounds with low activity, such as cysteine (3) and thiocyanate (8).

In this report, we describe the growth inhibition of *B. levii* by D- and L-cycloserine (4-amino-3-isoxazolidinone), the inhibition of 3KDS synthetase by these compounds, and other inhibitors with different modes of action. Observations on the effect of L-cycloserine on sphingolipid metabolism in mouse brain (15) and in growing bacterial cultures (16) have been published previously.

MATERIALS AND METHODS

Bacterial cultures. The strain of *B. levii* was that used in previous studies (11) and was grown in a medium consisting of 3% Trypticase (BBL Microbiology Systems), 0.3% yeast extract (Difco Laboratories), and 0.5% NaCl (pH 7.2). This was supplemented with vitamin K₁ (final concentration, 0.1 µg/ml). Vitamin K₁ was added as a stable emulsion in water (7). Horse erythrocytes lysed in distilled water were added to 0.025% final concentration as a 5% suspension. For antimicrobial assays, cycloserine was sterilized by filtration, and various concentrations were added to the above medium. After inoculation, cultures were read after 48 h of incubation at 37°C.

Preparation of bacterial 3KDS synthetase. Cells were grown overnight, reaching 80 to 100 Klett units. They were centrifuged and washed two times in phosphate buffer (0.05 M, pH 7.2) and diluted to 1 mg/ml (wet weight) in buffer containing 1 mM dithiothreitol and 1 mM pyridoxal phosphate. The cell suspension was sonicated as described previously (6) and centrifuged for 90 min at 100,000 × g. The supernatant was precipitated with (NH₄)₂SO₄, 53% saturation. The precipitate was dissolved in the above buffer, washed, and concentrated in a membrane filtration cell with a 10,000 M_r cutoff. This material was further purified by a Sephacryl S200 column (8), and fractions containing activity were pooled, concentrated, and used for inhibitor studies.

Preparation of microsomal 3KDS synthetase. Microsomes were prepared by the method of Morrell and Radin (9) from the brains of 16-day-old Swiss male mice (weight 8 to 12 g). The effect of inhibitors on the 3KDS synthetases was measured by preincubating various concentrations of an inhibitor with the bacterial enzyme preparation or with a suspension of brain microsomes for 15 min (15). The enzyme activity was then assayed by adding palmitoyl coenzyme A (0.4 mM) and DL-[3-¹⁴C]serine (2 µCi, 18 mCi/mmol) to the tubes. After 20 min of incubation at 37°C, the radioactive 3KDS formed was determined after extraction, thin-layer chromatography separation, and autoradiography (8).

L-Cycloserine was a gift from P. Sorter, Hoffmann-LaRoche Inc., Nutley, N.J. α-Fluoromethylserine was a gift from J. Kollonitsch, Merck Sharp & Dohme, Rahway, N.J. D-Cycloserine and other inhibitors were purchased from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Eleven compounds were examined for inhibitory activity against 3KDS synthetase from bacterial and brain microsomal sources. These compounds were tested at three concentrations, 0.025, 0.1, and 1.0 mM. L-Cycloserine was a far superior inhibitor compared with the other compounds, since at 0.04 mM it produced a 50% inhibition in enzyme activity in both the bacterial and brain microsomal enzymes (Table 1). The bacterial and microsomal enzymes differed in their responses to L-cycloserine. With the bacterial enzyme, increasing the concentration from 0.025 to 1 mM increased inhibition from 58 to 94%, whereas with the microsomal synthetase enzyme, increasing the concentration did not

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TABLE 1. Effect of various inhibitor concentrations on the activity of 3KDS synthetase from *B. levii* and mouse brain microsomes

Inhibitor	Concn (mM) ^a	
	Bacterial enzyme	Microsomal enzyme
D-Cycloserine	0.34 ± 0.25	0.66 ± 0.41
L-Cycloserine	0.04 ± 0.01	0.04 ± 0.01
Vinylglycine	0.43 ± 0.29	0.57 ± 0.45
Propargylglycine	0.66 ± 0.50	0.59 ± 0.01
Azaserine	0.88 ± 0	0.50 ± 0.39
Ethanolamine- <i>O</i> -sulfate	0.58 ± 0.47	0.50 ± 0.39
<i>O</i> -Succinylhomoserine	0.93 ± 0.72	3.26 ± 2.99
β-Mercaptoethylamine	NI	1.00 ± 0.61
α-Fluoromethylserine	0.29 ± 0.18	0.53 ± 0.43
Cycloleucine	1.27 ± 1.15	0.92 ± 0.74
Cycloleucylglycine	0.73 ± 0.64	1.83 ± 1.58

^a For the enzyme assays, palmitoyl coenzyme A (0.4 mM) and DL-[¹⁴C]serine (2 μCi, 18 mCi/mmol) were added. After 20 min of incubation at 37°C, the radioactive 3KDS formed was determined by counting after extraction, thin-layer chromatography, analysis, and autoradiography (see text). Values indicate concentration required to produce 50% enzyme inhibition. NI, Not inhibitory at 1 mM.

significantly increase inhibition from the 83% found at the lowest concentration (data not shown).

Most compounds possessed a lower but significant degree of inhibitory activity compared with L-cycloserine, including D-cycloserine, α-fluoromethylserine, vinylglycine, propargylglycine, and ethanolamine-*O*-sulfate. Certain of these compounds showed marked differences in inhibitory activity when the bacterial and microsomal enzymes were compared. D-Cycloserine, *O*-succinylhomoserine, and cycloleucylglycine were more active against the bacterial enzyme and less active against the microsomal enzyme, whereas cycloleucine and azaserine showed higher degrees of inhibition against the microsomal synthetase and less inhibition against the bacterial synthetase at the concentrations tested. Propargylglycine, ethanolamine-*O*-sulfate, and vinylglycine showed approximately equal activity against both enzymes.

The growth responses of *B. levii* to increasing concentrations of D- and L-cycloserine are shown in Fig. 1. L-Cycloserine produced a biphasic curve: a sharp linear decrease in growth from 0 to 50 μg/ml and a shallow decrease in growth from 50 to 250 μg/ml. The microorganisms were much less susceptible to D-cycloserine, since only a 50% reduction in growth response was found at 500 μg/ml.

Since L-cycloserine was the most active compound tested, it was of interest to examine its mode of action. The irreversible nature of the inhibition of the bacterial synthetase by D- and L-cycloserine was demonstrated by incubating the enzyme with the inhibitor. The inhibitor was then removed by extensive washing in a membrane filtration cell, with a 10,000 *M_r* cutoff. Enzyme activity could not be recovered after this procedure (data not shown).

DISCUSSION

Since *B. levii* contains sphingolipids in its membranes and 3KDS synthetase is a pyridoxal phosphate-dependent enzyme (as is D-alanine racemase), it seemed of interest to determine the inhibition of 3KDS synthetase by D-cycloserine and other inhibitors of pyridoxal phosphate-dependent enzymes, since the inhibition of this specific enzyme has not been examined in any detail previously.

The inhibitors chosen were from three groups: (i) those

which had some similarity to serine, which is one substrate of the synthetase, such as D- and L-cycloserine and azaserine; (ii) those which were known to be suicide substrate inhibitors of other pyridoxal phosphate-dependent enzymes, such as propargylglycine and vinylglycine (α-fluoromethylserine is a compound which from chemical considerations was expected to be a suicide substrate of the 3KDS synthetase [5]); and (iii) compounds whose structure suggested a potential inhibitory action, such as ethanolamine-*O*-sulfate, cycloleucine, and cycloleucylglycine (ethanolamine-*O*-sulfate is also a known inhibitor for 4-aminobutyrate:2-oxoglutarate aminotransferase, as is L-cycloserine [2]).

Of the 11 compounds tested, the superiority of L-cycloserine as an inhibitor of the first enzyme of the sphingolipid pathway was demonstrated both for the bacterial enzyme and for the brain microsomal synthetase. Moreover, the action of D- and L-cycloserine as suicide substrate inhibitors was indicated, because of the lack of regeneration of enzyme activity after removal of the inhibitor from the enzyme. Similar results were obtained with α-fluoromethylserine (data not shown). The mode of action of L-cycloserine in animals suggests that its action against GABA-T and ALA-T is not of the suicide substrate type, since physiological effects caused by these compounds can be reversed by simultaneous pyridoxal phosphate administration (14). That is, the inhibition of different enzymes by L-cycloserine may involve differing modes of action.

A number of suicide substrate inhibitors of other pyridoxal phosphate-dependent enzymes (18), such as propargylglycine and vinylglycine, showed moderate inhibitory activity which was expected to be potent against 3KDS-synthetase, α-fluoromethylserine, possessed only moderate activity against this enzyme from both bacterial and microsomal sources.

It is of interest to note that some inhibitors affect the bacterial synthetase to a greater degree than the microsomal enzyme, whereas with others the reverse is true. These effects could be due to the nature of the two enzymes, in that the bacterial synthetase is solubilized by the treatment used in preparation, whereas the microsomal enzyme is membrane bound and has not been solubilized to date. Accessi-

FIG. 1. Inhibition of *B. levii* by D- and L-cycloserine. D- and L-cycloserine were sterilized by filtration and added to tubes of medium supplemented with vitamin K₁ and a dilution of horse erythrocytes. The tubes were inoculated with *B. levii* and incubated anaerobically as described in the text. Symbols: ○, D-cycloserine; ●, L-cycloserine.

bility could therefore be a factor for those compounds which show a reduced effect against the microsomal enzyme.

The greater susceptibility of *B. levii* to L- rather than D-cycloserine is very unusual. Neuhaus (10) has presented MICs of both forms of cycloserine to a number of bacteria, most of which show greatly increased tolerance of the L- compared with the D-cycloserine. Since D-alanine racemase is not inhibited by L-cycloserine (13), the enhanced susceptibility of *B. levii* to L-cycloserine may indicate that inhibition of sphingolipid synthesis could be a factor in the growth inhibition. Sphingolipid synthesis has been shown to be an obligate requirement for the growth of a mutant of *Saccharomyces cerevisiae* (19).

The result of this survey of 3KDS synthetase inhibitors has been the discovery of a highly potent inhibitor, L-cycloserine. This has had application in the reduction of in vivo accumulation of glycolipids in mouse brain (15) and in the inhibition of sphingolipid synthesis in growing bacterial cells (16). Certain *Bacteroides* spp. which synthesize sphingolipids are resistant to many commonly used antibiotics. The inhibition of sphingolipid synthesis could provide a model for a new class of microbial antagonists which would have application in the chemotherapy of these *Bacteroides* spp. and, conceivably, other sphingolipid-synthesizing bacteria.

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