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Dihydrofolate Reductases in Some Folate-Requiring Bacteria with Low Trimethoprim Susceptibility

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Dihydrofolate reductases from the folate-requiring strains *Streptococcus faecalis* ATCC 8043, *Lactobacillus casei* ATCC 7496, and *Pediococcus cerevisiae* ATCC 8081, as well as from *Lactobacillus arabinosus*, which is not dependent on exogenous folate, were isolated, and their properties were compared to reductases of *Escherichia coli* B, *Staphylococcus aureus*, and rat liver reductase. An inhibition profile with six different inhibitors revealed significant differences among all enzymes. All lactobacilli reductases are less sensitive to trimethoprim than the enzymes of *E. coli* and *S. aureus*, the reductase of *P. cerevisiae* requiring a concentration at least 1,000 times higher for 50% inhibition. Inhibition of growth of *S. faecalis* by pyrimethamine and 2,4-diamino-6,7-diisopropyl-pteridine was seen to be much stronger than was predicted from the enzymatic data.

Strains such as *Streptococcus faecalis, Pediococcus cerevisiae*, and *Lactobacillus casei* are often used for evaluating and characterizing antifolate compounds (e.g., references 9, 10, and 21). They are unable to synthesize their own folate cofactors and require preformed folates or, as in the case of *P. cerevisiae*, reduced folates for growth. These strains are therefore also used for very sensitive assays of several folate compounds, as they differ in their ability to utilize particular derivatives. They are strongly inhibited by antifolate compounds such as methotrexate or aminopterin, which may be actively transported (14, 27). In *P. cerevisiae*, the uptake system has been shown to be not identical with that for the transport of folinate (13). The strains are of course resistant to sulfonamides which interfere with the biosynthesis of dihydrofolate at dihydropteroate synthetase.

During work on dihydrofolate reductases (EC 1.5.1.3) of different bacteria, investigated as target sites for trimethoprim-like compounds, we also studied the reductases of some folate-requiring strains, since their folate auxotrophy suggested possible alterations in this enzyme as well. Dihydrofolate reductases from *S. faecalis* ATCC 8043 (syn. *S. faecium* var. *durans*), *P. cerevisiae* ATCC 8081, *L. casei* ATCC 7496, and *L. arabinosus* ATCC 8014, which, however, is not dependent on exogenous folate, were partially purified and their response to some structurally different inhibitors was compared to that of the enzymes from *Escherichia coli* B, *Staphylococcus aureus*, and also to rat liver enzyme as an example of an eucaryotic enzyme. Inhibition profiles, which have been successfully used for detecting subtle differences in dihydrofolate reductases of different origin (e.g., reference 6), revealed major differences in these enzymes.

MATERIALS AND METHODS

Propagation of cells. Cells were grown in batches of 200 ml up to 6 liters in either Erlenmeyer flasks under agitation or, in the case of the lactobacilli, without agitation. The following media were used: sensitivity test broth (Oxoid Ltd., London, England) for *E. coli* B, *S. aureus*, and *S. faecalis;* lactobacilli AOAC broth (Difco Laboratories, Detroit, Mich.) for *L. casei* and *L. arabinosus*; folic AOAC broth (Difco) containing 1 μg of folinic acid per ml for *P. cerevisiae*. Cells were harvested in the late log phase or early stationary phase and stored frozen at −20°C.

Enzyme purification. Frozen cells were resuspended in 10 mM phosphate buffer, pH 7.0, and disrupted by either grinding with aluminum powder (Alcoa Chemicals) or by sonic treatment for 3 to 5 min in intervals. The crude extract, obtained after centrifugation at 20,000 rpm, was treated with 1/10 the volume of a 10% streptomycin sulfate solution to remove nucleic acids. Ammonium sulfate was added to the clear supernatant, and the 50 to 90% fraction was recovered. After dialysis against 50 mM phosphate buffer, pH 7.0, the dialysate was applied to a Sephadex G 75 or G 100 column (1.5 by 90 or 2.5 by 100 cm). The active fractions, which are free of reduced nicotinamide adenine dinucleotide phosphate oxidase, were pooled and used as the enzyme preparation, which was approximately 50- to 100-fold purified. Alternatively, the enzyme was purified by applying the dialyzed ammonium sulfate preparation to a methotrexate affinity column. After washes with buffered 1 M NaCl, elution

occurred with 3 mM folate in 10 mM tris(hydroxymethyl)aminomethane-hydrochloride containing 1 M NaCl. Folate was removed by extensive dialysis. This preparation, which was approximately 200- to 300-fold purified, was also used as enzyme source, and no differences were observed in the 50% inhibitory concentrations (I_{50}) obtained in either case. The enzyme from rat liver was purified as described by Burchall and Hitchings (5).

Assay of dihydrofolate reductase. The assay was carried out in a volume of 3 ml at 37°C. The reaction mixture contained: 50 mM phosphate buffer, pH 7.0, reduced nicotinamide adenine dinucleotide phosphate, 11 mM β-mercaptoethanol, 0.06 mM dihydrofolate, inhibitor as indicated, water, and enzyme. Enzyme was added to give a decrease in absorbancy at 340 nm of about 0.06/min, corresponding to 14.6 nmol of dihydrofolate reduced per min. The reaction rates were corrected for the nonenzymatic decrease in absorbancy. Controls were run without substrate and in the presence of 10^{-5} to 10^{-4} M aminopterin. After a 4-min incubation time, the reaction was started by adding substrate.

Inhibitor test. The concentration of inhibitor leading to 50% inhibition of the control rate was determined graphically after a titration, usually with five different concentrations of inhibitor. To correct for experimental errors, all determinations were carried out repeatedly on different days with different enzyme preparations.

Chemicals. Aminopterin was purchased from Serva (Heidelberg, West Germany), 2,4-diamino-6,7-dimethylpteridine from Merck-Schuchardt (Munich, West Germany), 2,4-diamino-6,7-diisopropylpteridine from Calbiochem (Los Angeles, Calif.), and triamterene from Lederle (Pearl River, N.Y.). These compounds were of >95% purity as determined by microanalysis. Reduced nicotinamide adenine dinucleotide phosphate was purchased from Boehringer (Mannheim, West Germany), and mercaptoethanol was from Fluka (Buchs, Switzerland). Dihydrofolate was prepared from folate as described by Blakley (4) and stored frozen at −20°C as a thick suspension. The actual concentrations were determined spectrophotometrically with a molar absorption coefficient of ϵ_{282} of 28,000 (25).

Statistical treatment of inhibition data. (i) Statistical model. We consider the logarithms of the I_{50} values as our basic data in the analysis of inhibition profiles. The log transformation is mainly justified by the fact that the variance of log (I_{50}) appears to be similar for any inhibitor/enzyme combination occurring in this study. This suggests the following statistical model. The log values (I_{50}) are random variables with equal variances and with mean values depending on the particular inhibitor and enzyme source. Moreover, we assume the log (I_{50}) values to be independently and normally distributed. These assumptions allow the standard techniques of statistical estimation and hypothesis testing by analysis of variance to be applied (23).

Note that due to the assumption of equal variances, a single variance estimate of log (I_{50}) valid for any inhibitor/enzyme combination is obtained, which is used in the computation of standard errors of means, confidence intervals, and tests. s^2 is the mean square error $\sum_{i,j} (n_{ij} - 1)s_{ij}^2 / \sum_{i,j} (n_{ij} - 1)$, where the index i,j runs over all inhibitors i and enzymes j, n_{ij} and s_{ij} denoting the corresponding number of determinations and standard deviations.

(ii) Description of inhibition profiles. According to the above assumptions, the basic statistical parameters describing the inhibition profile of some enzyme would be the arithmetic means (AM) and their standard errors calculated for the different inhibitors. These parameters, however, relate to transformed variables. For this reason, we use the geometric mean (GM) of the I_{50} values and its coefficient of variation. The geometric mean is by definition: GM of I_{50} = exp(AM of log I_{50}). The coefficient of variation (CV) of a random variable equals its relative standard error, expressed as a percentage of its mean value. Under the assumption of a normal distribution for log I_{50}, the coefficient of variation of the geometric mean is given by: $CV = \sqrt{\exp(SE^2) - 1} * 100\%$ where SE denotes the standard error of mean of log I_{50}.

Comparison of inhibition profiles. The difference between the inhibition profiles derived from two enzymes, A and B say, can be quantified by means of the set of ratios: λ = GM of I_{50} from enzyme A/GM of I_{50} from enzyme B, obtained from different inhibitors. λ can be calculated from the log I_{50} values by $\lambda = \exp(\Delta)$, where Δ is the difference of arithmetic means: Δ = {AM of log I_{50} from enzyme A} − {AM of log I_{50} from enzyme B}. Now, lower and upper 90% confidence limits Δ_- and Δ_+ for Δ can be obtained in the usual way (23), and so we get 90% confidence limits for λ by putting $\lambda_\pm = \exp(\Delta_\pm)$.

Finally, we wanted to test whether the two inhibition profiles are identical with respect to some (or all) inhibitors; i.e., we wanted to test the hypothesis "λ = 1 for some inhibitors," which obviously is equivalent to "Δ = 0 for some inhibitors." This results in testing equality of several mean values (*F*-test) by analysis of variance.

RESULTS

Enzymatic data. The I_{50} values obtained for the inhibition of dihydrofolate reductases together with the coefficients of variation and the number of determinations are summarized in Table 1. Differences in the I_{50} values between the *E. coli* enzyme and the other enzymes can be quantified, for each inhibitor, by means of the ratio: λ = I_{50} from enzyme under investigation/I_{50} of *E. coli* reductase. λ values significantly different from 1.0 indicate differences in the I_{50}. Table 2 contains estimates of these ratios together with their 90% confidence limits (for computations, see Materials and Methods).

The enzymes from *E. coli* and *S. aureus* were seen to be different by the aminopterin value only; i.e., the hypothesis "λ = 1 except for aminopterin" cannot be rejected. Using other types of inhibitors, more drastic differences have however been found (6). The other enzymes are all very different from the *E. coli* reductase. Indeed,

TABLE 1. *Inhibition of lactobacilli dihydrofolate reductases by different inhibitors*[a]

Enzyme source	Aminopterin			Trimethoprim			Pyrimethamine			Triamterene			2,4-Diamino-6,7-diisopropylpteridine			2,4-Diamino-6,7-dimethylpteridine		
	GM	CV	n	GM	CV	n	GM	CV	n	GM	CV	n	GM	CV	n	GM	CV	n
E. coli B	0.00225	35%	2	0.0087	22%	5	1.37	28%	3	4.10	35%	2	0.0706	28%	3	30.1	28%	3
S. aureus	0.000349	24%	4	0.0125	24%	4	1.64	35%	2	4.23	35%	2	0.0647	28%	3	38.8	28%	3
S. faecalis ATCC 8083	0.00351	24%	4	0.0335	28%	3	3.15	35%	2	3.74	35%	2	0.211	28%	3	44.9	28%	3
P. cerevisiae ATCC 8081	0.0031	28%	3	14.2	28%	3	11.4	28%	3	29.0	28%	3	0.459	28%	3	>100		
L. casei ATCC 7496	0.00476	28%	3	0.522	28%	3	14.9	28%	3	153	28%	3	26.3	28%	3	166	28%	3
L. arabinosus ATCC 8014	0.0017	35%	2	0.0548	51%	1	0.707	28%	3	3.34	28%	3	0.271	28%	3	50.9	28%	3
Rat liver	0.0065	22%	5	486	28%	3	2.03	35%	2	7.14	35%	2	2.37	35%	2	>100		

[a] Geometric mean (GM) of several independent determinations with coefficient of variations (CV) of I_{50} (μM) and number of determinations (n).

TABLE 2. *HA values for lactobacilli dihydrofolate reductases and inhibitors (with 90% confidential limits)*

Enzyme source	Inhibitor λ value for:					
	Aminopterin	Trimethoprim	Pyrimethamine	Triamterene	2,4-Diamino-6,7-diisopropylpteridine	2,4-Diamino-6,7-dimethylpteridine
S. aureus	0.157 (0.079–0.312)	1.44 (0.84–2.45)	1.20 (0.58–2.48)	1.03 (0.466–2.29)	0.92 (0.48–1.76)	1.29 (0.673–2.47)
S. faecalis ATCC 8083	1.58 (0.79–3.14)	3.85 (1.79–6.90)	2.29 (1.11–4.74)	0.913 (0.412–2.02)	2.98 (1.56–5.72)	1.45 (0.778–2.86)
P. cerevisiae ATCC 8081	1.40 (1.03–4.42)	1,630 (911–2,910)	8.33 (4.35–16.0)	7.08 (3.43–14.7)	6.50 (3.39–12.5)	
L. casei ATCC 7496	2.14 (1.63–4.42)	60.1 (33.6–107)	10.9 (5.70–20.8)	37.4 (18.0–77.3)	373 (195–714)	5.50 (2.87–10.5)
L. arabinosus ATCC 8014	0.764 (0.345–1.69)	6.29 (2.37–16.7)	0.515 (0.269–0.987)	0.816 (0.394–1.69)	3.83 (2.0–7.34)	1.69 (0.88–3.23)
Rat liver	2.93 (1.51–5.70)	55,900 (31,300–100,000)	1.48 (0.717–3.07)	1.74 (0.78–3.85)	33.5 (17.5–64.2)	

the hypotheses "$\lambda = 1$ for all inhibitors" has to be rejected in the case of these enzymes (error probabilities <1%).

Whereas all enzymes are highly sensitive to the structural analog aminopterin, major differences were revealed by trimethoprim, pyrimethamine, and the pteridine inhibitors. The enzymes from *P. cerevisiae, L. casei,* and *L. arabinosus* are much more resistant to trimethoprim than those from *E. coli* and *S. aureus.* Great variations are also observed in the response to pyrimethamine and the pteridine inhibitors. A comparison with rat liver enzyme shows that the enzymes from lactobacilli not only differ from those of common bacteria but that they are also strikingly different from the eucaryotic enzyme.

In vitro data. The in vitro activity of these compounds was also determined to correlate them with the enzymatic inhibition. I_{50} values were measured as they are more reliable than minimal inhibitory concentrations (Table 3). Due to different growth requirements of these strains, different media had to be used; all were however free of thymidine, which would antagonize the activity of antifolates. For *L. casei* and *L. arabinosus,* folic acid casei medium (Difco) supplemented with 1 μg of folic acid per ml was used; for *P. cerevisiae,* CF-assay medium (Difco) supplemented with 1 μg of folinic acid per ml. and for *S. faecalis,* folic acid assay medium (Difco) supplemented by 1 μg of folic acid per ml were used. For *L. casei,* the I_{50} values were also determined in the presence of 1 ng of folic acid only per ml, but no significant differences were seen. For *E. coli* B, mineral salts medium M9 containing 0.1% Casamino acids (Difco) and for *S. aureus,* sensitivity test broth (Oxoid) were used. The I_{50} values of *E. coli* B in the latter medium were seen to be very similar to those obtained in the semisynthetic medium.

Whereas *E. coli* and *S. aureus* were little affected by aminopterin due to the poor penetration of this compound, the other organisms were highly susceptible to this antifolate. *P. cerevisiae,* however, was less susceptible to aminopterin than the other lactobacilli, which is in accordance with the findings on methotrexate (9). Trimethoprim was an effective inhibitor in vitro, with the exception of *P. cerevisiae.* This correlates well with the presence of an insensitive dihydrofolate reductase. The low concentration of trimethoprim required to inhibit *L. casei* is somewhat surprising with respect to the relatively insensitive enzyme of this strain. Pyrimethamine is surprisingly active in *S. faecalis* as is the isopropylpteridine, both being much more active than can be assumed from the enzymatic data.

TABLE 3. *In vitro activity of several dihydrofolate reductase inhibitors in lactobacilli, S. aureus, and E. coli B*

Strain	I_{50} (μM) of:					
	Aminopterin	Trimethoprim	Pyrimethamine	Triamterene	2,4-Diamino-6,7-diisopropylpteridine	2,4-Diamino-6,7-dimethylpteridine
E. coli B	80	0.52	116	140	13	1,500[a]
S. aureus	68	1.7	56	28	81	1,500[a]
S. faecalis ATCC 8083	0.11	0.76	0.11	8.8	0.037	38
P. cerevisiae ATCC 8081	1.25	230	150	91	8.6	400
L. casei ATCC 7496	0.0034	0.86	11	47	6.1	195
L. arabinosus ATCC 8014	0.15	0.41	2.2	9.9	0.35	126

[a] Higher concentrations not soluble.

DISCUSSION

The characterization of several dihydrofolate reductases has recently progressed to the determination of the amino acid sequence, as e.g., determined for the enzyme from *E. coli* (24), from a methotrexate-resistant *L. casei* (2), and for *S. faecalis* (19). Although considerable homologous sequences exist, these enzymes are all different, despite their very similar biochemical properties (3). However, comparatively little data are available on the response of reductases to structurally different inhibitors, though these are able to reveal subtle differences and so provide valuable additional information on enzymatic differences (5, 6).

The compounds used here to obtain inhibition profiles were chosen according to their practical importance, differences in structure, and their accessibility. Triamterene and 2,4-diamino-6,7-dimethylpteridine also have diuretic activity (8, 20); the diisopropylpteridine was recently used as a vibriostatic agent (7, 17).

The enzymes from the four lactobacilli studied here show considerable variation with respect to the inhibition produced by the compounds selected. They differ significantly among themselves and from the *E. coli* reductase.

All lactobacilli reductases are less sensitive to trimethoprim, a powerful 2,4-diaminopyrimidine in most pathogens as compared with the enzyme, for which a trimethoprim concentration at least a 1,000 times higher was required to zyme, for which a trimethoprim concentration at least a 1000 times higher was required to produce the corresponding inhibition. The value of 14 μM for I_{50} agrees with the figures determined by Mandelbaum-Shavit (12). The trimethoprim mean value of I_{50} = 8.7 nM for *E. coli* is very close to several values reported in the literature, varying from 0.5 to 1.8 nM (1, 5, 16, 18).

Studies with a great variety of dihydrofolate reductase inhibitors in our laboratory have shown that, with a few exceptions, there is a good correlation between inhibition on the enzymatic level and the in vitro inhibition of growth. In general this is also true for the strains tested here. There are, however, remarkable exceptions: pyrimethamine and the isopropylpteridine are much more active in *S. faecalis* and also in *L. arabinosus* than was predicted from the enzymatic inhibition. The higher potency of pyrimethamine in comparison to trimethoprim was also observed by others (9). The high susceptibility of *S. faecalis* to pyrimethamine has been exploited for the microbiological determination of this compound (22). However, due to different media and testing procedures, the in vitro data cannot be compared directly. As there were no indications of an active transport for pyrimethamine in *S. faecalis* (26, 27), this high activity is still to be accounted for. Though lactobacilli do not play an important role as pathogens, it seems interesting that lactobacilli, isolated as causative agents in urinary tract infections, were found to be resistant to trimethoprim (11). This may be based on insensitive reductases of these strains. *P. cerevisiae* is less susceptible to aminopterin than the other lactobacilli, despite similar I_{50} values of the enzymes. This is also true for methotrexate (9) and can be explained by the uptake properties of *P. cerevisiae* (15).

One could think of active transport processes to explain the high in vitro activity of the isopropylpteridine inhibitor in *S. faecalis* as compared with the activity of trimethoprim, which enters the cell by passive diffusion. However, knowledge is limited on this subject. Active transport of folate antagonists has been described (13, 27); the transport systems, however, are often highly specific, and it remains open whether these 2,4-diaminopteridines are transported by folate uptake systems. Bacterial reductases greatly differing from those of common pathogens have been described for some strains, as a meningococcus and marine bacterium, *Caulobacter* (6). The data given for lactobacilli here suggest such variations to be fairly common not only in evolutionary distant species, such as bacteria and mammals, but also within bacterial enzymes.

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