Inhibition of Folate Enzymes by Sulfasalazine

JACOB SELHUB, G. JEELANI DHAR, and IRWIN H. ROSENBERG, Section of Gastroenterology, Department of Medicine, The University of Chicago, Pritzker School of Medicine, Chicago, Illinois 60637

A B S T R A C T Sulfasalazine (salicylazosulfapyridine), an agent widely used for the treatment of ileitis and colitis, is also a competitive inhibitor of intestinal folate transport (1, 2). The mechanism of action of sulfasalazine remains uncertain. To further explore the mechanism of sulfasalazine action, the interaction of the drug with the folate recognition site was tested with three enzymes: dihydrofolate reductase, methyl-L-enetetrahydrofolate reductase, and serine transhydroxymethylase, each catalyzing a reaction involving a different folate derivative. Each of these enzymes was inhibited by sulfasalazine in the same concentration range as that previously observed to inhibit intestinal folate transport; the kinetic data are consistent with a competitive mode of inhibition. Specificity of inhibition was demonstrated by the finding that the reduction of the pteridine ring of pteroylheptaglutamic acid by dihydrofolate reductase was subject to inhibition, whereas the hydrolysis of the γ-glutamyl peptide side chain by chicken pancreas conjugate was not affected. These results are interpreted to indicate that sulfasalazine interferes with a folate recognition site which is common to these enzymes and to the intestinal transport system. Sulfasalazine, therefore, has certain properties of an antifolate drug.

INTRODUCTION

Sulfasalazine, commonly employed in the treatment of inflammatory bowel disease, is composed of sulfapyridine and 5-aminosalicylate linked through an azo bond (Fig. 1). There is no convincing structural similarity between this compound and folic acid. Yet earlier data from this laboratory (1, 2) has shown that sulfasalazine is a competitive inhibitor of folate transport by the intestine. Sulapyridine or 5-aminosalicylate, singly or in combination, were without effect.

Inasmuch as the intestinal transport process is shared by all folate derivatives tested (2, 3), it was assumed that sulfasalazine blocks a site that is responsible for the binding of folate analogues irrespective of the state of oxidation and one carbon substitution.

This assumption predicts that sulfasalazine may interact with similar sites on folate enzymes. To test this prediction, three enzymes, each catalyzing a reaction of different folate substrate, were tested for inhibition by sulfasalazine.

METHODS

Radioactive compounds. [3H]Pteroylglutamic acid (34 Ci/μmol), [3HCH3]Nγ-methyltetrahydrofolate (53 μCi/μmol), and L-[-3-14C]serine (60 μCi/μmol) were purchased from Amersham/Searle Corp. (Arlington Heights, Ill.). [3H]Pteroylheptaglutamate (132 μCi/μmol) was prepared by the procedure described by Godwin et al. (4). Tetrahydrofolate was prepared from folic acid by reduction with sodium dithionite (5) and purification on a DEAE-cellulose column (6). This compound was stored in evacuated vials in a refrigerator (7).

Dihydrofolate reductase was prepared from rat liver as described by Peterson et al. (8) with the exception of the affinity chromatography step. The enzyme was assayed by the method of Rothenberg (9) except for [3H]pteroylglutamic acid and NADPH concentrations, which were increased to 5 and 50 μM, respectively. For study of [3H]pteroylheptaglutamate reduction, the substrate concentration was 1 μM and the ZnSO4 concentration required for coprecipitation of the unreduced substrate with unlabeled folic acid (9) was increased to 0.35 M.

Methylenetetrahydrofolate reductase and serine transhy-
The three enzymes and the intestinal folate transport system are given in Table 1.

In the preceding experiments sulfasalazine was tested against enzymes, each employing a different monoglutamyl folate derivative as a substrate. In this experiment the inhibitor was tested for its effectiveness against enzymes which act on different sites of the same substrate. Reduction of the pteridine ring of pteroylheptaglutamate by dihydrofolate reductase was inhibited (Fig. 4) whereas sulfasalazine had no effect on the hydrolysis of the γ-glutamyl peptide chain by chicken pancreas conjugase.

**DISCUSSION**

Three enzymes, each with a specific folate substrate, were inhibited by sulfasalazine, and the inhibition was competitive with respect to the folate substrate with inhibition constants within one order of magnitude (Table 1). Dihydrofolate reductase reduced both monoglutamyl and heptaglutamyl folate, and sulfasalazine was inhibitory in both systems. We have shown previously that the intestinal transport of folic acid and N$_5$-methyltetrahydrofolate were both equally inhibited by sulfasalazine (2). This suggests that the folate binding sites of these enzymes and the folate intestinal transport system possess common structural features that are independent of the state of oxidation.

**FIGURE 2** Effect of increasing sulfasalazine concentration on the activities of folate enzymes. Methylenetetrahydrofolate reductase (1 mg protein), dihydrofolate reductase (1 mg protein), and serine transhydroxymethylase (0.24 mg protein) were incubated with their respective substrates, under conditions described in Methods with increasing concentrations of sulfasalazine. The effect of the drug is expressed as percent of enzyme activity without sulfasalazine. Actual amount of product (100% of control activity) was 9.1, 2.2, and 29.2 nmol, respectively, for the three enzymes.
methyl group substitution, or the presence of extra glutamic acid residue on the folate molecules.

According to current concepts summarized by Blakley (15), dihydrofolate reductase possesses three distinct binding regions: an ionizable region that binds the p-carboxyl group by charge transfer, a hydrophobic region that binds a moiety located four to eight atoms away from the p-carboxyl group toward the pteridine ring, and a third region that is weakly acidic and interacts with the basic groups of the pyrimidine ring. It is possible that sulfasalazine possesses the proper structure for recognition by these regions. The carboxyl group of salicylic acid is a potential moiety for recognition by the first region. The basic nitrogen on the pyridine ring could recognize the third region whereas the benzene ring could be the primary target for the binding by the hydrophobic region. Further studies employing various derivatives of sulfasalazine

**TABLE I**

<table>
<thead>
<tr>
<th>System</th>
<th>Apparent $K_m$ (mM substrate)</th>
<th>Apparent $K_i$ (mM sulfasalazine)</th>
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</thead>
<tbody>
<tr>
<td>Folate transport*</td>
<td>0.004 PteGlu†</td>
<td>0.09</td>
</tr>
<tr>
<td>Dihydrofolate reductase</td>
<td>0.004 PteGlu</td>
<td>0.23</td>
</tr>
<tr>
<td>Methylene tetrahydrofolate reductase</td>
<td>0.23 1-methyltetrahydrofolate</td>
<td>0.02</td>
</tr>
<tr>
<td>Serine hydroxymethylase</td>
<td>0.100 1-tetrahydrofolate</td>
<td>0.15</td>
</tr>
</tbody>
</table>

* From Dhar et al. (2).
† Pteroylglutamic acid.

**Figure 3** Lineweaver-Burk plots of the inhibition of folate enzymes by sulfasalazine (SASP): (a) methylenetetrahydrofolate reductase (0.96 mg protein); (b) dihydrofolate reductase (1 mg protein); (c) serine transhydroxymethylase (0.12 mg protein) at changing tetrahydrofolate concentrations and constant amount of L-serine (0.5 mM); (d) serine transhydroxymethylase (0.12 mg protein) at changing serine concentrations and constant amount of tetrahydrofolate (0.2 mM). The velocities are expressed in nanomoles of product formed per incubation. Similar ordinate intercepts indicate a competitive mode of inhibition. Noncompetitive inhibition is depicted by similar abscissa intercepts.
will be required to confirm these possibilities. Sulfasalazine and its derivatives should be useful in probing the structural requirements for binding of folate substrates to enzymes and transport systems.

The pharmacological implications of these observations are uncertain. The mechanism of action of sulfasalazine in ileitis and colitis remains controversial (16). Current concepts emphasize the importance of bacterial lysis of the azo linkage with local release of 5-aminosalicylate and the sulfapyridine moiety in the intestine (17, 18). However, 10–15% of sulfasalazine is absorbed intact in the proximal intestine (16–18), and the possibility that the parent molecule, not its catabolites, is active as an inflammatory suppressant has not been excluded. On the basis of the enzyme inhibition reported here, sulfasalazine acts as a weak antifolate drug. As such, it could suppress proliferation of inflammatory cells or of epithelial cells turning over rapidly. Because both types of cell proliferation are elements in the pathogenesis of colitis, this hypothesis deserves further assessment.

ACKNOWLEDGMENTS

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REFERENCES