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Inhibition of Thymidylate Synthase by Non-Steroidal Anti-Inflammatory Drugs

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Abstract—Non-steroidal anti-inflammatory drugs (NSAIDs) have been known as inhibitors of the folate-requiring enzymes. In the present work, we have expanded on these observations and have investigated the inhibitory effects of NSAIDs on *Lactobacillus casei* thymidylate synthase expressed in *E. coli*. NSAIDs including sulphasalizine, salicylic acid, indomethacin and mefenamic acid were found to be competitive inhibitors with respect to folate of *Lactobacillus casei* thymidylate synthase. In contrast, aspirin and the antipyretic-analgesic drugs acetaminophen and antipyrine were weak inhibitors of the enzyme. Structure-activity correlation suggests that an aromatic ring with a side chain containing a carboxylic acid is a requirement for competitive inhibition of the thymidylate synthase. The results are consistent with the hypothesis that the antifolate activity of NSAIDs, and hence cytostatic consequences, are important factors in producing anti-inflammatory activity and aspirin exerts its anti-inflammatory effects after its conversion into salicylic acid, which possesses greater antifolate activity than its parent compound.

Keywords □ thymidylate synthase, non-steroidal anti-inflammatory drugs

Thymidylate synthase(TS) catalyzes the conversion of dUMP and 5,10-methylenetetrahydrofolate(CH_2-H_4 folate) to dTMP and 7,8-dihydrofolate(H_2folate). The amino acid sequences of TS from some 16 sources are known, and the primary sequences of TS have revealed that it is the one of most conserved proteins known(Climie *et al.*, 1992). Now that the crystal structure of TS is known(Hardy *et al.*, 1990), this capability has taken on new importance. Because of source limitations, most of biochemical work on TS has been performed with the enzyme obtained from methotrexate-resistant strains of *L. casei* that produced TS to levels of about 1% of the total protein. Recently, recombinant *L. casei* TS expressed in *E. coli* provides 10~20% of the total soluble protein as TS(Climie and Santi, 1990). Since the recombinant enzyme is highly overexpressed, large quantity of the pure enzyme has been obtained with no difficulties in purification. Catalytically active recombinant TS is indistinguishable from that isolated from *L. casei.*

Since TS is required for the de novo synthesis of dTMP, it has been a popular target of chemotherapeutic agents. Most inhibitors of TS are analogs of the substrates dUMP or CH_2-H_4folate; however, these analogs have deficiencies as potential therapeutics. From a number of standpoints on a development of new anticancer drugs, it would be desirable to have TS inhibitors which are structurally unrelated to TS substrates. It has been reported that non-steroidal anti-inflammatory drugs(NSAIDs) are inhibitors of the folate-requiring enzymes, phosphoribosylaminoimidazolecarboxamide formyltransferase and dihydrofolate reductase(Ha *et al.*, 1990). In the present work, we have investigated the inhibitory effects of NSAIDs on *L. casei* TS expressed in *E. coli.*

Materials and Methods

Materials

All NSAIDs and other drugs were purchased from Sigma Chemical Co. and were used without further purification. NSAIDs and other drugs were dissolved in 5 mM potassium phosphate buffer and the pH was adjusted to 7.4. Except for sulphasalazine, none of the drugs developed color with the modified Bratton-Marshall assay method. Folic acid was purchased from Si-

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gma Chemical Co., purified by butanol extraction(three times) of an aqueous solution(pH 6.5). Other materials have been reported(Climie *et al.*, 1990; Santi *et al.*, 1990) or were commonly available.

Enzyme Purification and Assay One liter of LB medium supplemented with thymidine(50 ug/m*l*) and ampicillin(50 ug/m*l*) was inoculated with 10 m*l* overnight cultures of *E. coli* strain x2913/recA$^-$ containing the synthetic TS gene and grown for 16~20 h at 37℃. Cells were harvested by centrifugation and washed with cold 100 mM NaCl. Cells were resuspended in 10 m*l* of 10 mM KH_2PO_4(pH 6.8), 0.1 mM EDTA, and disrupted by sonication in ice bath. Cellular debris was removed by centrifugation at 10,000×g for 15 min. Further purification of TS from the soluble cell extract was performed using hydroxyapatite column and ammonium sulfate fractionation by the method previously described elsewhere(Climie *et al.*, 1990). TS activity was assayed spectrophotometrically at 25℃ using the conditions of Santi *et al* (1990). One unit of activity is the amount of TS that catalyzes the formation of 1 μmol of product per min.

Gel Electrophoresis

Purity of the purified TS was demonstrated by 12 % SDS-polyacrylamide gel electrophoresis by the method of Laemmli(1970). Proteins were stained in Coomassie Brilliant Blue R-250.

Kinetic Studies and Drug Effects

Initial velocities and corresponding substrate concentrations were estimated by the method of Waley(1981) in the noncontinuous spectrophotometric assay. All kinetic parameters were estimated by the method of Cleland(1979). At least two drug concentrations were used to estimate K_i values and the concentration of dUMP was kept constant and CH_2-H_4folate was varied.

Results and Discussion

Since TS is required for the de novo synthesis of dTMP, it has been a popular target for chemotherapeutic agents(Santi and Danenberg, 1984; Benkovic, 1980). Most inhibitors of TS are analogs of the substrates dUMP or CH_2-H_4folate; however, these analogs have deficiencies as potential therapeutics(Mittelstaedt and Santi, 1986). In order to gain access across cellular membranes, nucleotides must be provided as the corresponding bases or nucleosides and, therefore, must undergo appropriate metabolic activation. CH_2-H_4folate analogs often require conversion to polyglutamylated forms in order to obtain potent inhibition(Lu *et al.*, 1984). From a number of standpoints, it would be desirable to have TS inhibitors which are structurally unrelated to TS substrates. It has been reported that non-steroidal anti-inflammatory drugs(NSAIDs) are inhibitors of the folate-requiring enzymes, such as phosphoribosyl-aminoimidazolecarboxamide formyltransferase and dihydrofolate reductase(Ha *et al.*, 1990). We, therefore, have expanded on these observations and have investigated the inhibitory effects of NSAIDs on *L. casei* TS. As a step toward that goal, we have used a synthetic gene constructed based on the amino acid sequence of TS from *L. casei*(Climie and Santi, 1990). Since the recombinant enzyme is highly overexpressed, large quantity of the pure enzyme has been obtained with no difficulties in purification. The enzyme was purified by hydroxyapatite chromatography using a potassium phosphate gradient, followed by ammonium sulfate precipitation(Table I). The purity was confirmed by Mono-Q showing one UV absorbing peak which had TS activity eluting at 300 mM NaCl. SDS-PAGE of the purified enzym showed a one protein band of Mr ~35,000 corresponding to that of TS (Fig. 1).

Inhibition of TS by NSAIDs is shown in Table II. High drug-to-substrate concentration ratio were attempted because the daily dose of many of these agents is large. The [I]/[S] ratios were limited by drug solubility. Most NSAIDs tested were inhibitors of TS. The following observations suggest that this property might related to anti-inflammatory activity; 1) Sulphasalazine, a compound which possesses substantial anti-inflammatory activity, was found to be a potent inhibitor of TS. On the other hand, its sulpha-drug metabolite, sulphapyridine, was found to be inactive. Thus the inhibition of the TS by sulphasalazine is not simply a general property shared by sulpha drugs, but is found in this particular compound which possesses anti-inflammatory properties. Selhub *et al.*(1978) have found that sulphasalazine, but not sulphapyridine, is an inhibitor of other folate-requiring enzymes.; 2) Penicillamine and hydroxychloroquine were inactive as TS inhibitors, even at very high [I]/[S] ratios. Antipyrine was inactive, whereas acetaminophen proved to be a relatively weak inhibitor. This again suggest that drugs with weak or no antifolate activity have little anti-inflamma-

Table I. Purification of thymidylate synthase from 50 m*l* cultures

Step	Protein (mg)	Specific Activity (units/mg)	Yield (%)	Purification fold
Crude	27	0.4	100	1.0
Hydroxyapatite	8	1.3	92	3.3
$(NH_4)_2SO_4$	4	1.9	65	4.8

tory effect, and that the antipyretic and analgesic effects are not related to antifolate activity.; 3) Aspirin was inactive as an inhibitor of the TS, in contrast with salicylic acid. This suggest that aspirin, when considered as an anti-inflammatory agent, is a pro-drug and that salicylic acid is the active compound.; 4) The inhibition was always competitive with respect to folate, suggesting that this was not due to a non-specific binding or denaturation(Fig. 2).

Table II shows that K_i values of sulphasalazine and salicylic acid for TS are lower than the peak plasma concentrations achieved with the usual daily dosages of these drugs. With the exception of piroxicam, other drugs tested had at least one K_i value within an order of magnitude of its peak plasma concentration or its intracellular concentration(Table II). Therefore inhibition of TS could occur in vivo under routine levels of drug administration. Panush(1976) has demonstrated that a number of NSAIDs, including aspirin, indomethacin and naproxen, are inhibitors of mitogen and antigen-mediated lymphocyte activation in vitro, whereas acetaminophen and penicillamine were found to be comparatively weaker inhibitors. These results could be explained by the relatively potent antifolate effect of

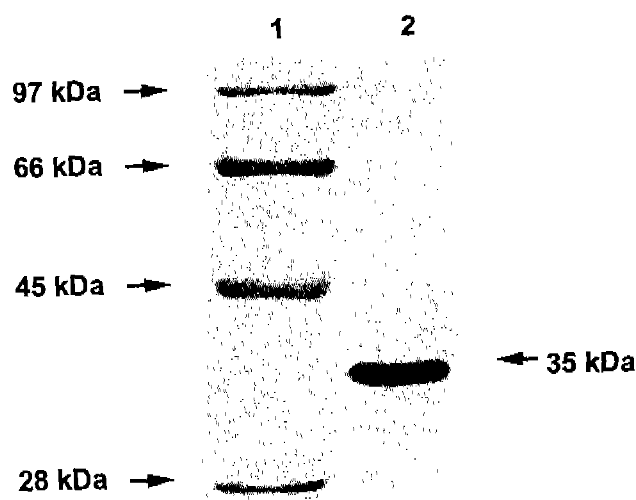

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of thymidylate synthase purified from *E. coli* strain x2913/recA⁻. Lane 1: Molecular weight marker proteins, Lane 2: purified thymidylate synthase.

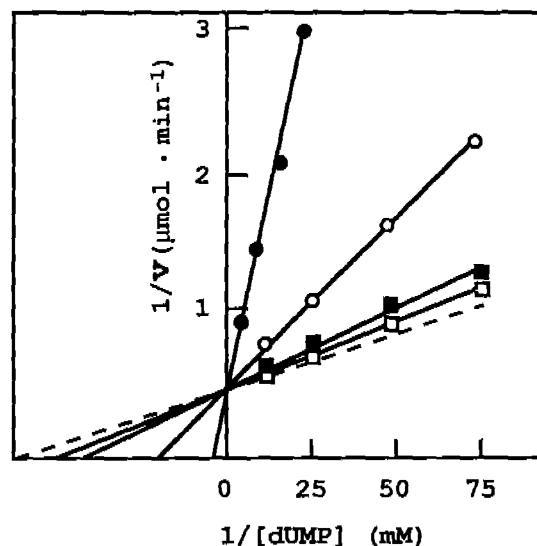

Fig. 2. Inhibition of thymidylate synthase activity by NSAIDs. For the determination of K_i values, the concentration of was kept CH_2-H_4folate constant and dUMP was varied. Results were obtained at concentrations of NSAIDs as indicated in Table II. K_i values were estimated by the method of Cleland (1979). Sulphasalazine(closed circle), indomethacin(open circle), salicylic acid(closed square), mefenamic acid(open square), and 5 mM potassium phosphate buffer, pH 7.4 as a control(dashed line).

Table II. Competitive inhibition of thymidylate synthase by NSAIDs

Drug	[I]/[S]*	Ki (mM)	Daily dose to treat inflammation(g)**	Concentration (mM)**	
				Peak plasma	Tissue
Sulphasalazine	5	0.013	3~4	0.05	
Indomethacin	50	0.77	0.2	0.02	0.06
Salicylic aicd	600	1.8	5~8	2.0	4~67
Mefenamic acid	100	2.7	1~2	0.08	1.3
Aspirin	300	Inactive***	5~8	2.0	12
Acetaminophen	400	10.3	1~4	0.5	
Piroxicam	50	Inactive	0.02	0.02	
Sulphapyride	100	Inactive			
Antipyrine	140	Inactive			
Penicillamine	800	Inactive	1.5	0.2	
Hydroxychloroquine	500	Inactive	0.4	0.001	

*The maximum drug-to-substrate concentraion ratio tested. **From Flower *et al.*(1985). ***Inactive means that less than 10% inhibition was found at highest [I]/[S] ratios.

indomethacin and naproxen, in contrast with the weak effect of acetaminophen and penicillamine. It has been reported that blood mononuclear cells(BMCs) from both methotrexate-treated rheumatoid arthritis(RA) patients and NSAIDs-treated RA patients have a blunted proliferative response to phytohaemagglutinin when compared with a population not using prescription drugs. The blunted response was more pronounced when the test was conducted in a medium containing physiological levels of folic acid as opposed to a medium with very high folate levels. This finding is mechanistically consistent with high intracellular folate coenzyme concentrations relieving, in part, the competitive inhibition produced by the NSAIDs.

A knowledge of the interaction of NSAIDs with TS may provide insights into approaches for the design of a new class of inhibitors. All inhibitors of TS listed Table II contain a carboxylic acid group and an aromatic ring. The carboxylic acid group may be proximal(e.q. salicylic acid) or distal(e.q. sulindac) from the aromatic ring. The data suggest that both a carboxylic acid and an aromatic ring are necessary for competitive inhibition of TS, since penicillamine has a carboxylic acid moiety but not aromatic ring; piroxicam, acetaminophen and chloroquine have aromatic ring, but no carboxylic acid moiety, and none of the above agents is an effective inhibitor. A nitrogen in the para position to the carboxylic acid group produced relatively tight binding, possibly better mimicking the structure of the *p*-aminobenzoylglutamate moiety of folate coenzymes.

The results presented are consistent with the hypothesis that the anti-inflammatory properties of NSAIDs results from interference with folate-coenzyme metabolism. Interference of folate metabolism will have a cytotoxic or cytostatic effect and may explain why general cytotoxic agents are useful in the treatment of diseases of inflammation. This finding may also explain why a variety of non-prostaglandin-mediated cytostatic and anti-proliferative effects are observed when cells are exposed to NSAIDs(Panush, 1976; Weissman, 1991). NSAID antifolate activity, anti-prostaglandin activity and other properties(Weissman, 1991; Selhub *et al.*, 1978), therefore, might work together to produce the entire complement of anti-inflammatory effects. A precise mechanistic description of the intimate interaction of the NSAIDs with TS remains to be elucidated.

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