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Inhibition of soybean lipoxygenase by sulfasalazine and 5-aminosalicylic acid: a possible mode of action in ulcerative colitis

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For over 30 years, sulfasalazine [1] has been the drug of choice in the treatment of ulcerative colitis. The exact mode of action remains unclear, but it has been hypothesized that the action of sulfasalazine and its metabolite, 5-aminosalicylic acid (5-ASA), is through inhibition of colonic mucosal prostaglandin synthesis [2, 3], inhibition of mucosal prostaglandin metabolism [4–6], inhibition of absorption of folates [7] or inhibition of DNA synthesis [8]. The apparent contradictions about the mode of action of sulfasalazine and 5-ASA suggest that a totally different mode of action might be involved.

Ulcerative colitis is characterized by an acute mucosal inflammation dominated by polymorphonuclear leukocyte accumulation by random migration [9]. Sulfasalazine and 5-ASA inhibit such random migrations, phagocytosis and oxidative metabolism [10].

Leukotrienes, hydroperoxyeicosatetraenoic acids (HPETE), and hydroxyeicostetraenoic acids (HETE) are products of arachidonic acid metabolism via the lipoxygenase pathway. They have been shown to be highly potent and stereospecific factors stimulating polymorphonuclear leukocyte (PMNL) migration [11–14] and enhancing vascular permeability [15]. Since sulfasalazine and 5-ASA inhibit the migration of PMNL, it seems possible that sulfasalazine and 5-ASA might be working in part through inhibition of the lipoxygenase pathway. To test the above hypothesis, we evaluated sulfasalazine and 5-ASA for possible lipoxygenase inhibitor activity.

Materials and methods

Sulfasalazine and 5-ASA were tested against the lipoxygenase of arachidonic acid using soybean lipoxygenase (EC 1.13.11.12) and linoleic acid as substrate.

Sulfasalazine was a gift from Salsbury Laboratories, Charles City, IA, and 5-ASA was obtained from the Aldrich Chemical Co., Milwaukee, WI. Soybean lipoxygenase, linoleic acid and Tris were purchased from the Sigma Chemical Co., St. Louis, MO.

Determination of lipoxygenase activity. The conversion of linoleic acid to hydroperoxylinoleic acid was followed spectrophotometrically by the appearance of a conjugated diene at 234 nm. The enzymatic reaction was monitored using a Gilford model 250 spectrophotometer at 24°. Each assay had a total volume of 1 ml and contained sodium linoleate, 100 μM; 0.1 M Tris hydrochloride, pH 9.0; 2% ethanol; 4.2% propylene glycol; and sufficient enzyme to give an easily measurable initial rate of reaction [16, 17]. Substrate solutions were prepared fresh prior to assays with 20% ethanol in Tris buffer, and inhibitors were dissolved in a 5.25% propylene glycol–Tris buffer solution in such a manner that an aliquot of each yielded a final concentration of 4.2% propylene glycol and 2% ethanol in each assay. The effects of inhibitors on the enzymatic reaction were compared against controls under identical conditions. The substrate concentration used for all assays was 100 μM. Under the conditions of this assay, an IC_{50} value of 6.1 μM was obtained for the known lipoxygenase inhibitor, nordihydroguaiaretic acid (NDGA). The reported IC_{50} values for NDGA are 2.4 to 10 μM [16, 18].

Results and discussion

The inhibitory activity of sulfasalazine and 5-ASA was measured against soybean lipoxygenase, an enzyme source shown to be predictive of human lipoxygenase [18]. In the presence of sulfasalazine, there was a dose-related inhibition of lipoxygenase enzyme. Based upon three separate determinations, the IC_{50} for sulfasalazine was found to be 66.2 μM, while 5-ASA, the metabolite of sulfasalazine, inhibited the enzyme with an IC_{50} of 170 μM. A representative IC_{50} determination is shown in Fig. 1.

Sulfasalazine inhibits prostaglandin biosynthesis with an IC_{50} of about 1500 μM [5], whereas 5-ASA is weaker than sulfasalazine [5]. While the IC_{50} concentrations are very high, local or topical application of these agents such as with enemas [19–21] might have therapeutic effect.

Sulfasalazine has been shown to inhibit the breakdown

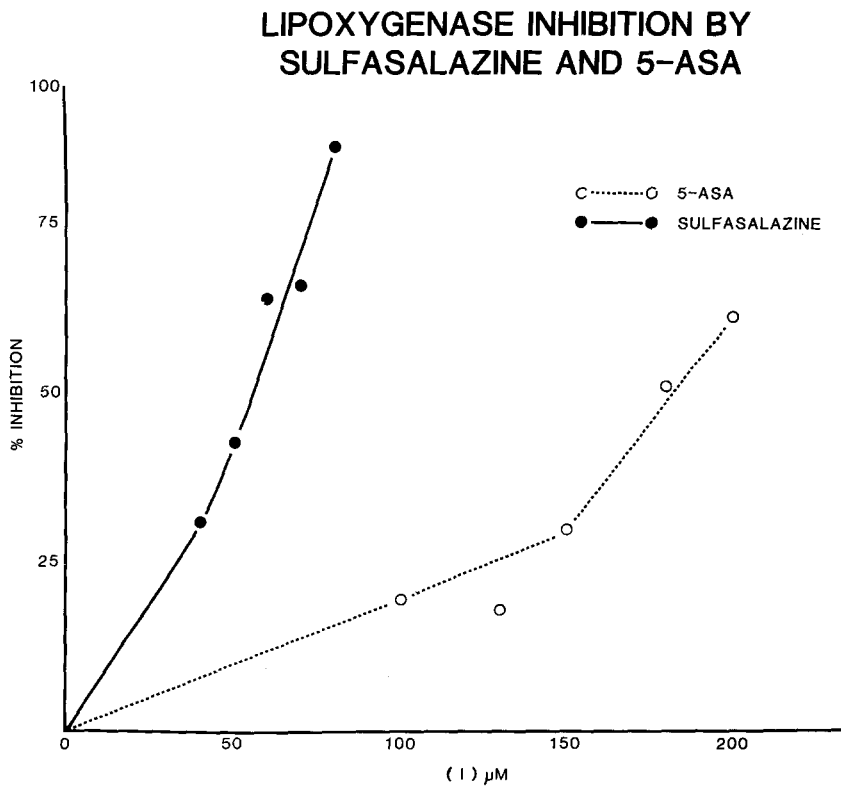

Fig. 1. A representative IC_{50} curve for sulfasalazine (—) and 5-ASA (----) against soybean lipoxygenase. Enzyme reaction was followed spectrophotometrically at room temperature, by the appearance of conjugated diene at 234 nm. Each point is the mean of at least eight determinations. The standard deviation (S.D.) of the mean was less than 10% of the mean value in all experiments. Sodium linoleate was used as substrate at 100 μM in all experiments.

of prostaglandin $F_{2\alpha}$ at 50 μM and prostaglandin E_2 at 50–100 μM through inhibition of prostaglandin-15-hydroxydehydrogenase [5] at concentrations comparable to that of lipoxygenase inhibition. However, 5-ASA is inactive as a prostaglandin-15-hydroxydehydrogenase inhibitor [5] and must have been acting through another, yet undefined, mechanism.

The inhibition of lipoxygenase by sulfasalazine and 5-ASA represents a single mechanism which could explain the action of both agents. The estimated binding affinities of the drug with the enzyme (K_i) for sulfasalazine and 5-ASA are 16.6 and 42.5 μM, respectively, based on the relation $IC_{50}/S = K_i/K_m$ [22] where the reported K_m for soybean lipoxygenase is 25 μM [23]. The K_i values of sulfasalazine and 5-ASA are less than the average concentration of the drugs found in stools of sulfasalazine-treated patients (78 and 150 mg/100 ml, respectively) [24]. Thus, the K_i values of sulfasalazine and 5-ASA may be at more physiologically significant concentration levels which could explain their mode of action in ulcerative colitis better than the previously suggested modes of action [2–7] of these two drugs.

In summary, sulfasalazine and 5-ASA, two drugs of choice for the treatment of ulcerative colitis, inhibited the arachidonic acid-metabolizing enzyme, lipoxygenase, with estimated K_i values of 16.6 and 42.5 μM respectively. Since these two drugs inhibited lipoxygenase at concentrations lower than that needed to inhibit either PG synthetase or prostaglandin-15-hydroxydehydrogenase, lipoxygenase inhibition might explain their activity against ulcerative colitis by a single mechanism. This conclusion has just been verified using [^{14}C]arachidonic acid and purified human neutrophils [25]. Lipoxygenase inhibition in the target organ, colon or large bowel, offers a new and rationale approach to the design of potent drugs in the treatment of ulcerative colitis.

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Effects of 1,7- and 1,10-phenanthroline dione on tissue culture cells*

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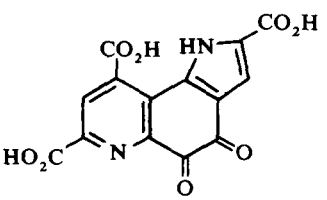
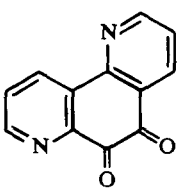
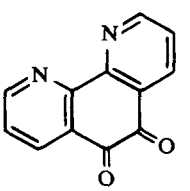
Methoxatin is a recently discovered coenzyme which functions in various bacterial NAD(P)-independent alcohol, glucose, aldehyde and, perhaps, methylamine dehydrogenases [1–4]. Because of the scarcity of this coenzyme, 1,7-phenanthroline dione (1,7-PAD) and 1,10-phenanthroline dione (1,10-PAD) have been studied as chemical models of methoxatin [5]. Both analogs were found to participate in diverse reactions such as 1-electron transfer, transamination, redox reactions, and condensation with nucleophiles. 1,10-PAD also has obvious structural analogy with *ortho*-phenanthroline which impairs cell proliferation [6, 7], and both have a quinone moiety found in a number of potentially useful anti-neoplastic agents [8–10]. Indeed, phenanthrenequinone and a number of related analogs have been shown to be cytotoxic towards cells in culture and to have activity in some experimental animal tumor models [9]. With these considerations, we undertook a study of the effects of 1,7- and 1,10-PAD on cells grown in tissue culture. A preliminary report of our findings is described here.



Methoxatin



1,7-PAD



1,10-PAD

Materials and methods

1,10-PAD was obtained from Alfa-Ventron and 1,7-PAD was prepared as previously described [5]. [CH_3-^{3}H]dThd (50 Ci/mmole) and [5-^{3}H]Urd (20 Ci/mmole) were purchased from Moravek Biochemicals, and [4,5-^{3}H]Ileu (100 Ci/mmole) was obtained from New England Nuclear. All other materials were of the purest grade commercially available. S49 mouse lymphoma cells were grown as suspensions in Dulbecco's Modified Eagle (DME) H16 media supplemented with 10% heat-inactivated horse serum. S180 mouse cells were grown as suspensions in DME H21 supplemented with 10% heat-inactivated fetal calf serum. Cell number was determined daily using a Coulter counter ZBI. The EC_{50} values refer to the concentration of drug which inhibited the growth rate of cells by 50% as compared to controls not containing drug.

Cloning assays of S49 cells were performed by a modfication of a reported procedure [11]. Primary mouse epithelial fibroblasts were grown as monolayers in DME H21 media supplemented with 10% heat-inactivated fetal calf serum. One day prior to cloning, confluent cultures were trypsinized, resuspended in media, and replated in 2.4 × 1.7 cm tissue culture wells at *ca.* 50–70% confluency as determined microscopically. The cloning medium was freshly prepared by adding 100 ml DME H16 medium (at 44°) containing 10% horse serum, 25 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) buffer (pH 7.4), 100 units/ml of penicillin, and 0.1 mg/ml of streptomycin to an autoclaved solution of 0.44 g agarose in 10 ml water. The medium was kept at 44°. At least 1 hr before use, media from primary mouse epithelial cell cultures were aspirated, and cells were overlayed with 2.0 ml of the cloning media. Plates were briefly cooled and kept at 37° until use. S49 cells, treated as specified, were diluted

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