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AN EXTREMELY POTENT ANILINOACRIDINE INHIBITOR OF ALDEHYDE OXIDASE

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An acridine antitumor agent, 4'-(9-acridinylamino)methanesulfon-m-anisidide, has been found to be an extremely potent competitive inhibitor of aldehyde oxidase (EC 1.2.3.1). The inhibitor constant (K_i) was determined to be 0.06 μM. The degree of enzyme inhibition was quite sensitive to small changes in the structure of the inhibitor's anisidide moiety. Drug inhibition was specific for aldehyde oxidase and inhibition was not detected with the other mammalian molybdenum iron-sulfur flavoenzyme, xanthine oxidase (EC 1.2.3.2). Members of the 4'-(9-acridinylamine)methanesulfonanilide series might be useful probes in the study of the structure and function of aldehyde oxidase.

The mammalian molybdenum, iron-sulfur, flavoenzymes form a small group of enzymes consisting of aldehyde oxidase (EC 1.2.3.1) and xanthine oxidase (EC 1.2.3.2). Xanthine oxidase is important in the catabolism of purines (1). Aldehyde oxidase has no known comparable biological function, but its broad range of acceptable substrates has lead to suggestions that it functions as part of the hepatic detoxification mechanism (2). Because of their very large and very complex structure, these enzymes have been intensively investigated and remain an area of active investigation.

Acridines, and particularly quinacrine, are known to inhibit the molybdenum, iron-sulfur flavoenzymes (3,4). Cain and co-workers have recently synthesized a series of acridine ring containing compounds, the 9-anilinoacridines, many of which possess antitumor activity (5-7). The prototype drug of this class, (4'- acridinylamino) methanesulfon-m-anisi-

Abbreviations: mAMSA, 4'-(9-acridinylamino)methanesulfon-m-anisidide;
oAMSA, 4'-(9-acridinylamino)methanesulfon-o-anisidide.

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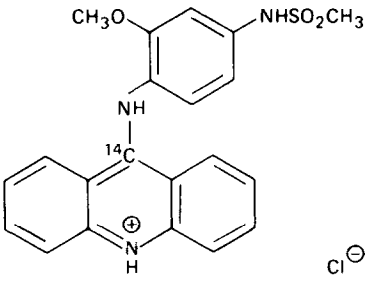

Fig. 1. Structure of 4'(9-acridinylamino)methanesulfon-m-anisidide.

dide, mAMSA, is undergoing extensive clinical trials and appears active against leukemias (Fig. 1) (8). This report presents data showing that mAMSA is an extremely potent inhibitor of aldehyde oxidase, possibly the most potent competitive inhibitor so far described.

MATERIALS AND METHODS

Aldehyde oxidase was prepared from the livers of New Zealand White rabbits. The liver was homogenized with a polytron in an iced buffer of 0.05M potassium phosphate pH 6.8 containing 0.001M EDTA. The homogenate was heated to 55°C for 12 minutes, rapidly cooled to 4°C and centrifuged at 12,000g x 45 minutes. An ammonium sulfate precipitation was performed on the supernatant and the protein precipitating between 35 - 55% saturation was dissolved in 0.05M potassium phosphate pH 7.8 buffer containing 0.3mM EDTA. At this stage of purification the enzyme exhibited a Km (using N^1-methylnicotinamide as substrate) of 7×10^{-4}M, which is consistent with published values (9). Any contaminating xanthine oxidase effects, as determined by the addition of allopurinol, were undetectable. The assay for enzyme activity consisted of a 0.05M potassium phosphate buffer pH 7.8 (0.3mM EDTA) containing N^1-methylnicotinamide, with or without inhibitors, plus enzyme. Activity was followed spectrophotometrically at 300nm. Activity was linear for at least 10 minutes under assay conditions.

Xanthine oxidase was purchased from Sigma and used without further purification. Enzyme was suspended in 0.05M potassium phosphate buffer pH 7.8 containing 0.3mM EDTA. Xanthine, with or without inhibitor, was dissolved in the enzyme buffer at appropriate concentrations and enzyme added to start the reaction. The reaction was followed spectrophotometrically at 295nm.

Analysis of the enzyme reaction mixture for mAMSA was by HPLC. The enzyme reaction mixture, containing [^{14}C] mAMSA, was frozen in a dry-ice/ethanol bath and then lyophilized. The lyophilized powder was extracted with methanol and 50ul aliquots injected onto a Waters C-18 ODS reverse phase column using a Gilson HPLC system. The mobile phase was run as a linear gradient: initial conditions were 80% solution A (0.02M sodium citrate with 20% acetonitrile, pH 3.8) and 20% methanol; final conditions were 20% solution A and 80% methanol. The gradient developed over 30 minutes at a flow rate of 3 ml/min and thereafter the mobile phase remained fixed at final conditions with a flow rate of (3ml/min.) mAMSA eluted at 33 minutes. Column effluent was constantly monitored at 254nm with a Gilson Model 111 LC detector and was collected in 3ml aliquots with a LKB 2112 Redirac fraction collector. Fractions

were mixed with 15ml of ACS scintillation fluid and counted in a Beckman LS 9000 scintillation counter.

4'-(9-acridinylamino)methanesulfon-m-anisidide, 4'-(9-acridinylamino) methanesulfon-o-anisidide, and 4-amino-3-methoxymethanesulfonanilide were obtained from Division of Cancer Treatment, NCI. N^1-methylnicotinamide and 9-aminoacridine were obtained from Sigma.

RESULTS AND DISCUSSION

When aldehyde oxidase was added to a mixture of N^1-methylnicotinamide and mAMSA, rapid and potent enzyme inhibition was observed. A double reciprocal plot of mAMSA inhibition of aldehyde oxidase is shown in Figure 2. The pattern of inhibition is classically competitive; all lines have a common point of intersection on the y-axis. A plot of the slope of the inhibited reactions against inhibitor concentration is linear, providing further confirmation of the competitive nature of the inhibition. Pre-incubation of the enzyme with mAMSA did not increase the observed inhibition. Replotting the data in the form of a Dixon plot determined the Ki to be 6×10^{-8}M (Fig. 3).

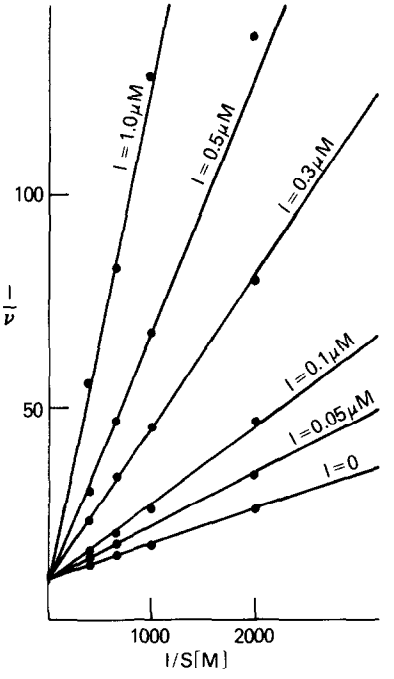

Fig. 2. A double reciprocal plot of the inhibition of aldehyde oxidase by mAMSA in 0.05 M potassium phosphate buffer, pH 7.8. Enzyme substrate was N^1-methylnicotinamide.

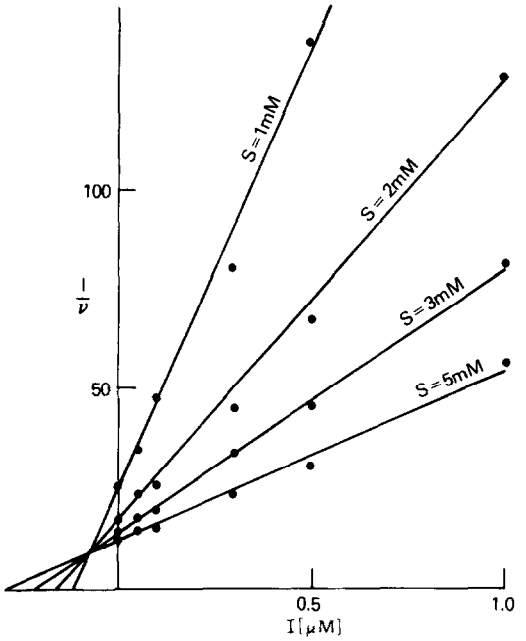

Fig. 3. Dixon plot of the inhibition of aldehyde oxidase by mAMSA. Substrate utilized was N^1-methylnicotinamide.

Competitive inhibition allows for the possibility that mAMSA is a substrate for aldehyde oxidase. This possibility was tested by incubating enzyme and radiolabeled (^{14}C) mAMSA for 1 hour at 37°C and analyzing the result by HPLC. When analyzed by UV absorbance, only a single peak, corresponding to parent drug, was seen on the chromatogram. All applied radioactivity could be accounted for in a single peak that co-eluted with the UV peak. There was no evidence of any enzymatic alteration of mAMSA by the aldehyde oxidase enzyme.

To examine the inhibitory ability of mAMSA, we studied separately the inhibitory properties of its acridine moiety, 9-aminoacridine, and its anilino side arm, 4-amino-3-methoxymethanesulfonanilide. The inhibition exhibited by 9-aminoacridine was competitive and had a K_i of 3×10^{-6}M. In contrast, the anilino side arm was not inhibitory at micromolar concentrations but did show inhibition at concentrations above 1×10^{-4}M. The inhibition, however, was noncompetitive.

To further study the relationship of aldehyde oxidase inhibition to the structure of the mAMSA anilino side arm, we examined a structural

isomer of mAMSA which has a methoxy group positioned ortho rather than meta on the anilino ring. This structural change reduced the inhibition by 10 fold, yielding a Ki for oAMSA of 6×10^{-7}M. Interestingly, oAMSA has proven to be less active than mAMSA in several other biochemical and biological test systems (7, 10).

No inhibitory activity of mAMSA against xanthine oxidase could be detected at any mAMSA concentration up to the solubility limit of the drug in buffer.

The anilinoacridines and particularly mAMSA have been extensively studied for their antitumor properties. Evidence to date suggests that the antitumor activity is the result of drug intercalation and interaction with cellular DNA (10,11,12). It is unlikely that inhibition of aldehyde oxidase plays a direct role in the cytotoxicity of mAMSA. However, it appears that the position of the methoxy group on the anisidide moiety effects biological activity, whether measured by cytotoxicity, DNA strand breaks, or aldehyde oxidase inhibition. The importance of the position of the methoxy group in both the induction of DNA damage and the inhibition of aldehyde oxidase suggests that the molecular interactions that produce one effect may be chemically similar to the interactions that produce the other effect.

Although the inhibition of aldehyde oxidase probably plays no role in the antitumor activity of mkAMSA, its inhibition might effect the use of mAMSA in combination chemotherapy with other antineoplastic agents. Aldehyde oxidase is thought to be the enzyme responsible for the conversion of methotrexate to 7-OH methotrexate (13) and aldophosphamide to carboxyphosphamide (14). Hence, the concurrent administration of mAMSA might effect the catabolism of methotrexate or cyclophosphamide and require alterations in the dose of either, or both, of these agents.

mAMSA is a very potent inhibitor of aldehyde oxidase. Its Ki of 6×10^{-8}M when compared to the published Ki values of other competitive inhibitors, quinacrine, 1.5×10^{-6}M, arsenite, 6×10^{-6}M, p-mercuribenzoate,

2×10^{-7}, suggests that it may be the most potent competitive inhibitor thus far described (3). More importantly, while major additions to the acridine ring at the 9-carbon can produce profound changes in enzyme inhibitor ability (9-aminoacridine, Ki = 3×10^{-6}M, and mAMSA, ki = 6×10^{-8}M), even subtle changes within the 9-carbon adduct can cause marked changes in enzyme inhibiitory ability (mAMSA versus oAMSA). Because of existing interest in the antitumor properties of mAMSA, hundreds of analogs exist which could be used for probing the active site of aldehyde oxidase.

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