The in Vitro Inhibition of Xanthine Dehydrogenase by 8-Azaguanine*

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The biochemical basis underlying the cytotoxic and carcinostatic effects of 8-azaguanine is still uncertain. The initial proposal that this compound acts as a guanine antagonist now seems less likely in view of recent findings that 8-azaguanine does not diminish the rate of incorporation of guanine-C\textsuperscript{14} into tissue nucleic acids (3). Observations that small amounts of isotopically labeled 8-azaguanine are incorporated into tissue nucleic acids have led some investigators to the belief that these fraudulent nucleic acids containing 8-azaguanine may be responsible for the growth-inhibitory effects (12, 18).

Recent studies in our laboratory concerning the mode of action of 8-azaguanine have explored the alternative hypothesis that 8-azaguanine inhibits one or more of the enzymes concerned with purine interconversions. The present report concerns our findings that even subtherapeutic concentrations of 8-azaguanine significantly inhibit xanthine dehydrogenase activity in vitro. A hypothesis is presented integrating these findings with other previously established phenomena induced by 8-azaguanine.

MATERIALS AND METHODS

The xanthine dehydrogenase assays employed were as previously described (6), wherein the enzyme catalyzes the transfer of hydrogen atoms from xanthine to 2,6-dichlorophenol indophenol. Xanthine dehydrogenase inhibition measurements were routinely carried out by the addition of the indicated amounts of 8-azaguanine to spectrophotometer cuvettes containing the appropriate reaction mixtures. The enzyme, cream xanthine oxidase, was added last, and the rate of dye reduction was followed by noting the decrease in optical density at 600 m\textmu (E\textsubscript{600 mm}) at 1-minute intervals. The extinctions were plotted against time, and the linear initial slopes were taken as a measure of enzyme activity. The assay conditions were arranged so that the reaction rates were linear functions of the enzyme concentration. Control flasks in which the enzyme was omitted were run, and in no case did significant changes in E\textsubscript{600 mm} occur. A Beckman DU Spectrophotometer and a Cary Model 11 Recording Spectrophotometer were employed in these kinetic studies. All analyses were conducted at room temperature. The experimental points presented are the means of duplicate determinations. The straight lines through these points were derived by the method of least squares (20).

Stock 0.03 M solutions of 8-azaguanine (Lederle) were prepared by the addition of NaOH until a pH of 9.5 was attained, and final 0.006 M 8-azaguanine solutions were prepared by dilution with water.

RESULTS

Chart 1 depicts the in vitro effects of 8-azaguanine on xanthine dehydrogenase activity. It is apparent that a 50 per cent enzyme inhibition occurred at a concentration of approximately 5 \times 10^{-4} M 8-azaguanine, and that the extent of inhibition was proportional to the drug concentration. The enzyme activity was never observed to decrease to below 25 per cent of the initial activity, even in the presence of drug concentrations several times higher than the highest concentration depicted in this figure. These findings suggest that a part of the catalytic activity, possibly one of the active centers of the enzyme, is less sensitive to this inhibitor.

To elucidate the nature of the 8-azaguanine-xanthine dehydrogenase interaction, a series of kinetic experiments were undertaken. Enzyme activities were determined over a wide range of substrate concentrations in the absence and presence of 5.6 \times 10^{-4} M 8-azaguanine. In Chart 2 the data obtained are plotted as the reciprocal of the enzyme activity (1/4) versus the reciprocal of the substrate concentration (1/4). The upper line ob
tained in the presence of 8-azaguanine clearly shows both an elevated ordinate intercept and an increased slope when compared with the lower line obtained in the absence of the inhibitor. This indicates that high concentrations of substrate do not reverse the 8-azaguanine-induced inhibition and that the inhibition is noncompetitive in nature (21). The calculated dissociation constant of the enzyme-substrate complex \( K_s \) is \( 2.6 \times 10^{-4} \) M, and the dissociation constant of the enzyme-8-azaguanine complex \( K_i \) is \( 5.6 \times 10^{-4} \) M. This indicates that 8-azaguanine has about one-half the affinity of xanthine for the enzyme.

To gain further insight into this enzyme-inhibitor relationship the number of moles of 8-azaguanine which combine per mole of xanthine dehydrogenase was estimated from a plot of \( \log (v - v_e) = R \log (I) + \log \frac{v}{v_e} \), where \( v \) and \( v_e \) are the enzyme activities observed in the absence and presence, respectively, of the inhibitor, 8-azaguanine \( (I) \); \( "R" \) is the number of moles of inhibitor combining per mole enzyme; \( K_i \) is the dissociation constant for the inhibitor-enzyme complex (21).

The data are depicted in Chart 3. Since the slope of the line \( "R" \) is 0.99, one may conclude that 1 mole of 8-azaguanine combines with 1 mole of enzyme over the entire inhibitor range tested. The calculated \( K_i \) is \( 4.5 \times 10^{-4} \) M, which is in fair agreement with \( K_i \) of \( 5.6 \times 10^{-4} \) M derived from the Lineweaver-Burk analysis.

Although the reactive center of xanthine dehydrogenase concerned with the oxidation of hypoxanthine has always been considered to be identical to that involved in xanthine oxidation, the effects of 8-azaguanine upon the former reaction were studied directly. With hypoxanthine as a substrate the disappearance of optical density at 255 m\( \mu \) upon incubation with cream xanthine dehydrogenase was measured in the absence and presence of 8-azaguanine. The drug was found to inhibit this activity of the enzyme to a degree similar to its inhibition of xanthine oxidation.

To ascertain the specificity and biological significance of the observed inhibition of xanthine dehydrogenase by 8-azaguanine, two approaches were utilized. It was first determined whether similar compounds would inhibit this enzyme. Thus the inhibitory influences of guanine and 8-azaxanthine were studied comparatively with isomolar levels of 8-azaguanine. The results of two such experiments shown in Table 1 indicate that both 8-azaxanthine and guanine exert but a fraction of the inhibitory activity on xanthine dehydrogenase shown by 8-azaguanine.

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The second test of the specificity of 8-azaguanine inhibitions of xanthine dehydrogenase involved studies of the effect of 8-azaguanine on a variety of other enzymes. It was found that the highest drug concentration tested \( (8 \times 10^{-4} \) M) exerted no significant inhibition on the following enzyme systems: yeast acid phosphatase, erythrocyte phosphohexose isomerase, erythrocyte nucleoside phosphorylase, and liver succinic oxidase. Thus, at a drug concentration 100 times that which significantly inhibits xanthine dehydrogenase, these other enzymes were uninfluenced. It was found, however, that one other enzyme, adenosine deaminase, was inhibited by 8-azaguanine in concentrations above \( 10^{-4} \) M (7). These data
clearly indicate that 8-azaguanine shows a high degree of specificity for the enzymes it will inhibit.

DISCUSSION

To have pharmacological significance, sensitivity of an enzyme to 8-azaguanine must satisfy at least two criteria: inhibition must take place at or below drug concentrations therapeutically achievable, and pharmacologically inactive catabolites of the drug should be less potent inhibitors of the enzyme than the drug itself. An effective carcinostatic dose of 8-azaguanine in mice is approximately 50 mg/kg body weight/day (18). Assuming an animal to be approximately 75 per cent water and assuming an equal distribution of the drug throughout the animal, the maximum drug concentration achievable in vivo is 4.4 x 10^{-4} M. As indicated in Chart 1, 50 per cent inhibition of xanthine dehydrogenase occurs at even one-tenth of this presumably pharmacologically attainable drug concentration.

8-Azaguanine is deaminated in vivo to 8-azaxanthine (17). As the latter compound possesses no carcinostatic activity (9), compliance with the second criterion above requires that the significant enzyme system be sensitive to 8-azaguanine and relatively insensitive to 8-azaxanthine. That this relationship holds for xanthine dehydrogenase was experimentally demonstrated (Table 1).

The specific conversion of inosinic acid to adenyllic acid and to xanthyllic acid by rabbit bone marrow extracts (1, 2). The same workers have also shown that the xanthyllic acid is aminated to form guanylic acid. Xanthine oxidase fits into this scheme in the interconversion of hypoxanthine and xanthine and in the diversion of xanthine to uric acid. Therefore, to the extent that conversion of hypoxanthine compounds to xanthine moieties occurs physiologically at the free base level, 8-azaguanine inhibition of xanthine oxidase will inhibit this conversion and limit the xanthine available for guanine moiety biosynthesis.
This hypothesis seems to help to explain several previously isolated phenomena described in the literature. The reported reversals of 8-azaguanine-induced inhibition of tumor growth by exogenous guanine and guanyclic acid (10, 13, 14) are readily accounted for as a replacement of the guanine moieties whose biosynthesis has been blocked. The carcinostatic synergisms observed when 6-formylpteridine or flavitin are administered together with 8-azaguanine are interpretable as a synergistic inhibition of the same enzyme, since the two former compounds have been found to be inhibitors of xanthine oxidase (4, 19).

In *Tetrahymena geleii*, adenine moieties are normally biosynthesized from guanine; however, in the presence of 8-azaguanine, adenine synthesis is blocked, and a requirement for exogenous adenine is induced. Hypoxanthine alleviates this 8-azaguanine-induced adenine requirement, whereas xanthine is completely ineffectual in this regard (8). These findings are compatible with the results here reported, that 8-azaguanine blocks the xanthine oxidase-catalyzed conversion of xanthine to hypoxanthine (Chart 4).

*In vitro* incubation of the purine precursor, glycine-2-Cl, with 6CSHED ascites cells in the presence of 8-azaguanine leads to inhibition of C14 incorporation into nucleic acid guanine and to stimulation of isotope incorporation into nucleic acid adenine (16). On the basis of the proposed hypothesis this might be rationalized as follows: Since 8-azaguanine inhibits xanthine dehydrogenase, the rate of conversion of the biosynthesized hypoxanthine to xanthine and then to guanine is depressed, resulting in diminished C14 incorporation into guanine; as the metabolic drain on the biosynthesized hypoxanthine-C14 is decreased, this compound is more readily available for conversion to adenine, thereby resulting in an elevation of C14 incorporation into adenine induced by 8-azaguanine.

It is to be emphasized that the proposed mechanism as to the mode of action of 8-azaguanine is strictly a working hypothesis which seems compatible with the findings reported in this study as well as those gleaned from the literature. Experiments are currently under way to test further the validity of this hypothesis.

**SUMMARY**

8-Azaguanine was found to inhibit purified xanthine dehydrogenase in *vitro*; 50 per cent inhibition of the enzymatic activity occurred at the concentration of 5 × 10^-4 M 8-azaguanine. The inhibition was noncompetitive with regard to substrate. Kinetic studies indicate that 8-azaguanine manifests approximately one-half the affinity for the enzyme as does the substrate (K1 = 5.0 × 10^-4 M; K2 = 2.6 × 10^-4 M). One mole of 8-azaguanine combines with each mole of enzyme.

A hypothesis is proposed localizing a major pharmacological effect of 8-azaguanine to the induction of a guanine moiety deficiency by interference with xanthine oxidase, an enzyme postulated as catalyzing an essential reaction along one metabolic route leading to guanine moiety biosynthesis. This hypothesis is applied toward the integration of several previously reported findings.

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