GLYOXALASE INHIBITORS AS POTENTIAL ANTICANCER AGENTS

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SUMMARY

A study of a series of S-alkylglutathiones as inhibitors of the enzyme, glyoxalase I, revealed that a non-polar region exists on the enzyme which plays an important role in the formation of an enzyme-inhibitor complex. The amount of inhibition of glyoxalase increases as the alkyl chain is lengthened from methyl to n-octyl. By taking advantage of the non-polar region of the enzyme, compounds have been prepared which are 435 times more active than the previously reported S-methylglutathione.

It has been known for several years that α-ketoaldehydes possess powerful carcinostatic activity (1). However, French and Freedlander (1) pointed out the possibility that these compounds may be converted to the corresponding inactive hydroxy acids by the enzyme, glyoxalase. Several reviews on the glyoxalase system (2) point out the fact that the cytotoxic methylglyoxal is the substrate for this enzyme and is converted to the non-toxic lactic acid in the presence of a cofactor, glutathione. These facts, along with the observation by Stern (3) that glutathione (GSH) is necessary for cell division, suggested to us that cells may regulate their own growth rate by utilizing the glyoxalase system in maintaining a proper concentration of methylglyoxal. In 1967, a proposal was submitted from our laboratory to the National Institutes of Health entitled "Glyoxalase Inhibitors as Potential Anticancer Agents"* in which we stated that inhibitors of the glyoxalase enzyme may be used to cause a build up of methylglyoxal in tumor cells and thus cause an inhibition of cellular growth. Recent publicity on a similar theory (4) has prompted us to present some of our preliminary studies on glyoxalase inhibitors at this time.

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The glyoxalase system comprises two enzymes which catalyze the reactions:

$$CH_3\text{-}CO\text{-}CHO + GSH \xrightarrow{\text{glyoxalase I}} CH_3\text{-}CH(OH)\text{-}CO\text{-}SG \xrightarrow{\text{glyoxalase II}}$$

$$CH_3\text{-}CH(OH)\text{-}COOH + GSH$$

(lactic acid)

The work described is concerned with glyoxalase I only. The sulfhydryl group of GSH plays an important role in the enzymatic reaction and S-methylglutathione is known to inhibit the glyoxalase I reaction (5). Our initial approach to the design of more potent inhibitors consisted of extending the S-alkyl chain of the GSH analogs in an attempt to find a possible non-polar region on the enzyme adjacent to the GSH binding site resulting in a much more stable enzyme-inhibitor complex. Consequently, a study of the factors which are important for the formation of a strong protein-inhibitor complex could lead to the design of compounds which would be selective in their biological action.

MATERIALS AND METHODS

Inhibitors. S-methylglutathione was purchased from Calbiochem Laboratories. All other S-alkylglutathiones were synthesized by reacting the appropriate alkyl iodide with reduced glutathione in aqueous NaOH. After three hours, the reaction mixture was adjusted to pH 3 with HI and refrigerated. The solid ppt. was recrystallized from water to obtain the analytical material. All compounds gave correct elemental analyses for C, H and N.

Reagents and Assay Procedure. A commercial 40% solution (3 ml) of methylglyoxal was diluted to 250 ml with distilled water and the acidic materials were removed by passing the solution through a Dowex 1-X8 (carbonate form) column. The solution was standardized by the method of Friedeman (6). Glyoxalase I was obtained from Sigma Chemical Company and was diluted to a concentration of 40 µg protein/ml with 30% glycerin containing 0.1% Bovine serun albumin. All

enzymatic reactions were performed at room temperature in 0.05M phosphate buffer at pH 6.6. A fresh glutathione solution was made before each assay using distilled water. For each assay the cell contained a total volume of 3.0 ml which was 5.0 mM with respect to methylglyoxal and 0.869 mM with respect to GSH. Sufficient amounts of glyoxalase were employed to give an easily measurable initial rate which was followed by increase in absorption at 240 mμ. Methylglyoxal, GSH and buffer were added to the cell and allowed to equilibrate for exactly 6 minutes before addition of the enzyme (see Results and Discussion for explanation). The ratio of the mM concentration of inhibitor to the mM concentration of substrate for 50% inhibition, $[I/S]_{0.5}$, i.e., the inhibition index was used to compare the inhibitory properties of the various compounds. In order to determine the concentration of inhibitor required for 50% inhibition, a plot of V_o/V_i vs I was made where V_o = initial velocity of the uninhibited enzymatic reaction, V_i = initial velocity of the inhibited reaction at various inhibitor concentrations (7).

RESULTS AND DISCUSSION

It is well known (5,8) that methylglyoxal and GSH react non-enzymatically to form a hemimercaptal according to the equation

$$CH_3\text{-}CO\text{-}CHO + GSH \rightleftharpoons CH_3\text{-}CO\text{-}CH(OH)\text{-}SG$$

Cliffe and Waley (8) have demonstrated that the adduct is the substrate for the enzyme. Kermack and Matheson (5) reported a value of 5×10^{-3} for the dissociation constant, K, in the above reaction using a spectrophotometric procedure. Cliffe and Waley, obtained a value of 2×10^{-3}M (8) which is in agreement with the value obtained by us using a different method which will be described in a more detailed account of this work. The hemimercaptal, q, forms immediately upon adding the methylglyoxal and reaches equilibrium in less than 5 minutes. Since the amount of q formed depends upon the initial concentration

of methylglyoxal, M, and GSH, G, according to equation 1,

$$\frac{(M - q)(G - q)}{q} = K \qquad (1)$$

we can simplify the equation by using a large concentration of M relative to G so that

$$q = \frac{M}{M + K} \times G \qquad (2)$$

This expression is equal to 5/7 x G when our concentration of methylglyoxal equals 5 mM.

TABLE I

INDEX OF INHIBITION OF GLYOXALASE

BY CERTAIN S-ALKYLGLUTATHIONES.

Compound	No.	mM Concentration for 50% Inhibition	$[I/S]_{0.5}$ *
G-S-Me	I	8.30 ± .50	13.8 ± .83
G-S-Et	II	0.964 ± .046	1.56 ± .070
G-S-Pr	III	0.187 ± .017	0.303 ± .030
G-S-Bu	IV	0.057 ± .002	0.093 ± .004
G-S-Pent	V	0.055 ± .003	0.091 ± .002
G-S-Hex	VI	0.032 ± .002	0.052 ± .008
G-S-Hept	VII	0.021 ± .002	0.034 ± .002
G-S-Oct	VIII	0.020 ± .002	0.032 ± .002

* The substrate, S, refers to the calculated concentration of hemimercaptal (0.618 mM) at the concentrations of methylglyoxal and GSH used in our assays. K_m for the hemimercaptal = 4.8×10^{-4} M.

An examination of Table I reveals that glyoxalase exhibits a significant hydrophobic region extending out from the S-alkyl chain of glutathione. The amount of inhibition increases as the alkyl group is lengthened from methyl to n-octyl (I - VIII). Further extension of the chain gave compounds which were too insoluble to assay. The reason for the large increase in binding of the

S-ethyl (II) over the S-methyl derivative (I) is probably a reflection of the fact that the methyl of I is falling into a hydrophilic region on the enzyme that would normally be occupied by the hydroxyl of the hemimercaptal. The addition of a methylene unit may allow the alkyl chain of II to reach out into a hydrophobic region of the enzyme and consequently enhance the binding within the enzyme-inhibitor complex. By taking advantage of the non-polar region of glyoxalase, we have been able to produce inhibitors up to 435 times more potent than S-methylglutathione. These compounds are competitive inhibitors and K_i values have been determined by the method of Dixon (9) or by Lineweaver and Burk plots (10). The K_i values may also be obtained from the $[I/S]_{0.5}$ plot from the slope according to equation 3 (11).

$$m = \frac{1/K_i}{1 + 1/K_m\,[S]} \qquad (3)$$

The above glyoxalase inhibitors are being evaluated as inhibitors of cellular growth in mammalian cell cultures. Other compounds are also being tested in an attempt to further explore the binding areas on the enzyme so that more potent inhibitors may be obtained.

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