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INVESTIGATION ON GLYOXALASE I INHIBITORS

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SUMMARY Several classes of compounds - flavones, coumarins, S- and N-substituted glutathione analogs, transition state analogs, porphyrins, nucleotides and nucleosides - have been reported to inhibit the enzyme glyoxalase I. In the current study, examination of some of the aforementioned compounds has revealed that squaric acid does not function as an inhibitor of glyoxalase I and several other compounds are much less effective in this regard than previously reported. Several new potent inhibitors of yeast glyoxalase I have been identified. Compounds containing the tropolone structure were especially inhibitory. Glutathione adducts of benzoquinone and naphthoquinone were also inhibitory and may be of particular interest with regard to the toxicology of normal aromatic metabolites in vivo.

The glyoxalase system consists of two component enzymes, glyoxalase I (S-lactoylglutathione methylglyoxal lyase, isomerizing; EC 4.4.1.5) and glyoxalase II (S-2-hydroxyacylglutathione-hydrolase; EC 3.1.2.6) (1,2). The substrate for glyoxalase I is the nonenzymatically formed hemimercaptal adduct of glutathione (GSH) and methylglyoxal (and other similar cytotoxic α-ketoaldehydes) which is converted to S-D-lactoylglutathione (3). Glyoxalase II catalyzes the hydrolysis of the thioester to free GSH and D-lactic acid (Fig. 1). Several studies have indicated the presence of the glyoxalase enzymes in all organisms and tissues known except for reduced or nonexistent glyoxalase II activity in tumor cell lines (4). However, the primary biological role of glyoxalase I is not known. It may be involved in cell growth regulation, threonine and glycine metabolism, protection against intestinal bacteria, heme biosynthesis, and detoxification of methylglyoxal (5). The product of glyoxalase I catalysis, S-D-lactoylglutathione, is known to mediate microtubule assembly in vitro, and to potentiate anti-IgE-induced histamine release (5).

Given the ubiquitous nature of the glyoxalase enzymes and the biocidal properties of methylglyoxal, inhibitors of glyoxalase I could prove to be potential anticancer agents and biochemical probes. GSH analogs have been synthesized and tested for in vitro and in vivo inhibitory ability but S-substituted analogs were found to be rapidly catabolized by glutathionases in vivo although inhibitory

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Abbreviations
β-CD, β-cyclodextrin; 2,3-bisGSH-1,4-NQ, 2,3-bisglutathione-1,4-naphthoquinone; 2,5-diOH-1,4-BQ, 2,5-dihydroxy-1,4-benzoquinone; 2-GS-1,4-BQ, 2-glutathione-1,4-benzoquinone; GSH, glutathione; S-methylGSH, S-methylglutathione; S-octylGSH, S-octylglutathione.

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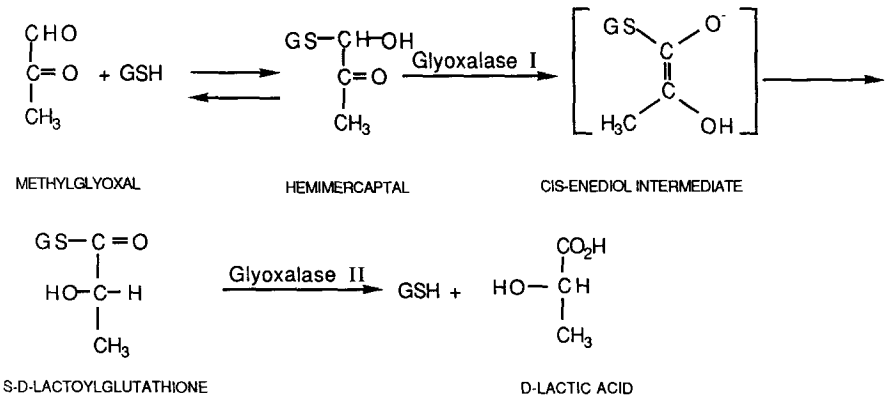

Figure 1. Metabolism of methylglyoxal by the glyoxalase system.

activity was observed in vitro (5,6). Of much greater interest are nonpeptide inhibitors which mimic the hypothesized cis-enediol transition state believed to be involved in the enzymatic mechanism of glyoxalase I (5,7). Douglas and Nadvi have reported on the inhibition of glyoxalase I by compounds containing a cis-enediol or paene structure (8).

Several reports have indicated that a surprising variety of structural types such as nucleotides, porphyrins, and polyhydroxylated compounds appear to inhibit yeast glyoxalase I. A recent report has suggested that nucleosides, nucleotides, D-isoascorbate, rhodizonic acid, and dihydroxyfumarate may not actually inhibit glyoxalase I but rather interfere with the absorbance at 240 nm of S-D-lactoyl-glutathione (9). The present report concerns the identification of new potent inhibitors of glyoxalase I, in addition to a reevaluation of several of the compounds that had been previously reported to have adverse effects on the enzyme.

MATERIALS AND METHODS The following chemicals were obtained from commercial sources: squaric acid, 2,5-dihydroxy-1,4-benzoquinone, tropolone, resorcinol, glutathione, purpurogallin, trifluoroacetic acid, and ethyl benzoylacetate (Aldrich); pyrogallol (Allied Chemical); trichloroacetic acid, catechol, and monobasic potassium phosphate (Baker Chemicals); 1,4-naphthoquinone and hemin (Fluka); hinokitiol (CTC Organics); β-cyclodextrin, mimosine, methylglyoxal, DTNB (Ellman's reagent), methotrexate, myricetin, S-octylglutathione, S-methylglutathione, and S-D-lactoylglutathione (Sigma).

The buffer employed was N_2-degassed 0.05 M potassium phosphate pH 6.6. GSH concentration in stock solutions were determined by titration with DTNB and these solutions were stored at 4°C (10).

Commercially available 40% aqueous solution of methylglyoxal was distilled under normal pressure to eliminate polymeric contaminants. Solution concentrations were determined enzymatically as per Bergmeyer (11a). Solutions were diluted and stored at 4°C. Hemimercaptal concentrations were determined assuming K_{diss}= 3.1 mM +/-0.2 mM (6). For the K_M determination, hemimercaptal concentrations of 0.200 mM, 0.300 mM, 0.500 mM, 0.700 mM, and 0.800 mM were used. Appropriate methylglyoxal and GSH solutions were allowed to equilibrate for 10 minutes. Actual substrate concentrations were taken as one-half that of the hemimercaptal to allow for the diastereomeric selectivity of glyoxalase I (12). Enzyme stock solutions consisted of commercial Sigma Grade X lyophilized yeast glyoxalase I (700 U/mg) dissolved in buffer containing 30% glycerol and 0.1% BSA. Water-soluble inhibitors were dissolved in buffer. Myricetin and coumarin-8 were dissolved in absolute ethanol. Hemin and 1,2-naphthalenediol were dissolved in DMSO. Mimosine was solubilized by the addition of β-cyclodextrin (1:5 molar ratio). Inhibitor stock solutions were diluted with buffer to achieve the desired concentration. Organic solvents accounted for 15% vol/vol or less of the final assay volume. No loss of enzymatic activity was observed under these conditions.

Spectrophotometric measurements for the K_M study and spectral interference were made using a Varian DMS-100 spectrophotometer connected to a Varian DS-15 Data Station. Hemimercaptal solutions were used as reference and reaction was initiated by the addition of glyoxalase I (0.187 U/ml/assay). Enzymatic production of S-D-lactoylglutathione, ε_{240}=3370 $M^{-1}cm^{-1}$, was followed at 240 nm at 25°C for 15 minutes (3). Initial rates were recorded at various substrate concentrations. K_M values were determined from Eadie-Hofstee plots. Linear regression analysis of the data indicate a K_M for the hemimercaptal with yeast glyoxalase I of 0.349 mM +/-0.117 mM, R^2=0.995, in excellent aggreement with previous reports (13,14). The UV spectrum of S-D-lactoylglutathione was measured in the presence and absence of the various potential inhibitors (200 µM). Compounds which appeared to strongly interfere with A_{240} absorbance were tested in decreasing concentrations in order to determine the point at which spectral interference was negligible. Spectrophotometric measurements for IC_{50} studies were performed with 1.6 mM hemimercaptal (with or without inhibitor as necessary) in reference and sample cuvettes. The reaction was initiated by addition of enzyme solution (0.233 U/ml/assay). After 3 minutes, the reaction was terminated by the addition of TCA (224 mM final concentration) and solutions were diluted ten-fold with buffer and the A_{240} nm absorbance was measured. All measurements were performed at 25°C and in triplicate.

ALTERNATE ASSAYS The following alternate assays were attempted: methylglyoxal derivatization, GSH derivatization, and D-lactate determination (10,11b,15,16).

INHIBITOR SYNTHESES Acetohydroxamic acid was prepared according to the method of Fishbein, Daley, and Streeter (17). 2-Glutathione-1,4-benzoquinone was synthesized utilizing the method of Tunek except the reaction time was 24 hrs (18). The solvent was removed under reduced pressure and the resultant crude material was purified by cellulose column chromatography (EtOH:H_2O 3:1). Fractions containing the 2-glutathione-1,4-benzoquinone were concentrated. ^1H NMR indicates the presence of an ABC system at 6.7 ppm, while the GSH portion of the spectrum was identical to that reported by Miller (19). Integration indicated a 1:1 benzoquinone:GSH complex and S-substitution of GSH was confirmed by DTNB and ninhydrin reactions. The compound 2,3-bisglutathione-1,4-naphthoquinone was prepared using the procedure of Nickerson (20). ^1H NMR and other physical characteristics were consistent with the proposed structure (19,20). Coumarin-8 was synthesized utilizing the procedure of Woods and Sapp (21). ^1H NMR,^{13}C NMR and IR data were consistent with published results and predictions (21-23).

RESULTS and DISCUSSION Glyoxalase I has attracted much attention as a target enzyme for anticancer drug development. Inhibition of glyoxalase I can lead to elevated levels of methylglyoxal, and if inhibition of the enzyme in a tumor cell can be achieved, reduction in tumor growth or perhaps tumor death may result. Thus, compounds which effectively inhibit glyoxalase I might be of potential chemotherapeutic importance as well as biochemical interest.

During the course of determining the IC_{50} values for a variety of potential inhibitors of glyoxalase I, Ray and Ray reported that the "inhibitory" ability of several nucleotides, D-isoascorbate, rhodizonic acid, and dihydroxyfumaric acid might be an artifact of interference of the absorbance of S-D-lactoylglutathione at 240 nm (9). Indeed, we found that several other compounds besides those mentioned in the above report also interfered substantially with the absorbance at 240 nm. The ultraviolet spectra of S-D-lactoylglutathione with increasing concentrations of various inhibitors were recorded. It was found that the severity of A_{240} interference was dependent upon the inhibitor to substrate ratio. Given the necessity of trying to determine unequivocally the efficacy of potential inhibitors, it was decided to modify the assay procedure for the detection of active inhibitors and to devise an assay applicable to all cases. Several methods were attempted to determine the effectiveness of a given compound as an inhibitor of glyoxalase I by measuring methylglyoxal, GSH, and

D-lactate (see Materials and Methods). The major components of the assay could not be readily isolated in acceptable yields and the 340 nm absorbance of NADH during the conversion of D-lactate to pyruvate was also subject to interference by several compounds. In this study, squaric acid, 1, tropolone, 16, and hinokitiol, 17, were found to exert extensive interference in the assay in view of their relatively high absorbance ($\varepsilon_M > 2x10^4 M^{-1}cm^{-1}$) at 240 nm. A modification of the published procedure (14) with linear kinetics prevailing during the entire period of assay proved to be a reliable method in the screening of compounds for inhibitory activity against glyoxalase I. (See Fig. 2.)

As yeast glyoxalase I is a Zn^{2+} metalloenzyme, compounds containing enediol or paene functions capable of chelating the metal have been considered as potential inhibitors of the enzyme. A compound that originally served as the prototypic cis-enediol inhibitor was squaric acid, 1. Douglas and Nadvi have reported an IC_{70} of 0.25 mM for squaric acid with yeast glyoxalase I (8). This compound has also served as the basis for the development of new squaric acid inhibitors of glyoxalase I (24). However,under the present assay conditions, squaric acid had no inhibitory activity on yeast glyoxalase I at concentrations up to 1 mM, an observation compatible with the predictions of Klopman and Dimayuga based on Computer-Automated Structure Evaluation (CASE) analysis (25). Three other cis-enediol/paene analogs not previously investigated, 2,5-dihydroxy-1,4-benzoquinone, 2, mimosine, 3, and acetohydroxamate, 4, were tested and found to be very poor inhibitors of the enzyme. (See Table 1.)

Various hydroxylated aromatic compounds such as coumarins and flavones have been reported to be potential inhibitors of glyoxalase I (26,27). In the present study, pyrogallol, 5, a compound previously reported to function as a glyoxalase I inhibitor was found to have no adverse effects on the enzyme. In this connection, it is pertinent to note that CASE analysis has predicted this compound, like squaric acid, to be ineffective as a glyoxalase I inhibitor (25). Other simple aromatic diols such as catechol, 6, resorcinol, 7, and hydroquinone, 8, were found to be very poor inhibitors of the enzyme. Coumarin-8, 9, and the flavone myricetin, 10, have been reported as good inhibitors of red blood cell glyoxalase I and in the present assay have IC_{50} values of 150 μM and 50 μM, respectively, on yeast glyoxalase I (26,27). A previously untested aromatic diol, 1,2-naphthalenediol, 11, was found to be a good inhibitor (IC_{50}= 34 μM), a result indicating that even simple bicyclic aromatic diols may have inhibitory ability.

Among the S-substituted glutathione analogs S-methylglutathione, 12, was found not to be inhibitory at concentrations up to 400 μM, while S-octylglutathione, 13, had an IC_{50} of 59 μM under the current assay conditions. Two S-substituted glutathione analogs not previously tested with glyoxalase I were the 2-glutathione-1,4-benzoquinone, 14, and 2,3-bisglutathione-1,4-naphthoquinone, 15, analogs which had IC_{50} values in the present assay of 156 μM and 32 μM, respectively. These two compounds may well be biologically produced adducts of the metabolites of benzene and 1-naphthol and hence our observations raise a hitherto unforeseen possibility of naturally occurring glutathione adducts interacting with the glyoxalase system (18,19).

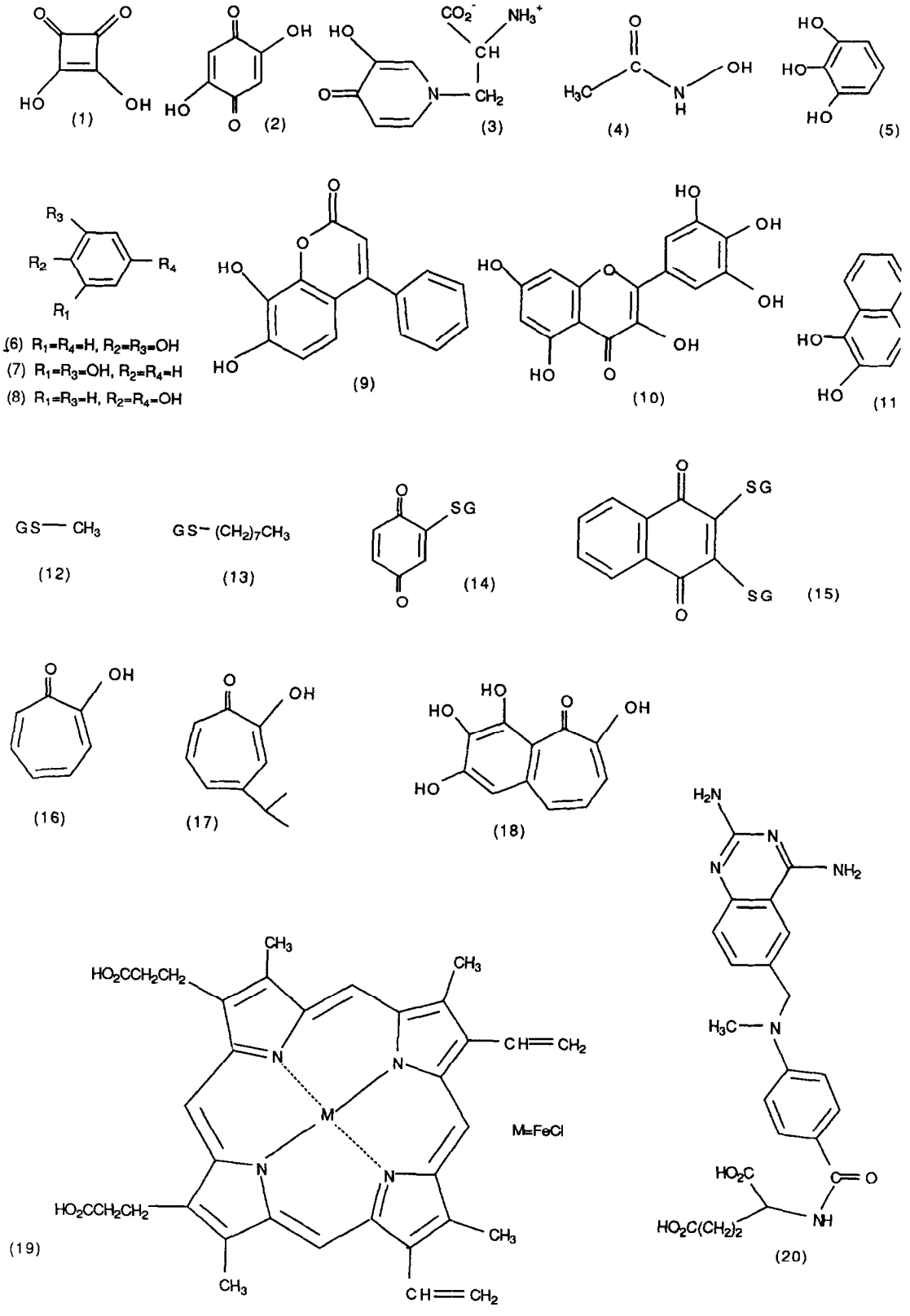

Figure 2. Compounds tested as yeast glyoxalase I inhibitors.

Table 1. 50% inhibition (IC_{50}) of glyoxalase I in vitro by compounds tested for inhibitory activity

Inhibitor	IC_{50} (μM)[a]
Squaric acid [1]	>1mM
2,5-diOH-1,4-BQ [2]	465+
Mimosine/β-CD [3]	1250+
Acetohydroxamate [4]	>20 mM+
Pyrogallol [5]	24% inhibition at 4 mM
Catechol [6]	5300+
Resorcinol [7]	24% inhibition at 10 mM+
Hydroquinone [8]	>10 mM+
Coumarin-8 [9]	150
Myricetin [10]	50
1,2-Naphthalenediol [11]	34
S-MethylGSH [12]	>400
S-OctylGSH [13]	59
2-GS-1,4-BQ [14]	156
2,3-bisGS-1,4-NQ [15]	32
Tropolone [16]	152
Hinokitiol [17]	36
Purpurogallin [18]	50
Hemin [19]	30% inhibition at 395
Methotrexate [20]	>500

[a]Compounds with + were performed at 0.400 mM total hemimercaptal, all other compounds were tested at 1.6 mM total hemimercaptal.

Several analogs of the tropolone structural class of compounds were found to be potential inhibitors of yeast glyoxalase I. The parent compound itself, tropolone, 16, and its 4-isopropyl derivative, hinokitiol, 17, have not been previously evaluated as inhibitors of glyoxalase I, although tropolones have been shown to have antifungal activity due to unknown mechanisms (28). Tropolone and hinokitiol have IC_{50} values of 152 μM and 36 μM, respectively. Purpurogallin, 18, which contains the tropolone nucleus, was found to have an IC_{50} of 50 μM in the present assay. While this work was in progress, Klopman and Dimayuga reported the good inhibitory activity of pupurogallin with glyoxalase I (25).

Interestingly, two miscellaneous structural types, hemin, 19, and methotrexate, 20, which have been previously reported to be good inhibitors of glyoxalase I (29,30) were found to have little effect on yeast glyoxalase I under the present assay conditions.

The results described herein identify several new potent inhibitors of yeast glyoxalase I and in addition described the lack of inhibitory activity of several previously reported inhibitors of yeast glyoxalase I.

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