irred with 1 *N* aq KOH (40 ml) under N_2 for 75 min. After eutralization with AcOH the soln was added to H_2O. The ppt as collected, dried, and chromatographed over neutral alumina Voelm, act. V, 40 × 4 cm). Elution with CH_2Cl_2 gave, after ystn from Me_2CO–Et_2O, 1.30 g of **8b**: mp 178–180°; $[\alpha]_D$ 93°; λ_{max} 237 mμ (ε 18,650), 244 (21,800), 252 (14,900); nr, δ 0.84 (C_{13}-CH_3), 1.09 (C_{10}-CH_3), 5.14 and 5.31 (C_{16}-=CH_2), 13 and 5.35 (C_{20}-CH_2F, J_{HF} = 48 Hz), 5.83 (C_7-H), and 6.10 C_4-H) ppm. *Anal.* ($C_{22}H_{28}O_3ClF$) C, H, Cl; F: calcd, 4.81; und, 4.39.

The mother liquor and the later fractions were rechromatoaphed over neutral alumina (Woelm, act. V, 20 × 4 cm). lution with C_6H_6 gave an additional 793 mg of **8b**.

6-Chloro-16-methylene-17α-hydroxy-21-fluoro-4,6-pregnadiie-3,20-dione (5a).—A soln of **8b** (1.3 g) in DMF (20 ml) was irred with activated MnO_2 (2.6 g) for 1.5 hr. The solids were moved by filtration, and the filtrate added to ice–H_2O. The ɔt was collected, dried, and crystd from CH_2Cl_2–*i*-Pr_2O arding 764 mg of **5a**: mp 223–225° dec; $[\alpha]_D$ −8°; λ_{max} 285 mμ (ε 22,200). *Anal.* ($C_{22}H_{26}O_3ClF$) C, H, Cl; F: calcd, 4.83; found, 4.39.

6-Chloro-16-methylene-17α-hydroxy-21-fluoro-4,6-pregnadiene-3,20-dione 17-Acetate (5b).—Trifluoroacetic anhydride (4 ml) was added to a soln of **5a** (607 mg) and *p*-TSA·H_2O (60 mg) in AcOH (6 ml) dropwise at 10° in a period of 10 min under N_2. The reaction mixture was allowed to warm up to 20° and stirred 5 hr. The soln was added to H_2O, the ppt collected, washed, and dried. Crystn from CH_2Cl_2–*i*-Pr_2O gave 384 mg of **5b**: mp 234–238° dec; $[\alpha]_D$ −147°; λ_{max} 284 mμ (ε 22,500); ν_{max} 1747 (sh), 1670, 1607, 1245 cm^{-1}; nmr, δ 0.82 (C_{13}-CH_3), 1.15 (C_{10}-CH_3), 2.20 (C_{17}-$OCOCH_3$), 4.98 and 5.06 (C_{20}-CH_2F, J_{H_1F} = 47 Hz, J_{H_2F} = 47.5 Hz), 5.47 and 5.58 (C_{16}-=CH_2), 6.26 (C_7-H), and 6.30 (C_4-H) ppm. *Anal.* ($C_{24}H_{28}O_4ClF$) C, H, Cl, F.

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Glyoxalase Inhibitors. A Possible Approach to Anticancer Agents[1]

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Some glyoxalase I enzyme inhibitors were assayed for cytotoxic activity against L1210 leukemia and KB cells in tissue culture. Sublethal concentrations of glyoxalase inhibitor caused a 14- to 18-fold increase in methylglyoxal toxicity in L1210 cells. A probable mechanism for the cytotoxic activity of methylglyoxal is discussed.

In a preliminary account of our work we presented ıe proposal that selective inhibition of the enzyme, yoxalase I, may provide carcinostatic activity by preenting the metabolism of the cytotoxic ketoaldehyde, ethylglyoxal, in tumor cells.[3] The carcinostatic acvity of α-ketoaldehydes, including methylglyoxal, was st reported by French and Freedlander.[4] However, ıese agents are metabolized to the corresponding α-hyoxy acids by the glyoxalase system, thus obviating ıeir use as effective anticancer agents. It has been ell established that the cytotoxic methylglyoxal is the ıbstrate for the glyoxalase enzymes and is converted to ıe nontoxic lactic acid in the presence of a cofactor, utathione (GSH).[5] These facts, along with the obrvation by Stern[6] that the GSH concentration in cells rapidly increased just prior to cell division, suggests ıat the glyoxalase system may be involved in the regution of cell growth by maintaining a proper concentraon of methylglyoxal.[3] The high concentration of lac acid[7] and the deficiency of methylglyoxal[8] in cancer lls further suggests that such cells, having lost the abily to maintain a proper balance of methylglyoxal, continue to grow at an uncontrolled rate. Recent interest in the possible role of methylglyoxal in cancer chemotherapy[9] has been concentrated on the preparation of ketoaldehydes. Since the toxicity of ketoaldehydes alone does not demonstrate the involvement of the glyoxalase system, we would like to report some results of our approach to this problem.

The glyoxalase system is widely distributed in cells of all forms of life[10,11] and comprises two enzymes which catalyze the reaction:

$$CH_3COCHO + GSH \xrightarrow{\text{glyoxalase I}} CH_3CHOHCOSG \xrightarrow{\text{glyoxalase II}} CH_3CHOHCOOH + GSH$$

Since the inhibition of glyoxalase I may result in a buildup of methylglyoxal in cancer cells, our efforts have been concentrated on the preparation of inhibitors of this enzyme. Preliminary investigations demonstrated that S-alkyl derivatives of GSH cause potent competitive inhibition of glyoxalase I by taking advantage of a nonpolar region adjacent to the binding region of the enzyme.[3] A wide variety of S-alkyl and S-aryl glutathiones was prepared to investigate further the binding requirements of these inhibitors to the enzyme[12] and to provide for greater penetration of the cell membrane. The compounds were then investigated for cell kill of L1210 leukemia and KB cell cultures.[13] Since the S-alkyl derivatives seem to have difficulty in penetrating the cell membrane, only those S-aryl glutathiones

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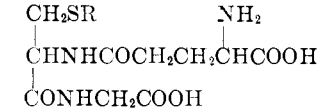
(13) We wish to thank Dr. Florence White of the CCNSC for the data obtained from Dr. Philip S. Thayer of Arthur D. Little, Inc.

with LD_{50} values below 0.20 m*M* are presented at this time and are listed in Table I.

TABLE I

INHIBITION OF GLYOXALASE I AND L1210 AND KB CELL CULTURES BY



No.	R	I_{50},[a] m*M*	LD_{50},[b] L1210 m*M*	LD_{50}, KB m*M*
1	$(CH_2)_2COC_6H_5$	0.077	0.08	0.07
2	$CH_2C_6H_3Br$-*p*	0.009	>0.20	0.11
3	$C_6H_2(NO_2)_3$-2,4-6	*c*	0.12	0.05
4	$C_6H_3(NO_2)_2$-2,4	0.766	>0.20	0.06

[a] I_{50} = concn for 50% inhibition of glyoxalase I when assayed as described in the Experimental Section. [b] Concn of compound for 50% kill of cell culture. [c] No inhibitory activity was detected.

Experimental Section

Inhibitors.—Where analyses are indicated only by symbols of the elements, analytical results obtained for those elements or functions were within ±0.4% of the theoretical values.

***S*-(2-Benzoylethyl)glutathione (1).**—Reduced glutathione (GSH) and chloropropiophenone were allowed to react according to the previously described procedure[3] and gave a 58% yield of **1**, mp 201–202°. *Anal.* ($C_{19}H_{25}N_3O_7S$), C, H, N.

***S*-(*p*-Bromobenzyl)glutathione (2).**—The same procedure[3] was followed using *p*-bromobenzyl bromide and GSH. The pure product was obtained as a white powder; yield, 74%, mp 206–207°. *Anal.* ($C_{17}H_{22}BrN_3O_6$) C, H, N.

***S*-(2,4,6-Trinitrophenyl)glutathione (3).**—Picryl chloride was condensed with GSH using a modification of the procedure described in ref 3 in which Na_2CO_3 was used in place of NaOH; yield, 89%, mp 198–199°. *Anal.* ($C_{16}H_{18}N_6O_{12}S$) C, H, N.

***S*-(2,4-Dinitrophenyl)glutathione (4).**—1-Chloro-2,4-dinitrobenzene and GSH were condensed as described for **3**; yield, 94%, mp 194–195°. *Anal.* ($C_{16}H_{14}N_5O_{10}S \cdot H_2O$) C, H, N.

Enzyme Assay.—A commercial 40% methylglyoxal soln was distd to remove polymerization products and diluted with distd H_2O. The acidic materials were removed by passing the distillate through Dowex 1-X8 (carbonate form) resin and the soln was standardized by the method of Friedemann.[14] Glyoxalase I was obtained from Sigma Chemical Co. and was diluted to 40 μg/ml with 30% glycerol containing 0.1% bovine serum albumin. All enzymatic reactions were performed at 30° in 0.05 m*M* phosphate buffer at pH 6.6. A fresh GSH soln was made before each assay using distd H_2O. For each assay the cuvette contained a total vol of 3.0 ml which was 5.0 m*M* with respect to methylglyoxal and 0.87 m*M* with respect to GSH. Sufficient amts 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 cuvette and allowed to equilibrate for exactly 6 min before addn of the enzyme (see refs 3 and 12). In order to determine the concn of inhibitor required for 50% inhibition, a plot of V_0/V_1 *vs.* [I] was made where V_0 = initial velocity of the uninhibited enzymatic reaction, v_i = initial velocity of the inhibited reaction at various inhibitor concns.

L1210 *in Vitro* Assay.[15]—Tenfold dilns of inhibitor were tested in duplicate sets of tubes inoculated with 200,000 L1210 cells in 4 ml of Fischer's medium with 10% horse serum. The tubes were rubber-stoppered and incubated for 48 hr at 37° without agitation. Cell growth was determined by cell count in the Coulter Counter, Model B, per cent inhibition calcd from the corresponding controls, and LD_{50} concn calcd from a least-squares regression line for inhibition *vs.* dose, correcting all counts for inoculum.

KB *in vitro* assay was performed according to CCNSC protocol.[16]

Results and Discussion

Tissue culture assay of the glyoxalase inhibitors indicates somewhat greater activity against KB cells than L1210 for those compounds which showed appreciable cytotoxicity. The enzyme inhibition and cytotoxicity data for 4 of the more active compounds from preliminary assays are listed in Table I. Methylglyoxal, which has been reported to inhibit 90–99% growth of ascites carcinoma, mammary carcinoma, leukemia, adenocarcinoma, lymphosarcoma, and sarcoma in mice,[17] gave an LD_{50} of 0.42 m*M* against L1210 in our assay. In comparison, **1** is 5.3 times more active than methylglyoxal against L1210.

Because cancer cells are deficient in methylglyoxal, a combination of the ketoaldehyde and a glyoxalase inhibitor may be a more effective means of chemotherapy than use of either compound alone. For example, when a concn of methylglyoxal required to inhibit L1210 growth 0–5% was added to the tissue cultures with **1**, a three- to fivefold decrease in LD_{50} concn for **1** was noted (Table II). If the cytotoxicity of **1** is due to a mechanism other than glyoxalase blockade, an increase in toxicity would not be expected in the presence of such a low concn of methylglyoxal. Also, the cytotoxicity of a compound such as **3**, that does not inactivate glyoxalase, should not be influenced by the same concn of methylglyoxal. When the LD_{50} of **3** was determined in the presence of the same concentration of methylglyoxal as used with **1**, no change in LD_{50} of **3** was noted (Table II). Since trinitrobenzene is cytotoxic, this compound may owe its activity to the trinitrophenyl moiety.

TABLE II

EFFECT OF PREINCUBATION WITH METHYLGLYOXAL $(LI)_{0-5}$ ON ACTIVITY AGAINST L1210

MeG Addn	**1**, μg/ml	**3**, μg/ml
0	31	40
t-2 hr[a]	12	39
t-6 hr[b]	6	35

[a] Methylglyoxal added 2 hr before inhibitor. [b] Methylglyoxal added 6 hr before inhibitor.

In another experiment the presence of a 2–5% cell growth inhibitory concentration of **1** caused a 14- to 18-fold increase in toxicity of methylglyoxal (Table III).

TABLE III

EFFECT OF INHIBITORS ON METHYLGLYOXAL TOXICITY (L1210)[a]

	% inhibition		
1, μg/ml	MeG, 0 μg/ml	MeG, 2.3 μg/ml	MeG, 7.7 μg/ml
0	0	1.7	3
3	2	14	44
10	5	25	54
3, μg/ml			
0	0	3	24
3	0	5	23
10	3.5	5	25

[a] The glutathione analog and methylglyoxal were added simultaneously.

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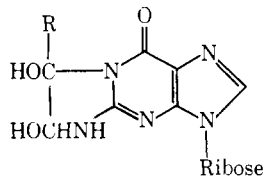
However, under the same conditions **3** did not seem to influence methylgloxal toxicity. Since methylglyoxal is rapidly destroyed by glyoxalase, the diffusion of the ketoaldehyde into the cells must reach a concentration necessary to overcome its rate of destruction by the enzyme. In the presence of glyoxalase inhibitor this destruction is greatly reduced and lower concentrations of ketoaldehyde are required for a given degree of toxicity.

Együd and Szent-Györgyi[18] observed that methylglyoxal inhibited proliferation of cells by interfering with protein synthesis. They also noted that certain thiols protected cells from the toxicity of methylglyoxal and thus suggested that ketoaldehydes "inhibit cell division by interacting with SH groups essential for proliferation."[18] The formation of hemimercaptals from methylglyoxal and certain thiols[3,5] would be expected to decrease the concentration of free ketoaldehyde available in the cells. Therefore, the protection by thiols does not necessarily establish that the mechanism of inhibition of cell division by methylglyoxal involves interaction with SH groups.

It is known that ketoaldehydes, including methylglyoxal, can bind to exposed guanine residues of t-RNA molecules.[19] The structure of the guanine–ketoaldehyde adduct depicted in structure I is supported by recent studies.[20,21] Furthermore, t-RNA labeled with



I

kethoxal (β-ethoxy-α-ketobutyraldehyde) did not exhibit amino acid acceptor activity.[20] The acceptor activity was restored after the t-RNA was stripped of the ketoaldehyde. In 1959, Staehelin reported[22] that glyoxal derivatives, including kethoxal, inactivated tobacco mosaic virus nucleic acid. Spectrophotometric studies showed that only the nucleic acids in which the guanine groups specifically reacted with the ketoaldehydes were inactivated.

Thus, it is reasonable to suggest that methylglyoxal may inhibit protein synthesis by binding reversibly with tRNA molecules and preventing their participation in protein synthesis. The protection against ketoaldehydes by thiols could be due to thiol displacement of the ketoaldehydes from the guanine moieties. Because the ketoaldehyde–guanine adduct is stable at slightly acidic pH (6.8) and less stable at slightly alkaline pH,[19] methylglyoxal or a glyoxalase inhibitor may show some selectivity toward cancer tissue over normal tissue. The preparation of glyoxalase inhibitors with greater cell penetration and the effect of the ketoaldehyde–guanine adduct formation on protein synthesis is being investigated.

Finally, a recent report describing a pathway for the biosynthesis of methylglyoxal[23] from glyceraldehyde supports the suggestion that ketoaldehydes are metabolic intermediates in the mammalian cells. This conversion may also explain the antitumor activity of glyceraldehyde.[24] Also, a recent discovery of an α-ketoaldehyde dehydrogenase in some mammalian cells[25] suggests the possible need for blocking this enzyme to further potentiate the effects of methylglyoxal on cancer cells. The effect of α-ketoaldehyde dehydrogenase inhibition is presently under investigation.

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