Glyoxal bis(guanyldihydrazone) as an inhibitor of polyamine biosynthesis in tumour cells

Pauli SEPPÄNEN,* Richard FAGERSTRÖM,* Leena ALHONEN-HONGISTO,* Hannu ELO,†
Paavo LUMME† and Juhani JÄNNE*

Departments of *Biochemistry and †Inorganic Chemistry, University of Helsinki, SF-00170 Helsinki, Finland

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Glyoxal bis(guanyldihydrazone), the parent compound of methylglyoxal bis(guanyldihydrazone), was synthesized and tested for its ability to inhibit the biosynthesis of polyamines. It was found to be a powerful competitive inhibitor of adenosylmethionine decarboxylase (EC 4.1.1.50), yet the lack of the methyl group at the glyoxal portion increased the apparent K_i value for the enzyme by about 30-fold in comparison with methylglyoxal bis(guanyldihydrazone). Glyoxal bis(guanyldihydrazone) inhibited diamine oxidase (EC 1.4.3.6) activity as effectively as did methylglyoxal bis(guanyldihydrazone). The cellular accumulation curves of glyoxal bis(guanyldihydrazone) in L1210 cells were practically superimposable with those of methylglyoxal bis(guanyldihydrazone), and the uptake of both compounds was distinctly stimulated by a prior treatment with 2-difluoromethylornithine. The drug decreased the concentration of spermidine in a dose-dependent manner and, in contrast with methylglyoxal bis(guanyldihydrazone), without a concomitant accumulation of putrescine. The fact that putrescine concentrations were decreased in cells exposed to glyoxal bis(guanyldihydrazone) was, at least in part, attributable to an inhibition of ornithine decarboxylase (EC 4.1.1.17) activity in cells treated with the compound. Under these experimental conditions equivalent concentrations of methylglyoxal bis(guanyldihydrazone) {1,1'-[(methylethanediyldine)dinitrilo]diguanidine} elicited large increases in the enzyme activity. When combined with difluoromethylornithine, glyoxal bis(guanyldihydrazone) potentiated the growth-inhibitory effect of that drug. Taking into consideration the proven anti-leukaemic activity of glyoxal bis(guanyldihydrazone), its effectiveness to inhibit spermidine biosynthesis (without raising the concentration of putrescine) as well as its suitability for combined use with inhibitors of ornithine decarboxylase, this drug is apparently worthy of further testing in tumour-bearing animals, especially in combination with difluoromethylornithine or related inhibitors of ornithine decarboxylase.

In connection with their initial report on the carcinostatic action of bis(guanyldihydrazone)s, Freedlander & French (1958) reported that the prototype compound, glyoxal bis(guanyldihydrazone) and its methylated derivative, methylglyoxal bis(guanyldihydrazone), exerted a distinct anti-leukaemic activity against L1210 murine leukaemia. When administered orally, both bis(guanyldihydrazone)s were roughly equally effective in increasing the life-span of leukaemic mice (Freedlander & French, 1958). Subsequent studies (French *et al.*, 1960) indicated that, if anything, the therapeutic index of glyoxal bis(guanyldihydrazone) may be broader than that of its methylated derivative, at least when administered in the diet.

Since the discovery of the profound inhibition of eukaryotic adenosylmethionine decarboxylase (EC 4.1.1.50) activity by methylglyoxal bis(guanyldihydrazone) by Williams-Ashman & Schenone (1972), several bis(guanyldihydrazone)s have been tested for their ability to inhibit the biosynthesis of spermidine and spermine. These include the multiply methylated and the ethyl derivatives of glyoxal bis(guanyldihydrazone) (Corti *et al.*, 1974; Pegg, 1983; Pegg & Jacobs, 1983; Jänne & Morris, 1984; Seppänen *et al.*, 1984). Even though many of these derivatives, most notably ethylglyoxal bis(guanyldihydrazone), appear to be even more potent inhibitors of adenosylmethionine decarboxylase than is methylglyoxal bis(guanyldihydrazone)

(Corti *et al.*, 1974; Pegg, 1983; Pegg & Jacobs, 1983; Jänne & Morris, 1984; Seppänen *et al.*, 1984), all these compounds were initially reported to be devoid of anti-leukaemic activity (Mihich, 1975). The lack of anti-leukaemic activity is in all likelihood related to the fact that ethylglyoxal bis-(guanyldiazide) and methylated derivatives of methylglyoxal bis(guanyldiazide) are only poorly taken up and/or retained by various animal cells (Jänne & Morris, 1984; Seppänen *et al.*, 1984).

In the light of the ever-expanding literature on methylglyoxal bis(guanyldiazide), surprisingly little is known about the actions of the parent compound, glyoxal bis(guanyldiazide), in general and about its influence on the metabolism of the polyamines in particular, especially since this compound is an even closer congener of spermidine than is methylglyoxal bis(guanyldiazide) (French *et al.*, 1960). Those authors observed that the therapeutic action of glyoxal bis(guanyldiazide) was not antagonized by spermine.

The metal-chelating properties, which may well be related to the biological action, of these two compounds are very similar, with the possible exception that glyoxal bis(guanyldiazide) is a better chelator of iron than is methylglyoxal bis(guanyldiazide), which in turn more effectively chelates copper than the parent compound does (Hodnett & Zelinski, 1970).

Glyoxal bis(guanyldiazide) is also reported to stimulate nucleohistone-primed DNA polymerase activity better than do the diamines putrescine and cadaverine (Schwimmer, 1968).

We show here that, although glyoxal bis(guanyldiazide) is a less potent inhibitor of putrescine-activated adenosylmethionine decarboxylase than is methylglyoxal bis(guanyldiazide), its apparent K_i value for the enzyme is still in the micromolar range and, owing to the efficient cellular accumulation of the compound, it is an equipotent [in comparison with methylglyoxal bis(guanyldiazide)] inhibitor of spermidine formation and does not lead to the strikingly enhanced accumulation of putrescine typical of the action of the latter drug. Just as with methylglyoxal bis(guanyldiazide), but not the ethyl derivative (Seppänen *et al.*, 1984), difluoromethylornithine or polyamine depletion in general can be used as a vector to enhance greatly the cellular accumulation of glyoxal bis(guanyldiazide).

Experimental

Animals and cells

Ehrlich ascites-carcinoma cells were maintained and propagated in female NMRI mice. Mouse L1210 leukaemia cells were cultured in RPMI 1640 medium supplemented with 5% (v/v) pooled

human serum (Transfusion Service, Finnish Red Cross, Helsinki, Finland), penicillin and streptomycin (50 µg/ml of each).

Enzyme preparations

Adenosylmethionine decarboxylase was partially purified from the livers of methylglyoxal bis(guanyldiazide)-treated DBA/2J mice by the method of Pösö *et al.* (1975) through the DEAE-cellulose chromatography step. Diamine oxidase was partially purified from mouse small intestine as described by Seppänen *et al.* (1980).

Chemicals

DL-[1-¹⁴C]Ornithine (sp. radioactivity 58 Ci/mol), L-[U-¹⁴C]leucine (sp. radioactivity 342 Ci/mol), [6-³H]thymidine (sp. radioactivity 30 Ci/mmol) and [1,4-¹⁴C]putrescine (sp. radioactivity 109 Ci/mol) were purchased from Amersham International (Amersham, Bucks., U.K.).

Methylglyoxal bis(guanyldiazide) was synthesized by and obtained from Orion Pharmaceutical Co. (Espoo, Finland). 2-Difluoromethylornithine was a gift from the Centre de Recherche Merrell International (Strasbourg, France).

The synthesis of glyoxal bis(guanyldiazide) sulphate tetrahydrate was based on the classical condensation reaction described by Thiele & Dralle (1898). The following protocol was developed. Aminoguanidine hemisulphate (Sigma Chemical Co., St. Louis, MO, U.S.A.) (70 mmol; 8.61 g) was dissolved in 80 ml of distilled water and the solution was heated to about 75°C. Then 6.8 ml of aqueous solution of glyoxal (about 30%, w/v) (Ega-Chemie, Steinheim, West Germany) was added with continuous stirring. A whitish precipitate rapidly appeared. Under continuous stirring (for about 2 h) the mixture was allowed to cool down to room temperature. The mixture was left overnight at room temperature, and then the precipitate was separated from the brown supernatant fraction by suction filtration. The precipitate was washed successively with cold water, ethanol and diethyl ether. The white product was dried in air and recrystallized from a large volume of water (about 1 litre of water was required for every 3 g of the product). Recrystallization gave poor yield and a brownish product if it was heated above 70°C. During the recrystallization, activated charcoal was added and the solution was filtered hot. The filtrate was allowed to cool down to room temperature and transferred to an ice/water bath. The crystals formed were filtered off and washed with cold water, then dried with ether and air-dried. The final product (very thin white needles) darkened on heating and melted with decomposition at 280–282°C. The content of crystal water (determined at 120°C) was consistent with the

tetrahydrate composition. U.v. spectra (at pH 7.0 and pH 11.0) were practically identical with those of authentic methylglyoxal bis(guanyldiazide).

Analytical methods

The activities of ornithine decarboxylase (Jänne & Williams-Ashman, 1971a), adenosylmethionine decarboxylase (Jänne & Williams-Ashman, 1971b) and diamine oxidase (Tryding & Willert, 1968) were assayed by published methods. The contents of methylglyoxal bis(guanyldiazide) and glyoxal bis(guanyldiazide) were determined by the enzyme inhibition assay of Seppänen *et al.* (1980) by using yeast adenosylmethionine decarboxylase for the former compound and mouse intestinal diamine oxidase for the latter. The syntheses of DNA and protein were measured as described by Seppänen *et al.* (1981). Polyamines present in the HClO_4 -soluble fraction were determined after dansylation (5-dimethylaminonaphthalene-1-sulphonylation) by the method of Seiler (1970), by using the solvent system of Hölttä *et al.* (1979) in the t.l.c. Protein content was determined by the method of Lowry *et al.* (1951). Cell densities were determined with an electronic particle counter (Coulter Electronics).

Results

Fig. 1 shows the structures of methylglyoxal bis(guanyldiazide), glyoxal bis(guanyldiazide) and adenosylmethionine (adapted from Porter *et al.*, 1981). There is a striking resemblance between the nucleoside and the two guanyldiazides, especially as regards the spacing of various atoms and atom groups. It was thus not unexpected that both drugs acted as competitive inhibitors of adenosylmethionine decarboxylase in respect to adenosylmethionine (Fig. 2). The omission of the methyl group at the glyoxal portion [giving glyoxal bis(guanyldiazide)] resulted in a marked decrease in the affinity for the enzyme. In fact, the apparent K_i value for methylglyoxal bis(guanyldiazide) was $0.2 \mu\text{M}$, whereas that for glyoxal bis(guanyldiazide) was $6 \mu\text{M}$, i.e. about 30 times higher.

Just like methylglyoxal bis(guanyldiazide), glyoxal bis(guanyldiazide) was also a powerful non-competitive inhibitor of the activity of diamine oxidase (Fig. 3). Both compounds were roughly equipotent in this respect, the apparent K_i values being $0.17 \mu\text{M}$ and $0.12 \mu\text{M}$ for methylglyoxal bis(guanyldiazide) and glyoxal bis(guanyldiazide) respectively.

The cellular accumulation of the diguanidines with or without a 'priming' of the cells with difluoromethylornithine is depicted in Fig. 4. The time curves for both drugs were almost super-

Fig. 1. Structures of glyoxal bis(guanyldiazide) (GBG), methylglyoxal bis(guanyldiazide) (MGBG) and *S*-adenosylmethionine (AdoMet)

The presentation is adapted from Porter *et al.* (1981).

impossible, with the possible exception that tumour cells treated with difluoromethylornithine and methylglyoxal bis(guanyldiazide) started to disintegrate (reflected as a rapid fall in the intracellular drug concentration) slightly sooner than in the presence of difluoromethylornithine and glyoxal bis(guanyldiazide) (Fig. 4). The cellular accumulation of both bis(guanyldiazides) was distinctly stimulated by a prior exposure of the tumour cells to difluoromethylornithine (Fig. 4).

The effect of increasing concentrations of glyoxal bis(guanyldiazide), with or without a preceding treatment with difluoromethylornithine, on cellular polyamines and cell growth in cultured L1210 leukaemia cells is presented in Table 1. The drug produced a dose-dependent decrease in the concentration of spermidine, a slight increase in spermine content, and a moderate decrease in the concentration of putrescine. The decrease in intracellular glyoxal bis(guanyl-

Fig. 2. Inhibition of adenosylmethionine decarboxylase activity by bis(guanyldihydrazone)s

Partially purified adenosylmethionine decarboxylase from mouse liver was used as the source of enzyme. Abbreviations used: MGBG, methylglyoxal bis(guanyldihydrazone); GBG, glyoxal bis(guanyldihydrazone); AdoMet, adenosylmethionine.

Fig. 3. Inhibition of diamine oxidase activity by bis(guanyldihydrazone)s

Partially purified diamine oxidase from mouse small intestine was used as the source of the enzyme. Abbreviations used: MGBG, methylglyoxal bis(guanyldihydrazone); GBG, glyoxal bis(guanyldihydrazone).

Fig. 4. Accumulation of bis(guanyldihydrazone)s in cultured L1210 leukaemia cells without or with a previous exposure to difluoromethylornithine

The leukaemia cells were first grown in the absence or presence of 1 mM-difluoromethylornithine for 24 h and then exposed to 5 μM-bis(guanyldihydrazone)s for time periods indicated. Abbreviations used: DFMO, difluoromethylornithine; MGBG, methylglyoxal bis(guanyldihydrazone); GBG, glyoxal bis(guanyldihydrazone).

hydrazine) at higher drug concentrations in cells treated with difluoromethylornithine solely reflects the disintegration of the cells as a result of the cytotoxicity of glyoxal bis(guanyldihydrazone). The decrease in putrescine content in response to glyoxal bis(guanyldihydrazone) is in striking contrast with the large increases (about 10-fold) in the concentration of the diamine in cells exposed to methylglyoxal bis(guanyldihydrazone) under identical experimental conditions (results not shown). It is also obvious from Table 1 that glyoxal bis(guanyldihydrazone) potentiated the anti-proliferative effect of difluoromethylornithine.

Methylglyoxal bis(guanyldihydrazone) consistently stimulates the activity of ornithine decarboxylase (Hölttä *et al.*, 1973; Heby *et al.*, 1973), as also shown in Table 2. In cultured L1210 leukaemia cells, methylglyoxal bis(guanyldihydrazone) enhanced ornithine decarboxylase activity 3-fold and in Ehrlich ascites cells grown in mice 6-fold. Similar concentrations of glyoxal bis(guanyldihydrazone) inhibited the enzyme activity (Table 2). When added directly into the assay system of the enzyme, the latter compound did not exert any inhibition.

Table 1. Effect of glyoxal bis(guanylhyazone) alone or in combination with difluoromethylornithine on polyamine accumulation in cultured L1210 leukaemia cells

Cultured L1210 leukaemia cells (initial cell density $0.15 \times 10^6/\text{ml}$), without or with a previous treatment with 1 mM-difluoromethylornithine for 24 h, were exposed to increasing concentrations of glyoxal bis(guanylhyazone) for an additional 24 h. For terminal cell densities, both viable and dead cells were counted. Abbreviations used: N.D., not detectable.

	Glyoxal bis(guanylhyazone)		Polyamines (amol/cell)			$10^{-6} \times$ Cell density (per ml)
	Extracellular (μM)	Intracellular (amol/cell)	Putrescine	Spermidine	Spermine	
No difluoro- methylornithine	0	—	70	1370	669	1.07
	1	376	39	1320	913	1.07
	5	1100	40	904	999	0.95
	10	1350	44	633	800	0.85
+ Difluoro- methylornithine	0	—	N.D.	52	483	0.79
	1	842	N.D.	48	548	0.70
	5	1430	N.D.	79	607	0.48
	10	376	N.D.	82	361	0.46

Table 2. Effect of methylglyoxal bis(guanylhyazone) and glyoxal bis(guanylhyazone) on ornithine decarboxylase activity of cultured L1210 leukaemia cells and of Ehrlich ascites-carcinoma cells grown in the peritoneal cavity of mice

L1210 leukaemia cells were grown in the presence of 1 μM -bis(guanylhyazone)s for 24 h, after which the activity of ornithine decarboxylase was determined from triplicate cultures. Mice, inoculated with Ehrlich ascites carcinoma cells 6 days earlier, received a single intraperitoneal dose (50 mg/kg) of bis(guanylhyazone)s and were killed 24 h later. The tumour cells were harvested from the peritoneal cavity, disintegrated and assayed for ornithine decarboxylase activity. Results are means $\pm$ S.D. for five to six animals in each group: * $P < 0.001$ compared with the untreated animals.

Treatment	Ornithine decarboxylase activity (pmol/mg of protein)	
	L1210 cells	Ehrlich ascites cells
None	409 $\pm$ 116	16.2 $\pm$ 4.8
Methylglyoxal bis- (guanylhya- zone)	1260 $\pm$ 90*	105 $\pm$ 28.6*
Glyoxal bis- (guanylhya- zone)	284 $\pm$ 141	4.4 $\pm$ 1.7*

Ornithine decarboxylase, as a metabolically unstable enzyme, is especially sensitive to an inhibition of general protein synthesis, and thus the effect of glyoxal bis(guanylhyazone) on the enzyme may have been mediated via an inhibition of protein synthesis. That this was not the case is illustrated in Table 3. An interesting difference existed between the two drugs. Methylglyoxal bis(guanylhyazone) more effectively inhibited protein synthesis than did glyoxal bis(guanylhyazone), to which DNA synthesis appeared to be more sensitive (Table 3).

Discussion

The present results and previous findings (Hölttä *et al.*, 1973; Corti *et al.*, 1974; Porter *et al.*, 1981) strongly suggest that methylglyoxal bis(guanylhyazone) acts as a structural analogue for adenosylmethionine and competitively inhibits adenosylmethionine decarboxylase. This view is further strengthened by the present finding indicating that the omission of the methyl group, spaced to correspond to the methyl group of adenosylmethionine (see also Fig. 1), from the glyoxal portion, strikingly decreases the inhibitory action of the bis(guanylhyazone) (Fig. 2). In line with this view is likewise the finding that replacement of the methyl group by an ethyl group enhances the enzyme inhibition (Corti *et al.*, 1974; Pegg, 1983; Pegg & Jacobs, 1983; Jänne & Morris, 1984).

The fact that bis(guanylhyazone)s act as true congeners of adenosylmethionine decarboxylase makes it much easier to design new inhibitors for adenosylmethionine decarboxylase. There is no need to design and synthesize cumbersome nucleoside analogues (with commonly encountered problems of their entry into the cells), but instead the relatively simple aliphatic bis(guanylhyazone) skeleton can be used as a model for new compounds.

Although glyoxal bis(guanylhyazone) is a substantially weaker inhibitor of adenosylmethionine decarboxylase than is methylglyoxal bis(guanylhyazone), it still remains as a compound of interest. By any definition, it is a strong inhibitor of the enzyme, with a K_i value in the micromolar range. It depletes polyamines, especially spermidine and putrescine, and apparently potentiates the effects of difluoromethylornithine on growth inhibition. Furthermore, it does not stimulate

Table 3. *Effect of methylglyoxal bis(guanyldrazone) and glyoxal bis(guanyldrazone) on DNA and protein synthesis in L1210 leukaemia cells*

Cultures (duplicate) of L1210 leukaemia cells were exposed to 5 μ M-bis(guanyldrazone) for 12h, after which the synthesis of macromolecules was determined.

Treatment	Thymidine incorporation (c.p.m./10 ⁶ cells)	Leucine incorporation (c.p.m./10 ⁶ cells)
None	49 300 (100%)	3150 (100%)
Methylglyoxal bis(guanyldrazone)	38 740 (79%)	1510 (48%)
Glyoxal bis(guanyldrazone)	29 570 (60%)	2270 (72%)

ornithine decarboxylase; on the contrary it decreases the enzyme activity *in vivo* without influencing the activity when added directly into the assay mixture. This, in fact, may indicate that its resemblance to spermidine is sufficiently close to produce some kind of repression-type inhibition of ornithine decarboxylase.

Moreover, the early experimental data (Freedlander & French, 1958; French *et al.*, 1960) indicated that glyoxal bis(guanyldrazone) displays an anti-leukaemic activity roughly comparable with that exerted by methylglyoxal bis(guanyldrazone), and may be better tolerated by the experimental animals (French *et al.*, 1960). Finally, glyoxal bis(guanyldrazone) interacts with difluoromethylornithine, and probably with any inhibitor of ornithine decarboxylase, just like its methylated counterpart.

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