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Inhibition of *S*-adenosylmethionine decarboxylase and diamine oxidase activities by analogues of methylglyoxal bis(guanylhydrazone) and their cellular uptake during lymphocyte activation

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Several congeners of methylglyoxal bis(guanylhydrazone) were tested for their ability to inhibit eukaryotic putrescine-activated *S*-adenosylmethionine decarboxylase (EC 4.1.1.50) and intestinal diamine oxidase (EC 1.4.3.6). All the compounds tested, namely methylglyoxal bis(guanylhydrazone), ethylglyoxal bis(guanylhydrazone), dimethylglyoxal bis(guanylhydrazone) and the di-N''-methyl derivative of methylglyoxal bis(guanylhydrazone), were strong inhibitors of both yeast and mouse liver adenosylmethionine decarboxylase activity *in vitro*. The enzyme from both sources was most powerfully inhibited by ethylglyoxal bis(guanylhydrazone). All the diguanidines likewise inhibited diamine oxidase activity *in vitro*. The maximum intracellular concentrations of the ethyl and dimethylated analogues achieved in activated lymphocytes were only about one-fifth of that of the parent compound. However, both derivatives appeared to utilize the polyamine-carrier system, as indicated by competition experiments with spermidine.

After the discovery of the anti-leukaemic activity of methylglyoxal bis(guanylhydrazone) {1,1′-[(methylethanediylidine)dinitrilo]diguanidine} (Freedlander & French, 1958), hundreds of bis(guanylhydrazones) were synthesized and screened for their anti-cancer activity (Mihich, 1975). These studies revealed that a relatively minor modification of the glyoxal portion of the molecule resulted in a loss of anti-leukaemic action (Mihich, 1963).

When methylglyoxal bis(guanylhydrazone) was found to be an extremely potent inhibitor of putrescine-activated *S*-adenosylmethionine decarboxylase (EC 4.1.1.50) (Williams-Ashman & Schenone, 1972), some of its close derivatives were tested for their ability to inhibit the decarboxylation of adenosylmethionine (Corti *et al.*, 1974). Single or double methylation (not involving the primary amino groups) of the parent compound, or replacement of the methyl group by an ethyl group, fully retained the strong inhibitory action on adenosylmethionine decarboxylase (Corti *et al.*, 1974), yet none of these compounds was reported to possess anti-leukaemic activity (Mihich, 1963).

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In addition to the inhibition of adenosylmethionine decarboxylase, methylglyoxal bis(guanylhydrazone) also strongly inhibits the activity of diamine oxidase (EC 1.4.3.6) (Hölttä *et al.*, 1973; Pegg & McGill, 1978). The inhibition of the latter enzyme is clearly disadvantageous when the drug is used to deplete polyamines *in vivo*, especially in combination with inhibitors of ornithine decarboxylase (Kallio & Jänne, 1983).

We show below that small modifications, such as extra methylation or ethyl replacement, of methylglyoxal bis(guanylhydrazone) enhanced the inhibition of adenosylmethionine decarboxylase activity, diminished the inhibition of diamine oxidase activity and profoundly decreased the cellular transport of these compounds in comparison with methylglyoxal bis(guanylhydrazone).

Experimental

Lymphocyte cultures

Bovine suprapharyngeal lymphocytes were prepared, purified and cultured in RPMI 1640 medium with 10% (v/v) newborn-calf serum as described by Seyfried & Morris (1983). The lymphocyte cultures (3 ml; 3×10^6 cells/ml) were activated by the addition of concanavalin A (18 μg/ml) to the cultures.

Analytical methods

The activity of adenosylmethionine decarboxylase was assayed as described by Jänne & Williams-Ashman (1971), by trapping CO_2 by the method of Morris & Pardee (1965). Dialysed $(NH_4)_2SO_4$ fractions from yeast (Seppänen *et al.*, 1980) and mouse liver (Corti *et al.*, 1974) were used as the source of the enzyme.

Dialysed cytosol fraction (Seppänen *et al.*, 1980) of mouse small intestine was used as the source of diamine oxidase. The activity of the latter enzyme was assayed by the method of Tryding & Willert (1968).

Cellular contents of the bis(guanylhydrazones) were determined by the enzyme-inhibition assay method for methylglyoxal bis(guanylhydrazone) of Seppänen *et al.* (1980).

The inhibition constants were determined by the method of Dixon (1953), by using least-squares fit.

Chemicals

Methylglyoxal bis(guanylhydrazone) was synthesized by and obtained from Orion Pharmaceuticals (Espoo, Finland). Dimethylglyoxal bis(guanylhydrazone) and the di-N''-methyl derivative of methylglyoxal bis(guanylhydrazone) were originally obtained from the late Dr. Chandrakant Dave (Roswell Park Memorial Institute, Buffalo, NY, U.S.A.). Ethylglyoxal bis(guanylhydrazone) was kindly provided by Dr. Carl Porter (Roswell Park Memorial Institute). 2-Difluoromethylornithine was generously given by the Centre de Recherche Merrell International, Strasbourg, France.

Results

Table 1 lists the inhibition constants of methylglyoxal bis(guanylhydrazone) and its three methyl or ethyl analogues for adenosylmethionine decarboxylase (from yeast and mouse liver) and for diamine oxidase (mouse intestine). Addition of an extra methyl group to the glyoxal portion [dimethylglyoxal bis(guanylhydrazone)] or a replacement of the existing methyl group by an ethyl group [ethylglyoxal bis(guanylhydrazone)] enhanced the inhibitory action of the bis(guanylhydrazone) on putrescine-activated adenosylmethionine decarboxylase from both baker's yeast and mouse liver. In fact, ethylglyoxal bis(guanylhydrazone) was 8 (liver) to 15 (yeast) times more potent an inhibitor of adenosylmethionine decarboxylase than was methylglyoxal bis(guanylhydrazone) (Table 1). The addition of an extra methyl group to the glyoxal portion of the molecule (dimethylglyoxal) likewise enhanced (about 2-fold) the enzyme inhibition (Table 1).

The di-N''-methylated derivative was a stronger inhibitor of the mouse liver enzyme, but less potently inhibited the yeast enzyme, than the parent compound (Table 1).

As also shown in Table 1, the modification of the glyoxal portion (ethyl replacement or extra methylation) had little effect on the inhibition of intestinal diamine oxidase, whereas the N''-dimethylation resulted in a striking decrease in the affinity for the latter enzyme. In fact the K_i value of di-N''-methyl derivative of methylglyoxal bis(guanylhydrazone) for diamine oxidase was 10 times that of methylglyoxal bis(guanylhydrazone) (Table 1).

With respect to the ratio of the inhibition constant for diamine oxidase to that for adenosylmethionine decarboxylase (from liver) (high ratio indicates preferential inhibition of adenosylmethionine decarboxylase), all the analogues of methylglyoxal bis(guanylhydrazone) had larger values than the parent compound. The ratio for methylglyoxal bis(guanylhydrazone) was close to 1:1, whereas that for the dimethyl analogue was about 4:1, that for the ethyl derivative 7:1 and that for the di-N''-methylated derivative more than 20:1.

Table 1. *Inhibition of eukaryotic adenosylmethionine decarboxylase and diamine oxidase by bis(guanylhydrazones)*
The K_i values are means for two to six experiments, ±S.D. when three or more experiments have been performed. Abbreviations used: MGBG, methoxyglyoxal bis(guanylhydrazone); EGBG, ethylglyoxal bis(guanylhydrazone); DGBG, dimethylglyoxal bis(guanylhydrazone); TGBG, di-N''-methylated derivative of methylglyoxal bis(guanylhydrazone). Significance of differences [in comparison with methylglyoxal bis(guanylhydrazone)]: $*P < 0.05$; $**P < 0.01$.

	K_i (μM)		
Compound	Yeast adenosylmethionine decarboxylase	Liver adenosylmethionine decarboxylase	Intestinal diamine oxidase
MGBG	0.74±0.40	0.47	0.42±0.11
EGBG	0.04±0.01**	0.06	0.42±0.21
DGBG	0.30±0.12*	0.17	0.74±0.12*
TGBG	1.57±0.50*	0.20	4.07±2.31

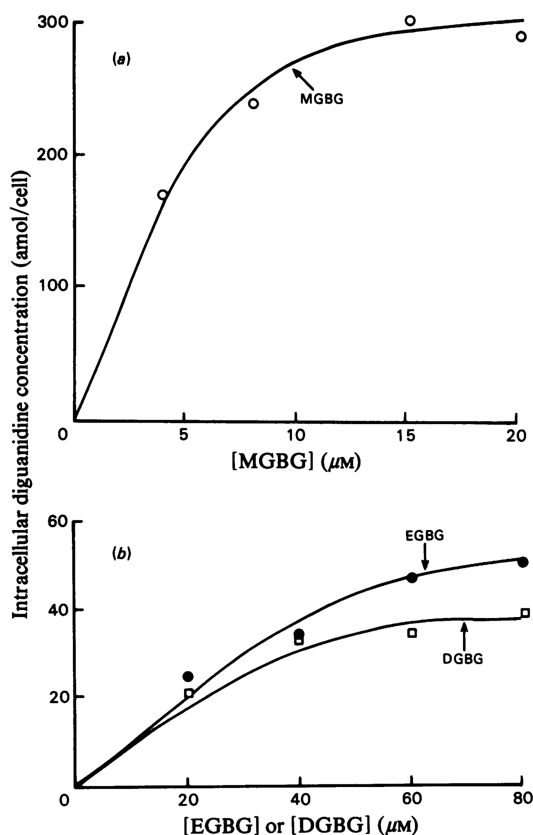

Fig. 1. *Cellular accumulation of bis(guanylhydrazones) by activated bovine lymphocytes*

Freshly prepared bovine lymphocytes (duplicate cultures) were incubated in the presence of concanavalin A for 20h, whereafter methylglyoxal bis(guanylhydrazone) (MGBG), ethylglyoxal bis(guanylhydrazone) (EGBG) or dimethylglyoxal bis(guanylhydrazone) (DGBG) was added at the concentrations indicated. After a further 20h incubation the cells were harvested, washed, and disintegrated by ultrasonication, and assayed for drug contents.

The fact that the derivatives of methylglyoxal bis(guanylhydrazone), except the di-N''-methylated compound, were even more potent inhibitors of yeast adenosylmethionine decarboxylase than the parent compound made it possible to use the enzyme-inhibition assay originally developed for methylglyoxal bis(guanylhydrazone) for the determination of their intracellular concentrations.

The cellular accumulation of methylglyoxal bis(guanylhydrazone), ethylglyoxal bis(guanylhydrazone) and dimethylglyoxal bis(guanylhydrazone) by activated bovine lymphocytes is illustrated in Fig. 1. In contrast with the inhibition of adenosylmethionine decarboxylase, which was enhanced by ethyl replacement or extra methylation of the glyoxal portion, the latter modifications resulted in a striking decrease in the cellular accumulation. The maximum intracellular concentrations achieved with ethylglyoxal bis(guanylhydrazone) and dimethylglyoxal bis(guanylhydrazone) (Fig. 1*b*) were less than one-fifth of that with methylglyoxal bis(guanylhydrazone) (Fig. 1*a*). Similarly, the external concentrations required to reach half-maximal intracellular concentrations were increased by a factor of 5–6-fold. The uptake of the di-N''-methylated derivative of methylglyoxal bis(guanylhydrazone) was even less than that of the ethylglyoxal and dimethylglyoxal analogues, the intracellular concentrations of the former compound being below the detection limit of the enzyme-inhibition method used.

In spite of the poor cellular transport of ethylglyoxal bis(guanylhydrazone), its intracellular concentrations were fully comparable with those of methylglyoxal bis(guanylhydrazone) as regards the potency of adenosylmethionine decarboxylase inhibition.

Although much less than the accumulation of methylglyoxal bis(guanylhydrazone), the cellular accumulation of ethylglyoxal bis(guanylhydrazone) and dimethylglyoxal bis(guanylhydrazone)

Table 2. *Accumulation of bis(guanylhydrazones) during lymphocyte activation*

Freshly prepared bovine lymphocytes were incubated in the absence or in the presence of concanavalin A without or with 5mM-difluoromethylornithine (DFMO) for 20h, whereafter methylglyoxal bis(guanylhydrazone) (MGBG; 10μM), ethylglyoxal bis(guanylhydrazone) (EGBG; 20μM) or dimethylglyoxal bis(guanylhydrazone) (DGBG; 20μM) was added with or without an equimolar concentration of spermidine. After a further incubation for 20h, the cells were harvested, washed, and disintegrated by ultrasonication, and homogenates were assayed for their diguanidine contents. The values are means ± S.D. for three parallel cultures, or means for duplicate cultures for the spermidine competition experiment. Significance of differences (in comparison with unactivated): $^*P < 0.05$.

	Bis(guanylhydrazone) uptake (pmol/culture)		
Treatment	MGBG	EGBG	DGBG
Unactivated	152±22	32±3	19±4
Activated	1810±396*	114±36*	31±4*
Activated + DFMO	1740±648*	134±65	30±2*
Activated + spermidine	793	39	25

apparently occurs via the polyamine carrier, as indicated in Table 2. The accumulation of ethylglyoxal bis(guanylhydrazone) and dimethylglyoxal bis(guanylhydrazone) was stimulated, yet not as much as that of methylglyoxal bis(guanylhydrazone), on activation of the lymphocytes by the lectin, and, more importantly, equimolar concentrations of spermidine effectively inhibited the process (Table 2). In fact, spermidine was most inhibitory to the ethyl derivative.

Unlike in many other cell lines (Alhonen-Hongisto *et al.*, 1980; Jänne *et al.*, 1981), a prior treatment of the lymphocytes with difluoromethylornithine did not stimulate the uptake of any of the bis(guanylhydrazones) (Table 2).

Discussion

The use of methylglyoxal bis(guanylhydrazone) as a specific inhibitor of polyamine biosynthesis is complicated by an array of factors. The contribution of depletion of spermidine and spermine to the profound anti-proliferative activity exerted by the compound may be a minor one in most cell lines in comparison with other cellular effects produced by the drug (Porter *et al.*, 1981). The fact that cells are capable of attaining millimolar intracellular concentrations of the drug (Mandel & Flintoff, 1978; Seppänen *et al.*, 1980), whereas only micromolar concentrations are needed for complete inhibition of synthesis of spermidine and spermine, makes it extremely difficult to attribute all the effects of methylglyoxal bis(guanylhydrazone) to the metabolism of polyamines.

A further disadvantageous feature of methylglyoxal bis(guanylhydrazone) is its capacity to inhibit diamine oxidase powerfully both *in vitro* and *in vivo* at micromolar concentrations (Hölttä *et al.*, 1973; Pegg & McGill, 1978). The latter inhibition is apparently responsible for the paradoxical restoration of normal or near-normal intracellular polyamine concentrations when methylglyoxal bis(guanylhydrazone) is combined with inhibitors of synthesis of putrescine and spermidine, such as difluoromethylornithine (Alhonen-Hongisto *et al.*, 1982; Kallio & Jänne, 1983).

In many respects, the ethyl and dimethyl analogues of methylglyoxal bis(guanylhydrazone) may be more specific inhibitors of polyamine biosynthesis than is methylglyoxal bis(guanylhydrazone) itself. Owing to their poor cellular uptake, in comparison with the parent compound, an accumulation of extremely high intracellular drug concentrations is unlikely to occur, thus minimizing unspecific toxic effects. All the analogues tested likewise possessed a more favourable ratio of the inhibition of diamine oxidase to that of adenosylmethionine decarboxylase. Thus the ethyl and dimethyl analogues may be more suitable candidates for combined drug regimens, including inhibitors of ornithine decarboxylase.

The present results, as regards the inhibition of mammalian adenosylmethionine decarboxylase, are in full agreement with those reported by Corti *et al.* (1974) and more recently by Pegg (1983). The only difference between the mammalian and yeast enzymes was the lesser sensitivity of the latter enzyme to the di-N''-methylated derivative of methylglyoxal bis(guanylhydrazone) (Table 1).

Although initially reported to be devoid of anti-leukaemic activity (Mihich, 1963), dimethylglyoxal bis(guanylhydrazone), and most notably the di-N''-methylated derivative of methylglyoxal bis(guanylhydrazone), inhibited the accumulation of spermidine and spermine as well as DNA synthesis in activated bovine lymphocytes, yet at concentrations 25 times higher than that of methylglyoxal bis(guanylhydrazone) (Seyfried & Morris, 1979). Our recent experiments (P. Seppänen & J. Jänne, unpublished work) also revealed that ethylglyoxal bis(guanylhydrazone) distinctly inhibits the growth of cultured L1210 cells in a dose-dependent fashion.

The approach of anti-cancer therapy based on specific inhibition of polyamine accumulation is so novel that the whole family of forgotten bis(guanylhydrazones) should be re-evaluated, irrespective of the initially reported anti-leukaemic activity, to find inhibitors of adenosylmethionine decarboxylase suitable for combined use with inhibitors of ornithine decarboxylase.

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