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DIETHYLGLYOXAL BIS(GUANYLHYDRAZONE): A NOVEL HIGHLY POTENT INHIBITOR OF *S*-ADENOSYLMETHIONINE DECARBOXYLASE WITH PROMISING PROPERTIES FOR POTENTIAL CHEMOTHERAPEUTIC USE

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SUMMARY

Diethylglyoxal bis(guanylhydrazone) (DEGBG), a novel analog of the antileukemic agent methylglyoxal bis(guanylhydrazone) (MGBG) was synthesized. It was found to be the most powerful inhibitor of yeast *S*-adenosylmethionine decarboxylase (AdoMetDC) so far studied (K_i approx. 9 nM). This property, together with the finding that the compound is a weaker inhibitor of intestinal diamine oxidase than are MGBG and its glyoxal, ethylglyoxal and ethylmethylglyoxal analogs, makes the compound a promising candidate as a polyamine antimetabolite for chemotherapy studies. DEGBG was also found to potentiate the antiproliferative effect of the ornithine decarboxylase inhibitor *α*-difluoromethyl ornithine against mouse L1210 leukemia cells in vitro. DEGBG increased several-fold the intracellular putrescine concentration of cultured L1210 cells, just as MGBG and its ethylglyoxal analog are known to do. The results strongly suggest that DEGBG is worth further studies. Combined with previous studies, they also made possible the construction of some empirical rules concerning the structure-activity relationships of bis(guanylhydrazone) type inhibitors of AdoMetDC. The identity of DEGBG was confirmed by a single-crystal X-ray analysis and by ^{1}H- and ^{13}C-NMR spectroscopy. It consisted of the same isomer as MGBG and several of its analogs are known to consist of.

Key words: Yeast adenosylmethionine decarboxylase inhibition; Intestinal diamine oxidase inhibition; Polyamine metabolism; Cultured mouse L1210 leukemia cells; Antiproliferative activity.

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INTRODUCTION

Various bis(guanylhydrazones) are potent inhibitors of *S*-adenosylmethionine decarboxylase (AdoMetDC), 1 of the 2 rate-limiting enzymes of polyamine biosynthesis, and are therefore potential agents for cancer chemotherapy, either as such or in combination with inhibitors of ornithine decarboxylase (ODC), the other rate-limiting enzyme of polyamine biosynthesis (for references, see Ref. 1). Although the first report [2] on the ability of a bis(guanylhydrazone), namely methylglyoxal bis(guanylhydrazone) (MGBG) to inhibit AdoMetDC appeared first in 1972, the antineoplastic properties of various bis(guanylhydrazones) and numerous analogs of them have been subject to intensive studies ever since the year 1958, when Freedlander and French reported [3] the antileukemic activity of 2 compounds of this class, namely glyoxal bis(guanylhydrazone) (GBG) and MGBG. In spite of intensive testing of derivatives, it appears that GBG and MGBG are the only aliphatic bis(guanylhydrazones) so far reported that have shown distinct antitumor activity in vivo (for references, see Refs. 1 and 4).

GBG and MGBG are far from ideal chemotherapeutic agents since they are highly toxic [5—11]. Further, they as well as any other bis(guanylhydrazones) so far studied are potent inhibitors not only of AdoMetDC but also of intestinal diamine oxidase (DAO) [1,12,13]. The latter property is considered to be a serious drawback in vivo since it appears to lead to a strikingly enhanced entry of intestinal (bacteria-derived) polyamines and diamines into general circulation and finally into tissues and tumor cells, this property being an obvious reason for the fact that although MGBG effectively decreases intracellular spermidine and spermine concentrations in cultured tumor cells, it is only moderately effective in decreasing polyamine levels in tissues of experimental animals [1,12]. Therefore, the bis(guanylhydrazones) so far reported are far from ideal also as inhibitors of polyamine metabolism. This fact has led to a search for novel compounds that would be potent inhibitors of AdoMetDC but whose ability to inhibit DAO would be lower than that of previously reported bis(guanylhydrazones). Preferably, the compounds should also be suitable for combination chemotherapy with *α*-difluoromethyl ornithine (DFMO) or other ODC inhibitors, i.e. they should have the ability to potentiate the antiproliferative effect of the latter drug in the same way as GBG and MGBG are known to do [14,15]. In this paper, we report the synthesis of the novel compound diethylglyoxal bis(guanylhydrazone) (DEGBG) and show that it is a more potent inhibitor of AdoMetDC than any other compound so far studied for this property, while its ability to inhibit DAO is lower than that of most other potent AdoMetDC inhibitors such as GBG, MGBG and their ethylglyoxal, dimethylglyoxal and ethylmethylglyoxal analogs (EGBG, DMGBG and EMGBG, respectively). Further, results are reported showing that DEGBG potentiates the antiproliferative effect of DFMO against tumor cells in vitro. All of these properties make the compound a promising candidate for further chemotherapy studies.

MATERIALS AND METHODS

Synthesis of DEGBG sulfate

Aminoguanidine bicarbonate (27.2 g, 0.2 mol) (Aldrich, Steinheim, F.R.G.) was dissolved in 200 ml of 0.5 M aqueous H_2SO_4 plus 10 ml of H_2O. To remove all bicarbonate, the mixture was heated to 80 °C and stirred for approximately 10 min. Then, a solution containing 12 ml (0.1 mol) of 3,4-hexanedione (Merck-Schuchardt, Hohenbrunn bei München, F.R.G.) in 200 ml of absolute ethanol was added dropwise with stirring. The mixture was refluxed for 30 min. On cooling to room temperature, a white precipitate began to form. The mixture was then cooled in an ice/salt bath for approximately 4 h. The white precipitate was filtered off, washed with water and ethanol and air-dried (yield 20.2 g, 50%). The crude product was dissolved in water (approx. 1 l) at approximately 70 °C, treated with activated charcoal and filtered hot. On cooling in an ice bath, no precipitate was formed. Therefore, the solution was concentrated in vacuo to approximately 200 ml. A white precipitate was formed. The mixture was cooled in an ice/salt bath, the precipitate was filtered off, washed with a small amount of ethanol and air-dried (recovery approx. 60%). M.p. 233.5–235 °C (dec.). Analsis: found C 26.64%, H 6.73%, N 30.7%; calculated for $C_8H_{18}N_8 \cdot H_2SO_4 \cdot 2H_2O$: C 26.66%, H 6.71%, N 31.1%. The identity of the product was confirmed by single-crystal X-ray crystallography and ^1H- and ^{13}C-NMR spectroscopy.

Other chemicals

[1,4-^{14}C]putrescine (spec. act. 230 Ci/mol) was obtained from Amersham International (Amersham, Bucks., U.K.). *S*-Adenosyl-L-[1-^{14}C]methionine (spec. act. 42.7 Ci/mol) was prepared enzymically from L-[1-^{14}C]methionine (Amersham International) as described by Pegg and Williams-Ashman [16]. Unlabelled *S*-adenosyl-L-methionine was prepared from L-methionine according to the same method. DFMO was a gift from the Centre de Recherche Merrel International (Strasbourg, France).

Biochemical measurements

AdoMetDC inhibition was studied essentially according to previously published procedures [17,18]. The measurements were carried out in a potassium phosphate buffer (pH 7.4, final concentration 0.1 M). The AdoMetDC used was partially purified from baker's yeast by the method of Seppänen et al. [19]. DAO inhibition was studied essentially according to the method of Tryding and Willert [20] using the enzyme of mouse small intestine. The above buffer system was used also in these studies. The DAO of mouse small intestine was isolated by the following procedure. The intestines were washed with 0.9% (w/v) NaCl (aq) and were homogenized in a 25 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 10 mM mercaptoethanol. After centrifugation (100,000 × *g*), the supernatant was dialyzed against a buffer similar to that used in the homogenization process. No further purification was performed.

Cell culture

Mouse L1210 leukemia cells were grown in Gibco's medium RPMI 1640 supplemented with 5% (v/v) of pooled human AB serum (Finnish Red Cross Transfusion Service, Helsinki, Finland), 2 mM glutamine, and antibiotics (the sodium salt of penicillin G and streptomycin, 50 mg of each per l). Cells were counted using an electronic particle counter (Coulter Counter, model Industrial D).

RESULTS AND DISCUSSION

DEGBG, a novel member of the bis(guanylhydrazone) family, was chosen for study and synthesized because DMGBG was known to be a more potent inhibitor of AdoMetDC than is MGBG [17,21], and also because the replacement of the methyl group of MGBG or one of the methyls of DMGBG by an ethyl group gave inhibitors (EGBG and EMGBG, respectively) with far lower K_i values than MGBG and DMGBG [17,21,22]. Thus, it was hoped that DEGBG would be an even more potent inhibitor of AdoMetDC than is EMGBG.

DEGBG sulfate was synthesized from the corresponding diketone, 3,4-hexanedione, and aminoguanidine sulfate essentially in the same way as has been described for GBG [23], DMGBG [23], EMGBG [22] and other related compounds. Because 3 different geometrical isomers of the compounds may exist in principle (i.e. the *anti-anti*, *syn-anti* and *syn-syn* isomers), and because different isomers would probably have different biological properties, the isomeric composition of the product was studied with the aid of ^{1}H- and ^{13}C-NMR spectroscopy and X-ray diffraction. The results of the NMR studies (data not shown) suggested the presence of one isomer only. Another possibility consistent with the NMR results would be a rapid isomerization process (on the NMR timescale) but this possibility seems improbable in the light of what is known about the isomerization rates of certain related compounds. Details of the NMR results will be published elsewhere, alongside with similar studies on other bis(guanylhydrazones). In the case of these related compounds, NMR results suggest either the presence of 1 isomer only or the occurrence of a rapid isomerization process, the former possibility being the more probable one.

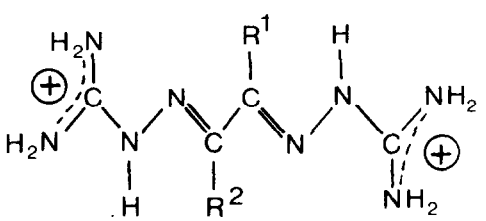

Fig. 1. A generalized structural formula of the dication forms of the bis(guanylhydrazones) of various glyoxals. In DEGBG, R^1 = R^2 = ethyl.

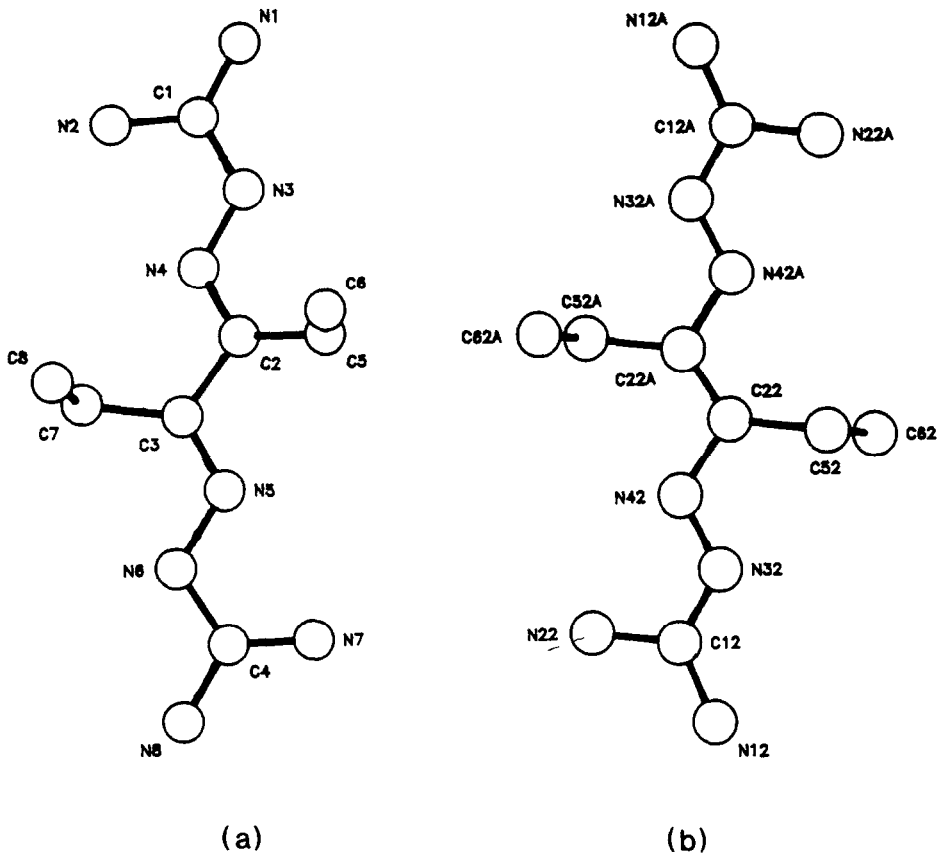

Fig. 2. The 2 rotamers found in the crystals of DEGBG sulfate. In rotamer (a) both ethyl side chains are directed to the same side of the molecular plane. In (b) the ethyl side chains are directed to different sides of the molecular plane.

A single-crystal X-ray crystallographic study was also performed on the product (data not shown). The crystal studied consisted of equal amounts of 2 different rotamers of one of the 3 possible isomers of the compound (see Fig. 2), namely the one in which the carbon-nitrogen double bonds between the diethylglyoxal part and the aminoguanidine moieties have the same configuration as they are known to have in the salts of GBG [24], MGBG [25], DMGBG [26], EMGBG [22] and propylglyoxal bis(guanylhydrazone) (PGBG) [27]. This isomer is the one whose structural formula is shown in Fig. 1. Taken together, the NMR and X-ray results suggest that there are no differences between the isomeric compositions of GBG, MGBG, DMGBG, PGBG, EMGBG and DEGBG. Thus, the differences between the biological properties of these compounds obviously are in no way due to any differences in their isomeric compositions. It is also worth mentioning that DEGBG constitutes the first case in which 2 dis-

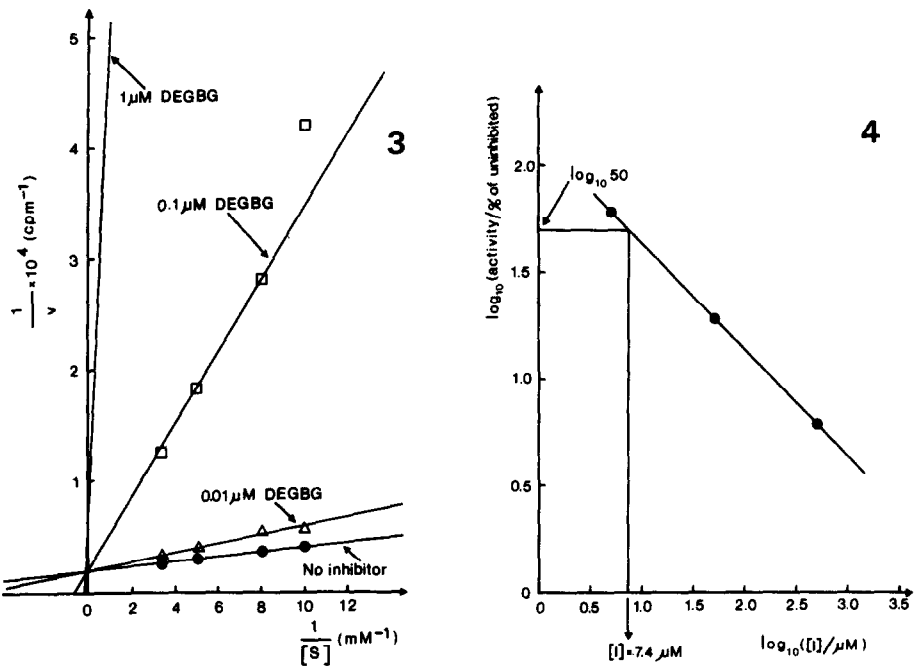

Fig. 3. Inhibition of yeast AdoMetDC activity by DEGBG. [S] refers to the concentration of *S*-adenosylmethionine, the substrate of the enzyme.

Fig. 4. Inhibition of DAO activity by DEGBG. The enzyme was obtained from mouse small intestine. The measurements were carried out at a 0.4 mM concentration of the substrate, putrescine.

tinctly different rotamers have been found to be present in the crystals of a bis(guanylhydrazone).

DEGBG was found to be a very potent competitive inhibitor of yeast *S*-adenosylmethionine decarboxylase (see Fig. 3), its K_i value being approximately 9 nM. Thus, the compound appears to constitute the most powerful inhibitor of this enzyme so far reported, since its K_i is slightly lower than that of the best previously known inhibitor, EMGBG (K_i approx. 12 nM) [22]. On the basis of the present results and other data so far accumulated [14,17,21,22,28], some conclusions can be drawn about the structure-activity relationships of bis(guanylhydrazone) type inhibitors of AdoMetDC: (1) the presence in the glyoxal moiety of an alkyl substituent with 1 or 2 carbon atoms drastically increases the inhibitory power of the compound, the effect of an ethyl group being more potent than that of a methyl group; (2) when the length of the alkyl substituent is increased above 2 carbons, the inhibitory potency does not increase further but instead is decreased to almost the same level as in the case of a methyl substituent; and (3) the presence of 2 alkyl substituents with no more than 2 carbon atoms in each makes the molecule a much more potent inhibitor than a corresponding compound with 1 substituent only, the inhibitory power

increasing with an increasing total number of carbons. Further, the present results indicate that the presence of 2 ethyl substituents in the glyoxal portion of the molecule obviously does not cause any dramatic increase of steric hindrance, suggesting that if the whole of the molecule of DEGBG is bound to the enzyme molecule, there must be enough bulk tolerance for an ethyl side chain at 2 different positions of the latter molecule.

DEGBG also inhibited the DAO of mouse small intestine (see Fig. 4). At a 0.4 mM concentration of the substrate, putrescine, the concentration of DEGBG required for 50% inhibition (IC_{50}) was approximately 7 μM, being thus approximately 10 times as high as that of EMGBG [22], the most potent AdoMetDC inhibitor so far reported. The less potent inhibition of DAO and the more potent inhibition of AdoMetDC by DEGBG, as compared to EMGBG, make the compound a promising candidate as a polyamine antimetabolite for chemotherapy studies. When compared to other previously studied bis(guanylhydrazones) such as MGBG, DMGBG, and EGBG [17], the difference is even larger, since in the case of these compounds, the ratios K_i (yeast AdoMetDC)/K_i (intestinal DAO) are approximately 1.8, 0.41 and 0.095, respectively, while for DEGBG, the ratio K_i (yeast AdoMetDC)/IC_{50} (intestinal DAO) is only approximately 0.0013.

Since Hibasami and his coworkers have reported [29] that methylglyoxal bis(butylamidinohydrazone), a structural analog of MGBG, DEGBG and related compounds, competitively inhibits also ODC, in contrast to GBG that does not inhibit this enzyme [14], we studied the effect of DEGBG on the activity of ODC.

TABLE 1

THE EFFECTS OF DEGBG ON THE GROWTH OF L1210 LEUKEMIA CELLS IN VITRO IN THE ABSENCE AND PRESENCE OF DFMO

In Experiment A, the initial cell density was 0.296 × 10^6 cells/ml, and in Experiment B it was 0.263 × 10^6 cells/ml.

Compound(s) added and their concentration(s)	Cell density × 10^6 (cells/ml)	
	After 24 h	After 48 h
Experiment A		
—	0.432	0.827
5 μM DEGBG	0.439	0.763
2 mM DFMO	0.348	0.761
2 mM DFMO + 5 μM DEGBG	0.290	0.671
Experiment B		
—	0.421	0.829
10 μM DEGBG	0.344	0.879
20 μM DEGBG	0.349	0.939
2 mM DFMO	0.339	0.832
2 mM DFMO + 10 μM DEGBG	0.254	0.554
2 mM DFMO + 20 μM DEGBG	0.275	0.629

The compound, up to the concentration of 0.5 mM, did not inhibit an ODC preparation of the liver of the thioacetamide-treated rat (data not shown). Thus, in this respect the compound is clearly similar to GBG, while distinctly different from the compound reported by Hibasami et al. [29].

Preliminary measurements were also performed on the effect of DEGBG on the growth of mouse L1210 leukemia cells in culture in the presence and absence of DFMO. The results (see Table 1) indicated that, in the concentration range 5–20 μM, the compound potentiates the antiproliferative effect of the latter drug. The results obtained after 1 day suggested that also as such, DEGBG may have a weak antiproliferative activity against the cell line used, but the 2-day results did not indicate any distinct inhibition of growth. In any case, the potentiation of the effect of DFMO by DEGBG further strengthens the view that the compound is a promising candidate for more profound chemotherapeutic studies. Since it appears that when going from the clearly antileukemic and antiproliferative compounds GBG and MGBG to derivatives with a higher degree of alkyl substitution (e.g. EGBG, PGBG and EMGBG), the antileukemic and antiproliferative activity is lost [1,22,28] or, in the case of EGBG, drastically weakened [30], it seems probable that the ability of the even more highly alkylated analog DEGBG to potentiate the antiproliferative effect of DFMO may have a mechanism different from that of the antiproliferative action of GBG and MGBG. Taking into account the very low K_i of DEGBG for AdoMetDC, it seems possible that the effect of DEGBG is purely due to AdoMetDC inhibition, which appears not to be the case with MGBG [1,31,32]. This point deserves further studies.

Preliminary studies were also carried out on the effects of DEGBG on the intracellular polyamine levels of tumor cells. The results (data not shown) indicated that when cultured leukemia L1210 cells were exposed to 10–20 μM DEGBG for 24 h, their intracellular putrescine concentration was increased several-fold (approx. 3.5–5 ×), as compared to that of untreated cells. Their intracellular spermidine content was possibly slightly decreased simultaneously. These changes are similar to those observed in cells treated with MGBG [14,30] or EGBG [30], while in striking contrast to those in cells treated with the parent compound GBG that causes a reduction of the intracellular putrescine content as well as of the spermidine content [14].

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