SENSITIVE ENZYMIC DETERMINATION OF METHYLGLYOXAL BIS(GUANYLHYDRAZONE) IN CULTURED CELLS AND IN ANIMAL TISSUES

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1. Introduction

Methylglyoxal bis(guanylhydrazone) (MGBG) is an antiproliferative compound used clinically in cancer chemotherapy [1–3]. Earlier the mechanism of action of MGBG was often related to the metabolism of polyamines, especially because its antiproliferative effects were counteracted by spermidine [4]; it was only in [5] that MGBG was shown to be a remarkably potent and specific inhibitor of eukaryotic *S*-adenosyl-L-methionine decarboxylase (EC 4.1.1.50), an enzyme directly involved in the synthesis of spermidine and spermine. Since then the compound has been widely used to produce intracellular polyamine deprivation in a number of experimental systems including cultured cells and animal tissues (for references see [6]). In all systems employed, the growth-inhibitory effect of MGBG can be prevented by μM levels of spermidine and spermine [6]. This finding has been taken as evidence indicating that the antiproliferative action exerted by MGBG is mediated through an inhibition of intracellular accumulation of spermidine and spermine. However, a straightforward interpretation of these studies is complicated by the fact that MGBG, spermidine and spermine appear to compete for a common cellular uptake system [7,8]. It is thus possible that the prevention of MGBG-induced toxicity by polyamines could partly result from a reduction of the intracellular concentration of the drug, and is not necessarily due to a normalization of cellular polyamine pattern. With the possible exception of a few studies [9,10], the effect of polyamines on the intracellular concentrations of MGBG has not been checked properly. This apparently is related to the lack of sensitive methods for determination of minute amounts of MGBG under cell culture conditions.

We have now developed a sensitive (pmol order) enzymic method for rapid determination of MGBG concentrations in cultured cells and in extracts of animal tissues. The method is based upon the findings that MGBG is a potent competitive inhibitor of putrescine-activated adenosylmethionine decarboxylase [5,11,12] and an equally effective noncompetitive inhibitor of mammalian diamine oxidase (EC 1.4.3.6) [11,13]. Using partially purified enzymes, MGBG contents from 25–250 pmol (adenosylmethionine decarboxylase) or from 50–400 pmol (diamine oxidase) could be easily measured.

When cultured cells (human lymphocytic leukemia cells and Ehrlich ascites carcinoma cells) were grown in the presence of μM levels of MGBG, the compound was so effectively taken up by the cells, that a 600–1500-fold gradient across the cell membrane was soon established. That is, in cells exposed to 5–10 μM MGBG the drug reached 4–6 mM intracellularly, which is probably sufficiently high to directly influence variety of cellular reactions and structures. Furthermore, the reversal of the antiproliferative effects of MGBG by spermidine and spermine was closely associated with strikingly decreased drug levels (by 85–90%) in cells cultured in the presence of MGBG and the polyamines.

2. Material and methods

2.1. *Cells*

Human acute lymphocytic leukemia cells (type B) (kindly donated by Dr Leif Andersson) and Ehrlich

ascites carcinoma cells (adapted to growth in suspension cultures) were cultured in RPMI 1640 medium (Orion Diagnostica, Helsinki) supplemented with 10% human serum (Finnish Red Cross Transfusion Service, Helsinki), L-glutamine (2 mM) and penicillin and streptomycin (50 μg/ml each).

2.2. *Partial purification of adenosylmethionine decarboxylase and diamine oxidase*

Adenosylmethionine decarboxylase from baker's yeast was extracted and purified through ammonium sulphate fractionation by the method in [14]. The dialyzed ammonium sulphate fraction was used in the MGBG assays. Diamine oxidase from day 11 rat fetuses was likewise refined through ammonium sulphate fractionation [15] and used after dialysis.

2.3. *Chemicals*

S-Adenosyl-L-[1-^{14}C]methionine was prepared enzymically essentially as in [16]. [1,4-^{14}C]Putrescine (spec. radioact. 116 Ci/mol) was obtained from the Radiochemical Centre, Amersham, Bucks. Methylglyoxal bis(guanylhydrazone) was the product of Aldrich-Europe (Beerse).

2.4. *Analytical methods*

Cell densities were measured in an electronic particle counter (Coulter Electronics Ltd). Cell diameters (for determination of the cellular volumes) were measured after standardization of the counter with latex particles as recommended in the instruction manual for the Coulter counter.

The activities of adenosylmethionine decarboxylase and of diamine oxidase were assayed as in [17,18]. Adenosylmethionine decarboxylase was always assayed in the presence of 2.5 mM putrescine.

Regression lines were computed by the least squares method.

2.5. *Determination of MGBG in cultured cells and rat tissues*

Leukemia cells and Ehrlich ascites carcinoma cells were harvested by low speed centrifugation, washed once with 2 ml physiological saline and suspended in distilled water. The cells were disintegrated ultrasonically by a Branson Sonifier (3 × 5 s, half-maximum power). Rat tissues were homogenized with 3 vol. Tris–HCl buffer (pH 7.4) containing 1 mM dithiothreitol.

Prior to the determination of MGBG, the cell or tissue homogenates were kept in a boiling water bath for 10 min in order to inactivate any endogenous adenosylmethionine decarboxylase (that can occur in substantial amounts in cells exposed to MGBG due to the stabilization of the enzyme). Any precipitate formed during heating was removed by centrifugation.

Aliquots (2 or 3 different dilutions) of the boiled homogenates were added to the adenosylmethionine decarboxylase [17] or diamine oxidase [18] assay system. A series of standards containing known amounts of MGBG were run in parallel. The amount of MGBG in unknown samples was obtained from a standard curve plotted according to [19], i.e., the plot of MGBG concentration versus the reciprocal of initial velocity.

The recovery of MGBG, added prior to the boiling, was virtually 100% in cell homogenates and 80% in tissue homogenates (due to large precipitations in protein-rich samples).

3. Results

As shown in fig.1, the sensitivity of the method allowed the determination of the drug starting from 25 or 50 pmol. The upper limit for linearity of adenosylmethionine decarboxylase assay was ~250 pmol (fig.1a), while with diamine oxidase (fig.1b) assay somewhat higher concentrations of MGBG (≤400 pmol) could be accurately measured.

When human lymphocytic leukemia cells were exposed to μM levels of MGBG, the compound was effectively concentrated intracellularly. The determination of the cellular concentrations of MGBG either

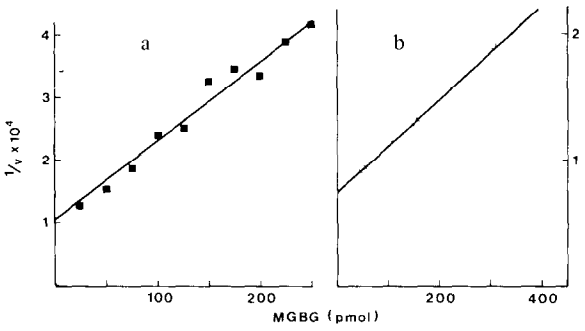

Fig.1. Standard curves for the determination of MGBG with adenosylmethionine decarboxylase (a, AMDC) assay or diamine oxidase (b, DAO) assay.

Table 1
Uptake of MGBG by human lymphocytic leukemia cells

MGBG in medium (μM)	Cellular uptake of MGBG			
	pmol/10^6 cells		Intracellular level (μM)	
	AMDC	DAO	AMDC	DAO
1	619 ± 148	770 ± 101	1480 ± 350	1850 ± 480
5	1940 ± 210	2441 ± 70	4660 ± 500	5860 ± 340
10	2380 ± 338	2350 ± 298	5710 ± 810	5630 ± 710

The cells were grown in the presence of the indicated concentrations of MGBG for 24 h. The concentrations of MGBG were measured by both adenosylmethionine decarboxylase (AMDC) and diamine oxidase (DAO) assays as in the text. The values are means (± SEM) of 4 determinations

with adenosylmethionine decarboxylase or diamine oxidase gave comparable results (table 1). When grown in the presence of 1 μM MGBG, the drug (assuming even distribution) in human leukemia cells reached 1.5–1.85 mM intracellularly, i.e., >1500-fold the concentration of the drug in the culture medium (table 1). In the presence of 10 μM MGBG, the intracellular level of the drug approached 6 mM that represents a concentration gradient of 600-fold across the cell membrane (table 1).

The leukemia cells appeared to take up the drug by a saturable transport process, since the intracellular MGBG concentration hardly increased when the extracellular concentration of the drug was raised from 5–10 μM (table 1). This is in reasonable agreement with earlier studies with L1210 cells, where the initial velocities of the transport of labeled MGBG were measured [8]. Although the initial velocities were not measured, the uptake process appeared to be rather slow occurring over a period of days.

In analogy with the leukemia cells, Ehrlich ascites tumor cells likewise effectively concentrated the drug intracellularly. An intracellular level of 4 mM was reached in cells exposed to 5 μM MGBG for 48 h (table 2). This concentration of MGBG produced a distinct antiproliferative effect in Ehrlich ascites cells, as also seen in table 2. Inclusion of 10 μM putrescine together with MGBG only slightly reduced the cellular concentration of the drug, while similar concentrations of spermidine and spermine decreased the cellular MGBG to 10–15% of that found in cells grown in the presence of MGBG alone (table 2). Spermidine and spermine added directly to the assay mixture of MGBG did not have any effect on the determination

Table 2
Uptake of MGBG by Ehrlich ascites carcinoma cells in the absence or presence of polyamines

Addition to medium	Conc. (μM)	Intracellular MGBG (μM)	Cell density (× 10^{-6}/ml)
None	–	0	0.892
MGBG	5	4050	0.424
MGBG + putrescine	5 10	3620	0.482
MGBG + spermidine	5 10	728	0.880
MGBG + spermine	5 10	525	0.920

The cells were grown in the absence or presence of MGBG with or without polyamines for 48 h. The concentration of MGBG was measured with adenosylmethionine decarboxylase assay as in the text

Table 3
Concentration of MGBG in some rat tissues and blood plasma after a single injection of MGBG

Tissue	MGBG (μmol/g)
Liver	0.790 ± 0.015
Kidney	0.410 ± 0.034
Spleen	0.180 ± 0.010
Plasma	0.024 ± 0.001

Rats (av. wt 275 g) received an intraperitoneal injection of 49 μmol MGBG 3 h before death. MGBG was determined in tissue extracts with the adenosylmethionine decarboxylase assay as in the text. The values are means (± SEM) obtained from 4 rats

of the drug. Interestingly, the latter reduction of cellular MGBG content by spermidine and spermine was closely associated with the disappearance of the MGBG-induced antiproliferative effect (table 2).

A single intraperitoneal injection of 49 μmol MGBG into adult rats produced relatively high drug concentrations in various tissues (table 3). The highest concentration of MGBG (~0.8 mM) was found in the liver, whereas in the plasma it was much lower (24 μM) (table 3).

4. Discussion

The cellular actions of MGBG are related to the metabolism of polyamines at least at two different levels:

(i) The drug apparently inhibits the transport of higher polyamines and vice versa [7,8];

(ii) It profoundly depresses the synthesis of spermidine and spermine by blocking the decarboxylation of adenosylmethionine [5].

The K_i for MGBG as a competitive inhibitor of eukaryotic adenosylmethionine decarboxylase is <1 μM [5,11,12]. MGBG in cell cultures at 1–10 μM are most used [6], producing a marked decrease in cellular spermidine and spermine contents associated with a distinct inhibition of cell growth. However, even though these concentrations of drug in the culture media appear to be only slightly higher than those needed for more or less complete inhibition of adenosylmethionine decarboxylase in vitro, the situation is strikingly different inside the cell, where the drug may be as high as 5 mM (tables 1, 2). Comparable intracellular concentrations of MGBG have also been reported [10] using an indirect assay method for the drug.

In analogy with the competition of MGBG with higher polyamines for a common transport system [7,8], it is probable, even likely, that mM intracellular levels of MGBG may influence the binding of polyamines (likewise occurring at mM levels to some cellular components and structures. In fact, at these concentrations MGBG has been shown to exert a number of effects in vitro. These include interaction with eukaryotic DNA and inhibition of template activity [20], inhibition of RNA polymerase activity [21] and inhibition of oxidative phosphorylation and cell respiration [22]. It thus seems possible, that in cells exposed to only μM levels of MGBG, the intracellular content of the drug is nevertheless sufficiently high to produce comparable effects also in vivo.

The observed close parallelism between the reduction of intracellular MGBG concentration and the reversal of its growth-inhibitory effects by spermidine and spermine, but not by putrescine, warrants more systematic studies to support the view that MGBG exerts its antiproliferative action exclusively through prevention of polyamine biosynthesis.

In addition to cultured cells, possessing such a remarkable concentrating capacity, this method is also suitable for determination of MGBG in tissue extracts. It could also be used clinically to monitor plasma concentrations of MGBG so as to achieve better control of cytostatic chemotherapy.

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