Acetylation of spermidine and methylglyoxal bis(guanyldihydrazone) in baby-hamster kidney cells (BHK-21/C13)

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Treatment of BHK-21/C13 cells with methylglyoxal bis(guanyldihydrazone) (MGBG) induced the cytosolic form of spermidine *N*¹-acetyltransferase. It stabilized the enzyme against proteolytic degradation, but the drug did not affect the enzyme activity *in vitro*. MGBG was itself acetylated by BHK-21/C13 cells, but at only one-tenth the rate at which spermidine was acetylated. Acetylation occurred almost exclusively in the nuclear fraction. The product was identified as *N*-acetyl-MGBG by h.p.l.c., by using [³H]acetyl-CoA and [¹⁴C]MGBG as co-substrates. The results suggest that the acetylation of MGBG by BHK-21/C13 cells occurs by a different acetyltransferase enzyme from that which acetylates spermidine.

INTRODUCTION

Methylglyoxal bis(guanyldihydrazone) (MGBG) is a synthetic polycarbonyl compound which has significant antiproliferative effects both on cells in culture (Seppanen *et al.*, 1980) and on some malignant tumours (Warrell & Burchenal, 1983). MGBG is a structural analogue of the polyamine spermidine, and consequently has a number of effects on polyamine metabolism. It is a potent reversible inhibitor of *S*-adenosylmethionine decarboxylase (EC 4.1.1.50) and thus depletes the cells of both spermidine and spermine (Williams-Ashman & Schenone, 1972; Wallace & Keir, 1986), which is believed to contribute to its antiproliferative activity. MGBG, however, also stimulates polyamine acetyltransferase activity (Erwin & Pegg, 1986), competes competitively with spermidine and spermine for uptake (Seppanen *et al.*, 1980; Nuttall & Wallace, 1987) and, in high concentrations, the drug produces severe mitochondrial damage (Porter *et al.*, 1979).

In the early 1960's MGBG was used in the treatment of acute myeloblastic leukaemia, but was soon abandoned as an anticancer agent because of its toxicity and narrow therapeutical index. It has, however, been re-evaluated more recently as a result of successful treatment of certain solid tumours by combination of MGBG with another polyamine antimetabolite, α -difluoromethylornithine, or with vinblastin-16 or cisplatin (Ask *et al.*, 1986; Warrell & Burchenal, 1983).

The pharmacokinetics of MGBG have been examined in cancer patients after a brief intravenous infusion (Marsh *et al.*, 1981). The drug rapidly distributes in the body water and is excreted slowly into the urine. There are, however, no reports of MGBG binding to plasma proteins or undergoing further metabolism (Warrell & Burchenal, 1983).

The present paper demonstrates that MGBG stimulates the acetylation of spermidine in BHK-21/C13 cells and that in the cytosol the stimulation is specific for the *N*¹-acetyl form of spermidine. We also show that MGBG stabilizes spermidine acetyltransferase. In addition,

MGBG itself can be metabolized by BHK-21/C13 cells. The drug is acetylated, and it appears that the enzyme responsible for this acetylation is different from the inducible cytosolic enzyme which acetylates the polyamines spermidine and spermine, previously observed in these cells (Wallace *et al.*, 1985).

EXPERIMENTAL

Materials

Horse serum and tissue-culture plastics were purchased from Gibco-BRL, Paisley, Scotland, U.K. Dulbecco's modification of Eagle's medium, penicillin and streptomycin were from Flow Laboratories, Rickmansworth, Herts., U.K. Acetyl-CoA was obtained from Sigma Chemical Co., Poole, Dorset, U.K., and [³H]acetyl-CoA (sp. radioactivity 5 Ci/mmol) and [¹⁴C]MGBG (12 mCi/mmol) were from Amersham International, Amersham, Bucks., U.K. MGBG was from Aldrich Chemical Co., Gillingham, Dorset, U.K.

Cell culture

BHK-21/C13 cells were grown routinely at 37 °C in monolayer culture in an atmosphere of CO₂/air (1:19) in Dulbecco's medium supplemented with 10 % (v/v) horse serum. MGBG was prepared as 100-times-concentrated stock solution in 0.9 % NaCl and was filter-sterilized before use. Cell cultures were checked routinely for bacterial and fungal contamination.

Analytical methods

Before extraction or preparation of homogenates, cell sheets were washed twice in ice-cold phosphate-buffered saline (Wallace *et al.*, 1984). Cells were harvested mechanically.

Polyamines were extracted in 0.2 M-HClO₄ and were quantified by h.p.l.c. by a modification of the method of Seiler & Knodgen (1980). The modifications to the published method are as follows: the wavelengths of excitation and emission are 347 nm and 465 nm respectively; buffer B was maintained at 100 % for 5 min before

Abbreviation used: MGBG, methylglyoxal bis(guanyldihydrazone).

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Table 1. Effect of MGBG on cell growth and polyamine content

Cells were seeded at a density of 1.3×10^4 cells/cm² and were grown in the presence or absence of 10 μ M-MGBG for 72 h. Cells were harvested and polyamine, MGBG and protein contents determined as described in the Experimental section. Values are means $\pm$ S.D. ($n = 3$); nd, not detected.

Cells	Protein content (mg/plate)	Content (nmol/mg of protein)				
		MGBG	Putrescine	Spermidine	Spermine	Total polyamines
Control	1.95 ± 0.70	nd	0.45 ± 0.01	8.05 ± 0.80	10.50 ± 0.75	19.00 ± 1.56
MGBG-treated	0.38 ± 0.02	10.42 ± 1.75	0.92 ± 0.01	2.08 ± 0.26	1.25 ± 0.13	4.25 ± 0.40

Fig. 1. Effect of MGBG on the acetylation of spermidine by BHK-21/C13 cells

Cells were seeded at a density of 1.3×10^4 cells/cm² and were grown in the absence (○) or presence (●) of 10 μ M-MGBG. At the times indicated, cells were harvested and assayed for acetyltransferase activity with spermidine as substrate. Results are means $\pm$ S.D. ($n = 3$).

re-introduction of buffer A to re-cycle the column. Intracellular MGBG content was determined by h.p.l.c. (Wallace & Cameron, 1986). After acetyltransferase assay products were analysed by h.p.l.c., fractions were collected after separation and the distribution of radioactivity in each fraction was determined.

Nuclei were isolated by the method of Furneaux (1978) and examined for cytoplasmic contamination by staining with Trypan Blue. Nuclei were then homogenized in 10 mM-Tris/HCl, pH 7.4. The non-nuclear fraction was centrifuged at 100000 g_{av} for 1 h, and the supernatant (the cytosolic fraction) was used for enzyme assays. Cell and nuclear homogenates and cytoplasmic extracts were used as the source of enzyme. Acetyltransferase activity was determined by the method of Matsui & Pegg (1980). Each assay contained (final concns.) 3 mM-substrate (spermidine or MGBG), 100 mM-Tris/HCl, pH 7.8, 25 μ M-[³H]acetyl-CoA and 0.2–1.0 mg of protein.

Protein content was determined by the method of Lowry *et al.* (1951).

RESULTS

Treatment of BHK-21/C13 cells with MGBG for 72 h prevented cell growth, as determined by the change in protein content (Table 1). MGBG was accumulated within the cells, with a concentration gradient of about 300-fold being generated (Table 1). The total polyamine content of the MGBG-treated cells was decreased by 78% compared with untreated cells, principally as the

Table 2. Spermidine acetyltransferase activity in BK-21/C13 cells with and without MGBG

Subcellular fractions of BHK-21/C13 cells grown for 72 h were prepared and spermidine acetyltransferase (1 unit = 1 pmol of acetylspermidine formed/min per mg of protein) activity was measured as described in the Experimental section. Values are means $\pm$ S.D. ($n = 3$). Fractions were collected and, after dilution, the radioactivity in each fraction was determined. Results are averages of two h.p.l.c. analyses (variation less than 10%).

Addition		Acetyltransferase sp. activity (units)	Relative amount of product (% of total)	
			N ¹ -Acetylspermidine	N ⁸ -Acetylspermidine
(a) Cell homogenates	None	0.48 ± 0.14	45.7	54.3
	10 μ M-MGBG	3.07 ± 0.49	64.9	35.1
(b) Nuclear fraction	None	0.53 ± 0.07	45.9	54.1
	10 μ M-MGBG	1.13 ± 0.14	49.3	50.7
(c) Cytosolic fraction	None	0.37 ± 0.04	12.5	87.5
	10 μ M-MGBG	1.53 ± 0.03	83.3	16.7

Fig. 2. Effect of MGBG on the half-life of spermidine *N*-acetyltransferase activity in the BHK-21/C13 cells

BHK cells were seeded at 1.3×10^4 cells/cm² and were grown for 24 h in the absence (□) or presence (■) of 10 μM-MGBG. At 24 h, cycloheximide (50 μg/ml) was added and cells were harvested at various times thereafter. Cell homogenates were prepared and spermidine acetyltransferase activity was measured as described in the Experimental section. The results are averages of two experiments; the range of values was less than 9%. Initial enzyme activity (100%) was 1.13 ± 0.33 and 1.80 ± 0.13 pmol of acetylspermidine formed/min per mg of protein, in control and MGBG-treated cells respectively.

Fig. 3. Acetylation of MGBG by BHK-21/C13 cells

Cells were grown as described in Fig. 1 in either the absence (○) or presence (●) of 10 μM-MGBG. Acetyltransferase activity was measured as described in the Experimental section with MGBG as a substrate. Results are means $\pm$ S.D. ($n = 3$).

result of significant decreases in both spermidine and spermine and despite an increase in the concentration of putrescine (Table 1).

Fig. 4. Chromatogram of the products from the acetyltransferase assay with MGBG as substrate

The acetyltransferase assay was carried out with [³H]-acetyl-CoA (5 Ci/mmol) and [¹⁴C]MGBG (12 mCi/mmol) as co-substrates. Portions of the assay product were analysed by h.p.l.c. (Wallace & Cameron, 1986); fractions (1 ml) were collected and diluted, and the radioactivity in each fraction was determined. Inserts: T₀ is the basal activity, the assay being stopped immediately; T₁₅ is the activity after 15 min incubation (—, ¹⁴C; ---, ³H). The results shown in the main Figure are T₁₄ - T₀. ●, ³H radioactivity; ○, ¹⁴C radioactivity.

Spermidine was acetylated by BHK-21/C13 cells with little change in the enzyme activity over 96 h in culture (Fig. 1). Treatment of the cells with 10 μM-MGBG increased the spermidine acetyltransferase activity in cell homogenates from 24 h, with a maximal 7-fold stimulation of 72 h (Fig. 1). Analysis of the products of the reaction showed a shift in the ratio of *N*¹- to *N*⁸-acetylspermidine from 0.84 to 1.85 (Table 2), suggesting specific stimulation of spermidine *N*¹-acetyltransferase. Separation of the cell homogenates into nuclear and cytoplasmic fractions showed that stimulation of the acetyltransferase activity by MGBG occurred in both fractions. However, the stimulation was 2-fold greater in the cytosolic fraction (Table 2). Analysis of the products of these assays indicated that only in the cytosolic fraction was the stimulation specific for spermidine *N*¹-acetyltransferase (Table 2). In the nuclear fraction the ratio of *N*¹- to *N*⁸-acetylspermidine remained relatively constant at 0.9–1.0 compared with the cytosolic fraction, where the ratio changed from 0.14 to 4.99 in the presence of the drug. MGBG itself at concentrations of 0.1–1000 μM had no effect on the acetylation of spermidine by spermidine acetyltransferase under the standard conditions used.

Treatment of BHK-21/C13 cells with MGBG produced a dramatic stabilization of the enzyme activity. In untreated cells the half-life of spermidine *N*-acetyltransferase was approx. 41 min (Fig. 2), whereas in the cells treated with MGBG there was less than a 5% loss of activity in 12 h.

MGBG was also a substrate for acetylation in these cells (Fig. 3), but acetylation occurred at only 14% of the

Table 3. Activity of MGBG acetyltransferase in nuclear and cytosolic fractions of BHK-21/C13 cells

BHK cells were seeded at a density of 1.3×10^4 cells/cm² and were grown as described previously. MGBG was added at 0 h to a final concentration of 10 μ M. After cell fractionation, acetyltransferase assays were carried out as described in the Experimental section; 1 unit = 1 pmol of acetyl-MGBG formed/min per mg of protein. Results are averages of duplicate experiments [variations for (a) less than 10% and for (b) less than 5%].

Fraction	Time in culture (h)	Addition	MGBG acetyltransferase activity (units)
(a) Nuclear	24	None	0.43
		10 μ M-MGBG	0.40
	48	None	1.07
		10 μ M-MGBG	0.63
	72	None	0.21
		10 μ M-MGBG	0.22
(b) Cytosolic	24	None	0.06
		10 μ M-MGBG	0.04
	48	None	0.08
		10 μ M-MGBG	0.05
	72	None	0.08
		10 μ M-MGBG	0.03

rate with which spermidine was acetylated. Treatment of the cells in culture with MGBG did not increase acetylation of the drug, but rather decreased the ability of the cells to conjugate MGBG (Fig. 3). Analysis of the products of this acetylation by h.p.l.c. showed a peak of radioactivity at 3.2 min, approx. 2 min before the peak of authentic MGBG (result not shown).

In order to identify this peak, the assay was repeated with [¹⁴C]MGBG and [³H]acetyl-CoA as cosubstrates. The peak of ¹⁴C radioactivity corresponded to the absorbance peak of authentic MGBG at 5.3 min, and the ³H/¹⁴C peak (acetyl-MGBG) corresponded to the ³H radioactivity peak obtained in the previous experiment (Fig. 4). The result shown in Fig. 4 is that obtained by subtraction of T₀ from T₁₅ (Fig. 4, insert). By a second h.p.l.c. method (Rosenblum & Loo, 1980), two peaks of radioactivity were again obtained: one containing ³H/¹⁴C, putative acetyl-MGBG, and one containing ¹⁴C only, authentic MGBG (results not shown). Fractionation of the cell homogenate showed that acetylation of MGBG was confined almost exclusively to the nuclear fraction (Table 3).

DISCUSSION

MGBG induced both the cytosolic and nuclear spermidine *N*-acetyltransferase when added to BHK-21/C13 cells growing in monolayer culture (Fig. 1, Table 2). Only in the cytosolic fraction, however, was the stimulation specific for the *N*¹-acetyltransferase (Table 2). It did not stimulate enzyme activity in the assay *in vitro*, which is consistent with the results of Erwin & Pegg (1986) and Pegg *et al.* (1985). The drug did stabilize the enzyme against intracellular degradation (Fig. 2), a result again consistent with previous observations in rat liver (Erwin & Pegg, 1986).

It seems likely that this increased acetylation of polyamines will, in some way, contribute to the decreased intracellular concentrations of spermidine and spermine and the increased intracellular putrescine observed (Table 1). Increased acetylation of spermidine did not result in polyamine depletion by causing an increase in the excretion of acetylspermidine, since, in MGBG-treated BHK-21/C13 cells, polyamine excretion was decreased (Wallace & Keir, 1986).

MGBG was acetylated in BHK-21/C13 cells by a nuclear enzyme, but the activity towards the drug was low, being only about 14% of that shown towards spermidine in cell homogenates (Figs. 1 and 3, and Table 3). It may be argued that the observed activity was the result of acetylation of endogenous spermidine. However, analysis of the assay products from the MGBG assay by the polyamine h.p.l.c. system revealed no *N*-acetylspermidine. Also, in view of finding of the peak corresponding to [³H]acetyl[¹⁴C]MGBG by MGBG h.p.l.c. (Fig. 4), this seems unlikely. There was no stimulation of MGBG acetylation in drug-treated cells; indeed, the activity was decreased in the cells grown in the presence of MGBG (Fig. 3), suggesting enzyme inhibition during cell culture. Erwin & Pegg (1986) have reported inhibition as well as induction of cytosolic spermidine *N*¹-acetyltransferase by MGBG in rat liver.

A more likely explanation of the difference is that the enzyme responsible for the acetylation of MGBG is not the same enzyme as that which acetylates spermidine. The MGBG-acetylating enzyme may be a non-inducible isoenzyme of the spermidine acetyltransferase. In a series of similar experiments with sulphadimidine as the substrate, BHK-21/C13 cells acetylated the drug in a pattern identical with that found with MGBG (F. C. Robinson & H. M. Wallace, unpublished work), suggesting that the enzyme responsible for the acetylation of the drugs may be an unrelated multisubstrate enzyme. Further evidence for separate enzymes comes from the work of Della Ragione & Pegg (1983), who showed that the spermidine acetyltransferase enzyme from rat liver will only react with molecules which have a primary amine group separated from an internal nitrogen atom by a C₃ aliphatic chain, a criterion which MGBG does not fulfil.

The results presented here show that the antiproliferative drug MGBG has marked effects on acetylation in BHK-21/C13 cells. In addition to stimulating the acetylation of spermidine, MGBG itself can be metabolized by acetylation by these cells. The pattern of acetylation suggest that the enzyme responsible for this is different from that which acetylates the polyamines spermidine and spermine. These results have important implications for the future clinical use of this drug.

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REFERENCES

- Ask, A., Persson, L., Oredsson, S. M. & Heby, O. (1986) *Int. J. Cancer* **37**, 465–470
- Della Ragione, F. & Pegg, A. E. (1983) *Biochem. J.* **213**, 701–706
- Erwin, B. G. & Pegg, A. E. (1983) *Biochem. J.* **238**, 581–587
- Furneaux, H. M. (1978) Ph.D. Thesis, University of Aberdeen

- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Marsh, K. C., Leismann, J., Patton, T. F., Fabian, C. J. & Sternson, L. A. (1981) *Cancer Treat. Rep.* **65**, 253–257
- Matsui, I. & Pegg, A. E. (1980) *Biochem. Biophys. Res. Commun.* **92**, 1009–1015
- Nuttall, M. E. & Wallace, H. M. (1987) *Biochem. Soc. Trans.* **15**, 450
- Pegg, A. E., Erwin, B. G. & Persson, L. (1985) *Biochim. Biophys. Acta* **842**, 111–118
- Porter, C. W., Mikles-Robertson, F., Kramer, D. & Dave, C. (1979) *Cancer Res.* **34**, 2414–2421
- Rosenblum, M. G. & Loo, T. L. (1980) *J. Chromatogr.* **183**, 363–366
- Seiler, N. & Knodgen, B. (1980) *J. Chromatogr.* **221**, 227–235
- Seppanen, P., Alhonen-Hongisto, L. & Janne, J. (1980) *Eur. J. Biochem.* **110**, 7–12
- Wallace, H. M. & Cameron, G. A. (1986) *J. Chromatogr.* **380**, 122–127
- Wallace, H. M. & Keir, H. M. (1986) *FEBS Lett.* **194**, 60–63
- Wallace, H. M., Gordon, A. M., Keir, H. M. & Pearson, C. K. (1984) *Biochem. J.* **219**, 211–221
- Wallace, H. M., MacGowan, S. H. & Keir, H. M. (1985) *Biochem. Soc. Trans.* **13**, 329–330
- Warrell, R. P. & Burchenal, J. H. (1983) *J. Clin. Oncol.* **1**, 52–65
- Williams-Ashman, H. G. & Schenone, A. (1972) *Biochem. Biophys. Res. Commun.* **46**, 288–295

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