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MITOCHONDRIAL EFFECTS OF THE GUANIDINO GROUP-CONTAINING CYTOSTATIC DRUGS, *m*-IODOBENZYLGUANIDINE AND METHYLGLYOXAL BIS (GUANYLHYDRAZONE)

CORNELIA LOESBERG, HENNY VAN ROOIJ, JOHANNES C. ROMIJN* and LOU A. SMETS†

Department of Experimental Therapy, The Netherlands Cancer Institute/Antoni van Leeuwenhoekhuis, 121 Plesmanlaan, NL-1066 CX Amsterdam; and * Department of Urology, Erasmus University, 50 Dr. Molewaterplein 50, NL-3015 GD Rotterdam, The Netherlands

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Abstract—The involvement of mitochondrial damage in the antiproliferative effects of *m*-iodobenzylguanidine [MIBG] and methylglyoxal bis (guanylhydrazone) [methylGAG] was studied in human neuroblastoma SK-N-SH, mouse neuroblastoma N_1E115 and mouse lymphosarcoma S49 cells. Proliferation of SK-N-SH cells was insensitive to MIBG (100 μM gave 15% inhibition), but sensitive to methylGAG (IC_{50} = 50 μM). MIBG and methlyGAG were approximately equitoxic to N_1E115 cells (IC_{50} of 92 and 87 μM, respectively). S49 cells were most sensitive to both MIBG (IC_{50} = 11 μM) and methylGAG (IC_{50} = 5 μM). In isolated sonicated mitochondria, MIBG inhibited respiration at complex I of the respiratory chain (EC_{50} = 0.5 mM), whereas methylGAG was much less effective (EC_{50} > 15 mM). In intact cells, MIBG at 31 μM impaired mitochondrial respiration and stimulated the glycolytic flux. In contrast, equimolar concentrations of methylGAG had no effect on oxygen consumption, ATP content, glucose consumption and lactate production. MethylGAG significantly increased putrescine levels in N_1E115 and S49 cells within 12 hr via inhibition of *S*-adenosylmethionine decarboxylase. No such effects were seen in SK-N-SH cells for up to 48 hr. Equimolar concentrations of MIBG had no effect on the putrescine levels in the various cell lines, suggesting that MIBG did not inhibit *S*-adenosylmethionine decarboxylase. It is concluded that the antiproliferative mechanisms of the guanidino compounds are essentially different. MIBG inhibited mitochondrial respiration at complex I with concomitant stimulation of the glycolytic flux but was essentially without effect on polyamine levels. On the other hand, cytotoxicity of methylGAG was not associated with mitochondrial dysfunction.

m-Iodobenzylguanidine (MIBG), a ring-iodinated alkylguanethidine, is a functional analog of natural norepinephrine. The drug is selectively accumulated in storage granules of chromaffin tissue [1]. Due to this property, radio-iodinated [^{131}I]MIBG is a radiopharmaceutical for diagnosis and targeted radiotherapy of tumors of neural crest origin [2, 3]. In micromolar concentrations, unlabeled MIBG itself appeared cytotoxic in several cell lines and the drug had anti-tumor effects in animal models in schedules that were well tolerated by the host [4]. Recent studies revealed that the antiproliferative properties of MIBG *per se* were due to guanidino-specific inhibition of mitochondrial respiration at complex I of the respiratory chain with compensatory stimulation of the glycolytic flux [5].

Several guanidino group-containing drugs have gained recognition for their anti-proliferative activity [6]. Of these, the diguanidino compound methylGAG or MGBG, which has entered several phase-II studies, is the best known [7]. MethylGAG is an effective inhibitor (K_i = 1 μM) of *S*-adenosylmethionine decarboxylase (EC 4.1.1.50), a rate-controlling enzyme in the biosynthesis of the polyamines spermidine and spermine from putrescine [8, 9]. At present, the effects of MIBG on polyamine levels are unknown.

Whether polyamine synthesis is the primary target of the cytostatic action of methylGAG action is still uncertain (for an extensive review, see Ref. 10). Several studies have demonstrated that methylGAG can also have profound effects on the ultrastructure and function of mitochondria *in vitro* and *in vivo* (e.g. Refs. 11–13). In fact, mitochondrial effects of methylGAG can precede alterations in polyamine content [14] and could be associated with reductions in cellular ATP levels and pyruvate oxidation and with depression of mitochondrial DNA replication [15]. However, others have pointed out that adverse effects of methylGAG on mitochondria only occur at relatively high, namely millimolar concentrations [16], while at low concentration the drug even protects mitochondrial membranes [17]. In Ehrlich ascites cells, methylGAG inhibits proliferation without detectable effects on mitochondrial respiration [18].

Since a correlation between mitochondrial and cytostatic effects of MIBG was recently established [5], a comparison between MIBG and methylGAG effects in the same cell system could provide some information on the relative contribution of mitochondrial and polyamine effects to the cytostatic and antineoplastic properties afforded by these drugs. In spite of considerable structural differences (Fig. 1), the two guanidino compounds share a number of functional properties. Both drugs are metabolically stable and accumulate in intact cells

† To whom correspondence should be addressed.

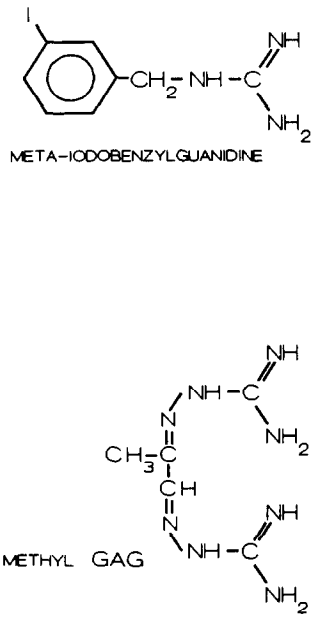

Fig. 1. Chemical structures of the mono-guanidino compound *m*-iodobenzylguanidine (MIBG) and the diguanidine methylglyoxal bis(guanylhydrazone) (methylGAG). MethylGAG is represented in a folded configuration to display guanidino groups at the same position as in MIBG.

[19, 20]; they are high-affinity substrates for cellular mono-(ADP)ribosyltransferases [21, 22] and affect the levels of endogenous acceptors of these enzymes [20, 23]. Mitochondrial proteins are abundantly mono-(ADP)ribosylated [24] and could conceivably be a common target of the two drugs. Moreover, mono- as well as di-guanidino compounds can reduce mitochondrial oxygen uptake by an interaction of protonated guanidino groups with negative regions of the mitochondrial membrane [16, 25].

We have, therefore, compared MIBG and methylGAG for their effects on cell proliferation, mitochondrial respiration and intracellular polyamine levels in human SK-N-SH and mouse N_1E115 neuroblastoma and in mouse S49 lymphosarcoma cell lines. Under comparable antiproliferative conditions, MIBG was essentially without effect on polyamine levels whereas methylGAG failed to demonstrate early effects on mitochondrial functions.

MATERIALS AND METHODS

Drugs. MIBG (Fig. 1) was synthesized from *m*-iodobenzylamine (obtained from Janssen Pharmaceutics, Beerse, Belgium) according to Wieland *et al.* [1]. The quality of the product was controlled by HPLC analysis and was identified as MIBG-sulphate with over 98% purity. MethylGAG was purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.).

Cells and culture methods. The mouse neuroblastoma N_1E115, the human neuroblastoma SK-N-SH and the mouse lymphosarcoma S49 cells were all grown in Dulbecco's modification of Eagle medium (Flow Laboratories, Irvine, U.K.) containing 4 g/L glucose and supplemented with 10% fetal calf serum and antibiotics [5]. Cell proliferations after the stated intervals was calculated from a protein determination and expressed as a percentage of the corresponding control culture.

Isolated mitochondria. Rat liver mitochondria were isolated from male Wistar rats (200 g) as described by Myers and Slater [26] with an isolation medium containing 0.25 M mannitol, 0.5 mM EDTA and 2 mM 4-morpholinopropanesulfonic acid; final pH 7.0. Contaminating lysosomes were removed according to Loewenstein *et al.* [27]. The mitochondria were washed twice and stored on ice at a concentration of approximately 25 mg protein/mL before use. Sonicated mitochondria (0.5 mg protein/mL) were incubated at 25° in a medium (total vol. 2 mL) containing the following standard components: 15 mM potassium chloride, 50 mM tris-(hydroxymethyl)aminomethane hydrochloride, 10 mM potassium phosphate, 5 mM magnesium chloride, 2 mM EDTA and 10 mM mannitol (derived from the mitochondrial suspension); final pH, 7.2. The mitochondria were preincubated for 2 min at 25° in a medium containing the standard components in the presence of different concentrations of either drug using 1 mM NADH as substrate. State 3 respiration was initiated by addition of 1 mM ADP. The rate of oxygen consumption, measured polarographically, was monitored continuously and the experiment was performed in 2 min, i.e. before the O_2 was completely consumed. The initial rate of respiration was measured, immediately after the addition of ADP, in sonicated mitochondria (0.5 mg protein/mL) incubated at 25° in a medium containing the standard components, with the exception of P_i.

Measurement of glucose metabolism. Uptake of D-[U-^{14}C]glucose (Amersham International, Little Chalfont, Amersham, U.K.; sp. act. 230 Ci/mmol) and 2-deoxy-D-[1-^{3}H]glucose (Amersham; sp. act. 20 Ci/mmol) was measured in 2.5×10^6 cells incubated in 1 mL growth medium containing 1 g/L unlabeled glucose and 0.25 μCi/mL [^{14}C]glucose plus 0.5 μCi/mL [^{3}H]deoxyglucose, as described previously [5]. Prior to the start of the experiment the cells were washed once with this low-glucose medium. After 2.5 hr at 37°, the cells were washed three times with phosphate buffered saline and harvested by centrifugation. The pellets were dissolved in sodium hydroxide (0.1 N), neutralized with hydrochloride (0.1 N) and the radioactivities were determined by differential liquid scintillation counting.

Glucose consumption and lactate production were measured in 2.5×10^6 cells incubated at 37° in 1 mL of phosphate buffered saline supplemented with 5% fetal calf serum and 1 g/L glucose. Samples of the culture medium were taken after 4 hr for the determination of lactate (using Du Pont AEA^{sx} discrete chemical analyser) and glucose (with Glucoquant, Boehringer, Mannheim, F.R.G.).

Oxygen consumption. Oxygen consumption was measured polarographically using a Clark-type electrode in single cell suspensions (total volume 330 μL; cell density 10^7/mL).

Determination of ATP. ATP was measured in cell suspensions (10^6/mL) incubated at 37° in phosphate buffered saline supplemented with 5% fetal calf serum. After 4 hr the cells were harvested by centrifugation and extracted with cold perchloric acid (final concentration 3%, w/v). The extracts were neutralized with 2 M potassium hydroxide in 0.3 M 4-morpholinopropane-sulfonic acid and stored at −70°. ATP was measured fluorimetrically using glucose, $NADP^+$, hexokinase and glucose-6-phosphate dehydrogenase [28].

Determination of polyamine content. For the determination of polyamine content, 10^6 cells/mL were grown in the presence of MIBG or methylGAG. After 12, 24 or 48 hr, the cells were collected, washed three times in phosphate buffered saline and extracted with 0.3 M perchloric acid. The protein content of the pellet was determined. Following the addition of the internal standard, 1,6-diaminohexane (approximately 1 nmol/10^5 cells), to the acid extracts, polyamines were benzoylated, as described previously [29]. Benzoylated polyamines were extracted from the incubation mixture using chloroform and separated by reversed-phase HPLC using ChromSpher C18 RP-column (Chrompack International, Middelburg, The Netherlands), isocratic elution (methanol/water, 60:40, v/v) and detection by UV-absorbance at 229 nm.

RESULTS

Effects on cell proliferation and cell survival

N_1E115, SK-N-SH and S49 cells were grown in various concentrations of MIBG or methylGAG (0–100 μM). The protein content of the cultures was determined daily to assess the effects of the drugs on cell growth. The results revealed that SK-N-SH cells were insensitive to MIBG ($IC_{50} > 100$ μM) but weakly sensitive to methylGAG ($IC_{50} = 50$ μM) (Fig. 2A). In contrast, N_1E115 cells were equally sensitive to MIBG and methylGAG (IC_{50} of 92 and 87 μM, respectively) (Fig. 2B), and S49 lymphosarcoma cells were sensitive to both drugs (IC_{50} of 11 and 5 μM for MIBG and methylGAG, respectively) (Fig. 2C). Overall, the sensitivity to both drugs was proportional to the population doubling times of 60, 36 and 12 hr for SK-N-SH, N_1E115 and S49 cells, respectively.

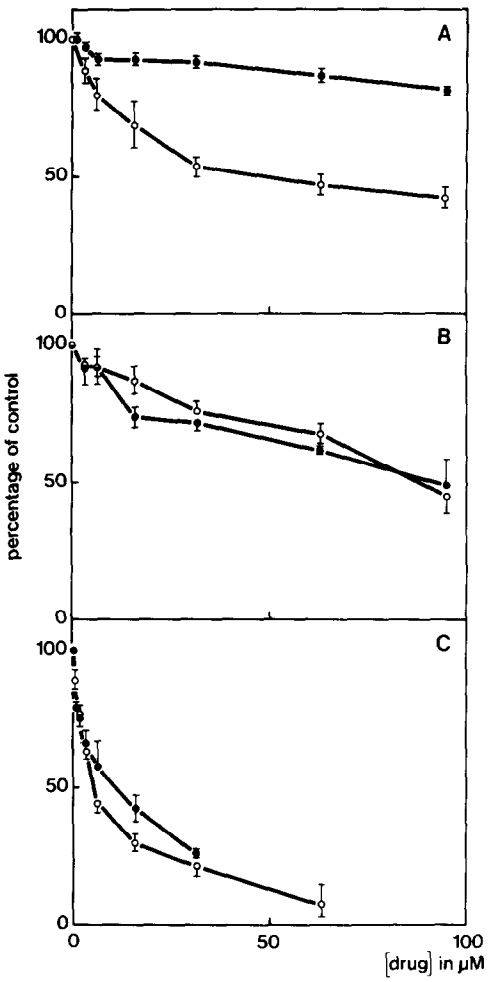

Fig. 2. Effects of different concentrations of MIBG (closed symbols) and methylGAG (open symbols) on the proliferation of (A) SK-N-SH cells, (B) N_1E115 cells and (C) S49 cells. Drugs were present for 48 hr and the results are expressed as per cent of control (mean ± SE of three different experiments).

Effects on isolated mitochondria

In isolated and sonicated rat liver mitochondria incubated with ADP and NADH as the respiratory substrates, MIBG almost instanteneously (within 2 min) reduced oxygen consumption (EC_{50} = 0.5 mM). Equimolar concentrations of methylGAG, however, had little or no effect. At 4 mM methylGAG, only a 10% inhibition of oxygen consumption was observed and the EC_{50} was greater than 15 mM.

Effects on mitochondria in intact cells

Addition of MIBG (31 μM) to the incubation medium of the three cell lines under investigation, resulted in a number of early and related metabolic effects. MIBG stimulated the glycolytic flux, as indicated by an increased deoxyglucose uptake (Fig. 3A); enhanced consumption of glucose from the medium (Fig. 3B); stimulated lactic acid release (Fig. 3C) and decreased the intracellular levels of radiolabeled glucose intermediates (Fig. 3D). The relative stimulation of glycolysis by MIBG in each of the three cell types was proportional to the cytostatic action, namely S49 > N_1E115 > SK-N-SH. In contrast, cells incubated in methylGAG (31 μM) maintained unchanged levels of deoxyglucose uptake, glucose consumption and lactate production, and of intracellular levels of glucose intermediates (Fig. 3A–D). Even 24 hr incubations with methylGAG did not significantly increase lactate production (not shown).

Oxygen consumption and cellular ATP content were determined after 4 hr of incubation with either drug at 31 μM. As in a previous study [5], oxygen consumption was significantly inhibited in cells incubated with MIBG to 22–63% of control values, with corresponding decreases in ATP levels in

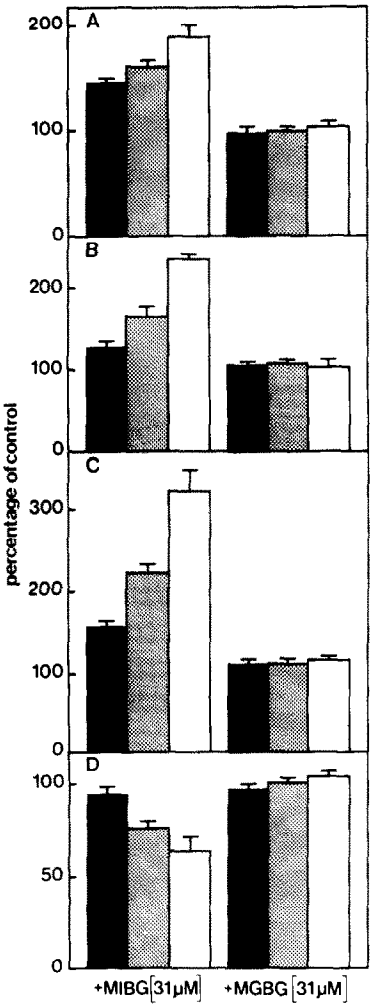

Fig. 3. Effects of MIBG and methylGAG (MGBG) (31 μM each) on glucose metabolism in SK-N-SH, N_1E115 and S49 cells. Results are expressed as the mean percentage of control ± SE of three different experiments. SK-N-SH cells are represented by closed bars, N_1E115 cells by dotted bars and S49 cells by open bars. $P < 0.01$ Student's *t*-test, control vs MIBG treated cells. (A) Uptake of [^{14}C]-deoxyglucose. Incubation time was 2.5 hr at 37°. (B) Glucose consumption. Mean glucose consumption in 4 hr at 37° of 2.5×10^6 control cells was 1.8, 2.9 and 0.2 mmol/L for SK-N-SH, N_1E115 and S49 cells, respectively. (C) Lactic acid production. Mean lactic acid production in 4 hr at 37° of 2.5×10^6 control cells was 2.6, 3.5 and 0.2 mmol/L for SK-N-SH, N_1E115 and S49 cells, respectively. (D) Level of intracellular glucose-derived intermediates. Retention of [^{3}H]glucose in the different cells was measured for 2.5 hr at 37°. Results are expressed as the mean percentage of control ± SE of three different experiments.

N_1E115 and S49 cells. With methylGAG, no effects on oxygen consumption and ATP content could be detected.

Electron microscopic investigations revealed considerable swelling and fusion of mitochondria in S49 cells grown for 24 hr in 31 μM MIBG. With methylGAG, swelling of mitochondria was only occasionally observed (courtesy of P. van Wachem).

Effects on polyamine levels

Polyamine levels were determined after several hours of incubation with the drugs. The data from 24 hr incubations are shown in Table 1. The cell lines differed considerably in basal putrescine levels. MethylGAG significantly increased putrescine levels, namely 3-fold in N_1E115 and 20-fold in S49 cells, and these changes were already apparent after 12 hr. Putrescine levels were unchanged in SK-N-SH cells incubated with methylGAG for up to 48 hr. The effect of methylGAG on other polyamines was insignificant except for a reduction of spermidine in N_1E115 and of spermine in S49 cells. MIBG was clearly without effect on polyamine levels except for a reduction in putrescine in SK-N-SH.

DISCUSSION

The purpose of the present study was to investigate whether methylGAG shared mitochondrial toxicity with MIBG, as a common mechanism of cytostatic action. It is obvious from the results that this was not the case. MethylGAG was without effect on several parameters of cellular respiration (oxygen consumption, ATP content) and of glycolytic flux (Fig. 3). In contrast, these parameters were markedly affected within hours of the addition of MIBG and the magnitude of the effects was proportional to the degree of growth inhibition in the various cell lines (Fig. 2). A similar discrepancy between MIBG and methylGAG was evident for oxygen consumption in isolated rat mitochondria and for mitochondrial ultrastructure in S49 cells.

Conversely, MIBG did not interfere with polyamine levels except for causing a reduction in putrescine in SK-N-SH cells (Table 1). This single effect is not well understood and is contrary to the stimulation expected from inhibition of *S*-adenosylmethionine decarboxylase. Preliminary and unpublished experiments (J. Holley and G. M. Cohen, University of London, School of Pharmacy; personal communication) revealed that DFMO potentiated markedly the toxicity of methylGAG, but not that of MIBG in Ehrlich ascites cells. Moreover, spermidine uptake in these cells was inhibited competitively by methylGAG whereas inhibition by MIBG was uncompetitive. It would appear that MIBG does not inhibit *S*-adenosylmethionine decarboxylase or compete with spermidine for a shared carrier system.

As such, the results in Table 1 do not prove (or exclude) that methylGAG acted through interference with polyamine metabolism. In S49 cells, the pools were atypically affected and there was no correlation between polyamine effects and inhibition of proliferation. Most importantly, the two neuroblastoma lines were fairly insensitive to methylGAG compared to published data, a finding which may well agree with the documented insensitivity of slowly growing cells to this drug [10]. Whatever the mechanism of methylGAG may be in these cells, it obviously differs essentially from the early mitochondrial effects of MIBG.

In view of the large structural differences (Fig. 1) between the two drugs, the conclusion that

Table 1. Effects of MIBG (31 μM) and methylGAG (31 μM) on intracellular polyamine levels

Cell type	Polyamine*	Control	MethylGAG	MIBG
SK-N-SH	PU	4.6 ± 0.3	5 ± 2	2.0 ± 0.4†
	SD	9 ± 2	9 ± 4.5	8 ± 3
	SM	12 ± 2	10 ± 5	11 ± 4
N_1E115	PU	1.7 ± 0.6	4.5 ± 0.4†	0.8 ± 0.5
	SD	11 ± 2	7.0 ± 0.4†	13 ± 4
	SM	17 ± 3	13 ± 6	25 ± 6
S49	PU	0.6 ± 0.2	11 ± 5†	0.5 ± 0.3
	SD	11 ± 2	10 ± 4	7 ± 3
	SM	10 ± 2	2 ± 2†	8 ± 3

Results are expressed in nmol/ng protein (in 24 hr) ± SE (mean of three different experiments).

* PU = putrescine, SD = spermidine, SM = spermine.

† $P < 0.05$; control vs treated (Student's *t*-test).

methylGAG, unlike MIBG, was without mitochondrial effects in our cell system, seems trivial. However, as was outlined in the introduction, the drugs share several functional properties related to mitochondrial integrity and functioning. Moreover, it is unlikely that the negative findings concerning methylGAG in this report could overrule the vast literature on the mitochondrial toxicity of this drug (Refs 11–13 and other citations in Ref. 10).

The finding of consistent, early mitochondrial effects with MIBG as compared to the variable responses obtained with methylGAG may well be due to differences in intracellular drug concentration. In isolated mitochondria, the manifestation of early effects of MIBG or methylGAG requires high concentrations in the millimolar range (Refs 5 and 16; this report) whereas effects in intact cells are usually achieved with micromolar extracellular concentrations. MIBG (Fig. 1) is an organic cation with a lipophilic benzyl moiety and a positively charged ($pK_a = 12$) guanidino group. Like rhodamine dyes [30], it accumulates considerably in cells and mitochondria by an electro-chemical gradient [20]. Recent studies applying electron spectroscopic imaging of MIBG-bound iodine have revealed a strong and almost exclusive accumulation of MIBG into mitochondria *in situ*, within 2 hr [31]. Conversely, the uptake of methylGAG, which is poorly protonated at physiological conditions, occurs by a high-affinity and saturable polyamine carrier mechanism [32]. Compared to the *non-specific* accumulation of MIBG, intracellular levels of methylGAG are much more dependent on variations in the capacity of the *specific* polyamine/methylGAG transport as a consequence of cell type, polyamine metabolism, cell proliferation rate and other physiological variables. Such variations, and notably the low proliferation rates of the neuroblastoma cells [13], might well determine whether, and to what extent, mitochondrial toxicity prevails over the other cytotoxic mechanisms of methylGAG.

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