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META-IODOBENZYLGUANIDINE INHIBITS COMPLEX I AND III OF THE RESPIRATORY CHAIN IN THE HUMAN CELL LINE MOLT-4

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Abstract—In this paper we report the effects of *meta*-iodobenzylguanidine (MIBG), a structural analogue of norepinephrine, on cell proliferation and several parameters related to mitochondrial respiration in Molt-4 cells. In micromolar concentrations, MIBG completely inhibited the proliferation of Molt-4 cells. In intact Molt-4 cells, a progressive increase in the lactate to pyruvate ratio was observed after incubation of these cells with glucose and increasing concentrations of MIBG. In Molt-4 cells permeabilized with digitonin, MIBG inhibited mitochondrial ATP synthesis when malate was used as a substrate. Succinate-driven synthesis of ATP was also inhibited by MIBG, although higher concentrations were required. These results indicate that apart from inhibition of complex I, MIBG inhibits at least one other complex of the respiratory chain. Measurement of the activities of the individual enzyme complexes in the presence of MIBG revealed that complex III is the other enzyme complex susceptible to inhibition by MIBG. Although maximal inhibition of ATP synthesis was observed at a concentration of 10 μM, maximal inhibition of cell proliferation was observed at a concentration of 50 μM of MIBG. This suggests that MIBG also influences other cellular processes apart from mitochondrial oxidative phosphorylation, resulting in additional inhibition of cell proliferation.

Key words: *meta*-iodobenzylguanidine; respiratory chain; Molt-4; cell proliferation; ATP synthesis; enzyme inhibition

MIBG§ is a structural and functional analogue of the neurotransmitter norepinephrine, and is therefore capable of competing with norepinephrine for uptake in chromaffin tissues [1]. In its radioiodinated form MIBG is used clinically as a tumour-seeking radio pharmaceutical agent for the diagnosis [2, 3] and treatment [3] of neuroendocrine tumours such as neuroblastoma and pheochromocytoma. It is a stable and non-metabolizable compound *in vivo* [4]. Unlabelled MIBG by itself has anti-proliferating effects against murine leukaemia (L1210) and neuroblastoma (N_1E115) tumours in mice and against a large number of cell lines of various origin [5]. It has now entered phase II trials in which elevated doses of unlabelled MIBG are given for potential palliation of carcinoid patients (neuroendocrine tumours, capable of MIBG uptake) (Taal BG, personal communication).

Comparison of the cytotoxicity of MIBG and MIBA showed that the guanidine moiety of MIBG is mainly responsible for these cytotoxic effects [5]. Recent studies suggest that the anti-proliferative action of MIBG is at least partially due to specific inhibition of complex I of the mitochondrial respiratory chain [6, 7]. Inhibition of complex I by MIBG was directly shown in rat liver mitochondria, in which state-3 oxidation of glutamate was decreased whereas state-3 oxidation of succinate, in which the electrons are donated at the level of coenzyme Q, i.e. after complex I, was unaffected by the addition of MIBG. In the human neuroblastoma cell line SK-N-SH and the murine cell lines S49 (lymphosarcoma) and N_1E115 (neuroblastoma) the presence of MIBG led to an enhanced glycolytic flux, decreased ATP levels and decreased oxygen consumption, suggesting inhibition of mitochondrial respiration [7].

So far, no direct evidence showing that MIBG inhibits the activity of complex I of the respiratory chain in human cells has been presented. Therefore, we studied the effects of MIBG on cell proliferation and several parameters related to mitochondrial respiration in the human T-lymphoblastic leukemia cell line Molt-4. This cell line was selected because of its human origin and high proliferation rate. Moreover, the effect of MIBG on mitochondrial respiration is likely to be a general one rather than an effect specific for a certain cell type.

The effect of MIBG on the proliferation of Molt-4 cells in different culture media was studied. This was followed by experiments in which the effects of MIBG on the end products of glycolysis (lactate and pyruvate) were studied. In order to test whether the effects observed were indeed due to specific inhibition of complex I, mitochondrial respiratory chain activity was studied in the presence of MIBG. Finally,

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§ Abbreviations: DCPIP, 2,6-dichlorophenol-indophenol; DNP, 2,4-dinitrophenol; MIBG, *meta*-iodobenzylguanidine; MIBA, *meta*-iodobenzylamine.

measurement of the activities of the individual enzyme complexes of the respiratory chain was performed to study whether complex I was the only enzyme complex affected by MIBG.

MATERIALS AND METHODS

Cell culture. The human lymphoblastic leukaemia cell line Molt-4 was cultured at 37° in a humidified atmosphere of 90% air with 10% CO_2. The culture medium used was either RPMI 1640 (Gibco, Paisley, U.K.) or DMEM (Gibco, Paisley, U.K.), both supplemented with 10% FCS (Sebak GmbH, Aidenbach, Germany), 10 mM L-glutamine (Gibco, Paisley, U.K.), 0.1 mg/mL penicillin and 100 IU streptomycin (Gibco, Paisley, U.K.). Cells were counted with the aid of a Coulter counter.

Before measurement of lactate to pyruvate ratios, ATP synthesis, aspartate production or the activity of the various enzyme complexes of the respiratory chain, cells were preincubated with appropriate concentrations of MIBG (Cis Bio International, Gif-sur-Yvette, France) in the culture medium (DMEM) for 24 hr.

Lactate to pyruvate ratio in Molt-4 cells. Measurement of lactate and pyruvate production from glucose in Molt-4 cells was carried out according to the method of Wijburg *et al.* [8].

ATP synthesis in Molt-4 cells. Spectrophotometric measurement of ATP synthesis in digitonin permeabilized Molt-4 cells with various substrates was performed according to the method of Wanders *et al.* [9].

Aspartate production in Molt-4 cells. Experimental conditions for the measurement of aspartate production from the oxidation of malate in Molt-4 cells were similar to conditions used during ATP synthesis with malate as substrate [9]. Aspartate was measured fluorimetrically according to the method of Kojima *et al.* [10].

Enzyme activity of complex I, II, III, IV and V of the respiratory chain in Molt-4 cells. For measurement of the activities of complex I, II and III, Molt-4 cells were solubilized (final concentration 10 mg cellular protein/mL) in PBS (0.14 M NaCl, 1.3 mM NaH_2PO_4 and 9.2 mM Na_2HPO_4) containing 1.5% (w/v) lauryl maltoside (Sigma Chemical Co., St Louis, U.S.A.). All activity measurements were performed in the standard incubation medium containing 25 mM KP_i (pH 7.2) and 5 mM $MgCl_2$ at 30°.

In case of complex I activity measurements the assay medium contained the standard components supplemented with 2.5 mg/mL BSA, 2 mM KCN, 20 μg/mL antimycin and 65 μM ubiquinone$_2$ (Sigma Chemical Co., St Louis, U.S.A.). For each assay 0.5 mg cellular protein was used. After a preincubation period of 5 min at 30° reactions were started by adding NADH at a final concentration of 100 μM. The activity of complex I was measured by following the rotenone-sensitive decrease in absorbance due to the oxidation of NADH at 340 nm.

In case of complex II activity measurements, cell suspensions (0.5 mg cellular protein/assay) were preincubated for 10 min in the standard incubation medium supplemented with 20 mM succinate.

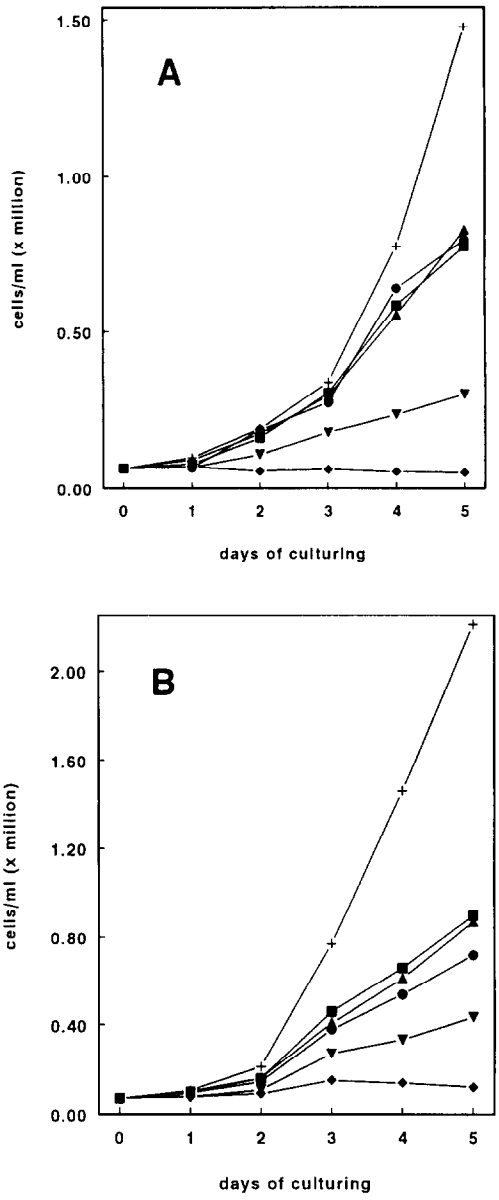

Fig. 1. Effect of MIBG on proliferation of Molt-4 cells cultured in DMEM or RPMI 1640 culture medium. Molt-4 cells were cultured in either DMEM culture medium (A) or RPMI 1640 (B) in the presence of various concentrations of MIBG. Each day small aliquots of the cell suspensions were taken and cell numbers determined with the aid of a Coulter counter. Results are given as the mean of two independent experiments. +, 0 μM; ■, 2.5 μM; ▲, 5.0 μM; ●, 10 μM; ▼, 25 μM; ◆, 50 μM MIBG.

Reactions were started by the addition of 20 μg/mL antimycin, 2 mM KCN and 50 μM DCPIP (Sigma Chemical Co., St Louis, U.S.A.). The activity of the complex was measured by following the decrease in absorbance due to the reduction of DCPIP at 600 nm.

In the case of complex III activity measurement, the assay mix contained the standard components

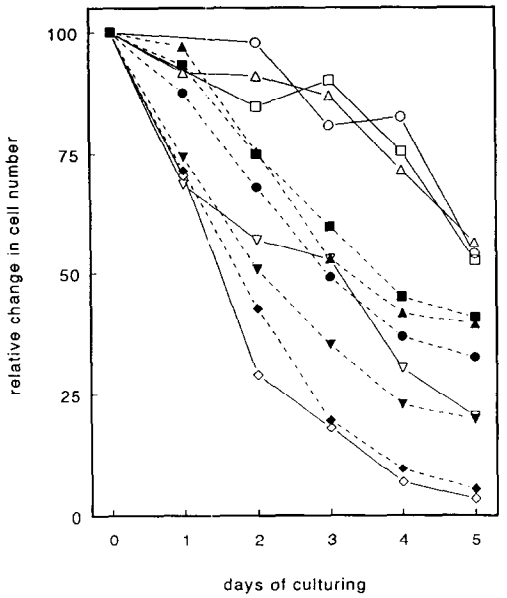

Fig. 2. Effect of MIBG on proliferation of Molt-4 cells cultured in DMEM or RPMI 1640 culture medium. Molt-4 cells were cultured in either DMEM culture medium (open symbols) or RPMI 1640 (closed symbols) in the presence of various concentrations of MIBG. Each day small aliquots of the cell suspensions were taken and cell numbers determined with the aid of a Coulter counter. Results are given as the mean of two independent experiments and represent the cell number as percentage of the number of control cells, i.e. cells cultured in the absence of MIBG. ■, 2.5 μM; ▲, 5.0 μM; ●, 10 μM; ▼, 25 μM; ◆, 50 μM MIBG.

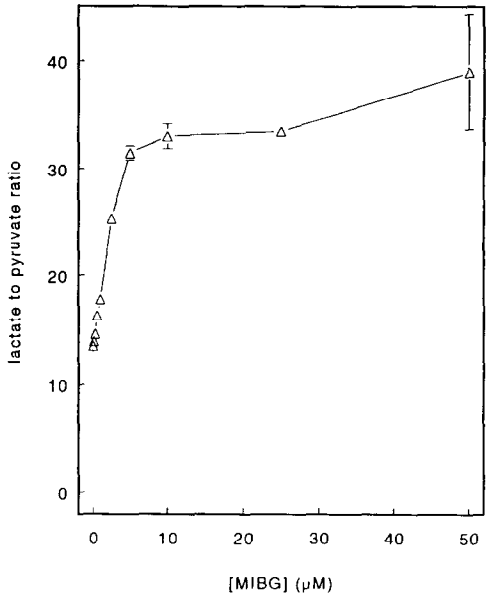

Fig. 3. Lactate to pyruvate ratio in Molt-4 cells after incubation with 10 mM glucose and various concentrations of MIBG. Molt-4 cells were incubated in the presence of 10 mM glucose and various concentrations of MIBG. After 4 hr reactions were terminated and the concentrations of lactate and pyruvate determined as described in Materials and Methods. Results are given as the means of three independent experiments ± SD.

plus 2 mM KCN, 20 μg/mL rotenone and 65 μM ubiquinol$_2$. Ubiquinol$_2$ was obtained by reducing 45 μL of a 13 mM ubiquinone$_2$ solution in ethanol with 15 μL 0.5 M Na-dithionite dissolved in 1 M Tris–HCl, pH 9, followed by centrifugation (10,000 *g*, 2 min, 4°). The supernatant was collected and centrifuged twice more in order to remove all traces of dithionite. For each assay 0.1 mg cellular protein was used. Reactions were started by adding oxidized horse heart cytochrome *c* (Janssen Chimica, Beerse, Belgium) at a final concentration of 20 μM. The activity of the complex was measured by following the increase in absorbance due to the reduction of cytochrome *c* at 550 nm.

Measurement of complex IV activity was performed essentially according to the method of Cooperstein and Lazarow [11]. Molt-4 cells were dissolved in 1% (w/v) lauryl-maltoside, 0.03 M Na_2HPO_4/KH_2PO_4, pH 7.4, and reactions were started by adding reduced cytochrome *c* at a final concentration of 20 μM, dissolved in the same buffer.

Complex V activity was measured by following the ATPase activity of the enzyme. This was done by adding 20 μL of a Molt-4 cell suspension in PBS (2 mg cellular protein/mL) to 80 μL of a solution containing 188 mM KCl, 31 mM Tris–HCl, pH 7.4, 2.5 mM EDTA, 0.05 mg digitonin/mL, 0.125% BSA (w/v) and 10 μM DNP (Merck, Darmstadt, Germany). Reactions were started by adding ATP at a final concentration of 1 mM (sufficient for a linear reaction of at least 30 min). After 30 min at 25° reactions were terminated by adding 30 μL 2 M PCA. After centrifugation (10,000 *g*, 2 min, 4°) the supernatant was neutralized with 30 μL 2 M KOH–0.6 M MOPS [3-(*N*-morpholino)propanesulfonic acid] and the ADP, formed due to the ATPase activity of the complex, was measured fluorimetrically according to the method of Williamson and Corkey [12].

RESULTS

Effects of MIBG on cell proliferation in various culture media

MIBG is known to affect cell proliferation in a large number of cell lines of both neuroblastoma and other origin [5]. Addition of low (micromolar) concentrations of MIBG to the culture medium can result in inhibition of cell proliferation. In order to study the effects of MIBG on the proliferation of Molt-4 cells, cells were grown in the presence of various concentrations of MIBG in either RPMI 1640 (a relatively poor medium) or DMEM. In both DMEM (Fig. 1A) and RPMI 1640 (Fig. 1B), increasing concentrations of MIBG led to progressive inhibition of proliferation of Molt-4 cells, eventually resulting in complete arrest of proliferation at a concentration of 50 μM. However, when RPMI 1640 was used as culture medium low concentrations of MIBG had a stronger effect on cell proliferation

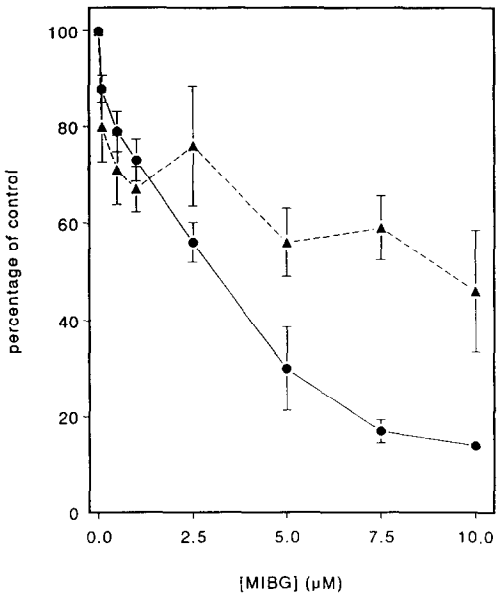

Fig. 4. ATP synthesis in Molt-4 cells with either malate or succinate as substrate, in the presence of various concentrations of MIBG. Digitonin permeabilized Molt-4 cells were incubated in the presence of 10 mM malate or 10 mM succinate and various concentrations of MIBG. After 30 min reactions were terminated and the concentrations of ATP determined as described in Materials and Methods. Results are given as the mean of three independent experiments ± SD, and represent the percentage of control values, i.e. no MIBG added (1204 nmol ATP/mg/hr) with malate as substrate and 610 nmol ATP/mg/hr with succinate as substrate). ▲, ATP synthesis with succinate as substrate; ●, ATP synthesis with malate as substrate.

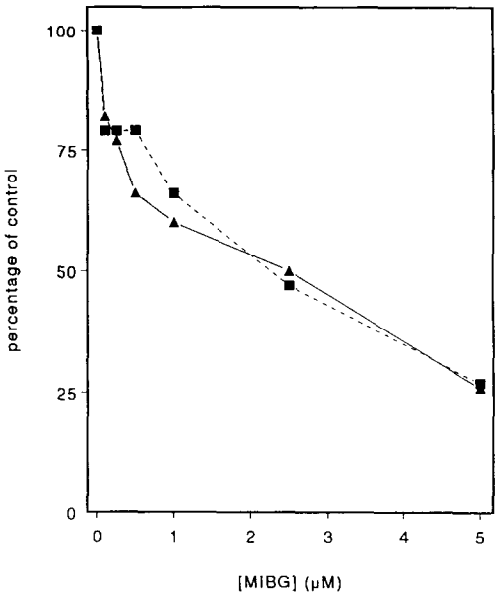

Fig. 5. Aspartate production in Molt-4 cells containing coupled or uncoupled mitochondria with malate as substrate, in the presence of various concentrations of MIBG. Digitonin-treated Molt-4 cells were incubated in the presence of 10 mM malate, various concentrations of MIBG and the presence or absence of 10 μM DNP. After 30 min reactions were terminated and the concentrations of aspartate determined as described in Materials and Methods. Results are given as the mean of two independent experiments, and represent the percentage of control values, i.e. no MIBG added (177 nmol aspartate/mg/hr). ■, Aspartate production in the presence of DNP; ▲, aspartate production in the absence of DNP.

than it did on the proliferation of cells cultured in DMEM (Fig. 2).

Effects of MIBG on lactate to pyruvate ratio

Measurement of the rates of production of pyruvate and lactate from glucose in intact cells can yield valuable information on the functional integrity of the respiratory chain [13]. In order to study the effect of MIBG on the respiratory chain of Molt-4 cells, we therefore measured the lactate and pyruvate production from 10 mM glucose. In the experiment depicted in Fig. 3 we studied the effect of increasing concentrations of MIBG on the lactate to pyruvate ratio in Molt-4 cells. The results show that increasing concentrations of MIBG led to a progressive increase of the lactate to pyruvate ratio in the Molt-4 cells, with a maximal ratio at 10 μM MIBG. This lactate to pyruvate ratio is comparable to ratios obtained in the presence of rotenone (20 μg/mL) or antimycin (20 μg/mL), which results in complete inhibition of complex I and III, respectively (data not shown).

Effects of MIBG on ATP and aspartate production in coupled and uncoupled mitochondria

In order to study the effects of MIBG on mitochondrial oxidative phosphorylation, we measured aspartate production as a result of state-3 oxidation of malate, and ATP synthesis as a result of state-3 oxidation of either malate or succinate, in the presence of various concentrations of MIBG. Figure 4 shows that ATP synthesis in digitonin-permeabilized Molt-4 cells was inhibited by MIBG when either malate (in the presence of glutamate) or succinate (in the presence of rotenone) was used as substrate for oxidative phosphorylation. This suggests that apart from the inhibition of complex I by MIBG at least one other complex of the respiratory chain is inhibited by MIBG. The extent of inhibition of ATP synthesis was significantly lower when succinate was used as substrate, compared with that observed when malate was used.

It is well established that oxidation of malate (in the presence of glutamate) yields aspartate in stoichiometric amounts. Accordingly, measurement of aspartate production gives direct information on the activity of the respiratory chain both in coupled and uncoupled mitochondria. When ATP production from ADP and phosphate is measured simultaneously, comparison of the rate of ATP production and aspartate formation provides valuable information on the degree of coupling between oxidation and phosphorylation within the respiratory chain.

In the experiment presented in Fig. 5 we studied the effect of MIBG on aspartate production with

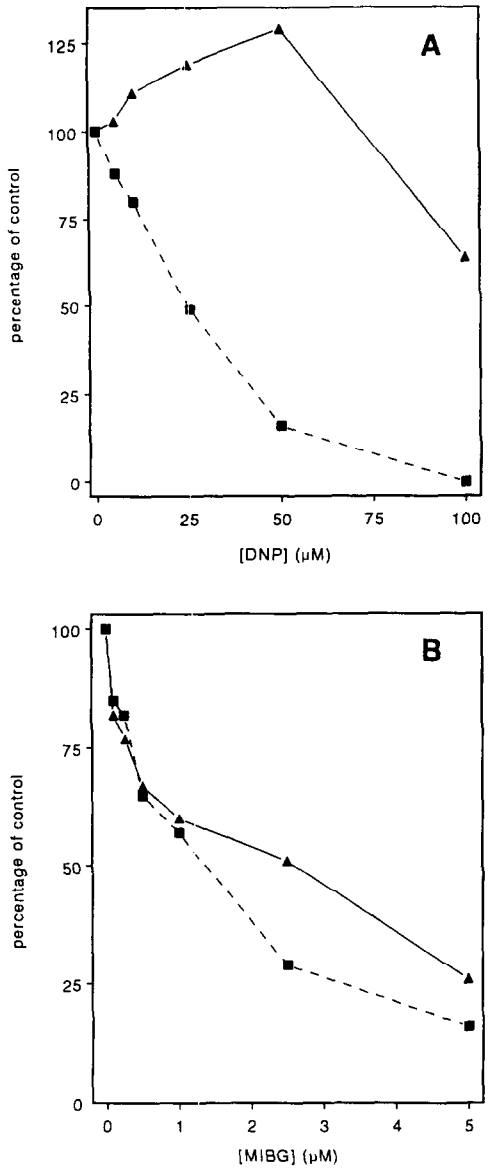

Fig. 6. Aspartate production and ATP synthesis in Molt-4 cells with malate as substrate, in the presence of various concentrations of DNP or MIBG. Digitonin-treated Molt-4 cells were incubated in the presence of 10 mM malate and various concentrations of DNP (A) or MIBG (B). After 30 min, reactions were terminated and the concentrations of aspartate and ATP determined as described in Materials and Methods. Results are given as the means of two independent experiments, and represent the percentage of control values, i.e. no DNP or MIBG added (219 nmol aspartate/mg/hr and 1237 nmol ATP/mg/hr). ▲, Aspartate production; ■, ATP synthesis.

malate as substrate in the presence of DNP (uncoupled mitochondria) or absence of DNP (coupled mitochondria). In both coupled and uncoupled mitochondria aspartate production is inhibited in a dose-dependent manner by MIBG. MIBG therefore does not seem to inhibit ATP synthesis by affecting complex V (F_1F_0 ATPase). To

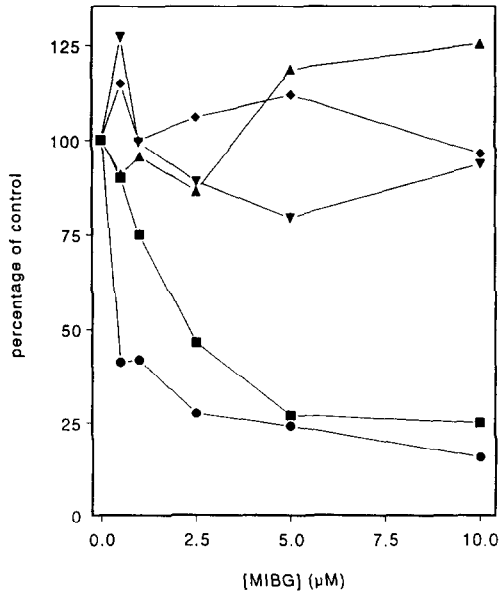

Fig. 7. Effect of MIBG on the activity of the respiratory chain complexes. Molt-4 cells solubilized with lauryl maltoside (complex I, ■; II, ▲; III, ●; IV, ▼) or permeabilized with digitonin (complex V, ◆) were used to measure the activities of the different complexes. Activities were determined as described in Materials and Methods, and are presented as percentage of control, i.e. no MIBG added. Complex I, 2.7 ± 0.7 nmol/mg/min (N = 3); complex II, 14.2 ± 2.8 nmol/mg/min (N = 4); complex III, 81.6 ± 3.5 nmol/mg/min (N = 4); complex IV, 8.2 ± 0.8 nmol/mg/min (N = 4); complex V, 2.9 ± 0.2 nmol/mg/min (N = 4). All curves represent typical examples of the effects of MIBG on the various enzyme complexes.

exclude the possibility that MIBG acts as an uncoupling agent, thereby inhibiting ATP synthesis, we compared the effects of the uncoupling agent DNP with those of MIBG on aspartate and ATP production in digitonin permeabilized Molt-4 cells. Figure 6 shows that MIBG causes a decrease in aspartate production as well as ATP synthesis. DNP does inhibit ATP synthesis; however, DNP at concentrations capable of inhibiting ATP synthesis showed a stimulatory effect on aspartate production.

Effects of MIBG on the activity of complex I, II, III, IV and V

In order to determine which complex of the respiratory chain apart from complex I is inhibited by MIBG, the activity of all enzyme complexes of the respiratory chain were measured separately. Measurement of the activity of complexes I, II, III and IV was performed in Molt-4 cells solubilized with the mild detergent lauryl maltoside, whereas measurement of the activity of complex V was performed in digitonin-treated Molt-4 cells (i.e. cells with a permeabilized plasma membrane but functional mitochondria, cf. Ref. 9). The oligomycin sensitivity of complex V activity was found to be 75% (data not shown).

Figure 7 shows the effects of MIBG on the activity

of the various respiratory chain complexes. The activities of complexes II, IV and V were unaffected by MIBG up to a final concentration of 10 μM. Complex I and III activity, however, decreased after addition of MIBG. Complex I showed a similar susceptibility to MIBG (50% inhibition at a MIBG concentration of approximately 1.8 μM) as compared to complex III (50% inhibition at a MIBG concentration of approximately 0.4 μM). This shows that apart from complex I, complex III is the other respiratory chain complex affected by MIBG.

DISCUSSION

This paper presents our studies on the effects of MIBG on cell proliferation and mitochondrial respiration in Molt-4 cells. In both RPMI 1640 and DMEM culture medium, MIBG at a concentration of 50 μM caused complete arrest of proliferation of Molt-4 cells. However, at low MIBG concentrations (2.5–10 μM), the anti-proliferating effect was more pronounced in RPMI 1640 than in the DMEM culture medium. Mitochondrial function is indispensable for proliferation of Molt-4 cells grown on relatively poor culture media such as RPMI 1640 [14]. Since MIBG at low concentrations had a more pronounced effect on proliferation, it would seem that the anti-proliferating action of MIBG is indeed, at least partially, associated with reduced mitochondrial function.

Measurement of the rates of production of pyruvate and lactate from glucose, a method used to identify defects in the oxidative phosphorylation system [13], showed that increasing concentrations of MIBG led to a progressive increase of the lactate to pyruvate ratio. This is probably the result of the inhibition of mitochondrial respiration. Loesberg *et al.* [7] reported an enhanced glycolytic flux in various cell types after addition of MIBG to the culture medium, and concluded that this was due to glycolytic compensation for inhibition of mitochondrial respiration. The increased glycolytic flux is accompanied by increased lactate production (and a reduced pH). As several anti-cancer drugs have a higher cytotoxicity at acidic pH, various strategies to improve the therapeutic index of these drugs are based on this phenomenon. *In vivo*, specific stimulation of glycolysis in tumors by MIBG leads to a reduced pH in these tumours. A further pH reduction is achieved by moderately increasing the glucose concentration in the plasma [15, 16]. Therefore the combination of MIBG and these anti-cancer drugs might improve the therapeutic index of these drugs.

When Molt-4 cells were grown for 24 hr in the presence of MIBG, mitochondrial ATP synthesis was inhibited with malate as well as with succinate as substrate. ATP synthesis with succinate as substrate, however, was less affected, indicating that MIBG inhibits at least one additional complex of the respiratory chain in addition to complex I. MIBG inhibits ATP synthesis without affecting complex V since MIBG influenced aspartate production in both coupled and uncoupled mitochondria. Comparison of the effect of MIBG and DNP (a mitochondrial uncoupler) showed that the inhibiting action of MIBG on ATP synthesis was not accomplished by dissipating the mitochondrial electrochemical gradient.

Loesberg *et al.* [7] did not find any effect of MIBG on state-3 oxidation of succinate in purified intact rat liver mitochondria, and concluded that MIBG merely inhibits complex I of the mitochondrial respiratory chain. They did, however, find a sudden decrease in the rate of oxygen consumption at a MIBG concentration of 0.5 mM when state-3 oxidation of succinate was measured. The absence of a clear effect of MIBG on the activity of oxidative phosphorylation with succinate as substrate in their experiments might be explained by the short (2 min) preincubation period with MIBG before oxygen consumption was measured. When we tried to measure ATP synthesis with malate or succinate as substrate after a preincubation period of 2 min with MIBG, we too detected no inhibition of ATP synthesis (data not shown). Moreover, we observed a more severe inhibition of ATP synthesis with malate as substrate after a 24 hr preincubation period with MIBG at a comparable mitochondrial protein to MIBG ratio, compared with the inhibition of state-3 oxidation of glutamate found by Loesberg *et al.* [7]. Therefore it appears that a preincubation period of 2 min is not sufficient for MIBG to enter the mitochondria in adequate concentrations for maximal inhibition of oxidative phosphorylation with malate or succinate as substrate.

Measurement of the separate enzyme complexes of the respiratory chain in the presence of MIBG revealed that apart from inhibition of complex I activity, MIBG is also capable of inhibiting complex III activity. Concentrations needed to achieve inhibition of complex III activity were of the same order of magnitude as those needed for inhibition of complex I.

ATP synthesis with malate as substrate was inhibited to a greater extent by MIBG than when succinate was used as substrate. This can easily be explained, since the inhibition of the activity of both complex I and complex III by MIBG contributes to the total inhibition of ATP synthesis when malate is used as substrate for ATP synthesis, whereas inhibition of ATP synthesis with succinate as substrate is only due to inhibition of complex III.

The concentrations of MIBG at which inhibition of mitochondrial ATP synthesis occurs are much higher than the recorded peak plasma levels (0.1 μM) obtained when neuroblastoma patients are treated with [^{131}I]MIBG (Voûte PA, personal communication). Since MIBG accumulates 30-fold in neuroblastoma cells [17], the theoretical MIBG concentration within the tumour will be approximately 3 μM. It is therefore not likely that the cytotoxic effects described in this paper contribute substantially to the effects observed on neuroblastoma *in vivo*. Recently, however, pilot studies have been performed in which high doses (approximately four times a [^{131}I]MIBG dose) of unlabelled MIBG are given to carcinoid patients (Taal BG, personal communication). Under these conditions, MIBG concentrations within the tumour are theoretically similar to concentrations leading to serious inhibition of mitochondrial ATP synthesis

described in this paper, and may have anti-tumour effects as described earlier by Smets *et al.* [5].

An intriguing phenomenon is the observation that although the activity of the mitochondrial respiratory chain is completely inhibited in the presence of 10 μM MIBG, only minor effects on the proliferation of the Molt-4 cells were observed, using DMEM as culture medium. Therefore, complete arrest of proliferation at MIBG concentrations of 50 μM suggests that MIBG influences other processes in addition to mitochondrial oxidative phosphorylation, resulting in a decreased proliferation of the Molt-4 cells.

Mono-ADP ribosylation, a covalent modification reaction of proteins, is another cellular process known to be inhibited by MIBG [18, 19]. The exact role of mono-ADP ribosylation is not yet clear, although it is likely that it plays a role in cellular signal transduction or metabolic regulation [20]. Further research needs to be done to reveal whether inhibition of mono-ADP ribosylation or other processes cause the non-mitochondrial effect of MIBG on cell proliferation.

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