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## Original Paper

# The Effect of the Neuroblastoma-seeking Agent *meta*-Iodobenzylguanidine (MIBG) on NADH-driven Superoxide Formation and NADH-driven Lipid Peroxidation in Beef Heart Submitochondrial Particles

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In this paper we report the effects of the neuroblastoma-seeking agent *meta*-iodobenzylguanidine (MIBG) on NADH-driven superoxide formation and NADH-driven lipid peroxidation in beef heart submitochondrial particles. MIBG is a structural analogue of noradrenaline and is capable of inhibiting complex I and complex III of the respiratory chain. The results of our studies show that MIBG enhanced both NADH-driven superoxide formation and NADH-driven lipid peroxidation at concentrations that are likely to exist inside mitochondria of the target cells of neuroblastoma patients treated with [<sup>131</sup>I]MIBG. The effect of MIBG is comparable to that of rotenone (an inhibitor of complex I) rather than that of antimycin (an inhibitor of complex III). These results suggest that the formation of superoxide and lipid peroxidation contributes to the cytotoxicity of [<sup>131</sup>I]MIBG. © 1997 Elsevier Science Ltd. All rights reserved.

**Key words:** *meta*-iodobenzylguanidine, superoxide, lipid peroxidation, respiratory chain, enzyme inhibition

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### INTRODUCTION

META-IODOBENZYLGUANIDINE (MIBG) is a structural analogue of the neurotransmitter noradrenaline. It is recognised by the active noradrenaline transport system (uptake 1) and accumulates, therefore, in tissues of neuroendocrine origin [1]. In its radio-iodinated form, MIBG is used as a tumour seeking radio-pharmaceutical agent for the diagnosis and treatment of neuroblastoma and pheochromocytoma [2, 3].

It has been suggested that at least part of the clinical responses with [<sup>131</sup>I]MIBG are not related to the radio-emitting properties of this agent [4]. Indeed, unlabelled MIBG is known to inhibit cell proliferation of a large number of cell lines [5]. The antiproliferative effect of unlabelled MIBG has recently attracted more interest since a phase II study was started in which a number of carcinoid patients were treated with high doses (34 mg/m<sup>2</sup>) of unlabelled

MIBG before administration of [<sup>131</sup>I]MIBG [6]. We demonstrated earlier [7, 8] that inhibition of complex I (NADH-ubiquinone-oxidoreductase) and complex III (ubiquinol-ferricytochrome *c*-oxidoreductase) of the mitochondrial respiratory chain by MIBG was partially responsible for the decreased proliferation rate of the human lymphoblastic leukaemia cell line Molt-4 and the human neuroblastoma cell line SK-N-BE(2c). During these studies, we observed that complete arrest of proliferation was achieved at MIBG concentrations that were approximately five times higher than the MIBG concentration required for complete inhibition of ATP synthesis. These findings strongly suggest that MIBG influences other processes in addition to mitochondrial oxidative phosphorylation.

Possible processes that may be influenced by MIBG in addition to mitochondrial ATP synthesis are the potentially harmful NADH-driven superoxide formation and lipid peroxidation that can be observed in submitochondrial particles [9, 10]. The rate of these two processes is enhanced when the electron transport from complex I to Q<sub>10</sub> is inhibited by

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specific inhibitors, such as rotenone, 1-methyl-4-phenylpyridinium (MPP<sup>+</sup>) or piericidin A [9–12]. Inhibition of electron transport from Q<sub>10</sub> to complex III by antimycin also enhances superoxide formation, while NADH-driven lipid peroxidation is inhibited in the presence of antimycin [10, 12].

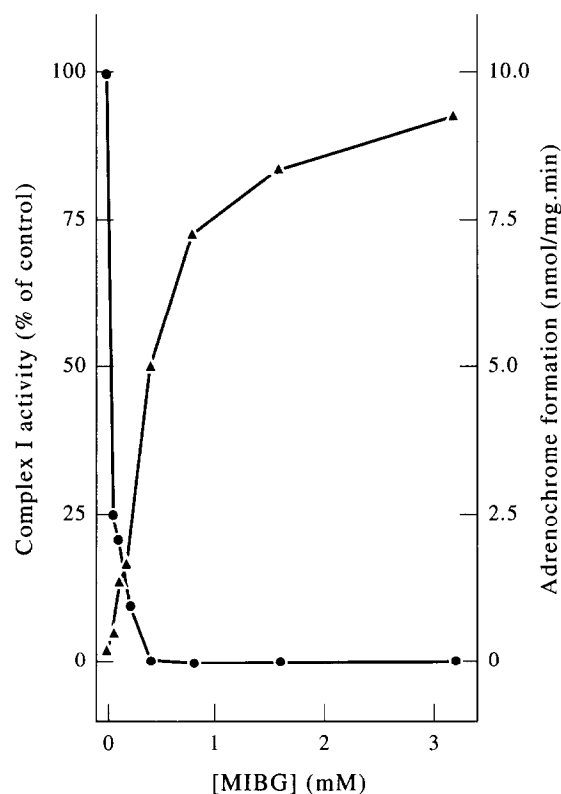
In order to determine whether inhibition of complex I and III by MIBG leads to an increase of NADH-driven formation of superoxide and NADH-driven peroxidation, we studied the effect of these two processes in submitochondrial particles (SMPs) by following the oxidation of adrenaline to adrenochrome as a measure of superoxide formation and the formation of malondialdehyde (MDA) as a measure of lipid peroxidation.

### MATERIALS AND METHODS

SMPs were prepared according to Crane and associates [13]. The assay medium for superoxide production contained 1 mM adrenaline, 5 mM MgCl<sub>2</sub> and 25 mM K<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub>, pH 7.4. SMPs (0.12 mg/ml) were pre-incubated with the various respiratory chain inhibitors (50 μM–3.2 mM MIBG or 10 μg/ml of either rotenone or antimycin) for 15 min at 37°C before reactions were started with 0.1 mM NADH. The rate of superoxide formation was determined by following the increase of absorbance due to the oxidation of adrenaline at 485–575 nm. The lipid peroxidation assay medium contained 0.2 mM FeCl<sub>3</sub>, 2 mM ADP and 50 mM Tris-HCl, pH 7.4. SMPs (0.35 mg/ml) were pre-incubated with the various respiratory chain inhibitors (50 μM–3.2 mM MIBG or 10 μg/ml of either rotenone or antimycin) for 15 min at 37°C before reactions were started with 0.5 mM NADH. After 5 min, reactions were terminated with 1 ml 50% acetic acid, and MDA concentrations were measured by the thiobarbiture acid method [14]. The effect of superoxide dismutase (SOD) on superoxide formation was measured by adding 10 μg/ml SOD to the assay medium just before reactions were started with NADH. The enzyme activity of complex I was measured as described before [7]; the assay mixture contained 5 mM MgCl<sub>2</sub>, 40 μM ubiquinone<sub>2</sub> (Sigma Chemical Company, St. Louis, U.S.A.), 10 μg antimycin, 0.05% lauryl maltoside (Sigma Chemical Company) and 25 mM K<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub>, pH 7.4. For each assay, 0.3 mg SMP protein was used. Reactions took place at 30°C and were started by adding NADH at a final concentration of 300 μM. The activity of the complex was measured by following the linear decrease in absorbance due to oxidation of NADH at 340 nm. MIBG was purchased from EMKA-Chemie, Markgröningen, Germany.

### RESULTS

Figure 1 illustrates the results of the effect of MIBG on NADH-driven superoxide formation in SMPs. The formation of superoxide was maximal at an MIBG concentration of approximately 3 mM. This concentration was much higher than the concentration needed for maximal inhibition of the complex I activity (approximately 0.4 mM, Figure 1). The maximal effect of MIBG on superoxide formation was comparable to that of rotenone and antimycin (Table 1). Inhibition of both complex I and III by MIBG is unlikely to lead to an extra increase in the formation of superoxide, since a blockage of the electron flow at complex I leads to a lack of electron supply to complex III and thus



**Figure 1.** NADH-driven superoxide formation and complex I activity in SMPs in the presence of various concentrations of MIBG. The oxidation of adrenaline to adrenochrome as a measure of NADH-driven superoxide formation and the complex I activity in SMPs were measured in the presence of various concentrations of MIBG as described in Materials and Methods. Results are given as the mean of two independent experiments and represent on the left y-axis the complex I activity as percentage of control values, i.e. no MIBG added (95 nmol NADH/mg/min) and on the right y-axis superoxide formation. —●—, complex I activity; —▲—, adrenochrome formation.

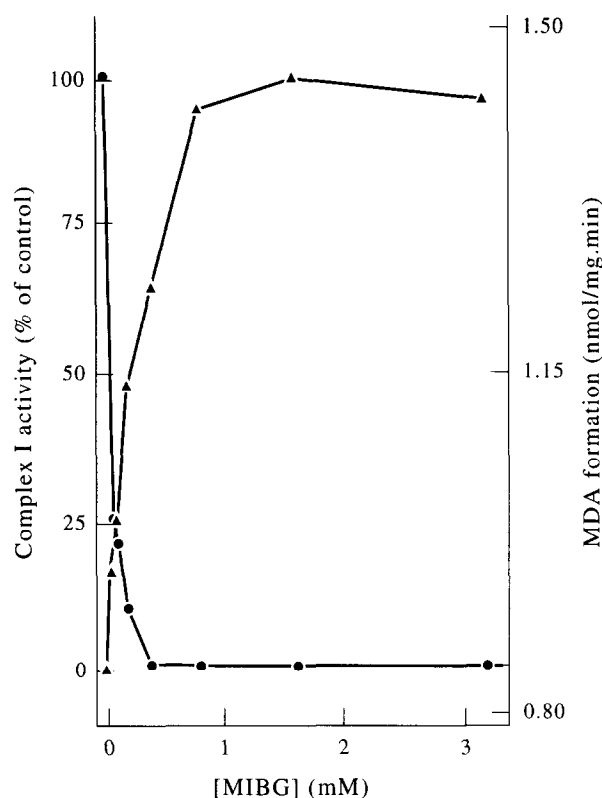
little or no effect due to inhibition of complex III by MIBG. This can also be observed when both rotenone (an inhibitor of complex I) and antimycin (an inhibitor of complex III) are present during the reaction (Table 1). Addition of SOD to the assay medium showed that superoxide is indeed the cause of the oxidation of adrenaline (Table 1).

Figure 2 shows the effect of MIBG on the NADH-driven lipid peroxidation in SMPs. Increasing concentrations of MIBG led to an increase in the MDA formation from 0.85 to 1.45 nmol/mg/min. This increase was correlated to a

**Table 1.** Effect of various respiratory chain inhibitors and SOD on superoxide formation in SMPs

Conditions	Adrenochrome formation (nmol/mg min)
Control (0.1 mM NADH)	0.2 ± 01
+SOD (10 μg/ml)	N.D.
+rotenone (10 μg/ml)	7.6 ± 0.2
+antimycin (10 μg/ml)	7.9 ± 0.8
+rotenone + antimycin	8.2 ± 0.2
+MIBG (800 μM)	7.4 ± 0.6
+SOD + MIBG	N.D.

Results are given as the mean of three to four independent experiments ± S.D. N.D., not detectable.



**Figure 2.** NADH-driven lipid peroxidation and complex I activity in SMPs in the presence of various concentrations of MIBG. The formation of MDA as a measure of NADH-driven lipid peroxidation and the complex I activity in SMPs were measured in the presence of various concentrations of MIBG as described in Materials and Methods. Results are given as the mean of two independent experiments and represent on the left y-axis the complex I activity as percentage of control values, i.e. no MIBG added (95 nmol NADH/mg/min) and on the right y-axis MDA formation. —●—, complex I activity; —▲—, MDA formation.

decrease in the complex I activity caused by MIBG. The same correlation has been found with other complex I inhibitors [11, 12]. The maximal effect of MIBG on the lipid peroxidation in SMPs was comparable to that of rotenone (Table 2). Inhibition of complex III by antimycin decreased the rate of NADH-driven lipid peroxidation, as has been reported before [10, 12]. The overall effect of MIBG on lipid peroxidation in SMPs was dominated by its effects due to inhibition of complex I rather than by the effects due to inhibition of complex III. The same can be observed when lipid peroxidation reactions take place while both complex I

**Table 2.** Effect of various respiratory chain inhibitors on MDA formation in SMPs

Conditions	MDA formation (nmol/mg min)
Control (0.5 mM NADH)	0.79 ± 0.11
+rotenone (10 µg/ml)	1.62 ± 0.30
+antimycin (10 µg/ml)	0.36 ± 0.18
+rotenone + antimycin	1.24 ± 0.13
+MIBG (800 µM)	1.42 ± 0.13

Results are given as the mean of four to six independent experiments ± S.D.

and III are inhibited by rotenone and antimycin, respectively (Table 2).

## DISCUSSION

This paper presents our studies on the effects of MIBG on NADH-driven superoxide formation and NADH-driven lipid peroxidation in beef heart submitochondrial particles. Both processes can be enhanced by inhibitors of complex I of the respiratory chain [9, 12]. MIBG, being an inhibitor of complex I and III [7, 8], did indeed enhance NADH-driven superoxide formation and lipid peroxidation (Figures 1 and 2).

The increase in lipid peroxidation correlated to the decrease in complex I activity, which is in agreement with similar experiments performed with other complex I inhibitors [11, 12]. There was no exact correlation between the inhibition of complex I and the formation of superoxide, with superoxide formation increased above concentrations of MIBG that inhibited complex I completely (Figure 1). This phenomenon has also been observed when the complex I inhibitors, rotenone, piericidin A or MPP<sup>+</sup> are used to enhance superoxide formation [9–12]. As a result of this, Ramsay and Singer [11] suggested that enhanced superoxide formation was due to binding of the inhibitors to a yet unknown site, rather than to inhibition of complex I.

MIBG concentrations leading to enhanced NADH-driven formation of superoxide and lipid peroxidation in SMPs are much higher than maximal plasma concentrations achieved when neuroblastoma patients are treated with [<sup>131</sup>I]MIBG. However, neuroblastoma cells are capable of accumulating MIBG approximately 30-fold [15]. Once inside the cell, MIBG further concentrates into the mitochondria [16]. In this paper, we reported that MIBG concentrations needed for complete inhibition of complex I in SMPs (mitochondrial inner membranes turned inside out) are approximately 40 times higher than those needed for the same effect in intact mitochondria in digitonin-treated cells [7] (0.4 mM and 10 mM, respectively), which provides further evidence that MIBG indeed accumulates in mitochondria. It is therefore likely that MIBG concentrations capable of enhancing NADH-driven superoxide formation and lipid peroxidation indeed occur in the mitochondria of neuroblastoma cells in patients treated with [<sup>131</sup>I]MIBG.

Furthermore, lipid peroxidation reactions in neuroblastoma cells are likely to occur because of the high ferritin content of the cells [17], which will enable the cells to provide the high iron concentrations needed for the reactions.

The formation of superoxide inside mitochondria of neuroblastoma cells will probably not lead to high concentrations of superoxide inside the mitochondria or the cytosol because of the SOD located in the matrix, which catalyses the conversion of superoxide to hydrogen peroxide, a molecule capable of crossing membranes. It has been observed, in intact insect mitochondria treated with the respiratory chain inhibitor phosphine (PH<sub>3</sub>), that hydrogen peroxide leaks out of these mitochondria leading to cytotoxic effects for the insect [18]. Hydrogen peroxide can be transformed into the highly reactive hydroxyl radicals in the presence of Fe<sup>2+</sup> in the Fenton reaction [19], or in the presence of Fe<sup>3+</sup> and superoxide in the iron-catalysed Haber-Weiss reaction [19]. These reactions can easily occur in neuroblastoma cells due to the high ferritin concentrations and the low activity of H<sub>2</sub>O<sub>2</sub>-detoxifying enzymes (catalase and glu-

tathione peroxidase), which will not efficiently prevent intracellular accumulation of H<sub>2</sub>O<sub>2</sub> [20].

In conclusion, the experiments described in this paper indicate that enhancement of NADH-driven superoxide formation and lipid peroxidation by MIBG most likely contributes to the cytotoxicity of the agent. Enhanced NADH-driven superoxide formation, at MIBG concentrations that were higher than those required for complete inhibition of ATP synthesis, is probably the cause of complete proliferation arrest of the human lymphoblastic leukaemia and human neuroblastoma cell line, as we previously reported [7, 8].

Combining radiolabelled and non-radiolabelled MIBG in order to increase the MIBG concentration in the target cells and/or the addition of Fe<sup>2+</sup> liberating agents are strategies that, in principle, enhance the effects described in this paper and thus improve the efficacy of MIBG therapy. These approaches are currently under investigation in our hospital (Prof. P.A. Voûte, Academic Medical Centre).

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