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Cytotoxic and antitumor effects of the norepinephrine analogue meta-iodo-benzylguanidine (MIBG)

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Summary. Meta-iodo-benzylguanidine (MIBG) is an analogue of the neurotransmitter norepinephrine. In its radioiodinated form, MIBG is clinically used as a tumor-targeted radiopharmaceutical in the diagnosis and treatment of adrenergic tumors. The potential cytotoxicity of the unlabeled drug was tested. MIBG appeared cytotoxic in a large panel of histogenetically different cell lines without preference against tumor cells of neural origin. The cytotoxicity of MIBG was higher than of the related mono-amine precursor, meta-iodo-benzylamine (MIBA). Drugs that block adrenergic receptors and inhibitors of tyrosinase or tyrosine hydroxylase had no effect on the cytostatic properties of MIBG. However, its activity was potentiated by the pharmacological inhibition of catecholamine degradation and by inhibitors of intracellular storage. MIBG had antitumor effects on L1210 leukemia and N_1E115 neuroblastoma, grown as subcutaneous tumors in animals treated with MIBG in non-toxic schedules. The observations suggest that MIBG is cytotoxic in its native form and may contribute by this property to the clinical responses obtained with the radiolabeled drug at high concentrations.

Introduction

Meta-iodo-benzylguanidine (MIBG), a ring-substituted alkylguanethidine, is a structural and functional analogue of natural norepinephrine. The drug is selectively accumulated in storage granules of chromaffin tissues [16]. Due to this property, radio-iodinated [^{131}I]-MIBG is used as a tumor-seeking radiopharmaceutical for scintigraphic detection and, at elevated doses, for radiotherapy of tumors derived from adrenergic tissues, such as pheochromocytoma [9, 10] and neuroblastoma [4, 5]. During the clinical application of [^{131}I]-MIBG with radiotherapeutic intent, patients receive milligram amounts of a potentially active amine. Up to 20% of the injected dose may be selectively stored in the tumor tissue [5, 10]. To our knowledge, the subsequent possibility that the accumulated drug has a pharmacological effect on tumor tissues has not been studied.

Yet a number of reports have indicated that catecholamines can be deleterious to cells in vitro with a clear preference for cells of neural origin. For instance, L-dopa is selectively toxic for melanoma cells [15]. Mouse melanoma cells are also killed by 2,4-hydroxyphenylalanine due to hydroxylation by the tissue-specific enzyme tyrosinase, eliciting a cascade of auto-oxidation and the generation of reactive oxygen radicals [8]. Similarly, 6-hydroxydopamine is accumulated and hydroxylated by neuroblastoma cells [2], resulting in tissue-specific toxicity. Since MIBG could have similar effects on neuroblastoma or pheochromocytoma cells, clinical responses obtained with [^{131}I]-MIBG at a high dose might in part be attributable to antitumor effects of the drug per se.

For this reason, the present investigation was started to investigate the possible cellular and antitumor effects of the unlabeled drug. The antiproliferative and cytotoxic effects were investigated comparatively in cell lines derived from neural crest tumors and control tissues, as well as in some tumors established from these cell lines. Pharmacological intervention was applied to obtain indications as to the mechanisms of the action of drug. All effects in vitro and in vivo were compared with those obtained using the monoamine precursor meta-iodo-benzylamine (MIBA) to probe the structural property most relevant in the observed effects.

Materials and methods

Synthesis. MIBG was synthesized from meta-iodo-benzylamine (Fig. 1) according to Wieland et al. [16]. The quality of the product was controlled by NMR spectrophotometry, infrared spectrophotometry and HPLC analysis, and it was identified as MIBG-sulfate with over 98% purity. MIBG was soluble in Tris-HCl buffered saline at 4° C up to 4 mg/ml.

Cells and culture methods. N_1E115 neuroblastoma cells were obtained by courtesy of Dr. W. H. Moolenaar, Hubrecht Laboratory, Utrecht. The other cells were from stocks routinely grown in this laboratory. The leukemic lines L1210 and K562 were grown in RPMI 1640 medium and the adherent cell lines in Dulbecco's modification of Eagle medium, all supplemented with 10% fetal calf serum and antibiotics. Cell numbers were monitored by counting appropriately diluted cell samples or trypsin-released suspensions using a Sysmex particle counter. Single-cell survival was assessed by plating cells in drug-containing medium with 20% fetal calf serum in six-well multitrays (Co-

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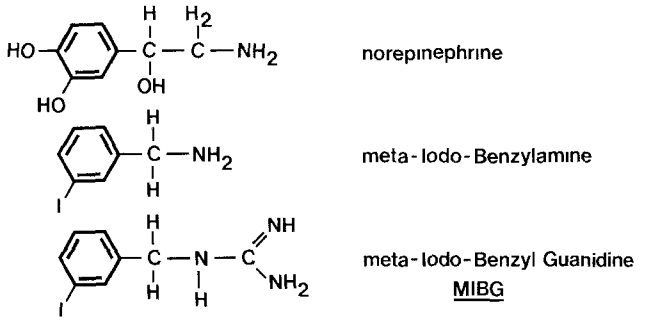

Fig. 1. Structure of norepinephrine, meta-iodo-benzylamine and meta-iodo-benzylguanidine. MIBA is the mono-amine precursor used for the synthesis of MIBG

star) and then counting the visible colonies after 10 days. With the poorly adhering N_1E115 and the non-adherent L1210 leukemia cells, 0.6% carboxymethylcellulose was included to obtain a semi-solid plating medium.

Incorporation of [^{131}] I-MIBG was measured after incubation of exponentially growing cells for 120 min in 2×10^{-8} M radiolabeled MIBG with or without 10^{-6} M norepinephrine. Subsequently, the cells were washed three times with cold saline and extracted with 0.5 *N* perchloric acid. Radioactivity in the extracts was determined by liquid scintillation counting.

Drug intervention studies. The interaction of various drugs, active in the metabolism or the hormonal action of catecholamine was tested on MIBG cytotoxicity in clonogenic assays with L1210 cells. The survival after combined treatment was compared with the effect of MIBG alone and expressed by a dose-modifying factor. This factor was defined as the ratio between the MIBG concentration required to equal the effect of combined treatment versus the concentration of MIBG actually used in the combination. Significance of differences between combined and single drug treatment groups was calculated by Student's *t*-test.

Animal experiments. All animal experiments were performed with syngeneic male strain AF (N_1E115) or DBA/2 (L1210) mice, aged 3 months. Acute toxicity (death, diarrhea and weight loss) was monitored daily in both strains during injections with MIBG in 0.25 ml of vehicle (5–9 injections, i.p., push, daily). Peripheral blood samples were analyzed by a Coulter Haemalog at 24 h following the last injection. Tumor cells were implanted s.c. on the back under light ether anesthesia. The latency period for tumors to reach an estimated size of 2.5 cm diameter was scored as survival time. The animals were then killed and the tumors excised and weighed to ascertain that the average tumor weight was similar (i.e., a median weight of 3.5 g) in the control and treated groups.

Chemicals. Meta-iodo-benzylamine and cyanamide were obtained from Janssen Pharmaceutics, Beerse, Belgium. Prazosin was kindly provided by the Pfizer Company, formulated reserpine (Serpacil) was a gift from Ciba, and [^{131}] I-MIBG (40 mCi/mg) was made available by Amersham. All other drugs were obtained from Sigma.

Results

Effects on cell proliferation and survival

Various cell lines were grown in the presence of MIBG and the percentage of growth inhibition was determined daily. The panel of test cells consisted of five neural crest-derived and seven unrelated control lines. Table 1 summarizes the results on day 3 following drug addition. All cell lines were inhibited to different degrees on a dose-related basis, but collectively the neural crest-derived neuroblastoma (LAN-1, N_1E115 and CHP212) and melanoma (M5A, B16) cells were no more susceptible than unrelated cell lines.

The sensitivity of B16 melanoma, N_1E115 neuroblastoma, control L1210 leukemia, and RIF1 fibrosarcoma to MIBG was tested quantitatively in clonogenic assays. Furthermore, the effect of MIBG was compared to that of the parent mono-amine MIBA (Fig. 1). Figure 2 displays the survival curves for L1210 cells plated in increasing con-

Table 1. Effect of MIBG on cell proliferation

Cell line	Description	Cell number[a] after 72 h	
		In MIBG:	
		2 μg/ml	20 μg/ml
3T3	Mouse fibroblasts	45	7
3T3-T13	Mouse fibroblasts	51	35
BHK	Hamster fibroblasts	47	15
Py-BHK	Hamster fibroblasts	58	11
K562	Human leukemia	90	44
L1210	Mouse leukemia	76	13
RIF1	Mouse fibrosarcoma	80	38
M5A	Human melanoma	67	37
B16	Mouse melanoma	58	21
LAN-1	Human neuroblastoma	60	37
N_1E115	Mouse neuroblastoma	34	17
CHP212	Mouse neuroblastoma	83	13

[a] Expressed in percentage (%) of control cultures grown without drug

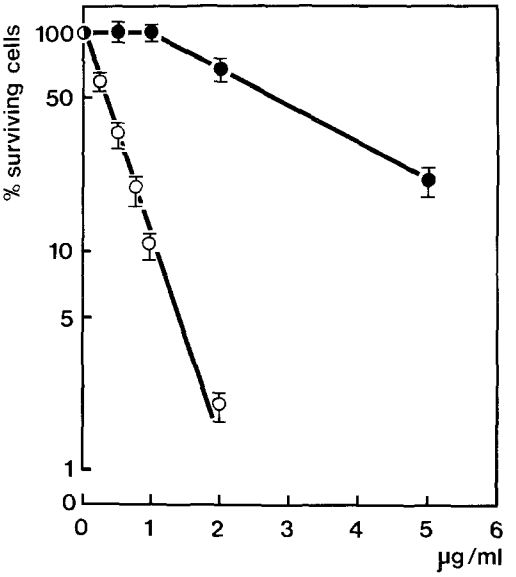

Fig. 2. Clonogenic survival of L1210 leukemia cells plated in semi-solid medium in the presence of MIBG (○-----○) or the mono-amine precursor MIBA (●-----●). Mean ± SE from three experiments

Table 2. The effect of catecholamine-active drugs on the cytotoxic potential of MIBG in L1210 cells

Drug tested	Concentration ($\times 10^{-6}$ M)	Main mechanism of action[a]	Dose-modification factor[b]
Phenylthiocarbamide	13.1	Tyrosinase inhibition	1.0
1-Phenyl-3-(2-thiazolyl)-2-Thiourea	8.5	Dopamine β-hydroxylase inhibition	1.1[c]
α-Methyltyrosine	51.2	Tyrosine hydroxylase inhibition	1.2[c]
3-Iodo-L-tyrosine	16.3	Inhibition	1.1[c]
Yohimbine	28.2	α_2-Blockade	0.9[c]
Prazosin	15.1	α_1-Blockade	0.8[c]
Propranolol	16.7	$\beta_{1,2}$-Blockade	1.0
Pyrogallol	15.8	Catechol-*o*-methyltransferase inhibition	3.5[d]
Pargyline	25.6	Monoamine oxydase inhibition	3.0[d]
Reserpine	8.2	Blockade of storage	4.4[d]
Reserpine + pargyline		–	7.2[d]

[a] For references, see [14]

[b] Calculated as described in Materials and methods to reflect the modification of the effect of MIBG alone (0.25 and 0.50 μg/ml; 0.66 and 1.32×10^{-6} M, respectively) by the added drug. Values < 1.0 indicate protection; 1.0 = no effect; > 1.0 potentiation. From triplicates in two experiments

[c] Not significantly different from 1.0 ($P > 0.25$)

[d] Significantly different at $P < 0.01$

centrations of MIBG or MIBA. From these curves ED_{50} values were calculated. These values varied between 1.2 ± 0.5 and $11.8 \pm 1.5 \times 10^{-6}$ *M* (mean ± SE) for L1210 and B16 cells, respectively. The corresponding values of MIBA were on average 5 times higher in all four cell lines tested, indicating that cytotoxicity was predominantly associated with the guanidinylated side chain of MIBG.

All cell lines were tested for specific, receptor-mediated uptake of MIBG by comparing its incorporation with or without a 50-fold molar excess of unlabeled norepinephrine [12]. In no cell line could a significant, norepinephrine-inhibitable uptake be established.

Pharmacological intervention

MIBG was tested on the survival of L1210 cells in the simultaneous presence of several drugs with known effects on the metabolism or on the hormonal activity of catecholamines (Table 2; for a review on drug actions, see ref [14]). All drugs were used at the highest concentration possible, which on their own would not affect the plating efficiency of the test cells; this was established in separate experiments. The molar excess of the selected concentrations (Table 2) was a minimum of six times (i.e., with phenylthiourea and reserpine) that of MIBG in combined treatments. On average, the concentrations of the inhibitors of hydroxylating enzymes exceeded by fivefold the concentrations for 50% inhibition of enzyme activity in vitro [7], and the predetermined concentrations of adrenergic receptor blockers were at least twice the highest and most effective therapeutic plasma concentrations [14]. Pyrogallol and pargyline concentrations were tested for complete inhibition of metabolite formation from [^{131}I]-MIBG during incubation with activated rat liver microsomes; reserpine completely blocked the selective, norepinephrine-inhibitable uptake of radiolabeled MIBG by SK-N-SH cells (R. Huiskamp, personal communication). Blockers of α- and β-adrenergic receptors and the inhibitors of catecholamine hydroxylation had no effect on the cytotoxicity of MIBG. This was not an unexpected finding, as the lymphoid cells may not contain receptors for catecholamine hormones or the tissue-specific enzymes tyrosinase and tyrosinehydroxylase. In contrast, pargyline and pyrogallol, the inhibitors of the degrading enzymes monoamine-oxydase and catechol-O-methyl-transferase respectively, and also reserpine, potentiated the action of MIBG. Combined exposure to pargyline and reserpine had an additive effect. These data indicate that for the manifestation of cytotoxicity, MIBG does not require metabolic activation by hydroxylation and is active when freely available in the cytoplasma in its native form.

Antitumor activity. In view of its cytostatic action in vitro, MIBG was tested for possible antitumor activity in vivo against N_1E115 neuroblastoma as a model for neural crest-derived tumors and L1210 as an unrelated control. First, MIBG was tested for its toxicity on host animals with 5 daily injections (Fig. 3). At 50 mg/kg body wt., all animals died after 1–4 doses and 4/16 animals died on a schedule of 44 mg/kg. However, doses of 40 mg/kg or lower were completely non-toxic and, after a total accumulated dose of 360 mg/kg (i.e., nine doses of 40 mg/kg, daily), no toxic effects were obvious from the parameters weight loss, diarrhea, white and red blood cell and platelet counts. Apparently, MIBG toxicity is a threshold phenomenon at a dose of approximately 44 mg/kg. Unlike most cytotoxic drugs. MIBG below this dose was without cumulative effects, preventing the establishment of precise LD10 values in these experiments.

Figure 4 shows the survival of DBA/2 mice inoculated with 10^6 L1210 cells and treated with MIBG on days 3–6 (arrows) with 20 mg/kg per dose. Considerable prolongation of survival (ILS = 177%) and some cures (4/20) were obtained. Figure 5 summarizes the survival of AF mice inoculated with N_1E115 neuroblastoma and treated according to NIH protocols for new drug testing [3] with 9 daily injections of MIBG (40 mg/kg). Marked prolongation of survival (ILS = 186%) but no cures were observed. MIBA tested at 50 mg/kg in the same schedules had no toxic or antitumor effects, confirming the observations in cell lines. Studies with tumor-bearing animals injected with 2 mCi of [^{131}I] I-MIBG failed to show specific uptake and retention

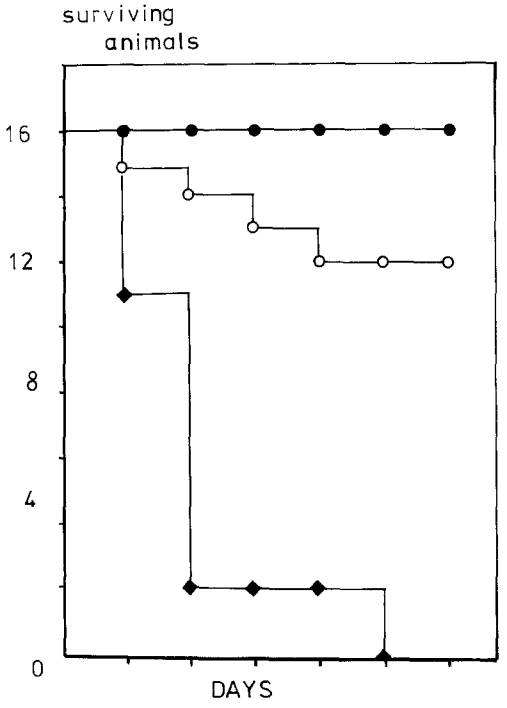

Fig. 3. The survival of mice injected daily on days 0–5 with MIBG at a dose of 40 mg/kg (● – ●), 44 mg/kg (○ – ○) and 50 mg/kg (◆ – ◆). Survival was scored 24 h following each injection. The data are combined from two experiments with 2 × 8 animals in each experimental group

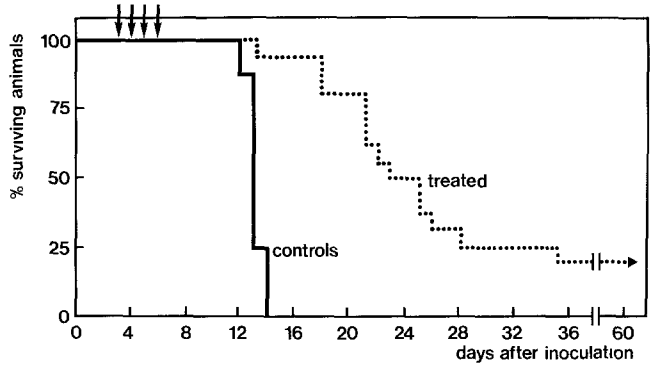

Fig. 4. Survival of mice inoculated s.c. with 10^6 L1210 cells and treated with MIBG (20 mg/kg, i.p., push, daily) on days 3–7 as indicated by *arrows*. Data from two experiments; 2 × 10 animals in each group

of the drug by L1210 and N_1E115 tumors, as reported by others [11], and confirming the in vitro observations.

Discussion

Catecholamines can be preferentially cytotoxic for tumor cells of neural origin such as melanoma and neuroblastoma [2, 8, 15]. Therefore, we investigated whether the norepinephrine analogue meta-iodo-benzylguanidine, which in its radio-iodinated form is used in the management of tumors of neural crest origin, could have similar effects. Unlabeled MIBG appeared (moderately) toxic to a wide variety of cell lines and had no preference for cells derived from neural tissues (Table 1).

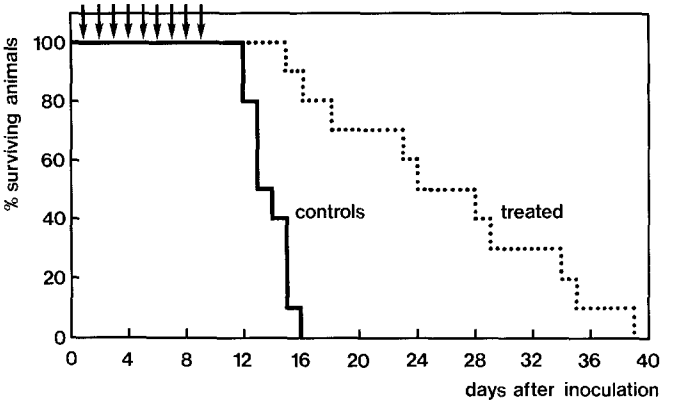

Fig. 5. Survival of mice inoculated with 2×10^6 N_1E115 cells and treated with MIBG (40 mg/kg, i.p., push, daily) on days 1–9 as indicated by *arrows*. Data from ten animals in each group

Apparently, cytotoxicity of MIBG was not related to the tissue-specific effects of catecholamines such as observed with 6-hydroxydopamine and 2,4-hydroxyphenylalanine in neuroblastoma and melanoma [2, 8, 15]. Moreover, in L1210 cells, various inhibitors of hydroxylation of the benzyl ring – a crucial step in the generation of a toxic auto-oxidative cascade – did not antagonize the cytotoxicity of the drug (Table 2), unlike their protective effect on catecholamine cytotoxicity in melanoma cells [8]. These experiments are not entirely conclusive because of the absence of a positive control on interfering drug action. The much lower cytotoxicity of the monoamine analogue MIBA, however, confirmed that the iodinated ring structure is indeed of minor importance in the observed effects.

In spite of a structural and functional analogy with natural norepinephrine, MIBG did not act as a false hormone. Cytotoxicity in L1210 cell was not abolished by blockers of α- and β-adrenergic receptors, let alone the fact that such receptors are probably not present on the L1210 cells that were the most sensitive to the drug so far. On the other hand, inhibitors of catecholamine catabolism and intracellular storage potentiated the activity of MIBG (Table 2), suggesting that MIBG is inactivated by catecholamine-degrading enzymes. This is conceivable in the case of the ubiquitous enzyme, monoamine oxidase. On the other hand, catechol-O-methyltransferase (the enzyme inhibited by pyrogallol) catalyzes the methylation of ring hydroxy groups, but such acceptor sites are not present in MIBG (Fig. 1). However, a pyrogallol-sensitive pathway of MIBG degradation was indicated by the inhibition of the formation of MIBG metabolites during incubation with activated rat liver microsomes. The potentiating mechanism of reserpine, which blocks the translocation of cytoplasmic norepinephrine into specialized compartments [14], is less clear since L1210 cell may not contain such structures. Anyhow, the additive effect of pargyline and reserpine (Table 2) is consistent with the assumption of different mechanisms of action by these two drugs.

Collectively, these pharmacological intervention data indicate that MIBG acts in the cytoplasma in its native form. Considering the low biological activity of the parent monoamine MIBA in vitro (Fig. 2) and in vivo, it is evident that the guanidine group is essential for both cytotoxicity and antitumor effects of MIBG. For the time being, one can only speculate as to the mechanisms operating in

the action of MIBG. In its side chain, the drug resembles the arginine receptor for the enzyme ADP-ribosyltransferase [13]. In fact, MIBG is very similar to some synthetic substrates for this enzyme, e.g., meta-iodoguanyltyramine [6]. Ongoing studies in our laboratory reveal that the drug is indeed an efficient acceptor for cholera toxin-catalyzed transfer of NAD. Incubation of cells with MIBG may, therefore, deplete cellular NAD pools. NAD depletion as a consequence of endogenous ADP-ribosylation has been proposed as a more general cytotoxic mechanism in the action of various drugs [1].

The question of whether MIBG contributes through this or other pharmacological mechanism, to the clinical responses obtained with the radiolabeled compound cannot be answered conclusively by the present study. Plasma concentrations of MIBG during treatment with [^{131}] I-MIBG are certainly lower than those causing responses in the cell lines and the transplantable tumor systems investigated to date. However, none of these tumors or their parent cell lines concentrate and store the drug, as is the case in human tumors eligible for this type of treatment. Here a combined action of radiation-induced cell kill and local release of accumulated drug may be conceivable, a possibility that is now under investigation.

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