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Original Paper

Influence of Cisplatin and Doxorubicin on ^{125}I-Meta-iodobenzylguanidine Uptake in Human Neuroblastoma Cell Lines

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The combination of ^{131}I-meta-iodobenzylguanidine (MIBG) with chemotherapy has recently been employed in the treatment of advanced stage neuroblastoma with encouraging results. However, the mechanisms underlying the interaction between these two different modalities of treatment have not yet been explored. In this study, human neuroblastoma cell lines pretreatment with cisplatin and doxorubicin increased cellular ^{125}I-MIBG accumulation in a dose-dependent manner. Cell cycle analysis showed that increased ^{125}I-MIBG accumulation correlated with the drug-induced G_2/M phase block. Northern blot analysis demonstrated an increase in gene expression of the noradrenaline transporter induced by doxorubicin, but not by cisplatin treatment. Increased ^{125}I-MIBG accumulation was also observed in murine xenografts of the human neuroblastoma cell line SK-N-DZ or BE(2)M17 treated intraperitoneally (i.p.) with cisplatin or doxorubicin, respectively. These results suggest that the combination of ^{131}I-MIBG and these drugs could selectively increase radiation doses delivered to neuroblastomas. © **1999 Published by Elsevier Science Ltd. All rights reserved.**

Key words: meta-iodobenzylguanidine, neuroblastoma, cisplatin, doxorubicin, noradrenaline transporter

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INTRODUCTION

ALTHOUGH INTENSIVE chemotherapy regimens have been applied to patients with advanced neuroblastoma, the 5-year survival rate of the patients still remains very disappointing [1]. Therefore, it is essential to introduce new agents and to improve the existing treatments. Meta-iodobenzylguanidine (MIBG), a structural analogue of the neurotransmitter norepinephrine, is selectively taken into neuroadrenergic tissue through noradrenaline transporter (hNET) [2–4]. Radioinodinated MIBG is currently used in the diagnosis of the neuroblastoma. In addition, ^{131}I-MIBG has increasingly been used in the treatment of disseminated neuroblastoma, since high radiation doses can be selectively delivered to tumour cells, with acceptable systemic toxicity [5].

Targeted radiotherapy with ^{131}I-MIBG has given encouraging results not only in the treatment of patients with disseminated neuroblastoma resistant to conventional therapy but also in patients at diagnosis [6, 7]; however, its effectiveness may be further improved by new therapeutic approaches.

One of the innovative therapeutic strategies for advanced stage neuroblastoma combines ^{131}I-MIBG treatment with conventional therapies [8, 9]. The rationale for combining MIBG and chemotherapy derives from the heterogeneity of neuroblastoma tumours, composed of cell populations with different capabilities of taking up MIBG. In addition, it has been reported that micrometastases may be unaffected by ^{131}I therapy and lead to treatment failure [10, 11]. On the basis of these considerations, ^{131}I-MIBG was integrated with cisplatin in a pilot study [12]. Cisplatin was selected because of its high activity against neuroblastoma and its relatively mild haematological toxicity [8, 13]. Furthermore, a synergism between cisplatin and radiation has been well documented in both *in vitro* and *in vivo* pre-clinical studies [8, 14, 15]. This new form of treatment combination deserves further evaluation in a larger number of patients, together with more detailed knowledge of the effect of chemotherapy on MIBG uptake mechanisms.

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Adequate intracellular concentration of the drug is a critical point in radiometabolic therapy with ^{131}I-MIBG. Therefore, it is of crucial importance to clarify whether chemotherapy administered before MIBG can induce biochemical and physiological alterations of the cell membranes, thus jeopardising the uptake of a therapeutic dose of ^{131}I-MIBG. In this study, we measured ^{125}I-MIBG incorporation in three human neuroblastoma cell lines which possess a specific uptake system for MIBG [16], after exposure to cisplatin and doxorubicin.

MATERIALS AND METHODS

Chemicals and biological agents

^{125}I-MIBG (specific activity 1.5 mCi/mg) was a gift from Sorin Biomedica (Saluggia, Vercelli, Italy). Cisplatin was obtained from Bristol-Myers Squibb (Sermoneta, Italy), and doxorubicin from Pharmacia (Milan, Italy).

Cell lines and culture conditions

All neuroblastoma cell lines were of human origin. Cell lines SMS-KCNR and SK-N-DZ were kindly provided by M.A. Israel (University of California, San Francisco) and subline BE(2)M17 was a gift from J.L. Biedler (Memorial Sloan Kettering Cancer Center, New York, U.S.A.). SMS-KCNR, SK-N-DZ and BE(2)M17 cells were grown in RPMI 1640 (Bio-Whittaker, Verviers, Belgium) supplemented with 10% heat-inactivated FCS and 2 mM glutamine. Cells were maintained at 37°C in a 5% CO_2–95% air humidified incubator.

^{125}I-MIBG uptake experiments

Since ^{125}I-MIBG uptake is considerably dependent on culture cell density [17], neuroblastoma cells were seeded in 25 cm^2 Falcon flasks taking into consideration the growth rate of the different lines, their sensitivity to the different treatments and the duration of culture. The aim was to obtain a subconfluence condition (70%) in both control and treated cultures at the time of the uptake experiments. Before tracer addition, the culture medium was removed and replaced by new complete medium in the absence or the presence of different concentrations of cisplatin (range 50–400 ng/ml) and doxorubicin (range 50–200 ng/ml). The concentrations used were shown to exert only a mild cytotoxic effect in preliminary experiments in each cell line. SMS-KCNR cells were exposed to cisplatin concentrations ranging from 50 to 200 ng/ml, based on their greater sensitivity to this drug. After 48 h of treatment the culture medium was removed and both control and treated cells were washed twice with culture medium. ^{125}I-MIBG uptake was assessed by the addition of medium containing 0.1 mM ^{125}I-MIBG either at 37°C or 4°C. The incubation time was chosen in order to reach a plateau of uptake in each cell line: 1 h for BE(2)M17 and 2 h for SK-N-DZ and SMS-KCNR. At the end, the flasks were placed on ice, the solution containing the radiotracer removed and the cells washed twice with cold Hepes-buffered Krebs-Ringer solution (KRH). The cells were then lysed with two 1.5 ml aliquots of 0.3 N sodium hydroxide and the total intracellular radioactivity was quantitated with a gamma counter. Uptake was expressed as pmol MIBG/10^6 cells for each experiment. All experiments were conducted in triplicate and results were expressed as ± standard error of the mean (SEM).

Flow cytometry

Progression of cell cycle was analysed by flow cytometry. Exponentially growing cells exposed to both drugs were removed after 48 h of incubation, resuspended in 1 ml of PBS-EDTA, diluted with 3 ml of 95% ethanol to the final concentration of 3×10^6 cells/ml and stored at 4°C. Before analysis, the cells were washed twice in PBS, stained with 2 ml propidium iodide (50 μg/ml) in 0.1% sodium citrate together with 25 μl RNA-ase and 25 μl 0.1% Nonidet, and incubated overnight at 4°C in the dark. All samples were run through a FACScan flow cytometer (Becton-Dickinson, Palo Alto, California, U.S.A.) equipped with an argon laser emitting at 488 nm. The percentage of cells in G1, S and G_2/M cell cycle phases were calculated using a Cellfit Software (Beckton-Dickinson). A minimum of 10 000 events were acquired in list mode for each determination.

Statistical analysis

All data were examined by the analysis of variance model (ANOVA). Individual concentrations were compared with the values of the untreated samples using the LSD [18] post-test. Pearsons' linear regression coefficient was used for correlation analysis (r).

Northern blot analysis

Total RNA was extracted and purified from control and treated cells with the guanidine-thyocianate method (TRI Reagent, Sigma Chemical, St Louis, Missouri, U.S.A.). hNET cDNA (kindly provided by Dr Amara, Oregon Health Science University, Portland, Oregon, U.S.A.) [19] was labelled with [^{32}P]dCTP (50 μCi) by random primed synthesis using random oligonucleotides as primers. RNA (30 μg) was sized fractionated on a denaturing formaldehyde agarose gel and transferred to a nylon membrane (Hybond). Following prehybrisation of the membrane at 42°C in 50% formamide, 5×SSPE, 1×Denhard's solution, 1% SDS and 250 mg/ml salmon sperm DNA, hybridisation was initiated by addition of a cDNA probe and 10% dextran-sulphate and continued at 42°C for 48 h. The blot was washed at 65°C for 1 h in 0.1×SSPE buffer and 0.1% SDS and exposed to autoradiographic film with an intensifying screen for 3 days. After stripping, the membrane was hybridised with the GAPDH probe at 42°C for 24 h.

Neuroblastoma xenografts and ^{125}I-MIBG biodistribution

All experimental animal investigations complied with the guidelines of the Istituto Superiore di Sanita' (Italy) on experimental neoplasia in animals. Six-week-old male nude-athymic mice CD1 nu/nu were subcutaneously injected with 30×10^6 cells (SK-N-DZ or BE(2)M17). Mice bearing a SK-N-DZ xenograft or BE(2)M17 xenograft were injected i.p. with cisplatin (6 mg/Kg) or doxorubicin (7 mg/Kg), respectively. The nude mice in the control group received saline injections. Cisplatin and doxorubicin concentrations used were selected in preliminary experiments to obtain an acceptable toxicity, as determined by the animal weight loss. Control and treated animals were injected i.p. with approximately 100 μCi ^{125}I-MIBG/mouse, 2, 3 or 5 days after drugs administration. At least seven mice were used for each group. Mice were sacrificed 48 h after MIBG administration, when a blood sample was drawn and the tumour, heart, one lung, one kidney, spleen, skin, and liver were excised. Tumour and tissue sample were placed in screw-capped tubes and weighed.

The radioactivity in each tube was measured by an automated gamma counter. The concentration of ^{125}I-MIBG in each organ and in the tumour was expressed as per cent, in each gram of tissue, of the injected dose per mouse.

RESULTS

Cisplatin and doxorubicin increase on ^{125}I-MIBG uptake in vitro

Compared to the untreated control, cisplatin and doxorubicin dose-dependently enhanced the incorporation of ^{125}I-MIBG in three neuroblastoma cell lines studied. Cisplatin at a concentration of 400 ng/ml produced a 1.5- and 1.4-fold increase in ^{125}I-MIBG uptake in SK-N-DZ cells and BE(2)M17 cells, respectively (Figure 1a, $P<0.0001$ and $P<0.01$, respectively). Cisplatin significantly increased ^{125}I-MIBG uptake in KCNR cells at concentrations of 100 and 200 ng/ml (Figure 1a, $P<0.05$). Approximately a 3-fold increase in ^{125}I-MIBG uptake was observed in SK-N-DZ cells when cells were treated with doxorubicin at concentrations above 50 ng/ml (Figure 1b, $P<0.05$). Doxorubicin produced a 2-fold increase in ^{125}I-MIBG uptake at a concentration of 100 ng/ml in BE(2)M17 (Figure 1b, $P<0.01$). However, doxorubicin was so toxic that ^{125}I-MIBG uptake decreased when cells were treated with 200 ng/ml. Overall, at the highest effective dose, cisplatin or doxorubicin treatment increased MIBG accumulation from 1.4 to 2.8-fold, respectively. In neuroblastoma cells, MIBG is taken up by two mechanisms: a specific uptake system, that is sodium- and temperature-dependent, predominant at low concentrations of the drug, and a nonspecific process of passive diffusion, that is more significant at the higher concentrations [16]. Treatment with cisplatin affected the specific component of MIBG uptake, since no increase in MIBG uptake was observed when cells were incubated at 4°C (Figure 2). Doxorubicin also had no influence on the uptake at 4°C (data not shown).

Effect of cisplatin and doxorubicin pretreatment on hNET expression

Expression of hNET was examined in all the neuroblastoma cell lines before and after cisplatin and doxorubicin treatment by Northern blot analysis. For each drug, the

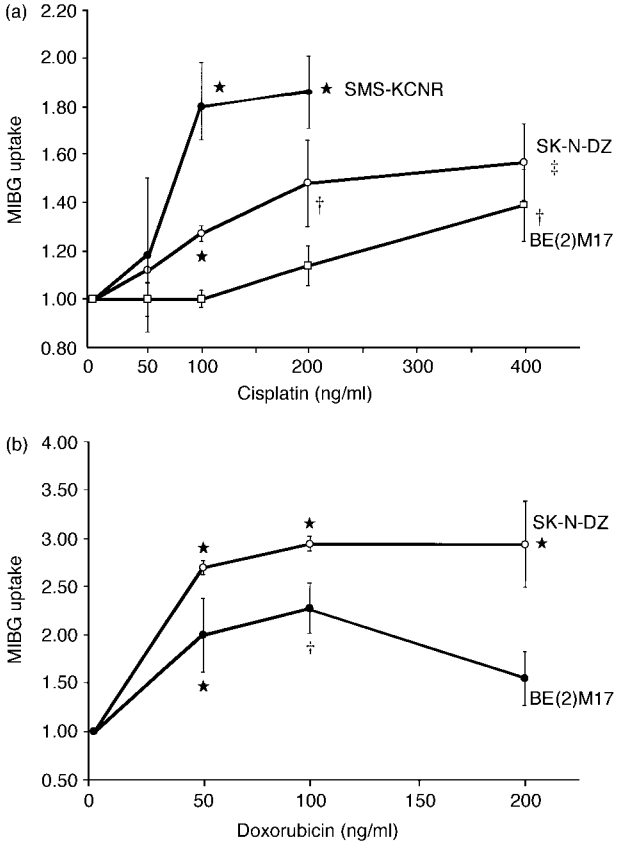

Figure 1. Effect of (a) cisplatin or (b) doxorubicin pretreatment on MIBG uptake in the human neuroblastoma cell lines. Cells were incubated with either cisplatin or doxorubicin for 48 h. MIBG uptake was measured after 1 h exposure to 0.1 mM of the radionuclide and expressed as relative ratio of treated to untreated cells. *Points*, means of triplicate determinations; *bars*, SEM. Uptake significantly different from control at *$P<0.05$, †$P<0.01$, ‡$P<0.0001$ (ANOVA).

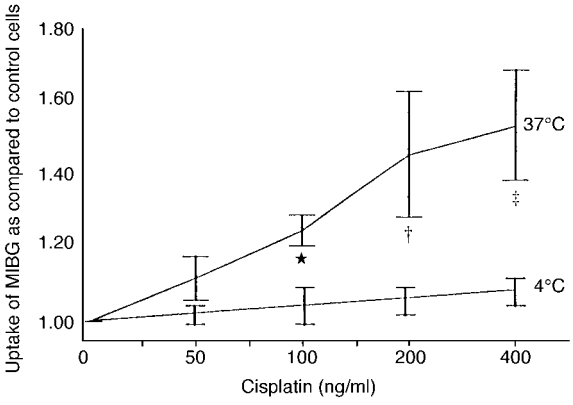

Figure 2. Cisplatin pretreatment affects the specific component of MIBG uptake in SK-N-DZ cell line. After cisplatin exposure, cells were incubated at 37°C or 4°C and MIBG uptake was evaluated as described in Figure 1. *$P<0.05$, †$P<0.01$, ‡$P<0.0001$.

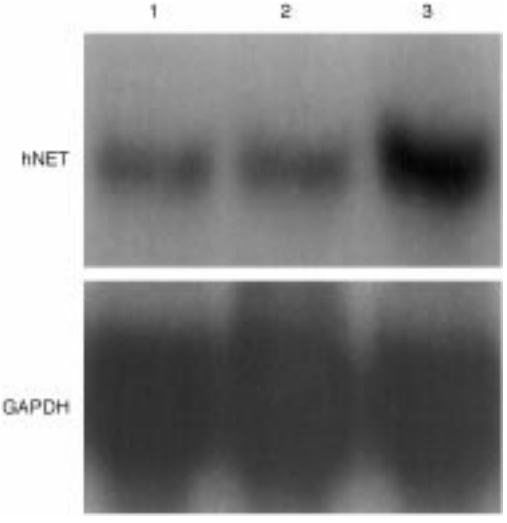

Figure 3. Northern blot analysis. Total RNA (30 μg) was isolated from BE(2)M17 cells that were untreated (*lane* 1), 400 ng/ml cisplatin-treated for 48 h (*lane* 2), 100 ng/ml doxorubicin-treated for 48 h (*lane* 3). All RNA samples were blotted on to a nylon membrane and hybridised to a ^{32}P-labelled human hNET cDNA probe. The lower panel indicates reprobing of the same membrane with a GAPDH probe to demonstrate equal loading.

concentration capable of inducing the highest stimulation of MIBG uptake was used. Only the BE(2)M17 cell line showed detectable levels of hNET and in these cells hNET expression, calculated as the ratio of target to reference (GAPDH) signal intensity, increased approximately 3-fold after treatment with doxorubicin (100 ng/ml), but it was similar to the control level after cisplatin treatment (400 ng/ml) (Figure 3).

The G_2/M block is significantly correlated with the drug-induced MIBG uptake increase

A possible correlation between the increase of MIBG uptake and the cell cycle changes induced by cisplatin and doxorubicin were examined. Flow-cytometric analysis demonstrated that both drugs induced a block in the G_2/M phase of the cell cycle in all neuroblastoma cell lines and this arrest was dependent on cisplatin and doxorubicin concentrations (Figure 4a and b, respectively). The correlation between MIBG uptake and G_2/M block was analysed by the Pearson test. In all cell lines, the increase in the amounts of MIBG uptake was correlated with the percentage of cells in the G_2/M phase. The strongest correlation was observed in SK-N-DZ cells, when cells were treated with cisplatin or doxorubicin: ($r = 0.82$, $P < 0.001$; Figure 5a and b). A significant correlation was also found after treatment with cisplatin or doxorubicin in BE(2)M17 cells (cisplatin: $r = 0.46$, $P < 0.05$; doxorubicin: $r = 0.62$, $P < 0.05$) (Figure 5c and d); and after cisplatin treatment in KCNR cells ($r = 0.47$, $P < 0.05$, data not shown).

To confirm a correlation between the arrest in a specific phase of the cell cycle (G_2/M phase) and the increase in MIBG uptake, MIBG uptake was measured in neuroblastoma cells treated with ara-C, a well established drug which blocks cells in S-phase. No significant difference in MIBG uptake was found between control and ara-C treated cells (data not shown).

The effect of chemotherapy treatment on MIBG uptake in neuroblastoma xenografts

To determine whether the effect of drug treatment on MIBG uptake observed in neuroblastoma cell cultures might

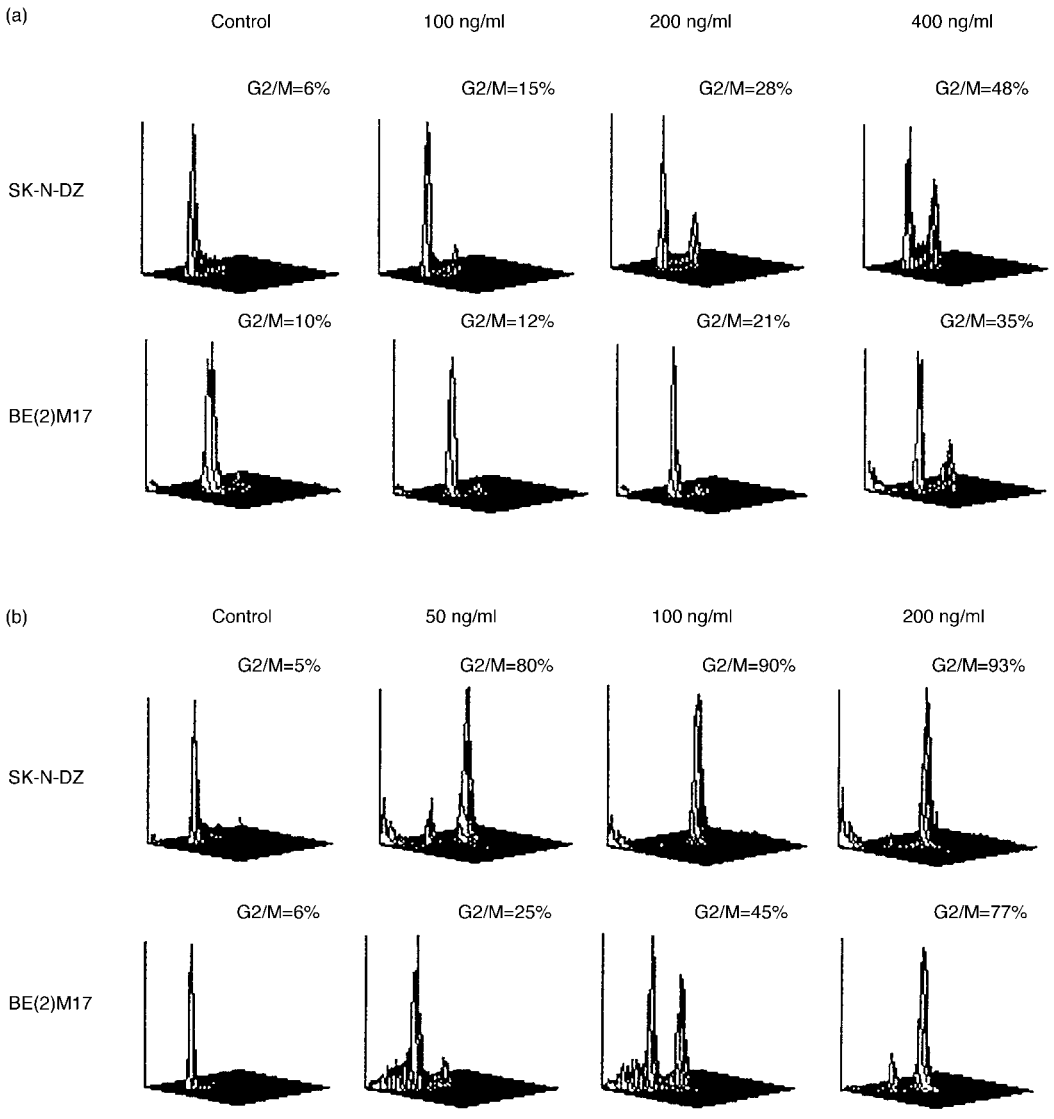

Figure 4. Flow cytometric analysis of cellular DNA content of SK-N-DZ and BE(2)M17 cells treated with cisplatin (a) and doxorubicin (b). Subconfluent cultures were incubated with different concentrations of drugs for 48 h, stained with propidium iodide and analysed for cell cycle distribution using a FACScan flow cytometer.

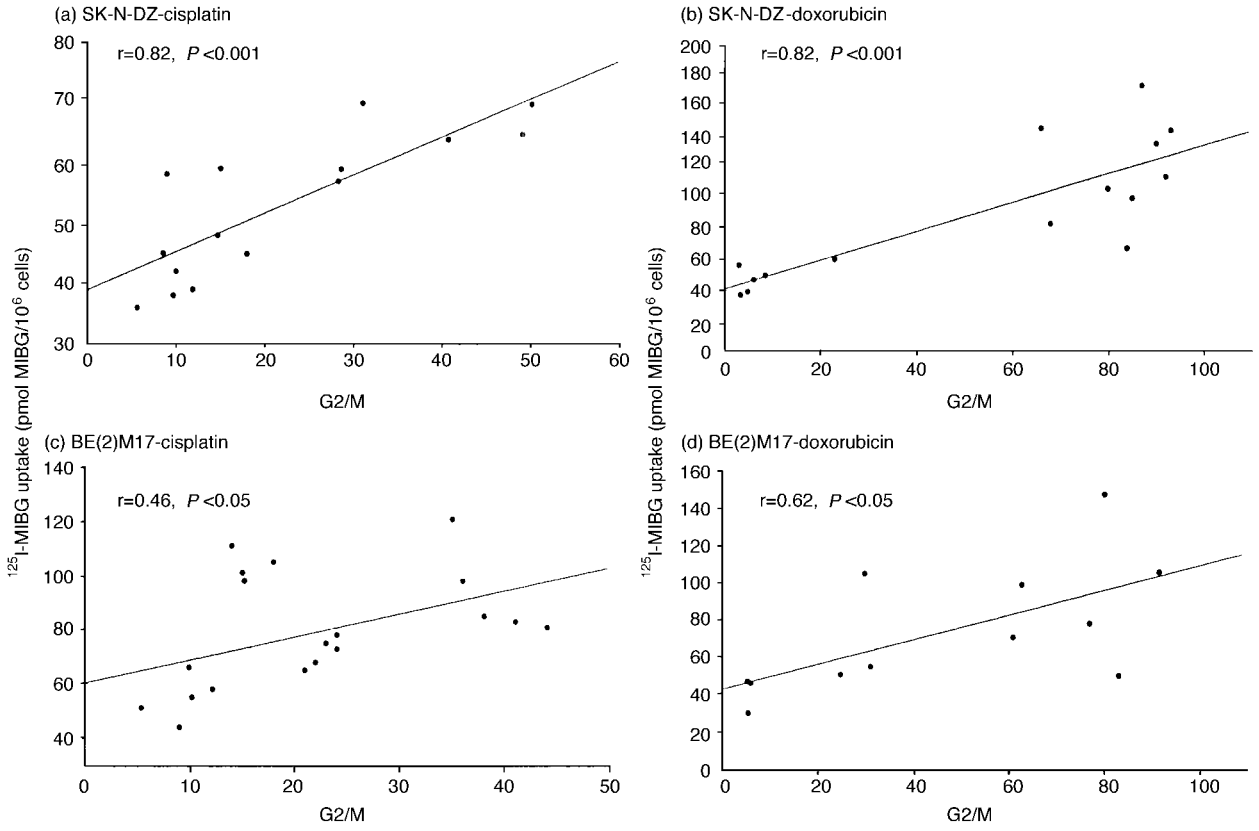

Figure 5. Correlation between drug-induced G_2/M arrest and MIBG uptake in SK-N-DZ and BE(2)M17 cells. Positive correlation was observed in SK-N-DZ (a–b) and BE(2)M17 (c–d) after treatment with cisplatin doxorubicin. The *lines* represent linear regression.

be relevant also *in vivo*, mice bearing SK-N-DZ xenografts and BE(2)M17 xenografts after cisplatin and doxorubicin treatment were used. MIBG incorporation increased in the tumours of mice treated with either drugs 2 days and 3 days before MIBG injection, but it was similar to control levels in animals treated with either drugs 5 days before MIBG injection (Figure 6 cisplatin; doxorubicin, data not shown).

Since the therapeutic index of targeted radiotherapy is determined by the target to non-target ratio of radionuclide accumulation, the ratio between tumour and liver, a representative normal tissue was used to better evaluate the effect

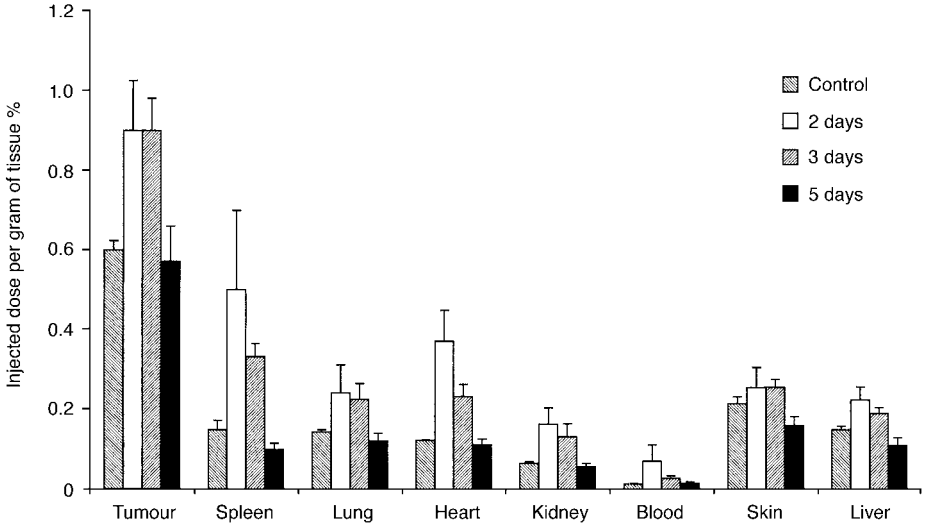

Figure 6. Tissue MIBG distributions in athymic mice bearing subcutaneous SK-N-DZ human neuroblastoma xenografts. Mice were untreated or treated with cisplatin (6 mg/kg) and injected with MIBG 2, 3 and 5 days after cisplatin administration. MIBG incorporation is expressed as per cent, in each gram of tissue, of the injected dose per mouse. Dates represent the average of seven mice. *Bars*, SEM.

of drugs on MIBG specific uptake [20]. The tumour to liver ratio for SK-N-DZ xenografts was significant only in animals given MIBG 3 or 5 days, ($P<0.05$), but not 2 days after cisplatin (Figure 7a). For BE(2)M17 xenografts, this ratio was significant in animals given MIBG 2 or 3 days but not 5 days after doxorubicin (Figure 7b). These data suggest that *in vivo* these drugs also selectively increase the specific component of the MIBG uptake system.

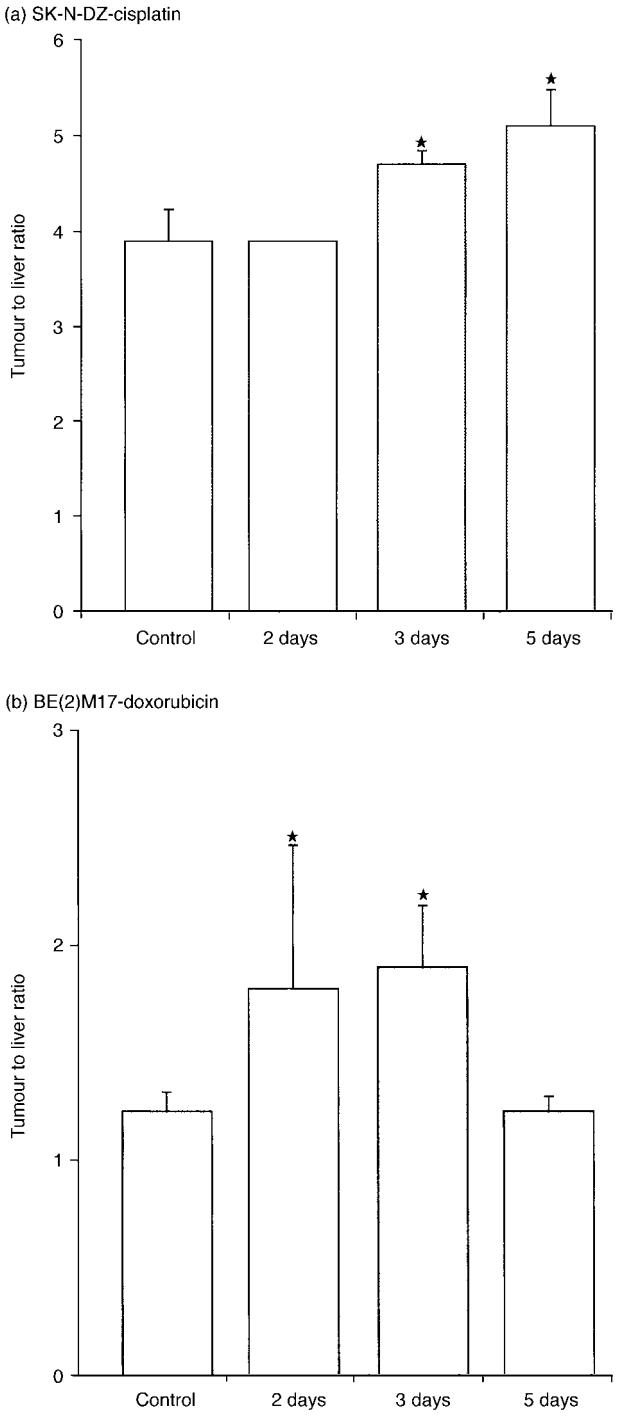

Figure 7. Tumour to liver ratio evaluated in athymic mice bearing subcutaneous SK-N-DZ (a) and BE(2)M17 (b) human neuroblastoma xenografts after cisplatin and doxorubicin treatment, respectively. Mice were untreated or treated with cisplatin (6 mg/kg) or doxorubicin (7 mg/kg) and injected with MIBG 2, 3 and 5 days after drugs administration. Data represent the average of seven mice. *Bars*, SEM. Asterisks indicate ratios significantly different from control at *$P<0.05$ (ANOVA).

DISCUSSION

To improve the efficacy of MIBG-targeted radiotherapy in the treatment of advanced stage neuroblastoma, new therapeutic strategies are currently being explored. The combination of MIBG with chemotherapy is one of the most promising alternatives, because it allows the administration of drugs with different mechanisms of action. Recently, cisplatin has been used in combination with MIBG for the therapy of relapsed neuroblastoma patients with progressive disease [12]. However, it is of concern that chemotherapy might damage MIBG uptake mechanisms in neuroblastoma cells, thus hampering the effectiveness of the radiometabolic treatment.

The present study focused on the effect of two drugs commonly used in the therapy of neuroblastoma, cisplatin and doxorubicin, on MIBG uptake in neuroblastoma cells. Our data demonstrated that the pretreatment of different neuroblastoma cell lines with both drugs induced a dose-dependent increase in MIBG accumulation by enhancing the specific component of MIBG uptake system. Indeed, the drug-induced increase in MIBG accumulation was lost when the cells were incubated at 4°C. Our data confirmed in three different cell lines the finding of Armour and colleagues who showed an increase of MIBG uptake following cisplatin exposure in BE(2)M17 cell line. We were not able to observe an induction of hNET mRNA after cisplatin at the doses that significantly increased MIBG accumulation. In contrast to our results, Armour and colleagues demonstrated an induction of hNET gene transcription in BE(2)M17 cells after cisplatin treatment at much higher concentrations using reverse transcriptase-PCR [21]. Difference in the technique and in the experimental conditions may explain this discrepancy. It is possible, therefore, that cisplatin-induced stimulation of MIBG uptake is not due only to the increased synthesis of new transporter molecules but also to other mechanisms related to already synthesised molecules. Surprisingly, pretreatment with doxorubicin did not result in stimulation of hNET gene transcription in BE(2)M17 cells, as detected by Northern blot analysis. Our findings indicate that cisplatin and doxorubicin do not hamper, but rather stimulate, MIBG uptake.

Neuroblastoma tumours are phenotypically heterogeneous, showing both undifferentiated, round cells, resembling primitive neuroblasts, and cells which display more differentiated features along glial or neuronal lineages. Neuroblastoma cells can be induced to differentiate by chemical agents in culture [17, 22] and therapy-induced maturation of neuroblastoma to benign ganglioneuroma has been reported [23, 24]. Neuronal differentiation of neuroblastoma cells has been shown to be associated with an increase in MIBG uptake and hNET induction in several studies [25]. Both cisplatin and doxorubicin induce differentiation of several cell lines; in particular, the neuroblastoma cell line N1E-115 displays neurite outgrowth after cisplatin treatment [26]. However, we have not observed any morphological sign of differentiation. It is possible that chemotherapy may select cells expressing a more differentiate phenotype, therefore, with a greater capability for MIBG uptake. In all neuroblastoma cell lines studied, both doxorubicin and cisplatin induced cell accumulation in the G_2/M phase, which significantly correlated with MIBG increase. Conversely, an

increase of S phase cells obtained by ara-c exposure did not influence MIBG uptake.

It is unclear why the G_2/M increase should affect MIBG uptake although this phenomenon was observed with all the cell lines tested. It has been reported that norepinephrine transport is regulated, in some parts, by protein kinase C-linked receptors that influence the transport capacity and transporter density in neuroblastoma cells [27, 28]. Folbergrova and colleagues [29] have reported that cisplatin increase intracellular cyclic AMP levels in glioma cells via an interaction with β-adrenergic receptors. Based on these reports, it is possible to hypothesise that cisplatin influences protein kinase C- and A-linked regulation of norepinephrine transporters capacity or their density in cell membranes during G_2/M phase.

To predict whether chemotherapy influence on MIBG accumulation may have clinical relevance, a neuroblastoma tumour xenograft model was used to evaluate the influence of pretreatment with cisplatin or doxorubicin on MIBG uptake *in vivo*. Several studies [30–32] have shown that, following intravenous administration, MIBG is recovered almost entirely in the urine. Since one of the main side-effects of cisplatin is nephrotoxicity, cisplatin pretreatment can influence MIBG renal excretion and, therefore, MIBG biodistribution [33–35]. Our data suggest that the nonspecific increase in MIBG accumulation observed in all organs of animals given MIBG 2 or 3 days after cisplatin may result from the higher MIBG plasma concentration in cisplatin-treated animals due to an impaired MIBG renal excretion. Interestingly, active MIBG accumulation reverted to control levels in those given MIBG 5 days after cisplatin, when presumably the renal function is again normal. Our data demonstrated that these drugs selectively increased the specific uptake of MIBG system as shown by the therapeutic index of targeted radiotherapy. Although the tumour to liver ratio was statistically significant higher in mice pretreated with either cisplatin and doxorubicin, the effect of doxorubicin appeared to be more potent. The mean increase of MIBG uptake induced by cisplatin pretreatment was approximately 26% whilst doxorubicin exposure was able to increase MIBG uptake by approximately 46%. Our data suggest that cisplatin and doxorubicin pretreatment affects both *in vitro* and *in vivo* the specific component of the MIBG uptake system, leading to increased tumour loading. Therefore, the combination of chemotherapy and targeted radiotherapy with MIBG may be of clinical significance, because not only does it destroy tumoral cells utilising agents with different mechanisms of action, but it may also increase the uptake of MIBG and, therefore, actual dose of radiation delivered to the tumour.

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