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Meta-iodobenzylguanidine Uptake in Platelets, Megakaryoblastic Leukaemia Cell Lines MKPL-1 and CHRF-288-11 and Erythroleukaemic Cell Line HEL

G.A.M. Tytgat, P.A. Voûte, S. Takeuchi, I. Miyoshi and M. Rutgers

The major toxicity encountered with [^{131}I]-Meta-iodobenzylguanidine (MIBG) therapy in neuroblastoma patients is an often isolated thrombocytopenia. We believe that this results from MIBG-induced radiotoxicity of the megakaryocytes. Since it is difficult to obtain enough human megakaryocytes for uptake studies, we investigated whether the megakaryocytic cell lines, MKPL-1, CHRF-288-11 and HEL, are good models to study serotonin and MIBG accumulation in human megakaryocytes. Compared with platelets, low levels of specific MIBG accumulation (imipramine-sensitive) were shown in all cell lines, but that of serotonin was negligible in MKPL-1 and CHRF-288-11. Furthermore, the proportion of specific uptake of both MIBG and serotonin appeared greatest in the HEL cells. Although these cells seem to be good candidates to study serotonin and MIBG uptake, they are not a good model to investigate MIBG and serotonin accumulation in human megakaryocytes since they have no functional storage granules.

Key words: meta-iodobenzylguanidine, serotonin re-uptake system, megakaryocytes, megakaryocytic cell lines
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INTRODUCTION

DESPITE SHORT-TERM improvement in remission rates, the long-term disease-free survival of children with advanced neuroblastomas has not substantially improved with multi-agent chemotherapy, surgery and local radiation therapy. Radioiodinated meta-iodobenzylguanidine (MIBG) therapy has been introduced for patients with advanced neuroblastoma after conventional therapy has failed. The major toxicity in this extensively pretreated group is thrombocytopenia [1]. This often isolated thrombocytopenia does not clearly correlate with the degree of MIBG-storing tumour deposits within the bone marrow, nor with the calculated whole-body radiation dose, although reports in the literature are conflicting [2, 3].

Platelets are well known for their extensive serotonin-accumulating capacity, by active uptake and subsequent granular storage. We have previously demonstrated that isolated human platelets can concentrate MIBG nearly 200-fold over extracellular concentrations, reaching nearly serotonin-equivalent levels [4]. From mutual inhibition studies of MIBG and serotonin uptake in platelets as well as in tumour cells expressing the norepinephrine Uptake-1 transporter, it was concluded that MIBG utilises predominantly the serotonin transporter for uptake in platelets [4].

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Thrombocytopenia that occurs after [^{131}I]MIBG treatment is characterised by a delayed appearance. Platelets have a lifespan of about 10 days, and do not possess a radiosensitive nucleus. It is, therefore, unlikely that the platelets are the primary targets of MIBG therapy-related (radio) toxicity, instead we believe that thrombocytopenia is caused by damage to the precursor cells of the platelets, the megakaryocytes.

To our knowledge, nothing is known about MIBG accumulation in bone marrow, but it has been shown that guinea-pig megakaryocytes isolated from bones accumulate serotonin at least as extensively as platelets *in vitro* [5]. Based on these data, we hypothesised that megakaryocytes can accumulate MIBG. It is difficult to obtain enough human megakaryocytes for quantitative uptake studies, since in human bone marrow only 0.5–0.05% of all the nucleated cells are megakaryocytes. To circumvent this methodological problem, continuous cell lines, originating from leukaemic marrow or peripheral blood and that express a range of megakaryocytic phenotypic properties, have been established. A selection of these cell lines are: a human erythroleukaemia cell line (HEL) [6, 7], and the human megakaryoblastic cell lines MKPL-1 [8] and CHRF-288-11 [9]. We investigated whether these cell lines can be suitable models to study MIBG accumulation in megakaryocytes. Since serotonin uptake is a marker for mature megakaryocytes, we compared the uptake of MIBG with that of serotonin.

MATERIALS AND METHODS

Cells and culture methods

The cell line MKPL-1 was obtained from Dr I. Miyoshi [8], and CHRF-288-11 was kindly donated by M. Lieberman [9]. All cells were grown at 37°C in a humidified atmosphere with

5% CO_2. HEL and MKPL-1 cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum and antibiotics, and CHRF-288-11 cells in Fishers' medium supplemented with 20% horse serum and antibiotics. Passages were performed approximately every 3 days to keep the cells growing exponentially.

(Radio)chemicals

Imipramine, iproniazide and pargyline were obtained from Sigman (St. Louis, Missouri, U.S.A.). MIBG was synthesised according to methods of Wieland and associates [10]. 5-Hydroxy[^{3}H]tryptamine creatinine sulphate ([^{3}H]serotonin) with a specific activity of 26 mCi/mg was purchased from Amersham Nederland ('s Hertogenbosch, The Netherlands).

[^{125}I]-MIBG was prepared by the Cu^{1+}-catalysed isotopic exchange method described by Mertens [11] with a specific activity varying from 19.1 to 61.9 mCi/mg.

[^{125}I]MIBG and [^{3}H]serotonin uptake studies

Uptake studies were conducted with approximately 2×10^6 cells in 2 ml of fresh, complete culture medium using 6 well culture dishes (10 cm^2). Experiments were started by adding 80 μl of [^{125}I]MIBG or [^{3}H]serotonin, adjusted with cold substrate if necessary to a final concentration of 10^{-7}M at a radioactive concentration of 0.05–1 μCi/ml. After an incubation period of 1 h, cells were collected by centrifugation (800 *g* for 10 min) and washed twice with ice-cold phosphate-buffered saline (PBS). The cell-associated radioactivity was measured by gamma ([^{125}I]MIBG) or beta scintillation counting ([^{3}H]serotonin) of the pellet. Parallel incubated controls without radioactivity were used to determine the number of cells in the pellet. Inhibition of uptake by the addition of 30 μM imipramine and by incubation at 0°C were studied to analyse the specificity of the uptake process.

RESULTS

We selected 3 cell lines with megakaryocytic properties to investigate MIBG (Figure 1) and serotonin (Figure 2) uptake. Since prolonged incubations (> 1 h) did not result in further increases in cellular radioactivity (data not shown), we compared overall accumulations after 1 h exposure of the cells to 10^{-7}M of each substrate. Co-incubations with imipramine (an inhibitor of active transport of biogenic amines) or on ice were performed to calculate the extent of specific uptake i.e. the total % uptake minus the imipramine-sensitive fraction representing the % accumulated due to passive diffusion.

Figure 1 shows the accumulation of MIBG in the 3 cell lines. The total percentage uptake is depicted as 100%. The highest level of MIBG accumulation was seen in the MKPL-1 cells amounting to 6.4% (± 1.1) of incubated [^{125}I]MIBG which was nearly twice the amount incorporated in the CHRF-288-11 cells. Since all incubations were performed at final substrate concentrations of 10^{-7}M, 1% of added radioactivity corresponds to 1 pmol per 10^6 cells.

With the inhibitor imipramine, we found a significant reduction in the MIBG uptake of 42% in the CHRF-288-11 cells, 37% in MKPL-1 cells and 51% in HEL cells. In general, total cellular radioactivities in cells incubated with serotonin (Figure 2) were low compared with all corresponding MIBG values. The relatively low levels of serotonin appeared not to be due to metabolic degradation by monoamine oxidase or catechol-O-methyltransferase since addition of 26 μM pargyline or 0.1 μM iproniazide (inhibitors of these two enzymes) respectively, had

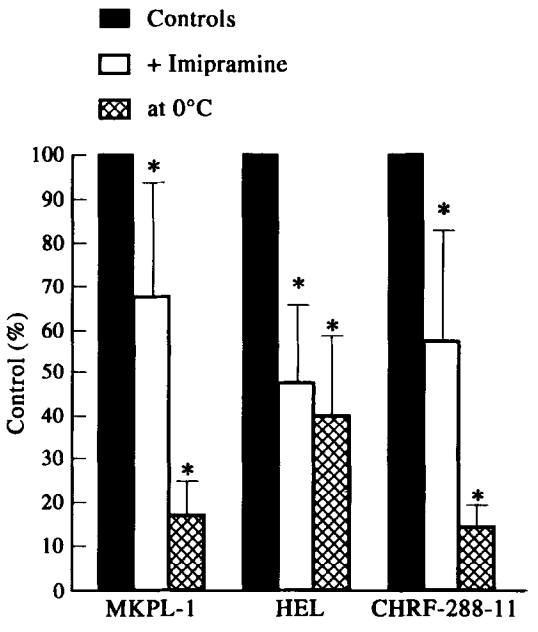

Figure 1. [^{125}I]MIBG accumulation in 3 megakaryocytic cell lines. Total accumulation at 37°C is given as 100% (solid bars). Open bars represent uptake in the presence of 30 μM imipramine, and hatched bars incubations at 0°C. Total MIBG accumulation as a percentage of added radioactivity/h/10^6 cells, amounted to 6.4% ± 1.13 (n = 7, MKPL-1), 4.1% ± 0.8 (n = 3, HEL) and 3.2% ± 0.3 (n = 3, CHRF-288-11). At 0°C, all cell associated radioactivities were significantly lower than controls $P < 0.005$. All data are given as mean ± SD. Statistical significant differences versus controls are indicated. * = $P < 0.05$ (Mann–Whitney U test).

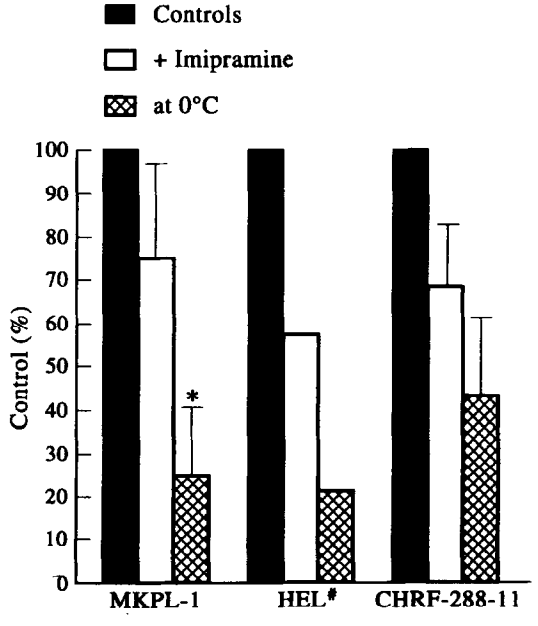

Figure 2. ^{3}H-Serotonin accumulation in 3 megakaryocytic cell lines. Total serotonin accumulation (solid bars) as a percentage of added radioactivity/h/10^6 cells amounted to 2.0% ± 1.0 (n = 6, MKPL-1), 1.9% (n = 2, HEL) and 0.6% ± 0.3 (n = 3, CHRF-288-11). At 0°C, the cell associated radioactivies were significantly lower than control for MKPL-1; for CHRF-288-11 this was not significant. * = $P < 0.05$ (Mann–Whitney U test.) The HEL data were from only 2 experiments and therefore not suitable for statistical analysis.

no effect (data not shown). Relative to MIBG accumulation, HEL cells showed the highest levels of total serotonin uptake. Moreover, the specific uptake of both serotonin and MIBG was the highest in the HEL cells. In most incubuations on ice, the incorporation of radioactivity was reduced by between 60 and 80% for both substrates.

DISCUSSION

Blood platelets contain two distinct transport systems for serotonin, a Na^+- and Cl-coupled serotonin transporter expressed on the plasma membrane and a monoamine transporter present in intracellular, dense (storage) granules, which uses a transmembrane H^+ gradient [12]. In our previous study, we investigated the uptake of serotonin and MIBG in human platelets. Serotonin accumulation in the platelets is very efficient, and although the accumulation of MIBG was much slower, serotonin equivalent levels were reached after prolonged (i.e., 4 h) incubation [4]. To demonstrate that in platelets MIBG is being taken up via the serotonin transporter, we studied the inhibition of MIBG transport with a serotonin re-uptake inhibitor, fluvoxamine. The uptake of MIBG was nearly entirely inhibited by fluvoxamine in concentrations that did not affect the MIBG accumulation by Uptake-1 (the re-uptake mechanism for norepinephrine) in neuroblastoma cells [4]. Another argument to support the idea that MIBG uses the serotonin uptake system is that serotonin effectively inhibited the uptake of MIBG, so MIBG can be considered as promiscuous for the two related but different monoamine transporters, i.e., that of serotonin and norepinephrine [4].

This view has been challenged by Glowniak and associates [13], who demonstrated low uptake of MIBG by cells with active serotonin transport or expressing transfected serotonin uptake transporter genes. This study included short, i.e. 5–10 min incubations and measured affinity of the uptake system rather than the overall uptake or storage capacity on prolonged incubations. As detailed in a previous study, the uptake of MIBG in platelets is characterised as low affinity, high capacity [4].

Because megakaryocytes accumulate serotonin, we hypothesise that they also accumulate MIBG via the serotonin uptake system. In order to protect the bone marrow stem cells from putative MIBG-induced radiotoxicity, a selective uptake inhibitor, such as fluvoxamine, is required which blocks MIBG accumulation in the platelets and the megakaryocytes, but does not interfere with the uptake of MIBG by the tumour.

In this study, much lower accumulation per cell of both MIBG and serotonin was found in the megakaryocytic cells than in the blood platelets. When considering the actual size of the platelets and the megakaryocytic cells, the difference in intracellular concentration will be even more pronounced. The cell lines represent immature megakaryocytes, with few dense (storage) granules, and it is not even known if these are functional. Because of the assumed paucity of the dense (storage) granules, the intracellular serotonin or MIBG can diffuse out of the cells, explaining the absence of a clear increasing cell-associated radioactivity with prolonged incubation time. This is in clear contrast with the effective storage in granule-rich human platelets [4].

When comparing the specific, imipramine-sensitive serotonin accumulation of the three cell lines, we found that only the HEL cells showed significant inhibition (40%) by imipramine, with little effect in CHRF-288-11 and MKPL-1 cells. The results with the HEL cells are in good agreement with the results of Vannucchi and colleagues [14] who demonstrated that HEL cells show appreciable incorporation of serotonin, although they did not test for inhibition by imipramine.

Total MIBG accumulation was higher in all cell lines compared with serotonin accumulation. It is known that MIBG, a lipophilic cation, accumulates in metabolically active cells by non-specific uptake. Smets and associates [15] showed a 10–15-fold nonspecific concentration in murine leukaemic L1210 cells which lack the specific Uptake-1 transporter of norepinephrine. Non-specific uptake can partly explain the difference in the accumulation of serotonin and MIBG between the cells.

When comparing the accumulation of MIBG and serotonin in HEL cells we found approximately 51 and 40% inhibition by imipramine, respectively. Further studies are needed to investigate the effect of specific serotonin re-uptake inhibitors on the MIBG accumulation in HEL cells. Now that the human serotonin transporter is cloned [16], expression of the transporter in the HEL cells needs to be confirmed. Although some of the results are, to some extent, preliminary, they indicate that the cell line HEL is a good candidate to study the uptake and inhibition of MIBG and serotonin.

It is not overlooked, however, that this model will underestimate the contribution of storage granules to total uptake in mature megakaryocytes. In two separate pilot experiments (data not shown) in which we investigated MIBG and serotonin accumulation in human megakaryocytes, a 10-fold higher serotonin uptake than that of MIBG was found, and inhibition by imipramine was about 90 and 30%, respectively. This is in plain contrast with the present findings in the HEL cells. As an efficient granular storage compartment appears to be lacking in HEL cells, we conclude that these cells are of limited value to investigate serotonin and MIBG accumulation in human megakaryocytes. However, radiotoxicity by the accumulated [^{131}I]MIBG in the megakaryocytes will be highest during active DNA replication associated with endoploidisation (endomitosis) of maturing megakaryocytes. Proliferating HEL cells do represent this aspect and may eventually become a suitable model after controlled maturation induction with recombinant growth factors.

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A New Approach in the Treatment of Stage IV Neuroblastoma Using a Combination of [^{131}I]Meta-iodobenzylguanidine (MIBG) and cisplatin

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The outlook for disseminated neuroblastoma (NB) continues to be dismal. NB is a radiosensitive tumour. Owing to its high concentration in NB lesions, [^{131}I]meta-iodobenzylguanidine [^{131}I]MIBG has the potential for specifically delivering very large radiation doses to the malignant cells. Encouraging results have been reported with [^{131}I]MIBG used alone in patients resistant to conventional therapy and at diagnosis. We report the first attempt to explore the integration of this new treatment modality with chemotherapy. Among the drugs effective in NB, cisplatin was chosen because of its high degree of activity against NB, its mild haematological toxicity and the known synergism between cisplatin and radiation. 4 patients, 3 with relapsed, heavily pre-treated, progressive stage IV NB, and 1 with stage IV NB at diagnosis, all with a good [^{131}I]MIBG uptake, were investigated with combined therapy (CO-TH). Two complete remissions and one partial remission were observed in these patients 4–6 weeks following only a single course of both cisplatin and [^{131}I]MIBG at "standard" dosage. The only toxicity was haematological, which was significant and relatively long-lasting, but was not associated with any serious infections or bleeding tendency. The general condition of these patients during the entire study period was excellent. The fourth patient, investigated at diagnosis with a modified less intensive treatment, obtained a partial remission with mild haematological toxicity. During the subsequent courses of intensive multidrug chemotherapy, this patient showed haematological toxicity comparable with that experienced by patients treated with an identical drug combination, but without previous treatment with CO-TH. The provisional conclusion of this ongoing study is that this new form of CO-TH appears most effective in obtaining a rapid and excellent response in heavily pretreated relapsed patients with progressive disease, and should be further investigated in earlier stages of the disease.

Key words: neuroblastoma, cisplatin, [^{131}I]meta-iodobenzylguanidine, synergism
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