211\textsuperscript{At}-Methylene Blue for Targeted Radiotherapy of Human Melanoma Xenografts: Treatment of Micrometastases\textsuperscript{1}

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ABSTRACT

Treatment of micrometastases of HX34 human melanoma grown as xenografts in nude mice represents an advanced stage of preclinical investigations concerning targeted radiotherapy of this neoplasm using 3,7-(dimethylamino)phenazathionium chloride (methylene blue (MTB) labeled with astatine-211 (\textsuperscript{211}At) (\alpha-particle emitter). The therapeutic effectiveness of \textsuperscript{211}At-MTB administered i.v. was determined by a lung colony assay combined with a search for metastases to organs other than the lungs. A single dose of \textsuperscript{211}At-MTB lowered the HX34 cell surviving fraction in lungs to below 10% almost independently of the time interval between cell inoculation and radioisotope injection and of \textsuperscript{211}At-MTB radioactivity within its investigated range. Radiation dose and the time of its administration did, however, influence the size of lung colonies. In contrast, the efficacy of \textsuperscript{211}At-MTB treatment as assessed by both surviving fraction and colony size was significantly dependent on a number of HX34 cells inoculated initially into mice. These results are explained by a short range of \alpha-particles emitted by \textsuperscript{211}At and a mechanism of growth of lung colonies from tumor cells circulating with blood and blocking lung capillaries. Metastases in organs other than lungs and characteristic of control animals were not found in mice treated with \textsuperscript{211}At-MTB. The high therapeutic efficacy achieved proved that \textsuperscript{211}At-MTB is a very efficient scavenger of single melanoma cells distributed through blood and micrometastases with sizes below the limit of clinical detection.

INTRODUCTION

Targeted radiotherapy is defined as a treatment selectively directed at a particular tissue, most often neoplastic, by using either a radioisotope exhibiting a high affinity to this tissue (e.g., iodine for the thyroid or its carcinoma) or a compound with such affinity used as a carrier for a suitable radioisotope. The selectiveness of the radionuclide uptake enables deposition of high radiation doses in the targeted tissue with only minor exposure of remaining structures, unlike exposure in the classical beam radiotherapy in which both the targeted and adjacent tissues undergo irradiation nonspecifically.

The idea of targeted radiotherapy derives from radiotracer studies which began in the 1910s when the Hungarian scientist G. Hevesy together with F. Paneth reported the use of \textsuperscript{125}Pb as a radioindicator in chemistry (1) and, subsequently, \textsuperscript{222}Pb to investigate lead metabolism in plants (2). These experiments were followed by first clinical studies in which a comparison between the velocity of blood circulation under physiological conditions and in patients with heart disease had been made by injecting a solution of radium into one arm of a patient and detection of a highly penetrating \gamma-rays in the other (3). Subsequent investigations concerned measurements of \textsuperscript{32}P uptake in different organs and tissues including bones (4), leukemia treatment with \textsuperscript{32}P (5), and a selective uptake of radiiodine in the thyroid (6) which resulted later in the use of the radionuclide for diagnosis and treatment of thyroid diseases including neo-

\textsuperscript{1}The abbreviations used are: MTB, 3,7-(dimethylamino)phenazathionium chloride (methylene blue); \textsuperscript{211}At, astatine-211.

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MATERIALS AND METHODS

Human Melanoma Xenografts. HX34 human melanoma xenografts were obtained by courtesy of Dr. G. G. Steel of the Institute for Cancer Research, Sutton, United Kingdom. The tumor was derived from a patient who had not undergone previous cytotoxic therapy and was established by J. Mills of the same Institute from biopsy samples of a metastatic lesion. The obtained samples contained 2-mm³ tumor pieces frozen in 10% dimethyl sulfoxide at its fourth passage previously grown s.c. in nude mice.

Thawed tumor fragments suspended in Ham’s F-12 medium (Flow Laboratories, Ltd., Irvine, United Kingdom) supplemented with 10% fetal bovine serum were transplanted into nude female mice (2 pieces in 0.15 ml of medium as a single s.c. injection) and subsequently passaged in vivo every 3–4 weeks.

Experimental Animals. Nude female mice (Crlnu-nu(CD/1TM)BR), 50–60 days old, were used for all experiments. The animals were kept in sterile cages and fed with sterilized food and water. The experiments were performed under sterile conditions using a laminar flow cabinet. Inoculations of melanoma cells i.v. and radioisotope injections were carried out on mice anesthetized with pentobarbitone sodium.

Injections of a Single Tumor Cell Suspension i.v. A single cell suspension from solid melanoma tumors was obtained according to the method described earlier (13, 14). Briefly, excised tumors were washed in serum-containing Ham’s F-12 medium and cut into very small fragments. Subsequently, a supernatant was removed and the remaining tumor fragments were placed in a conical flask containing 30 ml of Ham’s F-12 medium and three enzymes, 0.02% DNase, 0.05% Pronase, and 0.02% Collagenase (all from Sigma Chemical Co., Ltd., Poole, Dorset, United Kingdom). The suspension, slowly stirred with a magnetic stirrer, was incubated for 15–20 min at 37°C. After completion of the incubation, the obtained cells were recovered by centrifugation and washed 2–3 times with the serum-containing F-12 medium before filtering through a cotton gauze. Subsequently, the cells were counted in a Neubauer hemocytometer, further centrifuged, and diluted to the desired density in phosphate-buffered saline, and 0.2-ml aliquots were distributed into small test tubes. Afterwards, 0.1 ml of the suspension was injected into one of the tail veins of the recipient mice using a separate portion of the cell suspension for each individual inoculation.

²¹¹At-Methylene Blue: Synthesis and Therapeutic Trial. Astatine-211 production and the synthesis of ²¹¹At-astato-methylene blue (²¹¹At-MTB) were carried out according to the method described previously (15) in the Department of Physics, University of Birmingham, using a 28-MeV α-particle external beam from the Nuffield 1.52 m cyclotron. The radiolabeled compound was prepared before each experiment because of the very short half-life time of astatine-211 (7.21 h) and was characterized by a high specific activity of approximately 555 MBq/mg. ²¹¹At-MTB was dissolved in phosphate-buffered saline so that its initial activity amounted to approximately 296 MBq/ml. The radiolabeled compound was further diluted in phosphate-buffered saline to the desired radioactivity and 0.1 ml was injected i.v. into each mouse either 24 h or 7 days after the tumor cell inoculation.

Determination of Therapeutic Effectiveness of ²¹¹At-MTB Using Lung Colony Assay. Six to 11 weeks after cell injection, a careful necropsy was carried out to detect metastases in organs other than the lungs, particularly in lymph nodes and the liver. The lungs were removed and immediately fixed with 10% formal saline, and a total number of melanoma colonies were counted in all separated lobes using a dissecting microscope. Subsequently, two perpendicular diameters of each nodule were measured with a caliper. Final results were expressed as: (a) the surviving fraction calculated as the ratio of the colony-forming efficiency for treated cells to the colony-forming efficiency for control cells (colony-forming efficiency was calculated as the number of lung colonies divided by the number of inoculated cells); (b) the mean size of the counted colonies. Since the shape of lung nodules is ellipsoidal and it was not possible to measure their third diameter, the size of each colony was estimated by calculating an area of the ellipse according to the equation

\[
\frac{1}{4} \pi ab
\]

where \(a\) was the greatest colony dimension and \(b\) was the dimension perpendicular to \(a\); (c) the frequency of appearance of metastases in organs other than the lungs.

RESULTS

Our previous data revealed that the therapeutic effects of radiolabeled methylene blue are dependent on melanin content in melanoma cells with the highest efficiency observed for the highly pigmented tumors (10, 11). However, since human melanoma metastases are usually less pigmented than the primary lesion, the aim of the present studies was to investigate whether a limited ²¹¹At-MTB uptake conditioned by a lower pigmenta-

HX34 Human Melanoma Tumor Xenografts: Lung Colony-forming Efficiency

The lung colony-forming efficiency for poorly pigmented HX34 human melanoma tumor xenografts was 0.28 ± 0.06% and did not depend on either the time interval between cell inoculation and the sacrifice of mice (6–11 weeks) or number of colonies injected initially within the range of 1 × 10⁴–6 × 10⁴ (Fig. 1, A and B). The mean colony size increased almost exponentially with time and amounted to 0.8–2.4 mm² after a 6–11-week interval, respectively (Fig. 1C). Most colonies were nonpigmented as determined by macroscopic examination; however, highly pigmented but significantly smaller nodules were occasionally observed.

Most i.v. inoculations of HX34 cell suspension and all routine s.c. passages of the tumor fragments were accompanied by metastatic dissemination to lymph nodes. The number of such metastases correlated with significantly diminished colony-forming efficiency the value of which was lower than 0.1% (Table 1).

V. D. Courtenay and J. Mills, unpublished work.
Table 1 Influence of HX34 metastases growing in lymph nodes on the number of lung colonies

<table>
<thead>
<tr>
<th>No. of cells inoculated (x 10^4)</th>
<th>Colony-forming efficiency (%)</th>
<th>Metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.2</td>
<td>0.33</td>
<td>None</td>
</tr>
<tr>
<td>4.0</td>
<td>0.03</td>
<td>Lymph nodes of front and hind limbs</td>
</tr>
<tr>
<td>5.0</td>
<td>0.22</td>
<td>None</td>
</tr>
<tr>
<td>5.6</td>
<td>0.08</td>
<td>Lymph nodes of front and hind limbs</td>
</tr>
</tbody>
</table>

Fig. 2. Surviving fraction of HX34 cells and mean size of lung colonies for nude mice treated with 1.67 or 3.3 MBq of 211At-MTB. HX34 melanoma (5 x 10^4) cells were inoculated i.v. into nude mice. 211At-MTB was injected also i.v. 24 h after cell inoculation. Surviving fraction (•) and a size of the colonies (□) were determined 7.5 weeks after the cell inoculation. Bars, SD.

Fig. 3. Dependence of surviving fraction of HX34 cells (■) and mean size of lung colonies (□) on the number of inoculated cells for nude mice treated with 3.4 MBq of 211At-MTB injected i.v. 24 h after cell inoculation. Bars, SD.

Fig. 4. Time-dependent effectiveness of HX34 melanoma treatment with 3.55 MBq of 211At-MTB. HX34 cells (5.6 x 10^4) were inoculated i.v. into nude mice and injections of the radioisotope were carried out either 24 h or 7 days after cell inoculation. Surviving fraction (■) and size of colonies (□) were determined 7.5 weeks after cell inoculation. Bars, SD.

Therapeutic Effectiveness of 211At-MTB

The therapeutic effectiveness of 211At-MTB was investigated as a function of (a) the radioactivity of 211At-MTB injected i.v. into mice; (b) the number of cells inoculated into mice; and (c) the interval between cell inoculation and 211At-MTB injection.

211At-MTB Radioactivity and Therapeutic Effects. 211At-MTB of two different total radioactivities, 1.67 MBq and 3.33 MBq, was used for i.v. injections. The injections were carried out 24 h after inoculation of 5 x 10^4 cells into mice. The cell survival determined for mice treated with both 211At-MTB doses was approximately 95% lower than in controls but almost independent of the radioactivity used (Fig. 2). However, the size of the colonies was not only significantly smaller than in the controls but also differed by 20%, with the lower value for the higher dose of 211At-MTB (Fig. 2).

Influence of Inoculated Cell Number on 211At-MTB Effects. The therapeutic effectiveness of 211At-MTB was significantly dependent on the number of cells inoculated 24 h prior to the injection of the radioisotope (Fig. 3). The used radioactivity of 3.4 MBq resulted in the almost total inhibition of colony growth initiated by 1 x 10^4 cells (the surviving fraction decreased to below 1.7% of the appropriate controls). The size of the colonies found was below the accuracy of a caliper measurement although the lung examination was carried out 11 weeks after cell injection, unlike in the remaining experiments in which the necropsy was performed 7.5 weeks after cell injection. The inoculation of 5 x 10^4 and 5.6 x 10^4 cells caused a slight but progressive increase in cell survival; surviving fraction was 5 and 8%, respectively, and corresponded to the mean values of colony sizes amounting to 0.62 and 0.95 mm^2 (Fig. 3).

Dependence of 211At-MTB Effects on the Interval between Cell Inoculation and 211At-MTB Injection. The results of investigations concerning a time-dependent effectiveness of 3.55 MBq 211At-MTB are shown in Fig. 4. The surviving fraction decreased to 8 and 13% when the radioisotope was injected 24 h or 7 days, respectively, after the inoculation of 5.6 x 10^4 cells.
However, the application of $^{211}$At-MTB 7 days after cell injection appeared to be more efficient in diminishing colony size. The mean size of these colonies was one order of magnitude smaller than this found in controls, whereas for colonies growing in mice treated with $^{211}$At-MTB 24 h after cell inoculation its value decreased only by 21% (Fig. 4).

$^{211}$At-MTB Effects on Metastases Other Than Lung Colonies. Metastases disseminated in lymph nodes and characteristic of control mice were not detected in animals treated with $^{211}$At-MTB. Only one mouse developed metastases in the liver when 1.67 MBq of the radioisotope was injected 24 h after inoculation of $5 \times 10^6$ cells.

Additionally, it was conspicuous that none of the colonies growing in mouse lungs after treatment with $^{211}$At-MTB has been pigmented even if such colonies were observed in control mice. All colonies found in treated animals contained no melanin in a concentration allowing its macroscopic detection.

DISCUSSION

The experiments presented here initiate the advanced stage of our investigations aiming to establish whether $^{211}$At-astatinated methylene blue can be used in the clinic for targeted radiotherapy of disseminated melanoma to complement excision of the primary tumor, and to what extent the treatment could not only significantly improve but even replace already accepted clinical procedures.

HX34 and HX118 human melanoma xenografts grown in nude mice are very valuable models for such studies, not only allowing the investigation of the human tumor itself but also because both these melanoma xenografts imitate the clinical pattern of dissemination by metastasizing to lymph nodes and the liver, in addition to producing cutaneous tumors and lung colonies. These similarities justify extrapolation of the experimental results to the clinical situation.

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$^{211}$At-MTB administered i.v. 24 h after cell injection is distributed through blood similarly to the tumor cells and accumulated mostly in the proximal cell of the cellular embolus. Consequently, the uptake of $^{211}$At-MTB characterized by the appropriate radioactivity into a one-cell blockage is lethal for it and can be also lethal for any embolus consisting of up to 3 cells, if the number of $^{211}$At-MTB molecules accumulated by the top cell is sufficient to deposit sterilizing dose in every cell of the blockage (Fig. 6, A–C). This is due to the physical properties of astatine-211: a mean range of $\alpha$-particles emitted by this radioisotope does not exceed 65 $\mu$m corresponding to approximately 3 cell diameters. A blockage consisting of more than 3 cells will always result in a growth of a colony with its
The importance of the results obtained here is that the observed effects are not limited to the treatment of single cells or micrometastases distributed in lungs which are the prime target for $^{211}$At-MTB injected into a tail vein but extend to more distant neoplastic deposits such as lymph node and liver metastases. The lack of their appearance after exposure to $^{211}$At-MTB suggests that the amount of melanin characteristic of poorly pigmented metastatic lesions is still above the critical level below which the uptake of $^{211}$At-MTB will be insufficient to be lethal and, furthermore, that such targeted radiotherapy could be highly effective for treatment of melanoma metastases at a stage when their presence can be assumed with a very high probability but cannot be positively confirmed due to their small size preventing their detection.

The universality of the data and the lack of systemic toxicity of $^{211}$At-MTB encouraged us to investigate $^{211}$At-MTB treatment of cutaneous melanoma tumors and concomitant lymph node metastases. These results will be reported in the forthcoming paper.

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