211At-Methylene Blue for Targeted Radiotherapy of Human Melanoma Xenografts: Treatment of Micrometastases

Eva M. Link and Robert N. Carpenter

Department of Chemical Pathology, Academic Unit, University College and Middlesex School of Medicine, Cleveland Street, London W1P 6DB [E. M. L.], and Department of Chemistry, University of Birmingham, Edgbaston, Birmingham B15 2TT [R. N. C.], United Kingdom

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 (211At) (α-particle emitter). The therapeutic effectiveness of 211At-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 211At-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 211At-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 211At-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 α-particles emitted by 211At 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 211At-MTB. The high therapeutic efficacy achieved proved that 211At-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 210Pb as a radioindicator in chemistry (1) and, subsequently, 212Pb 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 γ-rays in the other (3). Subsequent investigations concerned measurements of 32P uptake in different organs and tissues including bones (4), leukemia treatment with 32P (5), and a selective uptake of radiodine in the thyroid (6) which resulted later in the use of the radionuclide for diagnosis and treatment of thyroid diseases including neoplasms (7). Increasing availability of radioisotopes due to a construction of first nuclear reactors led in the 1940s to a wide development of radiopharmaceuticals with the emphasis on polycyclic compounds (8). At present, monoclonal antibodies are of particular interest as the most selective carrier for radioisotopes. However, some difficulties such as a poor stability of the binding of a radioisotope to antibodies and insufficient penetration into the targeted tissue limit a choice of radioisotopes and suggest the use of those characterized by relatively long range radiation. The latter is associated with increased exposure of normal tissues. These shortcomings reduce the application and efficacy of radiolabeled monoclonal antibodies used for targeted radiotherapy and encourage a further search for alternative radioisotope carriers.

The main difficulties in human melanoma treatment are due to a wide dissemination often concomitant with the appearance of the primary tumor. It was important, therefore, to find a melanoma-specific compound which, labeled with an appropriate radioisotope, could be used as a scavenger of single melanoma cells distributed through blood, as well as tumors below the limit of clinical detectability. MTB3 seems to fulfill these requirements. Its strong affinity to melanin with a binding efficiency of 87% (9) enables a highly selective uptake of this compound in pigmented melanomas in vitro and in vivo; radiolabeled methylene blue was incorporated at least 5 times more effectively by cultured pigmented melanoma cells than by those of the nonpigmented tumor variety (10, 11). Its distribution in vivo showed the highest and most stable level in pigmented melanomas (12). The uptake by normal organs was particularly effective (59% of that in melanoma) in the eyes with their melanin-abundant choroid and less significant in the liver (12). Nonpigmented tissues revealed a negligible level of the compound; only the kidneys exhibit a transient peak due to the excretion of methylene blue through this organ (12). Further investigations concerning the anti-melanoma potential of radiolabeled monoclonal antibodies (13) and, subsequently, 212Pb-MTB (Auger electron emitter), and 211At-MTB (α-particle emitter), showed a significant and a dose-dependent therapeutic advantage both in vitro and in animal model system (10, 11). Moreover, the data proved that [211At]astatinated methylene blue was by far the most effective of those radioisotopes already investigated; while the average of 1.5 × 10−2 Bq of [32P]MTB or 1.4 × 10−2 Bq of [125I]MTB, respectively, must be accumulated by a single pigmented melanoma cell to diminish their survival below 4%, only 2.5 × 10−4 Bq of 211At-MTB was needed to achieve the same effect (10, 11).

The present examination of therapeutic efficacy of 211At-MTB for disseminated human melanoma xenografts is one of the series of our studies on this radiolabeled compound and its use to control the growth of cutaneous tumors, metastases arising from single cells circulating with blood, and micrometastases which remain undetected at the time when treatment for melanoma is first administered.

Received 9/19/89; revised 1/18/90.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Financial support from the Cancer Research Campaign is gratefully acknowledged.

2 To whom requests for reprints should be addressed.

Received 9/19/89; revised 1/18/90.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Financial support from the Cancer Research Campaign is gratefully acknowledged.

2 To whom requests for reprints should be addressed.

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

2963

Downloaded from cancerres.aacrjournals.org on January 22, 2018. © 1990 American Association for Cancer Research.
MATERIALS AND METHODS

Human Melanoma Xenografts. HX34 human melanoma xenografts were obtained by courtesy of Dr. G. G. Steel of the Institute of 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 (Cr1nu/nu(CD/1F1)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-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

\[ \frac{1}{4} \pi a b \]

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 pigmentation of such cells will still be sufficient to result in tumoricidal doses from the radioisotope accumulated and, therefore, diminish or even prevent a spread of the disease.

**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 cells injected initially within the range of \( 1 \times 10^4 - 6 \times 10^4 \) (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 appearance of such metastases correlated with significantly diminished colony-forming efficiency the value of which was lower than 0.1% (Table 1).

![Figure 1](image-url)
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 ^{211}At-MTB. HX34 melanoma (5 x 10^4) cells were inoculated i.v. into nude mice. ^{211}At-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.

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

^{211}At-MTB Radioactivity and Therapeutic Effects. ^{211}At-MTB of two different total radioactivities, 1.67 MBq and 3.3 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 ^{211}At-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 ^{211}At-MTB (Fig. 2).

Influence of Inoculated Cell Number on ^{211}At-MTB Effects. The therapeutic effectiveness of ^{211}At-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 mm² (Fig. 3).

Dependence of ^{211}At-MTB Effects on the Interval between Cell Inoculation and ^{211}At-MTB Injection. The results of investigations concerning a time-dependent effectiveness of 3.55 MBq ^{211}At-MTB are shown in Fig. 4. The surviving fraction decreased to 8 and 13% when the radioisotope was injected 24 h or 7 days after cell inoculation. Surviving fraction (•) and size of colonies (□) were determined 7.5 weeks after cell inoculation. Bars, SD.
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-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.

The data described in this paper unequivocally confirm the therapeutic effectiveness of $^{211}$At-methylene blue as a scavenger of either single melanoma cells distributed through blood or melanoma micrometastases and extend our previous observations on mouse B16 melanoma (11). The results are particularly promising since HX34 human melanoma xenografts used for these experiments are poorly pigmented, as are metastases usually found in humans. In such cases the amount of melanin present in the melanoma cells and available for $^{211}$At-methylene blue binding is very low. Therefore, taking into account that the effect from $^{211}$At-MTB is dependent on the number of its molecules accumulated by every cell in question (10, 11), it was possible that the radiation doses from $^{211}$At-MTB deposited in metastatic lesions would be too small to be lethal. However, the uptake and binding of the compound by human melanoma cells seem to be extremely efficient. Even a single i.v. injection of 3.55 MBq $^{211}$At-MTB diminishes the number of lung colonies to below 10% of controls almost independently of the time interval between cell inoculation and administration of the compound. Interestingly, $^{211}$At-MTB treatment performed 24 h after cell injection results in dramatic decrease in the number of colonies but without significant reduction of their size when $5-6 \times 10^4$ cells are inoculated initially. In sharp contrast, administration of the same dose of $^{211}$At-MTB 7 days after cell inoculation not only leads to the low surviving fraction but also considerably reduces the size of the fewer lung nodules formed. These results suggest that the scavenging of single cells, as the treatment at 24 h after cell injection can be viewed, takes place by an “all or none” mechanism. In other words, since the size of the colonies is comparable to that of controls, the nodules arise from cells which presumably failed to take up $^{211}$At-MTB. In contrast, in 7-day-old multicellular micrometastases in which a development of the vascular system, certainly initiated over this time, significantly increases the efficiency of $^{211}$At-MTB distribution, an uptake of the radiolabeled compound by a majority of cells present in every tumorlet results in their lethal damage and, consequently, in the colonies characterized by a very small size.

The observed relation between $^{211}$At-MTB actions and both $^{211}$At-MTB radioactivity and the number of cells injected support the reasoning. Furthermore, it suggests an explanation for a modification in dose-dependent effects of radiolabeled methylene blue (10, 11) when $^{211}$At-MTB is used. The mechanism applies to any targeted radiotherapy in which radioisotopes characterized by a short range of emitted radiation (equal to a few cell diameters) are used when both cells and the radioactive compound are distributed throughout blood (see Figs. 5 and 6).

The malignant cells injected into one of the tail veins are transported with blood through the right heart ventricle and distributed in lungs by their vascular system. Since the diameter of lung capillary vessels is less than the tumor cell diameter, melanoma cells present in blood block these vessels and initiate growth of colonies. The cell distribution is random throughout the pulmonary vascular bed; therefore, the number of occluded capillary vessels is proportional to the number of cells injected only when cell density is significantly lower than the number of all available capillary vessels (see Fig. 5A). With increased density of injected cells the vessels are blocked each with more than one cell (Fig. 5, B–D).

$^{211}$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 µm 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
size dependent on the number of cells beyond the range of \( \alpha \) particles from \(^{211}\text{At}\) and a degree of damage to the remaining cells beyond the range of \(^{211}\text{At}\) will be characterized by an increased surviving fraction with a size of colonies decreased by 20% with increased radioactivity compared with controls.

Consequently, to the abolishing of all pulmonary micrometastases and with their size comparable with controls.

When the density of injected cells will be increased by 3% only but the colony size by more than 50%, as well as the data obtained after administration of either 1.67 or 3.3 MBq \(^{211}\text{At}-\text{MTB} \) 24 h after injection of \( 1 \times 10^4 \) cells, resulting in a very low surviving fraction (1.7%) and a colony size below the accuracy of a caliper measurement. This will reach an intermediate stage in which surviving fraction will remain almost independent of the density of cells injected initially, whereas the colony size will gradually increase to almost the control value. Indeed, the results achieved by a treatment with 3.4 MBq \(^{211}\text{At}-\text{MTB} \) 24 h after i.v. inoculation of 5 \( \times 10^4 \) or 5.6 \( \times 10^4 \) cells, when the surviving fraction increased by 3% only but the colony size by more than 50%, as well as the data obtained after administration of either 1.67 or 3.3 MBq \(^{211}\text{At}-\text{MTB} \) 24 h after injection of 5 \( \times 10^4 \) cells, when surviving fraction remained almost unchanged but the mean size of colonies decreased by 20% with increased radioactivity of \(^{211}\text{At}-\text{MTB}, \) confirm the proposed mechanism. The last stage will be characterized by an increased surviving fraction with a number of colonies proportional to the density of injected cells and with their size comparable with controls.

It should be pointed out that the above mechanism describes the effects from a single dose of \(^{211}\text{At}-\text{MTB} \) only. Repeated injections of the radiolabeled compound properly synchronized with the physiological processes responsible for the resorption of necrotic cells from the vessels will lead gradually to the uptake of \(^{211}\text{At}-\text{MTB} \) by all melanoma cells lodged there and, consequently, to the abolishing of all pulmonary micrometastases.

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 \(^{211}\text{At}-\text{MTB} \) 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 \(^{211}\text{At}-\text{MTB} \) suggests that the amount of melanin characteristic of poorly pigmented metastatic lesions is still above the critical level below which the uptake of \(^{211}\text{At}-\text{MTB} \) 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 \(^{211}\text{At}-\text{MTB} \) encouraged us to investigate \(^{211}\text{At}-\text{MTB} \) treatment of cutaneous melanoma tumors and concomitant lymph node metastases. These results will be reported in the forthcoming paper.

ACKNOWLEDGMENTS

We are indebted for the cooperation extended to us by Dr. M. Scott, Dr. K. Randle, and the Staff of both the Radiation Centre and Nuffield Cyclotron Facility, Department of Physics, University of Birmingham, and to Dr. I. Brown of the Royal Marsden Hospital, Sutton, Surrey, United Kingdom, for rendering the target from which astatine-211 was extracted. We are very grateful to S. Downey of the Department of Physics, University of Birmingham, and G. Hansen and J. Bigge of the Biological Services, University College and Middlesex School of Medicine, London, for their excellent assistance.

REFERENCES

At-Methylene Blue for Targeted Radiotherapy of Human Melanoma Xenografts: Treatment of Micrometastases

Eva M. Link and Robert N. Carpenter


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/50/10/2963

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/50/10/2963. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.