211\(^{\text{At}}\)-Methylene Blue for Targeted Radiotherapy of Human Melanoma Xenografts: Treatment of Cutaneous Tumors and Lymph Node Metastases\(^1\)

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ABSTRACT

The next stage of our preclinical investigations of targeted radiotherapy for melanoma with \(3,7\)-dimethylaminophenazathionium chloride [methylen blue (MTB)] labeled with \(211\text{At}\) (\(\alpha\) particle emitter) concerns the treatment of cutaneous tumors and their metastases. Fragments of two human melanoma xenografts, highly pigmented HX118 and poorly pigmented HX34, implanted s.c. into nude mice, were treated with four doses of \(211\text{At-MTB}\) injected i.v. The growth rate of cutaneous tumors and the appearance and size of their lymph node metastases served as criteria of treatment effectiveness.

\(211\text{At-MTB}\) inhibited the growth of cutaneous tumors in a manner dependent on their pigmentation and initial size. Highly pigmented smaller melanomas were affected by \(211\text{At-MTB}\) to a greater extent than poorly pigmented and larger ones. Growth of the smallest HX118 lesions investigated was completely inhibited for 65 days, whereas the growth inhibition of HX34 tumors of the same size lasted 7 days only.

\(211\text{At-MTB}\) exhibited similar pigmentation-dependent effects toward lymph node metastases. The size of metastatic lesions derived from HX118 xenografts never reached that in control animals during the period of observation, whereas those grown from HX34 xenografts attained control values after a 50-day delay.

The results demonstrated the capacity of \(211\text{At-MTB}\) to control the growth of cutaneous melanomas and their metastases.

INTRODUCTION

Melanoma belongs to the most malignant neoplasms resistant to all currently available therapeutic modalities. This, together with a progressive increase in the overall tumor incidence during the past 2 decades, urged a search for new methods of early detection and more effective therapy of the neoplasm.

Recently, targeted radiotherapy attained wide recognition, particularly for tumors with limited response to other treatments. Since the main principle of such therapy is a high and selective uptake of a radioisotope in the tissue of interest, the effectiveness of targeted radiotherapy depends on both a proper choice of a carrier for radioisotope(s) and a judicious selection of the radionuclide itself (1). Some physical properties of the radioisotopes such as high linear energy transfer and the short range of emitted radiation, as well as their relatively short half-life and safe daughter elements to which the radionuclide decays, are of particular importance (1).

These features dictated the choice of three radioisotopes, namely, \(35\text{S}\) (\(\beta\) emitter), \(125\text{I}\) (Auger electron emitter) and \(211\text{At}\) (\(\alpha\) particle emitter), for our investigations as potentially very effective in melanoma treatment when attached to methylene blue (2–4). A selection of the latter as a carrier for radioisotopes was dictated by the properties of melanoma. This neoplasm has a variable melanin pigmentation, but its nonpigmented form is uncommon. A stably localized melanin in the tumor cells offers an easily distinguishable target for a radioisotope’s carrier. MTB\(^3\) possesses exceptional affinity to melanin (5) and is taken up by pigmented melanomas much more avidly than by most normal tissues (2–4) while remaining totally nontoxic at the doses required for targeted radiotherapy (6). Since our initial studies revealed (3, 4) that the radioactivity of \(211\text{At-MTB}\) that accumulated in pigmented melanoma cells and is needed to diminish their survival below 4% was 2 orders of magnitude lower than those required of \(35\text{S-MTB}\) and \(125\text{I-MTB}\), the subsequent experiments were carried out using \(211\text{At-MTB}\) only and concerned the treatment of human melanomas grown as xenografts in nude mice.

The main difficulties in melanoma treatment are due to a wide dissemination of the neoplasm. Therefore, it was important to investigate whether \(211\text{At-MTB}\) could be used as a scavenger of single melanoma cells distributed through blood, as well as tumors below the limit of clinical detectability to prevent such metastatic spread. Since human melanoma secondaries are usually less pigmented than the primary lesions, a poorly pigmented human melanoma was used to determine whether a lesser \(211\text{At-MTB}\) uptake conditioned by a lower pigmentation of such cells will still be sufficient to result in tumoridal doses from the radioisotope accumulated. \(211\text{At-MTB}\) proved to be a very efficient scavenger of melanoma cells circulating with blood (7): a single i.v. injection of the radiolabeled compound diminished the number of lung colonies grown from these cells by more than 95%. Since in these circumstances \(211\text{At-MTB}\) effectiveness was remarkable, it appeared essential to investigate the capacity of \(211\text{At-MTB}\) to prevent the growth of metastases derived, on this occasion, from cutaneous tumors so as to mimic clinical conditions, and to explore to what extent the efficacy of \(211\text{At-MTB}\) will be sufficient to treat cutaneous tumors themselves. The initial results of these investigations are discussed in the present paper.

MATERIALS AND METHODS

Human Melanoma Xenografts. Two human melanoma xenografts (a highly pigmented HX118 and poorly pigmented HX34) were obtained by courtesy of Prof. G. G. Steel (Institute for Cancer Research, Sutton, Surrey, England). Both melanoma lines were established by J. Mills of the same institute, and their properties included the response to ionizing radiation described (8, 9). HX118 and HX34 were derived from biopsy samples of secondary lymph node deposits, the former from cervical lymph node, the latter from inguinal lymph node. The samples contained 2-mm\(^3\) tumor pieces frozen in 10% dimethyl sulfoxide at their fourth passage.

The material used in current experiments was obtained from tumors grown s.c. in nude mice and passaged every 3–4 weeks (HX34) or 5–6

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\(^3\) The abbreviation used is: MTB, methylene blue.
weeks (HX118) by transplanting 2–4 tumor fragments suspended in 0.2 ml of Ham's F12 medium (Flow Laboratories, Ltd., Irvine, Scotland) supplemented with 10% fetal bovine serum.

Transplantation of Tumor Fragments. HX118 and HX34 melanomas excised from mice were placed in Petri dishes containing Ham's F12 medium supplemented with 10% serum. The tumors (either HX118 or HX34) were cut into small pieces (approximately 1 mm²), and one such fragment suspended in 0.1 ml medium was implanted s.c. into recipient mice.

Experimental Animals. All experiments were carried out using 50–60-day-old female nude mice [Crl nu-nu CD 1/BR] supplied by Charles River, U.K. The animals were kept in sterile cages covered with sterile filters and fed with sterilized food and water. The experiments were carried out under sterile conditions using a laminar flow cabinet (Flow Laboratories, Ltd). Some s.c. inoculations of tumor fragments and all i.v. injections of radiolabeled methylene blue were performed on animals anesthetized with either fluorothan or pentobarbital sodium.

211At-Methylene Blue. 211At was produced at the Department of Physics, University of Birmingham, using a 28-MeV α particle external beam from the Nuffield 1.52-m cyclotron. Labeling of methylene blue with 211At was carried out according to the thermal halogen isotope exchange method described by us in detail elsewhere (10). In short, a rapid preparation of 4-[211At]astato-methylene blue has been obtained by heterogeneous (211At → I) halogen isotope exchange in a molten solution of the inert iodo-analogue in 18-crown-6-ether at 80–100°C. Maximum radiochemical yields of 72 ± 10% (SD) were achieved after 5–30 min, and sequential chromatographic analysis was used to characterize the radiohalogenated product. 211At-MTB was isolated from the reaction mixture by a positive-pressure anion-exchange chromatography: a specific activity of the final preparation exceeded 555 MBq/mg. The obtained eluant was concentrated in vacuum and, subsequently, diluted in a required aqueous solution. Due to a short 211At half-life of 7.2 h, 211At-MTB was prepared before each experiment unless the injection was repeated after a 12-h interval, for which the radiolabeled compound from previous day was used. 211At-MTB was diluted in phosphate-buffered saline to a required radioactivity per unit volume, and 0.1–0.15 ml was injected i.v. into one of the tail veins of melanoma-bearing mice.

Determination of Therapeutic Effectiveness of 211At-MTB. Therapeutic effects of 211At-MTB were assessed in 23 mice (2 inguinal tumors/mouse) for both cutaneous lesions and lymph node metastases. Cutaneous melanomas, each grown from one tumor fragment of HX118 or HX34 implanted s.c., were treated with 211At-MTB injected i.v. The injections were started 10 days after tumor implantation and were continued on days 11, 17, and 18, with the consecutive doses amounting to 6, 3.7, 6.6, and 3.7 MBq, respectively. The choice of 211At-MTB dosages used was dictated by our previous calculations concerning tumoricidal doses from this astatinated compound deposited in pigmented tumors (4) and biodistribution studies (Ref. 2 and current data). An average of 3% of radiolabeled MTB injected i.v. is accumulated in a gram of pigmented cutaneous melanoma in nude mice of 25 g in weight. Since approximately 700 kBq of 211At-MTB/g of the tumor (if distributed homogenously) is required to diminish cell survival within the lesion to below 4%, 24 MBq of 211At-MTB should be administered i.v. to cause almost complete remission of the tumor. However, the above radioactivity seemed too high to be administered as a single injection, although parallel calculations concerning radiation doses expected in organs such as the liver or eyes, i.e., at a particular risk from the administered radioisotope, predicted their good tolerance for 211At-MTB treatment (see Ref. 4 for details). The required dose was split, therefore, into two fractions of approximately 10 MBq, and each fraction was given in two portions with a 12-h interval between them to diminish further radiation exposure to normal organs. Since, unexpectedly, pilot studies revealed that five fractions applied every 48 h were at best as effective as two injections of 211At-MTB given at 5–6-day intervals (despite the fact that the five fractions delivered about twice the total radioactivity of the two injections), a regimen with a 6-day interval between fractions was chosen. [Various patterns of 211At-MTB fractionations together with the mechanism underlying tumor response to them will be discussed in detail in a separate paper.]4

The rate of tumor growth prior to and after 211At-MTB injection, as well as the size of lymph node metastases, served as criteria of 211At-MTB therapeutic effectiveness.

The size of cutaneous tumors was determined by caliper measurements of three tumor diameters, the greatest one (a) and the two perpendicular to it (b and c), and was expressed as the mean diameter (d) calculated from the equation

\[ d = \sqrt{abc} \]

which is proportional to the tumor growth rate constant (k) and time interval between tumor growth initiation (t₀) and the measurement (t) as follows:

\[ d = k(t - t₀) \]

For details see Schreck (11).

The therapeutic effects of 211At-MTB on the growth of lymph node metastases were estimated by a caliper measurement of the mean size of front and hind limb lymph nodes rather than of the metastases themselves due to their small size and difficulties in distinguishing precisely between normal and malignant tissue, particularly with less pigmented or nonpigmented lesions. Lymph nodes possess a very regular, ellipsoidal shape, but their thickness is usually at the level of the instrumental error. Therefore, the node size was calculated from two reliably measurable diameters according to the equation

\[ 1 \over 4 \pi xy \]

where x is the greatest diameter and y is the diameter perpendicular to x. The measurements were carried out at the time of sacrifice, and some of the lymph nodes were preserved in formal saline for further examination.

We have shown previously that cold methylene blue at the concentrations used in the present experiments does not interfere with the growth of melanoma either in vitro or in vivo (3). Free 211At~ was therapeutically ineffective against melanoma cells as shown by lung colony assay (an experiment in which a suspension of single melanoma cells exposed in vitro to either 211At-MTB or 211At~ was injected i.v. into nude mice) (4), whereas at doses 2.5–4.5 times lower than those used for the in vivo treatment with 211At-MTB, it was toxic or even lethal to mice (12). Therefore, the control tumor-bearing animals could not be injected with high enough doses of 211At~ and were given none.

Development of Vascular System in Melanoma Xenografts. HX118 and HX34 human melanomas were implanted s.c. into nude mice (2 inguinal tumors/animal). Subsequently, two mice were chosen randomly every 24 h up to 216 h (9 days) and sacrificed, and the excised tumors were fixed in 10% formal saline. Afterward, the tissue was subjected to routine procedures of paraffin wax embedding, cutting, and eosin-hematoxylin staining (13). The slides were examined in the light microscope, and a time-dependent development of the vasculature in every tumorlet was investigated. Representative specimens derived from both melanoma xenografts were photographed using ×100 magnification.

RESULTS

Therapeutic Effectiveness of 211At-MTB

Cutaneous Tumors. The rate of growth of cutaneous tumors was significantly affected by 211At-MTB injected i.v. into one of the...
At-Methylene Blue For Human Melanoma Treatment

Highly Pigmented HX118

Fig. 1. Growth rate of cutaneous tumors and lymph node metastases derived from highly pigmented HX118 melanoma xenografts (A) or poorly pigmented HX34 melanoma xenografts (B), treated with 211At-MTB (○, □) or without treatment (●). The total 211At-MTB dose of 20 MBq was applied in 4 fractions of 6, 3.7, 6.6, and 3.7 MBq, respectively, at times indicated (arrows). Bars, SD.

POORLY PIGMENTED HX34

Development of Vascular System in Melanoma Xenografts

A selective uptake of radiolabeled methylene blue in pigmented melanomas is sometimes suggested to be due to a possible quantitative difference in the vasculature of the melanotic versus the nonpigmented form of the neoplasm rather than to an elevated pigmentation of the tumor.

Histological studies carried out on a highly pigmented HX118 and poorly pigmented HX34 human melanoma xenografts revealed an immediate development of the vascular system in poorly pigmented lesions; a dense network of vessels surrounded the tumor 48 h after its s.c. implantation, and single capillaries were found in the lesions at the same time (Fig. 3A), progressively increasing in number afterward (Fig. 3B).

The vascularization process in highly pigmented melanoma was delayed as compared to its poorly pigmented variety. First vessels within the tumor were observed 96 h after its s.c. implantation and, in contrast to the poorly pigmented lesions, their appearance was not preceded by a growth of the vasculature around the neoplastic tissue (Fig. 3C). Such "peripheral" vessels were found a few days later, but these were linked
Fig. 2. Differences in tumor size observed after treatment with $^{211}$At-MTB (A and C) as compared to controls (B and D) and dependence of the treatment effectiveness on tumor pigmentation. The photographs have been taken 48 days after s.c. melanoma implantation and 30 days after completion of treatment.

Table 1  Dependence of therapeutic effectiveness of $^{211}$At-MTB on pigmentation and size of cutaneous melanoma lesions

<table>
<thead>
<tr>
<th>Mean diameter of tumor (mm)</th>
<th>Highly pigmented HX118</th>
<th>Poorly pigmented HX34</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 days (first treatment)</td>
<td>23 days</td>
<td>45 days</td>
</tr>
<tr>
<td>Control</td>
<td>0.6</td>
<td>1.7</td>
</tr>
<tr>
<td>$^{211}$At-MTB</td>
<td>0.5</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>$^{211}$At-MTB</td>
<td>$P &lt; 0.01$</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>0.9</td>
</tr>
<tr>
<td>Control</td>
<td>1.0</td>
<td>4.5</td>
</tr>
<tr>
<td>$^{211}$At-MTB</td>
<td>1.0</td>
<td>2.2</td>
</tr>
</tbody>
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* $P$, Student's t test probability of no difference.

exclusively to melanoma cells infiltrating the surrounding normal tissues or, alternatively, were encircled by pigment-laden phagocytes. It should be emphasized that the neoplastic infiltration began immediately after tumor implantation and was observed only for highly pigmented lesions. The number of vessels within HX118 tumors increased progressively with time and, although comparatively delayed, proceeded in a manner similar to that observed for poorly pigmented melanoma (Fig. 3D).

Side Effects from $^{211}$At-MTB Treatment

Detailed light and electron microscope investigations concerning $^{211}$At-MTB effects on tissues other than melanoma are in progress to establish whether astatinated MTB at the optimal therapeutic doses could induce significant side effects in normal organs. However, a long-term (at least 7 months) careful observation of melanoma-bearing mice successfully treated with $^{211}$At-MTB and healthy animals that received injections of the...
compound as a control did not indicate any toxicity which would influence their life span or cause a loss of weight and other noticeable changes. Subsequent necropsies did not reveal any macroscopic organ damage.

**DISCUSSION**

Our long-term investigations are aimed at establishing whether methylene blue labeled with a suitable radioisotope(s) could be used in the clinic for the diagnosis and treatment of malignant melanomas. The potential ability of $^{211}$At-methylene blue in controlling the metastatic spread of melanoma and thus overcoming the most difficult aspect in the treatment of this neoplasm is of a particular interest to us.

The results presented here are a continuation of previous studies (7) in which $^{211}$At-methylene blue was shown to be a very effective scavenger of melanoma cells circulating with blood and prevented the development of lung and other metastases. In the present investigations clinical conditions were imitated more closely than previously (7); since HX118 and HX34 human melanoma xenografts disseminate to lymph nodes and other organs in addition to producing cutaneous tumors, the metastases derive from primary tumors implanted s.c., while under the former conditions lung and other secondaries developed directly from cells injected i.v. It was, therefore, possible to examine presently whether $^{211}$At-MTB could control the growth of cutaneous lesions, as well as their lymph node metastases.

The results demonstrate the therapeutic activity of $^{211}$At-MTB against both cutaneous melanomas and lymph node metastases derived from these primary tumors. The magnitude of $^{211}$At-MTB effects depends significantly on the pigmentation and size of the treated lesions with considerably better results for smaller and more pigmented tumors. It is evident that the growth of highly pigmented cutaneous melanomas with their mean diameter of 0.5–0.6 mm (which corresponds to the ellipsoidal lesion of approximately 2 mm x 1 mm x 0.1 mm) could be irreversibly inhibited by $^{211}$At-MTB, provided the fractionated treatment is applied during a specific period of time. However, $^{211}$At-MTB injections must be properly adjusted to the tumor age, at least in the animal model system. More specifically, an initiation of the treatment immediately after s.c. implantation of the tumor (i.e., within 24 h) does not increase the efficacy, i.e., there is no difference in the overall effects of $^{211}$At-MTB depending on whether such an early dose is or is not given. These and our previous (7) observations suggest that the significant efficiency of the treatment depends critically upon the effective distribution of $^{211}$At-MTB within the tumor, presumably conditioned by a fully developed vascular system in the lesion. The 48–96-h interval between s.c. implantation of melanoma xenografts and an appearance of the first vessels in the implant confirms the conclusion. At the same time, histological studies reveal quantitative similarities in the vasculature of both investigated melanoma subtypes except for the delay in the development of the vascular system in highly pigmented.
xenografts as compared with their poorly pigmented counterparts. This additionally argues for pigmentation as a critical factor underlying the significant effectiveness of $^{211}$At-methylene blue against HX118 melanoma.

Particularly important results concern the remarkable effectiveness of $^{211}$At-MTB against lymph node metastases which, in humans, are most common and appear usually almost concomitantly with the primary lesion. $^{211}$At-MTB significantly interferes with the growth of such metastases even in the absence of the major growth inhibition of cutaneous tumors. This finding could be incorrectly accounted for by a high affinity of either methylene blue or $^{211}$At for lymph nodes (12) rather than $^{211}$At-MTB affinity for pigmented melanoma cells deposited in the organ. However, if this were the case, $^{211}$At-MTB would affect lymph node metastases derived from highly pigmented HX118 and poorly pigmented HX34 cutaneous tumors at least equally, since the treatment was the same for both and, additionally, less pigmented melanomas are more sensitive to ionizing radiation than more pigmented tumors (14). The considerable differences in the $^{211}$At-MTB effects obtained, with much better results for metastases derived from highly pigmented than for poorly pigmented cutaneous tumors, lead to the conclusion that the effects of $^{211}$At-MTB are direct and pigmentation dependent.

The above considerations exclude also an involvement of free $^{211}$At− in the pigment-dependent effectiveness of $^{211}$At-MTB treatment of cutaneous tumors. Our biodistribution and autoradiographic studies (2–4) revealed a selective uptake of radio labeled MTB in highly pigmented melanomas and its negligible accumulation in amelanotic form of the neoplasm regardless of the radioisotope attached to methylene blue. A very low uptake of the radioisotopes themselves was equal in pigmented and nonpigmented melanomas (4). An association between the pigment-dependent effectiveness of $^{211}$At-MTB treatment and the pigment-dependent uptake of the radiolabeled MTB, as well as the lack of therapeutic efficacy of free $^{211}$At− for both forms of melanoma, which coincides with their negligible uptake of the radioisotope (4), exclude a contribution of $^{211}$At in its free form to the observed pigment-dependent therapeutic effects of $^{211}$At-MTB. A 70-day maximum tolerated dose of 1.5 MBq $^{211}$At−/mouse (12), i.e., a dose 2.5–4.5 times lower than that applied as a single fraction of $^{211}$At-MTB, prevented an introduction of $^{211}$At− control in vivo. However, since the observation time of animals after the treatment was limited exclusively by the tumor size or its ulceration, but not by the systemic toxicity of the injected radiolabeled compound, and lasted for several months in the case of successfully treated animals, the disappearance of the radiotoxicity of $^{211}$At upon binding to methylene blue argues further for a carrier-dependent therapeutic effectiveness of $^{211}$At for pigmented melanomas and, additionally, for a lack of substantial metabolic dehalogenation of $^{211}$At-MTB.

More analytical investigations concerning the efficacy of $^{211}$At-MTB treatment for cutaneous and lymph node melanoma lesions will be reported in a separate paper. However, the data presented here already demonstrate that the action of this radioactive compound is not limited to scavenging single melanoma cells circulating with blood (see previous paper, Ref. 7): $^{211}$At-MTB also affects solid tumors and, furthermore, combines the features of inhibiting growth of both cutaneous tumors and their regional metastases. Studies of normal tissues using light and electron microscopy carried out at present will enable a detailed assessment of side effects caused by $^{211}$At-MTB. The biodistribution of radiolabeled MTB (2) pointed at the liver and eyes as organs which could be at a potential risk from $^{211}$At-MTB administered systemically. However, subsequent calculations of radiation doses from $^{211}$At-MTB deposited in pigmented tumors and corresponding doses expected in normal organs indicated a good tolerance of the latter to $^{211}$At-MTB at a time of tumor exposure to its lethal dose of 30 Gy (see Ref. 4 for details). Indeed, long-term observations (200 days or more) of the animals successfully treated with $^{211}$At-MTB and subsequent careful necropsies did not disclose any functional impairment or macroscopic organ damage.

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