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## Original Paper

# [$^{211}$At]Methylene Blue for Targeted Radiotherapy of Human Melanoma Xenografts: Dose Fractionation in the Treatment of Cutaneous Tumours

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**3,7-(dimethylamino) phenazathionium chloride [methylene blue (MTB)] labelled with α-particle emitter astatine-211 ($^{211}$At) selectively accumulates in melanoma cells due to an exceptionally high affinity of MTB to melanin, and proves to be a very effective agent in targeted radiotherapy for pigmented human melanoma grown in mice. This study aimed at a selection of the most advantageous [$^{211}$At]MTB dose fractionation leading to irreversible regression of the treated lesions. Nude mice bearing subcutaneous human melanoma xenografts of either highly pigmented HX118 or poorly pigmented HX34 human melanoma were treated with [$^{211}$At]MTB administered intravenously. The treatment was performed using three different schedules of [$^{211}$At]MTB fractionation: a single large dose, five fractions delivered sequentially every 48 h and two to five fractions given with a mean frequency of one per week. The effectiveness of [$^{211}$At]MTB treatment was assessed by determination of the growth rate of cutaneous tumours and length of time between tumour implantation and killing of moribund mice. [$^{211}$At]MTB applied with a mean frequency of one fraction per week appeared to be the most efficient treatment for highly pigmented HX118 melanomas. Its effectiveness was dependent on [$^{211}$At]MTB activity used per fraction and the size of the cutaneous tumours at the beginning of the treatment. A total dose of [$^{211}$At]MTB seemed of less importance. An irreversible regression of the lesions was achieved. Poorly pigmented cutaneous melanoma xenografts were affected most significantly by [$^{211}$At]MTB applied as five fractions given every 48 h. The treatment caused a temporary inhibition of tumour growth after which the lesions regained the control growth rate. These and previous results suggest that [$^{211}$At]MTB could successfully control the growth of already formed lesions of pigmented melanoma, as well as prevent metastatic spread of the tumour, provided an appropriate fractionation régime of the radiolabelled compound is employed.** 

**Key words: melanoma, [$^{211}$At]methylene blue, targeted radiotherapy, α-particles, fractionation**
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## INTRODUCTION

MELANOMA is a skin cancer characterised by various levels of pigmentation, but with its entirely non-pigmented form very uncommon. This particular malignancy is often manifested by wide dissemination almost concomitant with the appearance of the primary tumour, as well as the notorious resistance of melanoma to all presently available therapeutic modalities. Thus, a search for new methods of effective diagnosis and treatment of the primary tumours and their metastases, extending to very early stages of their development, is justified.

The availability of a wide range of radioisotopes form the basis of nuclear medicine in which an *in vivo* tracing of tissues of interest has become possible provided that selective uptake of the appropriate radioisotope can be achieved. Selectivity is similarly the main problem in targeted radiotherapy of melanoma since neither the already investigated carriers for radioisotopes nor radionuclides themselves exhibit exclusive affinity for this neoplasm. Although phosphorus-32 ($^{32}$P) and gallium-67 ($^{67}$Ga) have attracted some interest as potential

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diagnostic radionuclides for melanomas [1, 2], both were eliminated from the routine screening of melanoma patients due to their limited diagnostic value.

Since none of therapeutically useful radioisotopes exhibit a specific affinity for melanomas, the use of an appropriate carrier is indispensable. Four classes of such carriers can be distinguished among those already investigated: (1) compounds with a high affinity to melanin [3–10]; (2) compounds which can serve as melanin precursors [11, 12]; (3) polyclonal or, more recently, monoclonal antibodies [13, 14]; and (4) melanocyte stimulating hormone (MSH) [15].

Currently, most advanced investigations concern clinical diagnosis of disseminated melanoma with radiolabelled monoclonal antibodies and MSH, boron neutron capture therapy of superficial, well localised tumours using [$^{10}$B]phenylalanine, and targeted radiotherapy of disseminated melanoma with [$^{211}$At]methylene blue (MTB). Boron neutron capture therapy attracts the greatest interest since the first clinical treatment of an inoperable melanoma on the left occiput with [$^{10}$B]para-boronophenylalanine hydrochloride, a melanin precursor, proved to be very successful [16]. Although neutron capture therapy is limited to superficial and well localised melanomas due to the poor penetration of thermal neutrons used and co-induction of $\gamma$-rays, some progress is being made to overcome these obstacles [17].

The radiolabelled compound for targeted radiotherapy which proves to be particularly suitable for disseminated melanoma is [$^{211}$At]MTB. The dye exhibits an exceptional affinity to melanin [10], and is taken up by pigmented melanomas grown in human and animal hosts much more avidly than by most normal tissues [18–22], while remaining totally non-toxic at the doses required for targeted radiotherapy [23]. Non-pigmented melanoma has neither affinity to MTB nor to radioisotopes used for labelling MTB in our studies [18, 21]. [$^{211}$At]MTB was selected from three radio-analogues of this compound, namely, $\beta$-emitting [$^{35}$S]MTB [20], Auger electron-emitting [$^{125}$I]MTB [21] and $\alpha$-emitting [$^{211}$At]MTB [21], since the therapeutic efficacy of the latter, as investigated both *in vitro* and *in vivo*, exceeded that found with the former two radio-analogues by two orders of magnitude [20, 21, 24]. $^{211}$At is an $\alpha$-particle emitter; the features of its densely ionising radiation consist of the optimal LET of almost 100 keV/$\mu$m at which oxygen enhancement ratio (OER) of approximately 1 and maximum relative biological effectiveness (RBE) are achieved, a short half-life of 7.2 h and, similarly to activated $^{10}$B, a short mean range of 60–65 $\mu$m in tissue which corresponds to three to six cell diameters. [$^{211}$At]MTB shows its exceptional effectiveness in scavenging single blood-circulating melanoma cells, even those poorly pigmented [24, 25]. This astatinated compound also manifests its therapeutic properties towards solid melanoma lesions, which significantly depend on the tumour pigmentation and size at the time of treatment [25]. These results encouraged more detailed investigations which would enable a selection of the most advantageous mode of [$^{211}$At]MTB application to melanoma. The aim of the present studies is, therefore, to analyse the effectiveness of treatment of solid tumours using various fractionation régimes and doses of [$^{211}$At]MTB. Data presented in a previous paper [25] provided strong evidence for the correlation between the effectiveness of [$^{211}$At]MTB treatment and the initial size of cutaneous tumours for a highly pigmented melanoma. This is investigated here in greater detail by analysing the influence of both the initial size of cutaneous melanoma and different fractionation of [$^{211}$At]MTB on the final outcome of the treatment. A comparison is made between highly pigmented HX118 and poorly pigmented HX34 melanomas to confirm the requirement of melanin presence in the tumour for curative effects from [$^{211}$At]MTB.

## MATERIALS AND METHODS

*Human melanoma*

Two human melanoma xenografts were obtained courtesy of Professor G.G. Steel of the Institute for Cancer Research, Sutton, U.K. after being established by J. Mills of the same Institute. Both highly pigmented HX118 and poorly pigmented HX34 were derived from biopsy samples of secondary lymph node deposits of patients who had not undergone cytotoxic therapy prior to the biopsy, and properties of these xenografts, including their response to ionising radiation, have been described in detail [26, 27].

The material subsequently used for experiments was obtained from tumours grown subcutaneously (s.c.) in nude female mice. The tumours were passaged *in vivo* every 3–4 weeks (HX34) or 5–6 weeks (HX118) by transplanting 2–4 tumour fragments suspended in 0.2 ml of Ham's F12 medium supplemented with 10% fetal bovine serum (FBS; both from Flow Laboratories, Ltd., Irvine, U.K.).

*Subcutaneous transplantation of tumours*

Small tumour fragments of either HX118 or HX34 melanoma, excised from mice, were suspended in Ham's F12 medium supplemented with 10% FBS; 0.1 ml medium containing fragments of approximately 1 mm$^3$ was injected s.c. into a recipient mouse to initiate tumour growth. Every experimental animal presented two cutaneous inguinal lesions. Neither a rejection of implanted tissue nor a spontaneous regression of cutaneous tumours was observed.

*Experimental animals*

Female nude mice 50–60 days old [Crl:nu-nu (CD/I$^{TM}$)BR] supplied by Charles River, U.K. were used for all experiments. The animals were kept in sterile cages covered with sterile filters and fed with sterilised food and water. Every procedure was carried under sterile conditions using a laminar flow cabinet (Flow Laboratories, Ltd.). Some s.c. inoculations of tumour fragments and all intravenous (i.v.) injections of radiolabelled MTB were performed on mice anaesthetised with either fluothan or pentobarbitone sodium.

[$^{211}$At]*Methylene blue*

Astatine-211 production and the synthesis of [$^{211}$At]astatomethylene blue ([$^{211}$At]MTB) were carried out as previously [21, 24, 25] in the Department of Physics, University of Birmingham, using 28-MeV $\alpha$-particle external beam from the Nuffield 1.52 m cyclotron. Due to a short astatine-211 half-life of 7.2 h, an aqueous solution of [$^{211}$At]MTB with a specific activity of at least 555 MBq/mg was prepared before each experiment unless the injection was repeated after a 12 h interval, for which the radiolabelled compound from the previous day was used. [$^{211}$At]MTB was diluted in phosphate-buffered saline to the desired radioactivity per unit volume, and 0.1–0.15 ml was injected i.v. into one of the tail veins of melanoma-bearing mice.

*Treatment with* [$^{211}$At]*MTB*

[$^{211}$At]MTB was administered at various times after tumour inoculation into mice, with a minimum interval of 6 days. The fractionation régime varied from a single dose to five fractions applied with a different frequency and dose per fraction. Detailed values of [$^{211}$At]MTB radioactivity injected per fraction and of a total [$^{211}$At]MTB radioactivity used for the particular treatment are shown in the Results section. To avoid the loss of radioactivity with time without changing experimental conditions ($t_{1/2}$ of $^{211}$At = 7.2 h) every variant of [$^{211}$At]MTB treatment was repeatedly performed in very small groups of two to three animals (two inguinal tumours/mouse) and investigated in a total of 8–12 animals. A relevant control group consisted of 6–10 mice (two inguinal tumours/mouse). We have previously shown that free $^{211}$At$^-$ is therapeutically ineffective against melanoma cells as determined by lung colony assay (an experiment in which a suspension of single melanoma cells exposed *in vitro* to either $^{211}$At$^-$ or $^{211}$At-MTB was injected i.v. into mice) [21], whereas at doses 2–5.3 times lower than those used as a single injection for *in vivo* treatment with [$^{211}$At]MTB, $^{211}$At$^-$ was toxic or even lethal to mice [28]. Therefore, the control animals could not be injected with high enough doses of $^{211}$At$^-$ and were given none.

*Therapeutic effectiveness of* [$^{211}$At]*MTB*

The effectiveness of [$^{211}$At]MTB treatment was assessed by determination of the growth rate of cutaneous tumours.

The rate of growth of cutaneous tumours was determined by calculating a ratio of tumour size at time-intervals after [$^{211}$At]MTB injection to a size of the tumour at the time of the introduction of the treatment. Subsequently, all curves were normalised to day 10 after tumour inoculation into mice to enable their comparison. The size of the lesions was calculated as previously described [20, 25], from repeated caliper measurement of three diameters of the tumour: the greatest one and the two perpendicular to it. The thickness of the skin in the close proximity of the lesion was determined in every mouse and subtracted from the thickness of the tumour. The measurements were initiated before an introduction of the treatment to ensure that the precise size of the tumour was known at the time of first [$^{211}$At]MTB injection.

In addition, a careful examination during the dissection of all experimental animals was carried out to detect metastases which could appear in other organs. Some of the organs were preserved in 10% formal saline for further studies.

*Statistical analysis and curve fitting*

Significance of differences between growth rate of control tumours and those treated with [$^{211}$At]MTB, as well as between tumours treated with various fractionation régimes was calculated using the Student *t*-test in which slopes of the relevant curves were compared [29]. The lines in all Figures were drawn directly through experimental points using computer software Microsoft Excel.

## RESULTS

Cutaneous lesions with various initial sizes respond differently to a given [$^{211}$At]MTB treatment, with smaller tumours being more sensitive to [$^{211}$At]MTB than the larger ones [25]. Therapeutic effectiveness of various modes of [$^{211}$At]MTB fractionation was, therefore, investigated in two groups of lesions as selected from the previous studies: (1) small tumours (approximately 2 mm × 1 mm perpendicular diameters and 0.2 mm thickness) affected by [$^{211}$At]MTB most profoundly, and (2) lesions 4–6 times larger (with a less pronounced response to [$^{211}$At]MTB).

The treatment was performed using three different schemes of [$^{211}$At]MTB fractionation, namely, a single dose of 7.9 MBq, five fractions delivered sequentially every 48 h resulting in total radioactivity of 35 MBq and, as a third mode of the treatment, two to five fractions given with the mean frequency of one per week and, occasionally, complemented by additional doses applied approximately 12 h after the previous one. Using the last régime, the total [$^{211}$At]MTB radioactivity administered amounted to 18–27 MBq (for details see Figures 2–6). The choice of [$^{211}$At]MTB radioactivity used

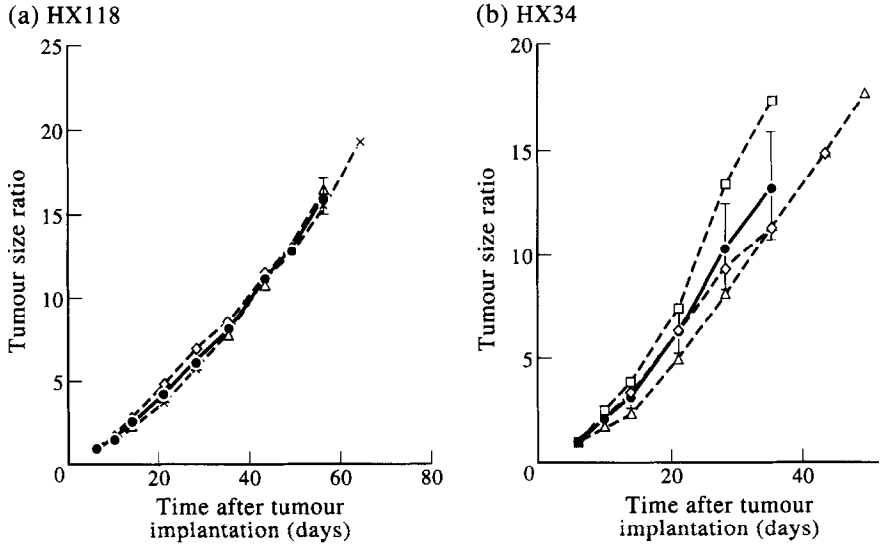


**Figure 1. Comparison of growth rates of control tumours characterised by various initial sizes (as measured on day 6 after their subcutaneous implantation). (a) HX118 melanoma: x 0.7 × 0.3 mm in perpendicular diameter and 0.1 mm thick; △ 1.9 × 1.4 mm in diameter and 0.2 mm thick; ◇ 3.1 × 1.1 mm in diameter and 0.5 mm thick; ● mean values. (b) HX34 melanoma: △ 1.1 × 1 mm in perpendicular diameter and 0.1 mm thick; ◇ 2.2 × 1.7 mm in diameter and 0.2 mm thick; □ 2.7 × 2.0 mm in diameter and 0.4 mm thick; ● mean values.**

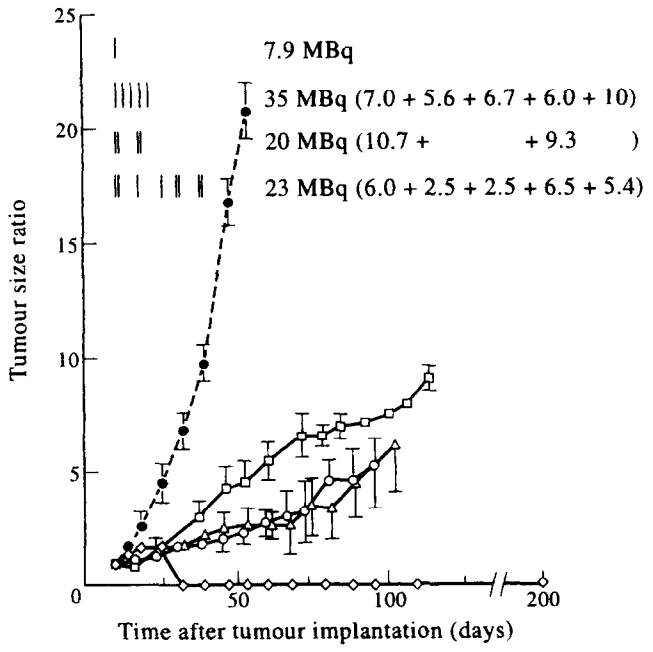


**Figure 2. Highly pigmented HX118 human melanoma xenografts grown in nude mice. A growth rate of small cutaneous tumours (approximately 2 × 1 mm in perpendicular diameters and 0.2 mm thick) after i.v. administration(s) of [211At]MTB given as a single injection of 7.9 MBq (□), 35 MBq in five fractions applied at 48 h intervals (△), 20 MBq in two fractions given with 6 days interval (○) and 23 MBq in five fractions applied as indicated by strokes (◇). ●, untreated tumours. Curves are normalised to day 10 after tumour implantation. ±S.D. bars are shown for all experimental points for which error bars exceed the size of symbols used. A time-dependent schedule of the treatment specifying total [211At]MTB doses and [211At]MTB doses used per fraction is shown at the top of the graph. Mean size of tumour at the time of mice culling was approximately 1.05 × 0.7 cm in perpendicular diameter and 0.4 cm thick (□), 0.55 × 0.6 cm in diameter and 0.25 cm thick (△), 0.6 × 0.5 cm in diameter and 0.3 cm thick (○), non-measurable pigmented stain without thickness (◇).**

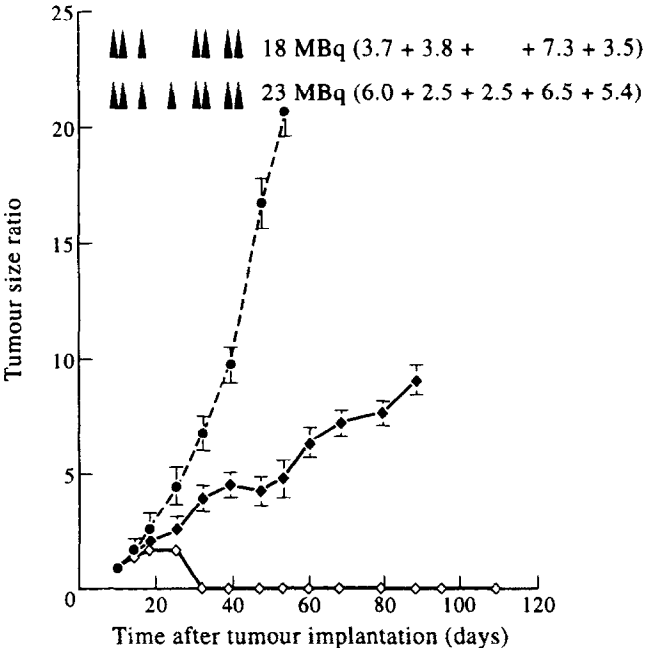


**Figure 3. Highly pigmented HX118 human melanoma xenografts grown in nude mice. A growth rate of small cutaneous tumours (approximately 2 × 1 mm in perpendicular diameter and 0.2 mm thick) after i.v. injection of either 18 MBq (◆) or 23 MBq (◇) of [211At]MTB given in four or five fractions, respectively, applied as indicated by arrowheads. ●, untreated tumours. Curves are normalised to day 10 after tumour implantation. ±S.D. bars are shown for all experimental points for which error bars exceed the size of symbols used. A time-dependent schedule of the treatment specifying total [211At]MTB doses and [211At]MTB doses used per fraction is shown at the top of the graph. Mean size of tumour at the time of mice culling was approximately 0.9 × 0.7 cm in perpendicular diameter and 0.55 cm thick (◆), non-measurable pigmented stain without thickness (◇).**

was dictated by our previous calculations concerning tumoricidal doses from this astatinated compound deposited in pigmented tumours [21] and biodistribution studies [18, 22]. An average of 3% of radiolabelled MTB injected i.v. is accumulated in a gramme of pigmented cutaneous melanoma in nude mice of 25 g in weight. Since approximately 700 kBq of [211At]MTB/g of the tumour (if distributed homogeneously) is required to reduce 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 tumour. However, the above radioactivity seemed too high to be given as a single injection, although parallel calculations concerning radiation doses expected in normal organs at a particular risk from the administered radioisotope predicted good tolerance of such treatment (see ref. [21] for details). The required [211At]MTB dose was therefore split into fractions with various doses per fraction and various fractionation régimes so as to choose the most advantageous course of treatment.

### 1. *Growth rate of untreated HX118 and HX34 cutaneous melanoma xenografts*

The growth rate of control cutaneous melanomas was dependent on their pigmentation, but remained similar between the subgroups regardless of their size at first measurement ($P > 0.1$; Figure 1).

### 2. *Growth rate of [211At]MTB-treated highly pigmented HX118 human melanoma xenografts*

*Small Tumours.*

*Single dose.* A single high dose of [211At]MTB (7.9 MBq) caused an immediate, temporary inhibition of tumour growth lasting 7 days (Figure 2). A subsequent growth rate of lesions so treated was irreversibly lowered to approximately 50% of control values ($P < 0.02$).

*Five fractions applied at 48 h intervals.* An initial lag phase followed by partial inhibition of tumour growth was observed after applying five fractions delivered every 48 h (total dose: 35 MBq) (Figure 2). The treatment lowered tumour growth rate by 75% as compared to controls ($P < 0.02$), i.e. only 25% more efficiently than a single dose of 7.9 MBq. The effect, therefore, was not proportional to the total dose of [211At]MTB administered.

*Two fractions applied at 6 days interval.* The response of lesions exposed to two fractions of [211At]MTB given with a 6 day interval between them was identical with that of tumours treated with five fractions applied every 48 h ($P > 0.1$), although the total dose of [211At]MTB administered was significantly lower and amounted to 20 MBq (Figure 2).

The equal response of tumours treated with two different modes of [211At]MTB fractionation (i.e. two fractions with a 6 day interval between them and five fractions applied every

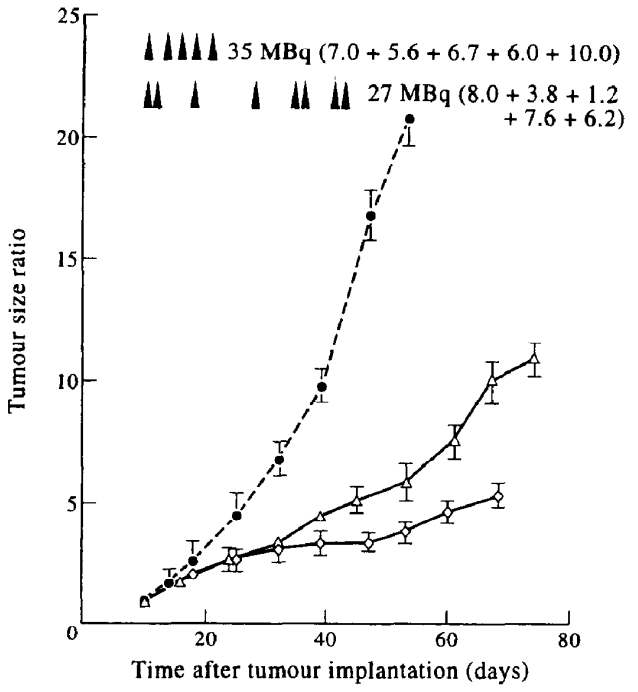


**Figure 4. Highly pigmented HX118 human melanoma xenografts grown in nude mice. A growth rate of larger cutaneous tumours (approximately 2 × 2.2 mm in perpendicular diameter and 0.4 mm thick) after i.v. injection of 35 MBq [$^{211}$At]MTB given in five fractions applied at 48 h intervals (△) and 27 MBq in five fractions applied as indicated by arrow heads (◇). ●, untreated tumours. Curves are normalised to day 10 after tumour implantation. ±S.D. bars are shown for all experimental points for which error bars exceed the size of symbols used. A time-dependent schedule of the treatment specifying total [$^{211}$At]MTB doses and [$^{211}$At]MTB doses used per fraction is shown at the top of the graph. Mean size of tumour at the time of mice culling was approximately 1.4 × 1.3 cm in perpendicular diameter and 1 cm thick (△), 0.85 × 0.8 cm in diameter and 0.5 cm thick (◇).**

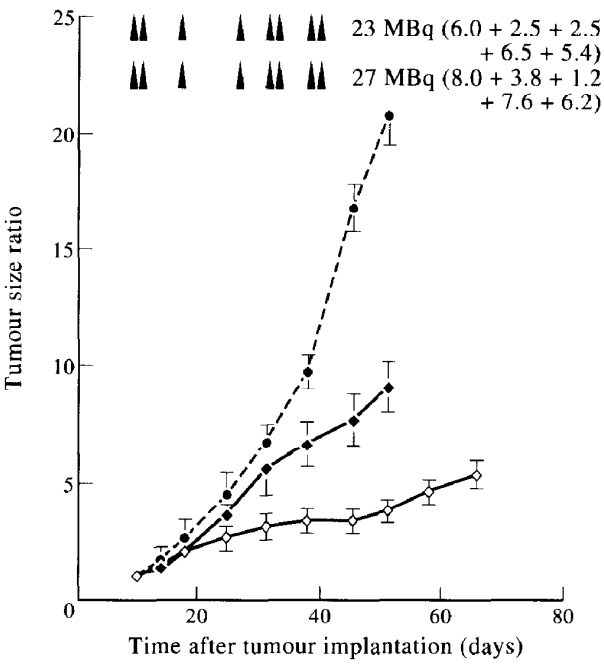


**Figure 5. Highly pigmented HX118 human melanoma xenografts grown in nude mice. A growth rate of larger cutaneous tumours (approximately 2 × 1.95 mm in perpendicular diameter and 0.4 mm thick) after i.v. injection of either 23 MBq (◆) or 27 MBq (◇) of [$^{211}$At]MTB in five fractions applied as indicated by arrow heads. ●, untreated tumours. Curves are normalised to day 10 after tumour implantation. ±S.D. bars are shown for all experimental points for which error bars exceed the size of symbols used. A time-dependent schedule of the treatment specifying total [$^{211}$At]MTB doses and [$^{211}$At]MTB doses used per fraction is shown at the top of the graph. Mean size of tumours at the time of mice culling was approximately 1.2 × 0.9 cm in perpendicular diameter and 0.4 cm thick (◆), 0.85 × 0.8 cm in diameter and 0.5 cm thick (◇).**

48 h) indicates that only the first and penultimate, given 6 days later, of five frequent fractions led to a significant damage to the lesions. Therefore, the effectiveness of five [$^{211}$At]MTB fractions applied at 6 day intervals was investigated.

*Five fractions applied at 6 day intervals.* [$^{211}$At]MTB treatment, in which fractions were administered with a mean frequency of one per week, proved to be the most effective for pigmented tumours, leading to the irreversible regression of the lesions (Figure 2). The employment of this fractionation scheme resulted in a gradual reduction of the tumour growth rate, progressing with every consecutive fraction of [$^{211}$At]MTB provided the time-interval between them was not greater than 10 days (Figure 3). An initial dose of 6 MBq delivered either once or, alternatively, as two fractions applied at less than 12 h interval, proved to be more effective than 3.7 MBq in initiating a significant ($P < 0.001$) inhibition of tumour growth (Figure 3).

The final outcome of [$^{211}$At]MTB treatment of pigmented melanoma was, therefore, dependent on [$^{211}$At]MTB dose per fraction and the time-interval between them (Figure 3). The frequency of delivery was discovered to be the predominant factor in achieving the maximum therapeutic efficacy, with an optimum time-interval between fractions of 5–7 days, and with a 10–14 day interval evidently too long for persistent inhibition of tumour growth (see the curve illustrating growth of lesions treated with a total dose of 18 MBq [$^{211}$At]MTB; Figure 3). A subsequent return to progressive growth of the tumours, if it occurred, was evidently due to interruption of the treatment (Figure 3). This allows the conclusion that the proper selection of the number and size of [$^{211}$At]MTB fractions, given with a mean frequency of one every 5–7 days, will result in the irreversible growth inhibition of solid pigmented melanoma.

*Larger tumours.*

The pattern of tumour response to each investigated fractionation régime of [$^{211}$At]MTB treatment remained the same for lesions 4–6 times larger. The tumour growth rate was affected identically ($P > 0.1$) by [$^{211}$At]MTB applied as five frequent fractions and by two fractions administered at a 6 day interval, although a total [$^{211}$At]MTB dose of the latter treatment amounted to only 12 MBq, whereas a total of 35 MBq of [$^{211}$At]MTB was injected sequentially at 48 h intervals (Figure 4; initial overlap of curves illustrating growth rate of tumours treated with five frequent fractions and with the first two of five fractions given at a 6 day interval).

Five fractions of [$^{211}$At]MTB administered with a mean frequency of one a week proved again to be therapeutically more efficacious than if delivered at 48 h intervals.

*Five fractions given at 48 h intervals.* A reduced tumour growth rate of 50% compared to controls was observed 12

days after completion of the treatment ($P < 0.001$). Within the subsequent 28 days, tumour growth rate returned to almost control values (Figure 4).

*Five fractions applied at 6 day intervals.* A gradual decrease in tumour growth rate caused by each [$^{211}$At]MTB fraction given led to a complete inhibition of the tumour growth upon delivery of all five fractions. This lag phase lasted for 9 days and the tumours, which were left without further treatment, resumed their growth at a rate reduced to approximately 50% of the control values ($P < 0.001$; Figure 4). The overall reduction in the tumour growth rate was strongly dependent ($P < 0.001$) on [$^{211}$At]MTB doses used per fraction (Figure 5).

The obtained results suggest once again that proper choice of the number and size of [$^{211}$At]MTB fractions, delivered once a week, will cause irreversible regression of pigmented melanoma regardless of the tumour volume at the start of [$^{211}$At]MTB treatment.

3. *Poorly pigmented HX34 human melanoma xenografts*

Poorly pigmented melanomas are known to be more sensitive to ionising radiation than their highly pigmented counterparts [30]. However, the response of HX34 cutaneous xenografts to [$^{211}$At]MTB treatment was very limited ($0.05 < P < 0.1$), and almost independent of the manner in which [$^{211}$At]MTB was applied. For this reason, only results obtained for the smallest investigated lesions, i.e. those with the best response to the treatment, are shown (Figure 6). The growth rate of these tumours was influenced by [$^{211}$At]MTB only temporarily, with the most significant ($P < 0.05$) effect observed after administration of five frequent fractions delivered at 48 h intervals. The treatment caused an intermediate lag phase in the tumour growth lasting for a maximum of 20 days (Figure 6). Subsequently, the tumour growth rate returned almost immediately to control values. A third régime of [$^{211}$At]MTB injections in which the radiolabelled compound was administered with a 6 day interval between fractions, and which proved to be the most effective form of treatment for highly pigmented lesions, appeared to be the least potent for HX34 cutaneous melanoma ($P > 0.5$) (Figure 6). It was, therefore, apparent that [$^{211}$At]MTB affected poorly pigmented melanoma proportionally to its dose delivered per fraction and a frequency of the fractions—the result characteristic for an almost uniform distribution of [$^{211}$At]MTB within the tumour without much affinity of the compound to the tissue.

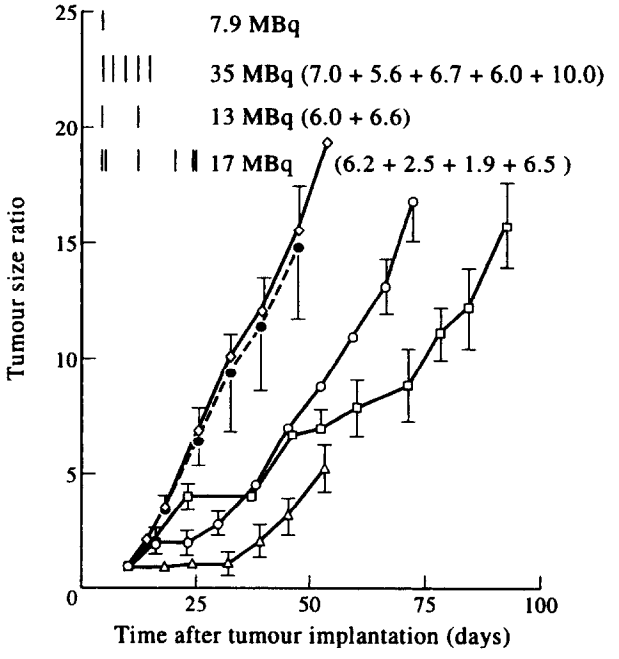


**Figure 6. Poorly pigmented HX34 human melanoma xenografts grown in nude mice. A growth rate of cutaneous tumours (approximately 1.8 × 1.75 mm in perpendicular diameter and 0.35 mm thick) after i.v. administration(s) of [$^{211}$At]MTB given as a single injection of 7.9 MBq (□), 35 MBq in five fractions applied at 48 h intervals (△), 13 MBq in two fractions given with a 6 day interval (○) and 17 MBq in four fractions applied as indicated by strokes (◇). ●, untreated tumours. Curves are normalised to day 10 after tumour implantation. ±S.D. bars are shown for all experimental points for which error bars exceed the size of symbols used. A time-dependent schedule of the treatment specifying total [$^{211}$At]MTB doses and [$^{211}$At]MTB doses used per fraction is shown at the top of the graph. Mean size of tumours at the time of mice culling was approximately 1.6 × 1.5 cm in perpendicular diameter and 1 cm thick (□), 0.9 × 0.8 cm in diameter and 0.6 cm thick (△), 1.0 × 1.1 cm in diameter and 0.5 cm thick (○), 1.8 × 1.6 cm in diameter and 1 cm thick (◇).**

## DISCUSSION

Although targeted radiotherapy with [$^{211}$At]MTB aims at preventing metastatic spread of melanoma rather than treating large, well formed tumours, our previous results revealed that radiolabelled MTB is capable of inhibiting growth of both solid melanoma lesions and their metastases [20, 21, 24, 25]. A high melanin content was required for the effective treatment of macroscopic tumours, but the growth of metastases was successfully prevented by [$^{211}$At]MTB, even though pigmentation of these circulating cells was limited [24, 25]. The present studies confirmed the previous observations and enabled selection of the optimal fractionation régime necessary to cause irreversible regression of the solid lesions.

Pigmented melanoma is considered to be a radioresistant neoplasm [31] and, consequently, requires fractional doses almost twice as high as the average employed in γ- and X-radiotherapy [32–34]. Thus, it was initially puzzling that highly pigmented HX118 xenografts responded best to smaller doses of [$^{211}$At]MTB delivered once a week, whereas large, frequently given fractions of [$^{211}$At]MTB proved to be most effective treatment for almost non-pigmented HX34 melanoma.

Although a vascular system develops similarly within HX118 and HX34 xenografts [25], and both xenografts are characterised by comparable radiosensitivity [26, 27], the maximum response of poorly pigmented HX34 melanoma to [$^{211}$At]MTB treatment (frequent, large fractions) was manifested by a temporary lag phase in the tumour growth followed by a growth rate equal to that observed for control lesions. This indicates lethal effects from [$^{211}$At]MTB towards a fraction of tumour cells (a lag phase in the curves) and minor or even no sublethal damage to the remaining population (the ultimate rate of the tumour growth almost or totally unchanged, respectively). For highly pigmented HX118 melanoma, [$^{211}$At]MTB treatment used in its optimal pattern (i.e. smaller fractions delivered once a week) progressively inhibited growth of cutaneous lesions, leading either to their irreversible regression or, if the treatment was not completed, to a recurrence whose reduced growth rate could reflect

sublethal damage. Such distinct patterns of tumour response to the maximally effective but completely different [$^{211}$At]MTB treatments are suggestive of the mechanism governing the tumour response to the treatment.

Administration of [$^{211}$At]MTB is a targeted radiotherapy selective for pigmented melanoma cells [18, 20, 21, 24, 25,35]. Radiolabelled MTB penetrates through cytoplasmic membranes very easily, having an easy access to the interior of melanoma cells [20, 21]. However, since the compound is exteriorised efficiently by these cells, its overall intracellular concentration remains low, unless MTB is bound to an internal structure(s) (i.e. melanin), resulting in its comparatively high and stable level in pigmented cells [18, 20]. Consequently, [$^{211}$At]MTB, once released from blood, is distributed almost uniformly in non-pigmented melanoma within a relatively short period of time, giving a small gradient in [$^{211}$At]MTB concentration throughout the tissue. As a result, a radiation dose delivered to every tumour cell remains low and consistent with the [$^{211}$At]MTB gradient within the lesion. Since the efficacy of cell damage is dependent on [$^{211}$At]MTB dose delivered to the cell, only frequently delivered large fractions of [$^{211}$At]MTB proved to be the most effective treatment for almost non-pigmented HX34 lesions.

In contrast, melanin present in pigmented melanoma cells serves as a "scavenger" of [$^{211}$At]MTB supplied by the vascular system of the tumour. If the distance between two adjacent vessels remains small, the amount of [$^{211}$At]MTB delivered to every intervening cell is sufficient to result in its receiving a lethal dose (Figure 7) (smallest tumours). However, with an increasing distance between the vessels, only a fraction of cells in the close proximity to the capillaries are exposed to lethal doses of the radioisotope (Figure 7). A longer time is needed for [$^{211}$At]MTB to reach remote cells and, since this process is in competition with the $^{211}$At decay ($t_{1/2}$ = 7.2 h), more cells remain underexposed (larger lesions). A frequent repetition of [$^{211}$At]MTB injections does not result in uniformity of its distribution within the tumour since the capacity of melanin to bind MTB is almost unlimited [20]. Therefore, frequent pulses of the radiolabelled compound are all "scavenged" by the same cells causing the so-called overkill effect and leaving the remaining tumour cells relatively undamaged. However, when the fractionation régime is timed so as to allow resorption of doomed cells between consecutive doses, [$^{211}$At]MTB is gradually taken up by all melanotic cells, thus leading to the observed irreversible resorption of the tumour. Indeed, only a properly chosen number of [$^{211}$At]MTB fractions delivered weekly inhibited tumour growth regardless of size of the lesions, whereas the response from pigmented tumours treated with five frequent fractions of [$^{211}$At]MTB was identical to that obtained for lesions exposed to two fractions with a 6 day interval between them. This indicates that only the first and penultimate, given 6 days later, of five frequent fractions contributed to tumour damage.

The data presented in this and previous papers [21, 24, 25, 35] demonstrate, therefore, that [$^{211}$At]MTB employed for targeted radiotherapy of pigmented melanoma can inhibit the progress of this neoplasm by scavenging single melanoma cells circulating in blood and eradicating micrometastases and small solid tumours. The larger size of the latter currently requires an increased number of [$^{211}$At]MTB fractions for the treatment of these lesions. However, the proposed underlying mechanism immediately suggests the modification appropriate for treatment of pigmented tumours regardless of their size, thus allowing the method to be introduced for therapy of not only micrometastases derived from primary tumours, but also the tumours themselves.

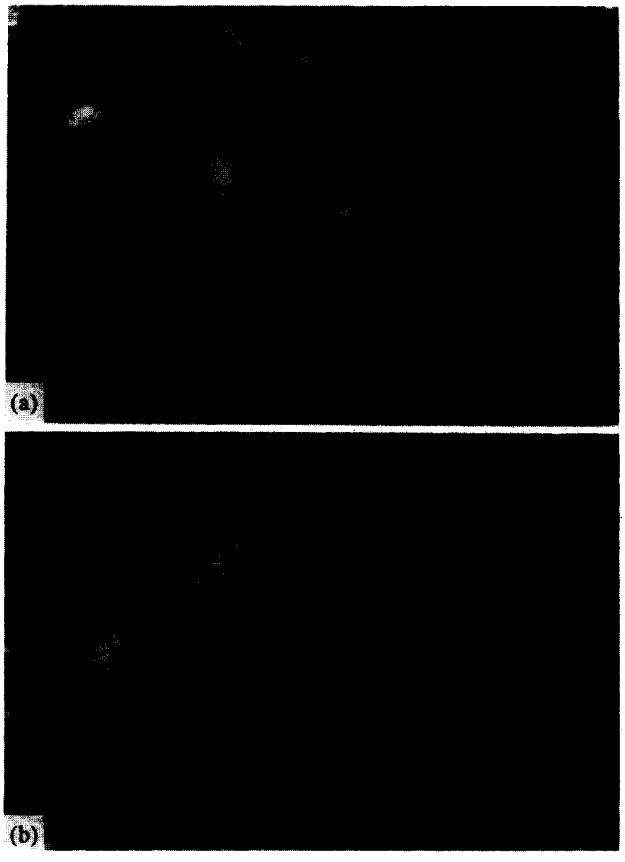


**Figure 7. Cross-section of highly pigmented HX118 human melanoma tumour taken from nude mouse 5 days after a single i.v. injection of [$^{211}$At]MTB and examined in light microscope. Blood vessels (indicated by arrows) are surrounded by a few layers of cells damaged by [$^{211}$At]MTB. If the distance between two adjacent capillaries is small, the layers of damaged cells join together and all cells surrounding the vessels are affected by the treatment. H&E staining; magnification (a) ×200, (b) ×400.**

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