Radioiodinated Methylene Blue – A Promising Agent for Melanoma Scintigraphy: Labelling, Stability and *In Vitro* Uptake by Melanoma Cells

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Abstract. *Melanoma is a tumor of continuously increasing incidence for which new methods of imaging and targeted therapy are widely sought. Radioiodinated methylene blue is a promising tracer, showing selective uptake in human pigmented melanoma cells. We performed ^{131}I-labeling of the tracer using 1% methylene blue injection United States Pharmacopeia (USP) and ^{131}I sodium iodide. For quality control, a Merck high performance liquid chromatography (HPLC) system was used. We developed a new HPLC procedure using 0.1% trifluoroacetic acid, 90% acetonitrile and 10% water as solvent for isocratic elution of the tracer and applied a TLC method using ITLC-SG strips and the same solvent. The stability of the preparation was studied for 15 min, 3 h and 6 h. In order to evaluate the potential relevance of ^{131}I-labeled methylene blue for melanoma detection, the in vitro uptake of ^{131}I-methylene blue was investigated in SK-MEL 28 and 518A2 melanoma cells. Time and a temperature influence on uptake of ^{131}I methylene blue by these two melanoma cells were investigated. The radiochemical purity obtained by the HPLC method was 99.97±0.08% (n=8), while that by the TLC method was 99.88±0.16% (n=8). This indicates the excellent agreement between these two methods. The stability was persistent over 6 h and amounted to 99.75% ±0.21% (n=8). The uptake of ^{131}I methylene blue was time and temperature dependent by both melanoma cells lines. The net cellular uptake on incubation at 37˚C of ^{131}I methylene blue by SK-MEL 28 cells was high at 56.3-61.8% and that by 518A2-cells was 36.3-56.0% . Uptake by these cells was also investigated at 22˚C. The uptake by both cell types was also high at this temperature, but lower than that at 37˚C, amounting to 45.0-51.7% and 25.6-36.3% , respectively. Due to its easy handling and quite high uptake by melanoma cells, we expect that this tracer could be successfully used in routine application for melanoma imaging or eventual radiotherapy.*

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It is known that the polycyclic phenothiazinium dye, methylene blue, possesses a high affinity for melanin, a pigment present in melanoma cells (1). Methylene blue forms a strong complex with melanin and may provide a means of selective delivery of radionuclides to melanoma cells, useful for noninvasive diagnosis as well as for therapy of disseminated disease. The fact that methylene blue is not directly toxic to the tumor and accumulates in melanoma tissue, showing a high concentration of melanin, allows tumor imaging using suitable radionuclides (2-4). According to data obtained by Blower *et al.* (3), the radiolabeled tracer consists of two main fractions which are more lipophilic than unlabeled methylene blue (MB) (Figure 1). Of this, the less lipophilic MBI comprised 85% of radioactivity and the more lipophilic MBI2 aproximately 15% of radioactivity.

The interest in this radiotracer continues to increase, since it was shown by use of the α-particle emitter ^{211}At instead of iodine that it offers the possibility for targeted radiotherapy of a tumor (5-8). A rapid and convenient method of radioiodination was developed and described by Blower *et al.* (9), with iodine ^{131}I or ^{123}I. This method is simpler than methods described in the literature for toluidine blue ^{131}I or ^{125}I labelling (10-11) and gives a higher labeling yield and radiochemical purity.

In the last few years, there have been many efforts in nuclear medicine to target radiopharmaceuticals to melanoma cells for detection and staging, as well as with the aim of using these agents for therapy in patients with malignant melanoma. Owing to its stable intracellular localization, melanin synthesised by melanoma cells forms a distinctive target, to which diagnosis and treatment may be addressed specifically and systemically (12-14). Agents with melanin affinity, such as quinolines (15) and radiolabeled derivatives of the melanin precursor tyrosine (16), as well as nonspecific tumor imaging agents, such as gallium-67 citrate (17),

^{99m}Tc-methoxyisobutylisonitrile (MIBI) (18), ^{99m}Tc-tetrofosmin (19), ^{18}F fluorodesoxyglucose (FDG) (20) and peptides (21-24), have been investigated as eventual diagnostic and/or therapeutic agents for patients with malignant melanoma. Radiolabeled monoclonal antibodies (25-28) and their fragments (29), that bind to specific melanoma antigens, compounds based on alpha-melanocyte stimulating hormone, in which peptide sequences were linked to a single molecule of diethylenetriamino-pentaacetic acid (DTPA) (30) and benzamide, an inhibitor of polyadenosinediphosphate [(ADP)-ribose] polymerase (31), have all been examined as well. However, to date, no radioisotopic method was shown to be clearly superior to the conventional imaging procedures available for melanoma.

In athymic mice bearing highly pigmented human melanomas, it was recently documented (2) that radioiodinated methylene blue has an exceptionally high affinity to and formation of a charge transfer complex with melanin, and therefore accumulates preferentially in melanoma cells. Targeted radiotherapy for pigmented melanoma with methylene blue labeled with ^{211}At (an alpha-particle emitter) proved to be very effective in animal models (7). A clinical study (32, 33) has recently reported some success with radioiodinated methylene blue in patients with melanoma metastases. These encouraging results prompted us to perform preclinical investigations of *in vitro* uptake kinetics and characteristics of ^{131}I methylene blue uptake into melanoma cells.

Materials and Methods

Labeling procedure. The labeling procedure of the tracer was performed according to the modified method of Blower *et al*. (9). The labeling was carried out using 1% methylene blue injection USP (Luitpold Pharmaceuticals, NY, USA) and ^{131}I sodium iodide (Trevi Health Care Product, Vienna, Austria). The reaction takes place in sealed 10 ml Technevials (Mallinckrodt, UK), by successively adding 0.5 ml 1% methylene blue solution to approximately 30 MBq of ^{131}I sodium iodide in 0.1 ml. Subsequently, 1 ml freshly prepared potassium iodide/iodate solution (585 μg KI and 3.85 mg KIO_3) and 0.5 ml of 0.18 M hydrochloric acid were added. The reaction time was 60 min under heating in a water-bath at 100˚C. After cooling to room temperature, the mixture was purified with a Lida SAX-sample preparation column (500 mg; Lida Manufacturing Corp., WI, USA), followed by filtration through a Millipore Millex-GS 0.22 μm filter (Bedford, MA, USA) into a vented Technevial. The column was eluted with 2×4 ml water.

Quality control. For performing quality control, a Merck (Darmstadt, Germany) high performance liquid chromatography (HPLC) system consisting of an L-6200A pump, L-4000 UV detector (λ=600 nm) and a 150 TR Flow Scintillation Analyzer (Packard, Meriden, CT, USA) was used with an RP-18 4×250 mm column. A new improved method was created for the quality control of the radiolabeled methylene blue tracer. A reversed-phase HPLC isocratic method was performed using a solvent system consisting of 0.1% trifluoroacetic acid, 90% acetonitrile and 10% water. The flow rate was 1 ml/min using a sample volume of 20 μl.

We also developed a new thin layer chromatography (TLC) method, using instant thin layer silica gel (ITLC-SG; Gelman, Ann Arbour, MI, USA) strips and the same mobile phase as the HPLC method. Samples of ^{131}I methylene blue were spotted 2 cm from the bottom of the strips and developed in the solvent to a distance of 8-10 cm. Impurities, consisting of free iodine and molecular iodine remained at the origin (R_f=0), while the ^{131}I methylene blue migrated with the solvent front (R_f=0.8-1.0). As it is more rapid to perform, we found this method more convenient and more economical.

Stability of the tracer. We performed extensive stability studies of the tracer up to 6 h after labeling. Using the HPLC method for radiochemical impurities estimation and, in parallel, the TLC method for stability estimation, we evaluated the stability of the tracer 15 min, 3 h and 6 h after preparation.

Cell lines. SK-MEL 28, a cell line derived from a human pigmented malignant melanoma and 518A2, another human cell line carrying wild-type *N-, H-* and *K-ras* genes were investigated. Cells were grown *in vitro* in Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL, Life Technologies Ltd., Paisley, UK) supplemented with 10% heat inactivated fetal calf serum (FCS; GIBCO BRL and antibiotics (penicillin 10 IU/ml and streptomycin 50 μg/ml) in a humidified (5% CO_2, 95% ambient air) atmosphere at 37˚C. Cells were trypsinized and washed once in medium containing FCS in order to remove the residual trypsin. Growth curves were generated after seeding of 1×10^3 cells/well from each melanoma cell line into 96-well plates. For the uptake studies, the cells were resuspended in DMEM/10%/FCS at a density of 1×10^6 cells/ml.

Uptake studies. ^{131}I methylene blue (10 μCi) was added to the cells at an incubation temperature of 22˚C or 37˚C. After different incubation times (10-180 minutes), the cells were separated from the incubation medium by simple differential centrifugation. The net (after subtraction of residual activity and activity related to adhesion of tracer to tubes) *in vitro* cellular uptake of ^{131}I methylene blue was measured by a gamma counter. The characteristics and kinetics of ^{131}I methylene blue uptake into the two cell lines were examined.

Statistical analysis. Results are expressed as mean±standard deviation (SD). Statistical analysis was performed using Student's *t*-test. A value of $p<0.01$ was considered as significant.

Results

Labeling procedure. According to our experience in this study and that of Soenarto (11), the same methodological limiting factors influence both labeling yield and radiochemical purity. Among them are order of reagent use, temperature, duration of incubation, pH and iodate:iodide ratio. Concerning the order of reagents, the highest labeling efficiency was obtained keeping the order as given in the Materials and Methods section. Moreover, the highest labeling yield was achieved at an incubation temperature of 100˚C for 1 hour. The optimal iodate:iodide ratio was 5:1. This reaction ratio corresponds to 0.009 M and 0.0018 M

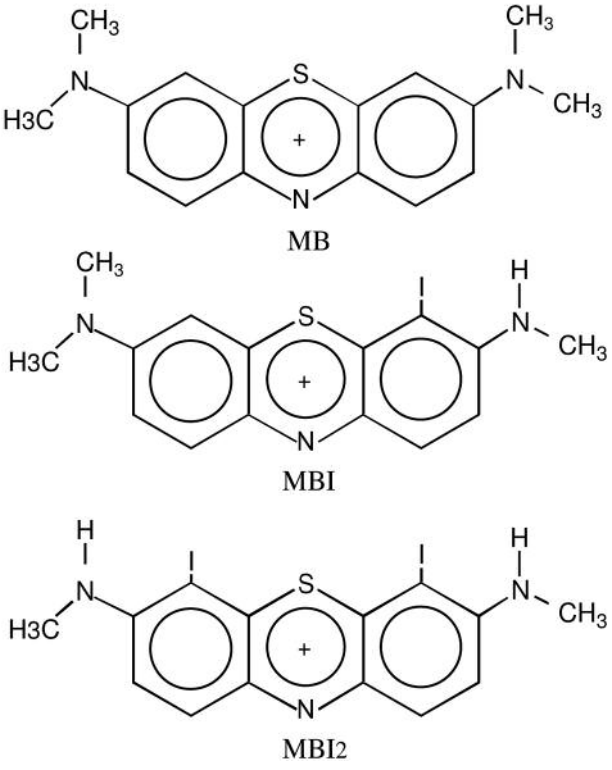

Figure 1. *Chemical structure of methylene blue (MB) and its labeled products (MBI, MBI2).*

end concentrations, respectively. The excess of iodate was removed after passage through the column additionally to the labeling procedure. The labeling yield of the final product after purification on the column amounted to 55.44±7.7% (n=8).

Radiochemical purity and stability. Using the HPLC method for radiochemical impurities estimation, we found 98.91±0.68% (n=8) radiochemical purity. The retention times (Rt) were free iodide=2.71 min and ^{131}I methylene blue=4.83 min for UV detection (600 nm) of the labeled product (Figure 2). For the radioactive detection by the gamma detector (calcium fluoride europium-doped), Rt ^{131}I methylene blue=5.09 min (Figure 3). The radiochemical purity amounted to 99.97±0.08% (n=8). The gamma detector signal was delayed for 0.26 min in relation to the visible absorbance signal. We also performed the TLC method using ITLC-SG strips and the same solvent. In this method, radiochemical purity was determined as 99.88±0.16% (n=8). This indicates the excellent agreement between these two methods. The stability of the preparation was studied at 15 min, 3 h and 6 h (Figure 4). The stability was persistent over 6 h and even after 24 h at room temperature, the preparation was still stable (Table I).

Table I. *Results of stability assessment of the tracer obtained by ITLC method.*

Time after labelling	Radiochemical purity %
15 min	99.88±0.16%
3 h	99.90±0.12%
6 h	99.75±0.21%
24 h	99.38±0.95%

Uptake studies. The net cellular uptake of ^{131}I methylene blue by SK-MEL 28 cells at 37˚C was high at 56.3-61.8% (Figure 5), while that of 518A2 cells was 36.3-56.0% (Figure 6). The uptake by both cell types was also high at 22˚C but lower than at 37˚C, amounting to 45.0-51.7% and 25.6-36.3%, respectively. A time- and a temperature-dependent uptake of ^{131}I methylene blue by these two melanoma cell lines was found (Figures 5 and 6).

Discussion

The described labeling method of methylene blue allows rapid performance of labeling and purification procedure of the tracer in the clinical routine. The tracer shows a high value of radiochemical purity that has been estimated by both reversed-phase HPLC and ITLC-SG.

In this study, we introduced a very simple isocratic HPLC method for estimation of the radiochemical purity after radiolabeling, in contrast to the more complex gradient solvent elution method by Blower *et al.* (3). This method required only few minutes to perform and gives very good reproducibility. Moreover, the ITLC-SG method, which we applied in parallel with the HPLC method gave very good agreement of the results, 99.88±0.16% *versus* 98.91±0.68%, (n=8), respectively.

We performed an extensive stability study of the tracer up to 6 h after labeling. This problem is very important in radiopharmacy because radiopharmaceuticals generally have only limited stability. The stability of radiotracers declines with time, while the amount of free ^{99m}Tc and impurites (*e.g.* colloid and hydrolysed products) increase. According to Pharmacopeia, the radiochemical purity for most radiopharmaceuticals should not be lower than 95% and this value should persist up to 6 hours.

For this reason, we were interested in how stable this tracer is over a longer period of time, as the stability of the tracer is such an important factor for daily clinical use. We found that radiolabeled methylene blue is a very stable tracer during extended time periods (15 min - 6 h). This allows the application of the tracer to the patients during a whole day. The tracer's stability allows scintigraphy to be performed all day with only one labeling dose given in the morning.

Some promising results with other tumor seeking agents for scintigraphic imaging or therapy of melanoma exist, but

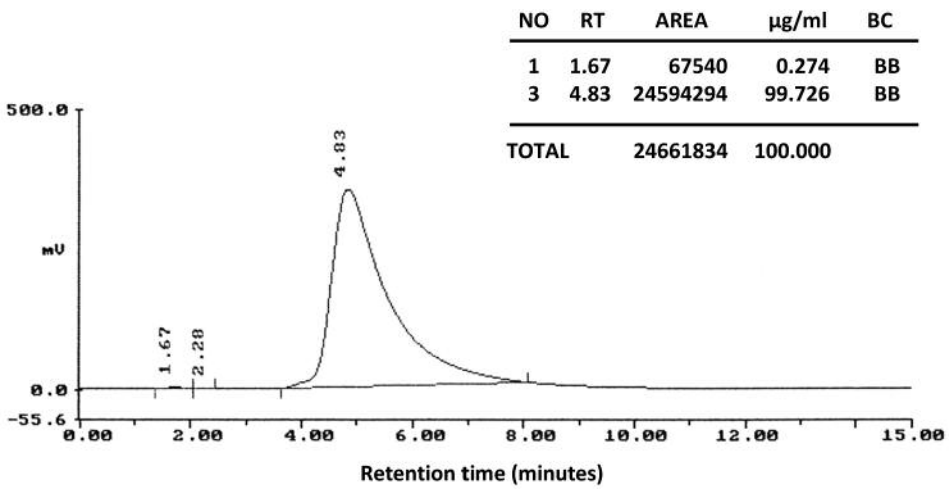

Figure 2. *Reversed-phase HPLC method for radiolabeled methylene blue with visible absorbance (λ=600 nm). The peak at the retention time of 4.83 min is iodinated methylene blue, while that at 1.67 min is free iodine.*

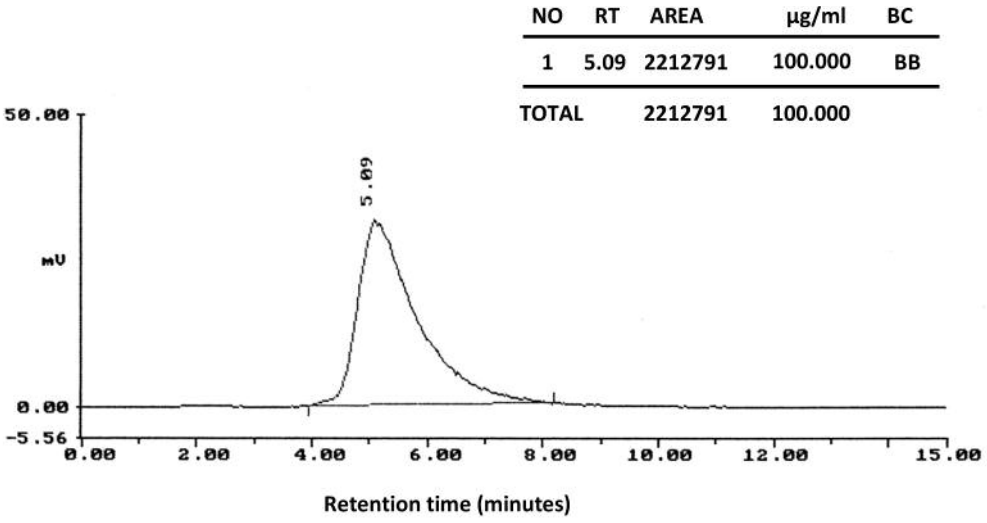

Figure 3. *Gamma-emmision scan for radiochemical impurities estimation. The peak at the retention time of 5.09 min consists of iodinated methylene blue. The gamma detector signal is delayed for 0.26 min in relation to the visible absorbance signal.*

no breakthrough as regards a radioisotopic imaging or radiotherapeutic agent of choice has yet been reported for malignant melanoma. Methylene blue is a low molecular weight, water-soluble compound that easily diffuses through the cellular membranes and accumulates selectively in melanoma cells (2-7).

Malignant melanoma is one of the most frequently occurring tumors and has shown a continuous rise over recent decades. Around 90% of patients with malignant melanoma present at primary diagnosis without recognizable metastases. The 10-year survival rate of patients with this tumor type ranges between 75% and 80%. However, if distant metastases are present, the prognosis is very poor, with a median life expectancy of only 4-6 months. Therefore, a fast, noninvasive diagnosis of eventual metastases and a systemic targeted treatment are of key interest and relevance.

We performed pre-clinical preliminary *in vitro* investigations of the uptake of ^{131}I methylene blue by two human melanoma cell lines: SK-MEL 28 and 518A2. *In vitro* data indicate a relatively high cellular uptake of ^{131}I

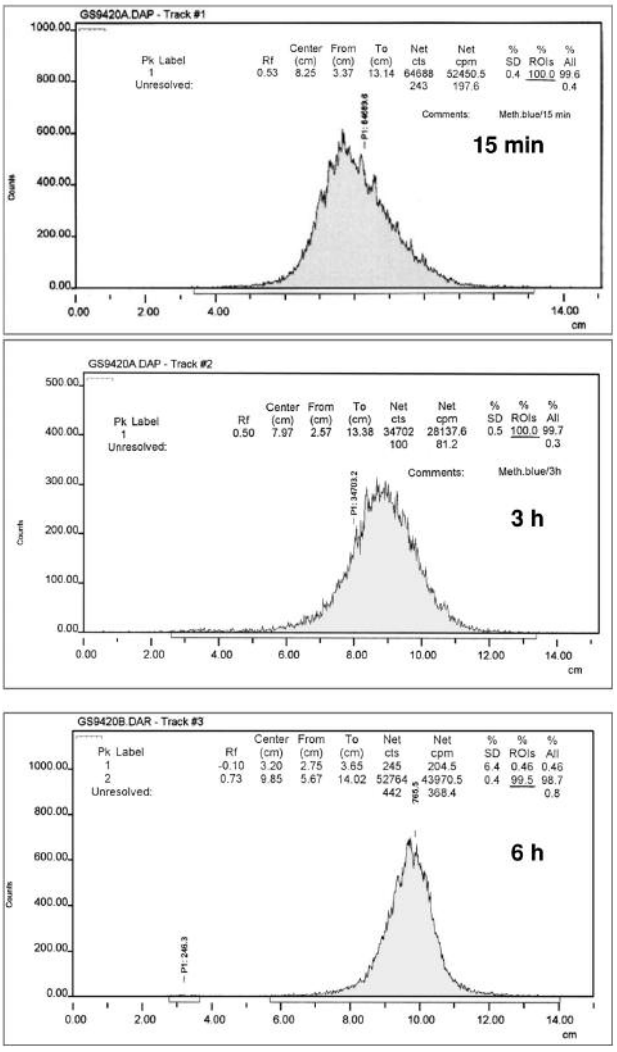

Figure 4. *Estimation of tracer stability by TLC method up to 6 h after labeling. Using a solvent system consisting of 0.1% trifluoroacetic acid, 90% acetonitrile and 10% water, free iodine remained at the origin (R_f=0), while the ^{131}I methylene blue migrated with the solvent front. The peak at R_f=0.5 consists of iodinated methylene blue.*

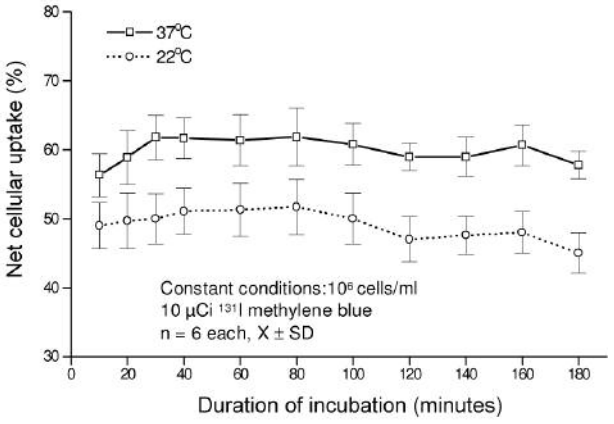

Figure 5. *Net cellular uptake of ^{131}I methylene blue by SK MEL 28 cells at 37˚C and 22˚C incubation.*

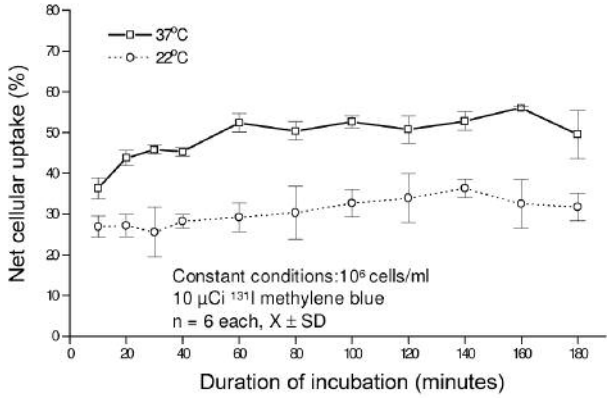

Figure 6. *Net cellular uptake of ^{131}I methylene blue by 518A2 cells at 37˚C and 22˚C incubation.*

methylene blue by the melanoma cells investigated. Therefore, this radiopharmaceutical seems to be very promising for scintigraphic imaging and for targeted therapeutic application in patients suffering from malignant melanoma.

Conclusion

The preliminary *in vitro* data indicate that this radiopharmaceutical is a promising tool for both scintigraphic detection and radiotherapy of malignant melanoma. *In vivo*, larger studies in humans are mandatory to evaluate the value of radiolabeled methylene blue for diagnosis and staging, as well as for therapy of disseminated melanoma.

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