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Preparation and biodistribution of [^{125}I]Melphalan: a potential radioligand for diagnostic and therapeutic applications

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This paper addresses the development of a new radiopharmaceutical for cancer imaging and therapy. The optimization of the labeling conditions of thymidine analogue, melphalan, with ^{125}I is described. High radiochemical yield 96.8% was obtained by reacting 0.2 mg melphalan with ^{125}I in the presence of choloramin-T as oxidizing agent in 0.5 M phosphate buffer, pH 7, at 70°C for 15 min. Preliminary *in vivo* study was done in non-tumor bearing mice. The results revealed that this new tracer, ^{125}I-melphalan, has a high affinity to be localized in the tumor site for a long period, which indicates the specificity of this tracer to the tumor cells. The labeled compound was cleared quickly from most of the body organs. These findings suggest that ^{125}I-melphalan allows imaging and treatment of cancer. ^{125}I-melphalan meets most of the requirements necessary to be used as a successful diagnostic and therapeutic agent: it is a low-molecular-weight molecule that diffuses readily in the tissues, and dose not induce an antibody response.

Keywords: melphalan; iodine-125; biodistribution

Introduction

Nitrogen mustards are bifunctional alkylating agents, and one of the main classes of clinically used anticancer drugs. These drugs react extensively with cellular macromolecules (DNA, RNA, and proteins), thus inducing multiple kinds of molecular lesions. The critical cellular target of these agents is DNA, which is alkylated primarily at the N-7 position of guanine with lesser reaction at the N-3 position of adenine.[1] Besides monofunctional binding of the drug to a single site in the DNA molecule (monoadducts), drug-induced cross-linking between bases in the complementary strands of a DNA molecule (DNA interstrand cross-links), as well as DNA-protein cross-links, also occur.[2,3] Nucleotide excision repair and base excision repair have a crucial role in the repair of monoadducts.[4,5] Furthermore, a number of multistep DNA repair pathways including nucleotide excision repair, homologous recombination, and postreplication/translation repair, all contribute to the DNA interstrand cross-link repair.[6]

Melphalan is a member of the nitrogen mustard class of chemotherapeutic agents and elicits its mechanism of action by the alkylation of DNA. The nitrogen mustard melphalan does not require metabolic activation to become an alkylating agent and forms ~38% N-7-guanine monoadducts, 20% N-3-adenine monoadducts, 20% N-7-guanine-N-7-guanine diadducts, and 13% N-3-adenine-N-7-guanine diadducts.[7] The role of melphalan today is essentially reserved for the management of multiple myeloma (MM). MM is a malignant plasma cell disorder accounting for about 10% of hematologic malignancies. This malignancy, although treatable, is considered incurable and accounts for 1% of all cancer deaths.[8] For several decades, melphalan and corticosteroids were the main drugs for the treatment of MM. Subsequently, high-dose melphalan supported by autologous stem cell transplantation was shown to improve progression-free and overall survival in some but not all studies.[9–11]

One of the chemotherapeutic agents,cytarabine, which was successfully labeled with iodine-125, allows imaging and treatment of cancer. ^{125}I-cytarabine meets most of the requirements to be used as a successful diagnostic and therapeutic agent.[12]

Iodomelphalan has been prepared by iodination. The position of the iodine as *ortho*-position to the alanine moiety.[13] The present work deals with the labeling of melphalan by iodine-125. The factors affecting the labeling yields were investigated and the bio-distribution in mice was performed.

The structure of ^{125}I-melphalan via the reaction of melphalan with iodine-125 in the presence of chloramine-T at pH 7 is shown in Equation (1).

Experimental

Materials

All chemicals and laboratory reagents used in this work were of the highest purity grade. In all cases, double distilled water

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was used. Melphalan and Chloramine-T (*N*-chloro-*p*-toluene sulfonamide sodium salt) (CAT) were purchased from Sigma-Aldrich, Germany. Iodine-125 was purchased from (H-1121 Budapest, Konkoly-Thege Miklòs ùt 29-33) as no-carrier added solution, radionuclidic purity > 99%.

Animals: Albino type mice, weighing 25–30 g, were used in groups containing three mice.

Method

Radioiodination

Ethanolic solution of melphalan (200 μg) was added to 150 μL of CAT (150 μg) and 150 μL phosphate buffer pH 7, in a screw caped reaction vial. Then 10 μL $Na^{125}I$ (3–5 MBq) was added and the reaction mixture was heated to 70°C for 15 min. The reaction was terminated by the addition of 50 μL of $Na_2S_2O_3$ (20 mg/mL, 0.12 M).

Radiochemical analysis

The radiochemical yield of the labeled melphalan was determined using aluminum-backed silica gel-60 (TLC). The strips were previously impregnated with $Na_2S_2O_3$ (20 mg/mL, 0.12 M) to inhibit the oxidation of the radioiodide to a volatile form. Volume of 5 μL of the reaction mixture was spotted on the start line, then the strip was developed using *n*-butanol:acetic acid:water (4:1:2, v/v/v) as a developing system. The strips were removed, dried, cut to 1 cm segments, and assayed for the radioactivity using a well-type Na/T1 crystal connected to the γ counter. The free iodide remains near the origin with R_f = 0.1–0.2, while the labeled melphalan migrates with the solvent front with R_f = 0.8–1.0. An HPLC analysis and purification was performed with a reverse phase C18 column and the elution was done using the methanol:water:acetic acid (49.5:49.5:1) system with a flow rate of 1 mL/min.[14]

$$\text{Radiochemical yield, } \% = \frac{\text{Activity of labeled product}}{\text{Total activity}} \times 100$$

Preparation and structure confirmation of unlabeled iodo-melphalan

The unlabeled iodo-melphalan is prepared by the same method on a larger scale (to provide enough iodo-melphalan) to allow isolation and characterization. Unlabelled iodo-melphalan is characterized by proton NMR.

^{1}H NMR spectrum of iodo-melphalan in (DMSO-d6) revealed signals at: δ (ppm) = 12.57(s, H,OH-carboxylic), 8.81(d, 2 H, NH_2-amine), 7.59(s, 1 H, CH(a)), 7.10(d, 1 H, CH(b)), 6.48(d, 1 H, CH(c)), 4.18(t, 1 H, CH-NH_2), 3.63(t, 4 H, methylene-N$(CH_2)_2$), 3.61(t, 4 H, methylene $(CH_2)_2$ Cl_2), 3.43(d, 2 H, CH_2-benzen), Figure 1.

Biodistribution studies

This experiment was done by diluting the neutral solution of the purified labeled melphalan with 1 mL saline for injection, and filtration of the solution through 0.22 μm Millipore filter into a sterile sealed vial. 100 μL (3.7 MBq) was injected in the tail vein of the healthy and tumor-bearing Albino mice, weighing approximately 30 g each (three groups each of three mice). The mice were maintained on normal diet in a metabolic cage, then sacrificed at 0.5 h, 2 h post injection, and in addition to 4 h and 24 h in the case of tumor-bearing mice. Samples of fresh blood, bone, and muscle were collected in pre-weighed vials and counted. The different organs were removed, counted, and compared to the standard solution of the labeled melphalan. The average percent values of the administrated dose/organ were calculated. Blood, bone, and muscles were assumed to be 7, 10, and 40% of the total body weight, respectively.[15]

Tumor implementation

For non-hypoxic tumor, 2.5×10^6 cells (200 μL) of Erlich culture was injected intraprotenial and the mice were kept for 10 days on normal diet in a metabolic cage until the tumor growth appeared.

Results and discussion

Effect of melphalan concentration

The radiochemical yield of ^{125}I-melphalan as a function of melphalan concentration was studied as shown in Table 1. The results indicate that the radiochemical yield of ^{125}I-melphalan increased from 81.2 to 96.8% by increasing the amount of melphalan from 50 to 200 μg. The radiochemical yield is not affected by the amount of melphalan higher than 200 μg. This may be attributed to the fact that the yield reaches the saturation value (96.8%) because the entire generated iodonium ions in the reaction are captured at that concentration.

Effect of chloramine-T concentration

Radioiodination of organic molecules has been performed by using a mild oxidizing agent such as chloramine-T, which decomposes to hypochlorite anion that acts as an oxidizing

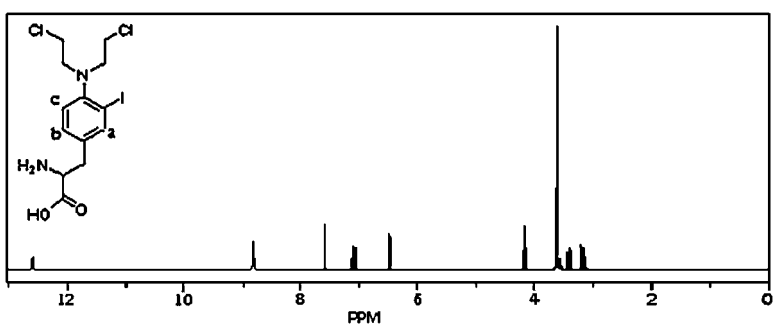

Figure 1. ^{1}H NMR spectrum of iodo-melphalan.

Table 1. Effect of the amount of melphalan on the radiochemical yield of ^{125}I-melphalan

Melphalan amount (μg)	^{125}I-melphalan (%)	^{125}I (%)
50	81.2 ± 2.2	18.8 ± 1.5
100	84.9 ± 1.9	15.1 ± 0.9
150	93.1 ± 1.5	6.9 ± 0.3
200	96.8 ± 1.8	3.2 ± 0.4
250	94.5 ± 1.2	5.5 ± 0.2
300	95.3 ± 0.9	4.7 ± 0.2

Mean ± SD (mean of three experiments). Reaction condition: X μg melphalan, 150 μg chloramine-T, 150 μL phosphate buffer at pH 7 and 10 μL Na^{125}I. The reaction mixture was kept in a water bath (70°C) for 15 min.

Table 2. Effect of the amount of chloramine-T on the radiochemical yield of ^{125}I-melphalan

Chloramine-T amount (μg)	^{125}I-melphalan (%)	^{125}I (%)
50	82.4 ± 1.8	17.6 ± 1.1
100	93.8 ± 2.2	6.2 ± 0.8
150	96.8 ± 2.4	3.2 ± 0.3
200	90.1 ± 1.2	9.9 ± 0.6
250	88.0 ± 1.4	12.0 ± 0.9

Mean ± SD (mean of three experiments). Reaction condition: 200 μg melphalan, X μg chloramine-T, 150 μL phosphate buffer at pH 7 and 10 μL Na^{125}I. The reaction mixture was kept in a water bath (70°C) for 15 min.

agent transforming iodine from I^- to oxidative state I^+. The influence of chloramine-T concentration on the radiochemical yield of ^{125}I-melphalan was studied. The experiment was carried out by the addition of 200 μg melphalan to different amounts of CAT (50, 100, 150, 200, and 250 μg), and 5 μL Na^{125}I. The reaction mixture was heated to 70°C for 15 min. The results of this experiment are presented in Table 2. As it is clear from this data, the radiochemical yield of ^{125}I-melphalan was low when the concentration of chloramine-T was not sufficient to oxidize all the free iodide present in the solution. Increasing the amount of chloramine-T to 150 μg caused an increase in the radiochemical yield of ^{125}I-melphalan to more than 96%. Increasing the amount of oxidizing agent above 150 μg leads to a decrease in the radiochemical yield of ^{125}I-melphalan due to the formation of undesirable oxidative side reactions like chlorination,[16] polymerization, and denaturation of substrate.

Effect of temperature

The reaction temperature has an important role in the electrophilic substitution reactions. The leaving hydronium ion requires some energy to break the C–H bond and to initiate the introduction of the radioactive iodonium ion into the phenyl ring. During this reaction, it was found that the kinetic energy required to breakdown C–H bond and introduce I^+ into the phenyl ring of melphalan was build up when the reaction mixture was heated to 70°C for 15 min. The labeled melphalan did not decompose on increasing the reaction temperature to 100°C, as it is clear from Table 3. This indicates that the labeled melphalan was stable against rise in temperature.

Table 3. Effect of the temperature of the reaction mixture on the radiochemical yield of ^{125}I-melphalan

Reaction temperature (°C)	^{125}I-melphalan (%)	^{125}I (%)
25	85.5 ± 1.5	14.5 ± 1.2
50	92.8 ± 1.3	7.2 ± 0.3
70	96.8 ± 0.9	3.2 ± 0.2
100	95.8 ± 0.8	4.2 ± 0.4

Mean ± SD (mean of three experiments). Reaction condition: 200 μg melphalan, 150 μg chloramine-T, 150 μL phosphate buffer at pH 7 and 10 μL Na^{125}I. The reaction mixture was kept in a water bath (X°C) for 15 min.

Table 4. Effect of reaction time on the radiochemical yield of ^{125}I-melphalan

Reaction time (min)	^{125}I-melphalan (%)	^{125}I (%)
5	84.2 ± 1.5	15.8 ± 1.2
15	96.8 ± 1.3	3.2 ± 0.3
30	93.1 ± 0.9	6.9 ± 0.2
60	94.2 ± 0.8	5.8 ± 0.4
120	95.3 ± 0.9	4.7 ± 0.2

Mean ± SD (mean of three experiments). Reaction condition: 200 μg melphalan, 150 μg chloramine-T, 150 μL phosphate buffer at pH 7 and 10 μL Na^{125}I. The reaction mixture was kept in a water bath (70°C) for X min.

Effect of reaction time

As the reaction temperature was effective in the build up of the energy required to breakdown the C–H bond and form the C–I bond, also the reaction time has the same role. As it is clear from Table 4, the minimum time required to get a maximum yield of ^{125}I-melphalan was 15 min, and no valuable benefits can be gained from increasing the reaction time.

Effect of pH

This experiment was carried out using different buffer systems to obtain the required pH values; citrate buffer for pH 2 and 4, phosphate buffer for pH 7 and 9, and bicarbonate buffer for pH 11. The optimum amount of melphalan was added to the buffer system and then 150 μL CAT (150 μg) followed by 5 μL Na^{125}I. The reaction mixture was heated to 70°C for 15 min. The results indicate to the effectiveness of pH of the reaction mixture on the labeling yield. The radiochemical yield of ^{125}I-melphalan was relatively poor at pH 2 and 4, as a result of the predominance of ICl species, which have low oxidation potential than HOCl species.[17] At pH 7, the radiochemical yield of ^{125}I-melphalan reaches a maximum value of 96.8%. When the pH increased towards the alkaline side (9 and 11) the radiochemical yield decreased, this may be attributed to the decrease in HOI*, which is responsible for the electrophilic substitution reaction.[18] The data is summarized in Figure 2. The high radiochemical yield of ^{125}I-melphalan at pH 7 maybe due to many factors; the good solubility of melphalan in neutral pH, and the efficiency of CAT at this pH value.[19]

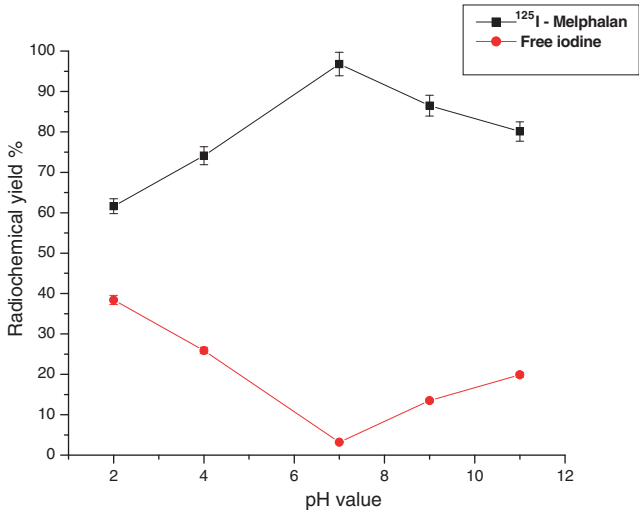

Figure 2. Effect of pH of the reaction medium on the percent labeling yield of ^{125}I-melphalan. Reaction condition: 200 μg melphalan, 150 μg chloramine-T, 150 μL phosphate buffer at pH X and 10 μL Na^{125}I. The reaction mixture was kept in a water bath (70°C) for 15 min.

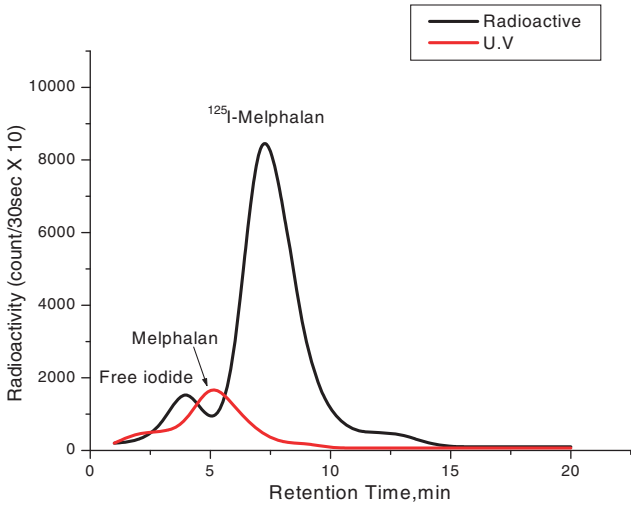

Figure 3. HPLC radiochromatogram of ^{125}I-melphalan

Table 5. The *in vitro* stability of ^{125}I-melphalan

Time post labeling (hour)	^{125}I-melphalan (%)	^{125}I (%)
1	94.8 ± 1.7	5.2 ± 0.3
2	93.0 ± 1.2	7.0 ± 0.3
4	94.1 ± 1.3	5.9 ± 0.2
8	94.3 ± 1.3	5.7 ± 0.1
12	92.9 ± 1.6	7.1 ± 0.2
24	90.6 ± 1.3	9.4 ± 0.5

Mean ± SD (mean of three experiments). Reaction condition: 200 μg melphalan, 150 μg chloramine-T, 150 μL phosphate buffer at pH 7 and 10 μL Na^{125}I. The reaction mixture was kept at room temperature (25°C).

Table 6. Biodistribution pattern of ^{125}I-melphalan in normal mice

Organs	% Injected dose/organ	
	0.5 h post injection	2 h post injection
Blood	15.5 ± 2.1	9.2 ± 1.3
Bone	2.7 ± 0.8	2.0 ± 0.4
Muscle	2.9 ± 0.7	1.9 ± 0.3
Spleen	0.6 ± 0.1	0.4 ± 0.1
Stomach	10.6 ± 1.4	13.7 ± 1.6
Kidney	4.5 ± 1.1	2.6 ± 0.8
Heart	0.9 ± 0.4	0.6 ± 0.2
Lung	1.7 ± 0.8	0.8 ± 0.2
Liver	12.1 ± 1.7	8.9 ± 1.2
Intestine	8.8 ± 0.9	15.6 ± 2.2
Urine	5.5 ± 1.1	9.0 ± 1.6
Thyroid	0.2 ± 0.1	0.8 ± 0.4

Stability test

The stability of ^{125}I-melphalan was studied in order to determine the suitable time for injection to avoid the formation of the undesired products. These undesired radioactive products may be accumulated in non-target organs. Table 5 shows the stability of ^{125}I-melphalan up to 12 h.

HPLC analysis

A radiochromatogram for the iodination of Melphalan obtained after HPLC separation on RP-18 column at the optimum conditions is shown in Figure 3. Two peaks were obtained: one at 4 min while the second at 7 min retention time. The first peak corresponds to free iodide, whereas the second peak corresponds to the ^{125}I-melphalan. The eluted fractions containing the labeled compound are pooled together and evaporated to dryness. The residue was dissolved in physiological saline and sterilized by filtration through 0.22 μm Millipore filter, and the ^{125}I-S6G is then suitable for use in biodistribution studies.

Biodistribution of melphalan

The biological distribution pattern and the ability of the melphalan compound to localize the therapeutic radionuclide, iodine-125, in cancer site were examined in mice. The mice were intravenously injected with 0.1 mL of the tracer. The biodistribution of ^{125}I-melphalan in normal mice is presented in Table 6. The data shows that the clearance of the tracer from the blood was high as the percentage reached ~9.2 ± 1.3% at 2 h post injection. Because the excretion route of this compound occurs via liver, the intestine content increased by time from 8.8 ± 0.9% to 15.6 ± 2.2% at 0.5 and 2 h post injection, respectively. In addition, part of this compound was excreted through the kidneys, as the activity detected in the urine was ~9.0 ± 1.6%. The uptakes of other organs (bone, muscle, lung, heart, and spleen) were within the normal values. The *in vivo* stability of the tracer can be seen, as the thyroid uptake did not increase by time.

The biodistribution of ^{125}I-melphalan in tumor-bearing mice is presented in Table 7. The uptake of the tracer in bone, muscle, lungs, heart, and spleen was similar to the uptake of the same organs in normal mice. In addition, the increased uptake of liver and kidneys may be due to the excretion pathway of the tracer.

Table 7. Biodistribution pattern of ^{125}I-melphalan in tumor bearing mice (Ascetics) at different times post injection

Organs	% Injected dose/ organ			
	0.5 h post injection	2 h post injection	4 h post injection	24 h post injection
Blood	16.4 ± 1.7	13.1 ± 1.3	9.9 ± 1.0	6.8 ± 1.4
Ascetic	38.0 ± 2.8	42.1 ± 2.2	39.0 ± 2.2	36.2 ± 2.4
Bone	2.7 ± 0.6	2.2 ± 0.5	1.1 ± 0.6	0.9 ± 0.7
Muscle	2.2 ± 0.5	1.9 ± 0.6	0.8 ± 0.4	0.5 ± 0.3
Spleen	0.1 ± 0.4	0.2 ± 0.2	0.2 ± 0.1	0.2 ± 0.1
Stomach	6.9 ± 0.9	9.5 ± 1.2	11.1 ± 1.8	7.2 ± 0.2
Kidney	3.1 ± 0.6	4.8 ± 0.9	5.3 ± 0.7	2.8 ± 0.8
Heart	1.6 ± 0.7	0.3 ± 0.1	0.4 ± 0.2	0.2 ± 0.3
Lung	1.0 ± 0.8	0.8 ± 0.3	0.7 ± 0.3	0.5 ± 0.4
Liver	9.4 ± 0.9	6.0 ± 1.1	5.6 ± 1.2	3.2 ± 0.9
Intestine	6.8 ± 1.0	8.0 ± 1.3	12.2 ± 1.4	9.7 ± 1.3
Urine	4.1 ± 0.6	6.2 ± 0.7	8.0 ± 0.9	23.0 ± 2.2
Thyroid	0.1 ± 0.3	0.3 ± 0.3	0.8 ± 0.6	1.3 ± 0.6

Total ascetic fluid has high activity reaching 42.1 ± 2.8% at 2 h post injection and decreased to 36.2 ± 2.4% after 24 h post injection. The localization of this tracer with this high percentage in the tumor site for this long period indicates the specificity of this tracer to the tumor cells.

All the obtained data demonstrate that the tracer was distributed rapidly through out the body after intravenous injection (2 h), and cleared rapidly through the hepatobiliary system (4 h). The liver was the organ with the highest radioactivity that was quickly excreted into the intestinal tract. The presence of activity in the urinary bladder suggests the excretion of the tracer through the kidneys to some extent. The low activity located in the thyroid gland indicates that ^{125}I-melphalane is stable *in vivo* against biological decomposition. Also, the biodistribution of the tracer in tumor bearing mice pointed to the possibility of the use of this tracer as imaging or therapeutic agent for cancer. However, many biological studies are required to establish these findings as the examination of the tracer *in vitro* preparation of hypoxic tissue and the quantitative determination of the tissue uptake of this tracer.

Conclusion

The labeling of melphalan with radioactive iodine-125 was done. The optimum conditions of the labeling of melphalan to give a radiochemical yield of 96.8% were 200 μg melphalan, 150 μL of CAT (150 μg), and 150 μL phosphate buffer pH 7 when the reaction mixture was heated at 70°C for 15 min. The ligand studied provides efficient labeling and good *in vitro* stability. All the obtained data demonstrate that the tracer was distributed rapidly throughout the body after intravenous injection (2 h) and cleared through the hepatobiliary system (24 h). In addition, the biodistribution of the tracer in tumor-bearing mice demonstrated the possibility of the use of this tracer as imaging or therapeutic agent for cancer.

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