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Preparation of ^{99m}Tc-metronidazole as a model for tumor imaging

Ismail Taha Ibrahim

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Abstract Metronidazole (MTNZ) is an antiprotozoa drug, could be labeled with the ^{99m}Tc. MTZL could be used as an ideal vehicle to deliver radioactive decay energy of ^{99m}Tc to the sites of tumor, thus facilitate tumor imaging. The process of labeling was done using tin chloride as reducing agent. The optimum conditions required to label 25 μg MTZL were 100 μg stannous chloride, 30 min reaction time, room temperature at pH 7–9 using 0.5 M phosphate buffer. The radiochemical purity of the labeled compound, at the above conditions, was determined using paper chromatography. The yield was about 93%. About 2.5×10^6 of Ehrlich Ascites Carcinoma (EAC) was injected intrapritoneally (i.p) to produce ascites and intramuscularly (i.m) in the right thigh to produce solid tumor in female mice. Biodistribution studies were carried out by injecting solution of ^{99m}Tc-MTZL in normal and tumor bearing mice. The uptake in ascites was over 5% of the injected dose per gram tissue body weight, at 4 h post injection and above 4% in solid tumor. These data revealed localization of the tracer in the tumor tissues with high percentage sufficient to use $^{99\ m}$Tc MTZL as promising tool for diagnosis of tumor.

I. T. Ibrahim (✉)
Hot Laboratories Center, Atomic Energy Authority,
P.O. Box 13759, Cairo, Egypt
e-mail: ismailtaha_73@yahoo.com

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Introduction

It was reported that the earlier diagnosis of tumor the more the % success in treatment of cancer [1]. The goal is to deliver labeled chemotherapeutic drugs or radioisotopes to the specific tumor site with decreased toxicity to other proliferating tissues as well as neighboring tissues [1]. Many drugs were labeled with radioisotopes to fulfill this purpose. These drugs may be Antimetabolites, antibiotics, antiinflamatories, antibodies or other drugs [2–4]. Many radioisotopes are widely used in cellular radiation studies like ^{99m}Tc, ^{131}I and ^{123}I [5–7].

Imidazole derivatives are widely used in medicine [8]. Metronidazole (MTZL) is a 5-nitroimidazole derivative with activity against anaerobic protozoa and anaerobic bacteria. It also has a radiosensitizing effect on hypoxic tumor cells. MTZL act by interfere with DNA by a metabolite in which the nitro group has been reduced [9, 10].

Previous work was done for labeling of many drugs with ^{99m}Tc for imaging study like MDP (methylene diphosphonates for bone imaging, DTPA (dithiopentaacetic acid for kidney scanning) [11, 12].

This study was conducted to label metronidazole as a vehicle to carry ^{99m}Tc to a tumor cells. This was achieved by injecting EAC to mice either intraperitoneally to induce ascites or intramuscularly in the right thigh to produce solid tumor. *Biodistribution study* of ^{99m}Tc-MTZL in normal mice was investigated. In addition in vitro stability of ^{99m}Tc-MTZL and its in vivo biodistribution in EAC mice was also investigated.

Materials and methods

Drugs and chemicals

1 Metronidazole was supplied as a gift from El-Kahera for Drugs Chemical Co. USA.
2 ^{99m}Tc was obtained as saline eluent of an expired Mo column.
3 Tin chloride was purchased from Sigma Chemical Company, USA.
4 All other chemical reagents were of analytical grade (AR), obtained from reputed manufacturers.
5 Ehrlich ascites carcinoma (EAC) was kindly supplied from National Cancer Institute, Cairo, Egypt.

Animals

Female Swiss Albino mice weighing 20–25 gm were purchased from the Institute of Eye Research Cairo, Egypt. The animals were kept at constant environmental and nutritional conditions throughout the experimental period and kept at room temperature (22 ± 2 °C) with a 12 h on/off light schedule. Female mice were used in this study due to their susceptibility to Ehrlich ascites carcinoma more than male mice [13]. Animals were kept with free access to food and water all over the experiment.

Labeling procedure and requirement

^{99m}Tc-metronidazole was prepared by the following procedures [14]. One milligram MTZL was dissolved in 3 mL purged distilled water with stirring. Tin chloride was added to MTZL solution in evacuated vial with Hamilton syringe and approximately 200–400 MBq ^{99m}Tc at room temperature. After a specified interval of time, chromatographic analysis was developed using paper chromatography ascending techniques [15]. The yield of the reaction and the radiochemical purity were determined by paper chromatography using acetone as mobile phase to distinguish between free at the top and both complex and reduced colloids near the point of spotting. On the other hand, 4 N NaoH as a mobile phase differentiate between reduced colloids which persist near the point of spotting and both complex and free, which move towards the front of chromatogram.

Factors affecting % labeling yield

This experiment was conducted to study the different factors that affect labeling yield such as: (1) tin content, (2) substrate content, (3) reaction temperature, (4) pH of the reaction and (5) reaction time.

In the process of labeling, trials and errors were performed for each factor under investigations till obtains the optimum value. The experiment was repeated with all factors kept at optimum changing except the factor under study, till the optimal conditions achieved. [16].

Paper chromatography

Paper chromatography was achieved using two mobile phases acetone and 4 N NaoH with ascending technique [17];

$$\mathrm{RF} = \frac{\text{Distance traveled by MTZL } ^{99m}\text{Tc}}{\text{Distance traveled by solvent}}.$$

In vitro stability

This experiment was conducted to determine the stability of ^{99m}Tc-MTZL after labeling and the impact of time on that compound. The yield was measured at different time intervals (1, 2, 4, 6 and 12 h) after labeling [18].

Induction of tumor in mice

The parent tumor line (Ehrlich Ascites Carcinoma) was withdrawn from 7 days old downer female Swiss albino mice and diluted with sterile physiological saline solution to give 12.5×10^6 cells/ml. About 0.2 ml solution was then injected in mice intraperitoneally to produce ascites, or intramuscularly in the right thigh to produce solid tumor. The animals were maintained till the tumor development was apparent for about 10–15 days [19].

In vivo biodistribution

In normal mice

In vivo biodistribution studies were performed using four groups each comprise six mice. Each animal was injected in the tail vein with 0.2 ml solution containing 50–100 KBq of ^{99m}Tc-MTZL. The mice were kept in metabolic cages for the required time. Each group was subjected to scarification by cervical dislocation at the recommended time (15 min, 1 h, 6 h or 12 h) after injection. Organs or tissues of interest were removed, washed with saline, weighted and counted. Correction was made for background radiation and physical decay during the experiment [20]. The weights of blood, bone and muscles were assumed to be 7%, 10% and 40% of the total body weight, respectively [21].

In tumor bearing mice

Biodistribution of ^{99m}Tc-MTZL was carried out in two groups of animals each group consists of 24 mice, one ascites bearing group and the other solid tumor bearing

mice. Each animal was injected in the tail vein with 0.2 ml solution containing 50–100 KBq of ^{99m}Tc-MTZL 2 weeks post inoculation. Each group subdivided to four subgroups of six mice each. Animals in each group were kept in metabolic cages for scarification at its required time, after 15 min, 1 h, 6 h or 12 h post injection of the labeled drug. Sacrification of mice was done by cervical dislocation and the organs or tissues of interest were isolated, weighted and counted for its uptake of radioactivity. Ascites fluid was drained and counted as a whole. The counting tubes, including a standard equivalent to 1% of the injected dose, were assayed in a well type NaI (TI) gamma counter and the results were calculated as percentages of injected dose (I.D) per gram tissue. The final results were expressed as mean ± one standard error [22].

Statistical analysis

The results are expressed as means ± SEM for the indicated number of different experiments. The statistical significance of differences was assessed by unpaired Student's *t*-test $p < 0.05$.

Results and discussion

Paper chromatography

The analysis of chromatographic data revealed the high percentage labeling yield of ^{99m}Tc-MTZL. Free ^{99m}Tc was obtained from paper acetone chromatogram. Colloid was obtained from 4 N NaoH chromatogram. Complex ^{99m}Tc-MTZL was obtained by subtracting colloid from activity obtained near the spotting in acetone chromatogram.

Factors affecting labeling yield

Tin content

Results obtained in this study showed the high yield obtained for ^{99m}Tc-MTZL sing tin chloride as reducing agent (Table 1). It was observed that the radiochemical yield significantly increased by increasing the amount of tin from 5 µg to 100 µg (optimum content) at which maximum labeling yield was obtained. By increasing the amount of tin to 200 µg, the yield showed significant decrease in % complex ^{99m}Tc-MTZL. A significant reduction in the labeling yield was noted by decreasing the concentration of tin below 100 µg may be explained as at low concentrations of tin, not all ^{99m}Tc-was reduced. While by increase the tin content to 200 µg colloid may be increased and hence, % complex decreased [22].

Table 1 Effect of tin chloride content on the radiochemical yield ^{99m}Tc-MTZL

Tin (µg)	% Labeled compound	% Free ^{99m}Tc	% Colloid
5	81.0 ± 0.40	18.0 ± 0.3	1.0 ± 0.3
10	85.1 ± 0.50*	5.5 ± 0.75	9.4 ± 0.75
25	87.5 ± 0.54*†	4.9 ± 0.06	7.6 ± 0.06
50	90.2 ± 0.32*	4.0 ± 0.35	5.8 ± 0.35
100	93.0 ± 0.32*	5.0 ± 0.35	2.0 ± 0.35
200	78.2 ± 0.32*	1.0 ± 0.35	2.9 ± 0.35

Values represent the mean ± SEM; $n = 6$

* Significantly different from the initial values using student's *t*-test ($p < 0.05$)

† Significantly different from the previous values using student's *t*-test ($p < 0.05$)

Effect of substrate content

The influence of MTZL content as a substrate on the labeling yield using tin chloride was shown in Table 2. The increase of the concentration of MTZL was accompanied by a significant increase in the labeling yield, where it reached above 90% at 25 µg of MTZL. Increasing the amount of MTZL above 25 µg produced no significant increase in the labeling yield. Increasing the concentration of starting material is usually increases the total incorporation of ^{99m}Tc-MTZL since there is a minimum limit to the volume used [23]. A total of 25 µg of MTZL was required to obtain maximum labeling yield, below this concentration significant decrease in the yield. On the other hand, using higher concentration did not significantly affect labeling yield.

Effect of pH

In order to reach the suitable pH value for maximum radiochemical yield, labeling of MTZL with ^{99m}Tc was carried out at different pH ranging from 2 to 12. The test was performed using 25 µg of MTZL, 100 μ1 of 0.5 M

Table 2 Effect of MTZL content on the labeling yield

Substrate (µg)	% Labeled compound	% Free ^{99m}Tc	% Colloid
5	83.0 ± 0.40	16.0 ± 0.3	1.0 ± 0.3
10	87.1 ± 0.50*	10.5 ± 0.75	1.4 ± 0.75
25	93.0 ± 0.32*†	5.0 ± 0.35	2.0 ± 0.5
50	93.3 ± 0.2*	4.0 ± 0.25	2.7 ± 0.45
100	92.7 ± 0.3*	3.0 ± 0.15	4.3 ± 0.35

Values represent the mean ± SEM; $n = 6$

* Significantly different from the initial values using unpaired Student's *t*-test ($p < 0.05$)

† Significantly different from the previous values using unpaired Student's *t*-test ($p < 0.05$)

Table 3 Effect of pH of the reaction medium on the labeling yield of ^{99m}Tc-MTZL

pH value	% Labeled compound	% Free ^{99m}Tc	% Colloid
2	83.0 ± 0.40	16.0 ± 0.3	2.0 ± 0.2
4	89.5 ± 0.50*	7.5 ± 0.75	3.0 ± 0.75
7	93.0 ± 0.32*†	5.0 ± 0.35	3.3 ± 0.5
9	92.3 ± 0.2*	4.0 ± 0.25	2.0 ± 0.45
12	71.7 ± 0.3*†	4.2 ± 0.15	24.1 ± 0.35

Values represent the mean ± SEM; $n = 6$

* Significantly different from the initial values using unpaired Student's t-test ($p < 0.05$)

† Significantly different from the previous values using unpaired Student's t-test ($p < 0.05$)

phosphate buffer of pH7 at 30-min reaction time. The experiment was repeated using 100 µl of each buffer at different pH values. As shown in Table 3, pH 7 is the optimum pH at which the maximum yield was obtained (94.8%). Also, it was observed that at pH 2 or 4, the yield was 8.4%, and 65%, respectively, while at pH values 9 and 11, the yield was 56.0%, 89.5%, respectively. There was significant difference between all pH values of the reaction mediums. The observation of this study that the optimum pH is 7, using phosphate buffers is constant with other previous work [22].

Effect of reaction time

Table 4 shows the relationship between the reaction time and the yield of ^{99m}Tc-MTZL. Radiochemical yield was significantly increased from 56.9% to 94.8% with increasing reaction time from 1 to 15 min. Extending the reaction time to 60 min, produced no significant change of the radiochemical yield. Extending the reaction time more than 1 h was associated with decrease in labeling yield. The efficiency of reducing agent may be affected by time and thus yield decreased [24].

Table 4 Effect of reaction time on the % labeling yield of ^{99m}Tc-MTZL

Time of reaction (min)	% Labeled compound	% Free ^{99m}Tc	% Colloid
1	53.0 ± 0.40	45.0 ± 0.3	2.0 ± 0.2
5	80.5 ± 0.50*	15.2 ± 0.75	4.7 ± 0.75
15	89.0 ± 0.32*†	10.4 ± 0.35	4.6 ± 0.5
30	93.3 ± 0.2*	4.0 ± 0.25	2.0 ± 0.45
60	92.7 ± 0.2*	4.0 ± 0.5	3.30 ± 0.4

Values represent the mean ± SEM; $n = 6$

* Significantly different from the initial values using unpaired Student's t-test ($p < 0.05$)

† Significantly different from the previous values using unpaired Student's t-test ($p < 0.05$)

Table 5 Effect of time on the stability of ^{99m}Tc-MTZL

Time post labeling (h)	% Labeled compound	% Free ^{99m}Tc	% Colloid
1/2	93.3 ± 0.2	4.7 ± 0.25	2.0 ± 0.45
1	93.3 ± 0.2	4.0 ± 0.25	2.07 ± 0.45
2	89.0 ± 0.32*†	10.4 ± 0.35	4.6 ± 0.5
6	85.3 ± 0.2*†	11.0 ± 0.25	3.7 ± 0.45
12	82.7 ± 0.2*†	14.0 ± 0.5	3.30 ± 0.4

Values represent the mean ± SEM; $n = 6$

* Significantly different from the initial values using unpaired Student's t-test ($p < 0.05$)

† Significantly different from the previous values using unpaired Student's t-test ($p < 0.05$)

In vitro stability of ^{99m}Tc-MTZL

In the present experiment, a significant decrease in the stability of ^{99m}Tc-MTZL from 94.8% to 91% at 12 h post labeling was observed. Further significant reduction was observed at 24 h post labeling, as the yield was 88%. The labeling yield was about 85% at 48 h post labeling (Tables 5).

Biodistribution of ^{99m}Tc-MTZL

In normal mice

Biodistribution study of ^{99m}Tc-MTZL in normal mice showed that ^{99m}Tc-MTZL was distributed rapidly in blood, stomach, heart and kidney at 15 min post injection. After 1 h, ^{99m}Tc-MTZL uptake was significantly decreased in organs like blood, heart, liver and intestine. However, ^{99m}Tc-MTZL uptake was significantly increased in bone, muscle, and thyroid after 1 h. At 12 and 24 h post injection, the majority of tissues showed significant decrease in ^{99m}Tc-MTZL uptake. Thyroid gland showed significant increase in ^{99m}Tc-MTZL uptake at 12 h post injection (Table 6).

In ascites bearing mice

The results of this experiment showed that the sites of greatest uptake of ^{99m}Tc-MTZL after 15 min post injection were the blood, heart and lung (16.5, 8 and 7.5), respectively. Table 7 shows that the concentration of ^{99m}Tc-MTZL was the lowest in thyroid, muscle and spleen at 15 min post injection. The uptake of ^{99m}Tc-MTZL in ascitic fluid was rapidly take place as each ml of ascitic fluid received 3.3% of total activity. The uptake of ascitic fluid was significantly increased after 1 and 12 h to reach 5.2% and 6.5% per 1 ml, respectively. No significant change in the uptake of ^{99m}Tc-MTZL at 24 h post injection was observed when compared to its previous value. The data

Table 6 Biodistribution of ^{99m}Tc-MTZL in normal mice

Organs and body fluids	Percent I.D./g organ			
	Time post injection			
	15 min	1 h	4 h	6 h
Blood	18.0 ± 1.10	14.6 ± 0.20*	8.2 ± 0.04*	4.7 ± 0.30*
Bone	2.0 ± 0.05	3.10 ± 0.10*	2.2 ± 0.10*	2.2 ± 0.2
Muscle	0.50 ± 0.01	1.50 ± 0.02*	1.3 ± 0.10	0.7 ± 0.02*
Liver	5.10 ± 0.05	6.50 ± 0.15*	3.5 ± 0.06*	2.0 ± 0.02*
Lung	4.00 ± 0.10	7.00 ± 0.12*	3.0 ± 0.20*	2.0 ± 0.01*
Heart	6.0 ± 0.80	6.50 ± 0.30*	5.0 ± 0.01*	1.5 ± 0.04*
Stomach	13.2 ± 0.90	17.2 ± 0.600	8.6 ± 0.16*	6.7 ± 0.2*
Intestine	7.40 ± 0.50	8.18 ± 0.30*	3.5 ± 0.10*	1.8 ± 0.03*
Kidney	6.90 ± 0.40	9.00 ± 0.600	4.1 ± 0.30*	2.1 ± 0.06*
Spleen	1.70 ± 0.02	3.00 ± 0.14*	3.0 ± 0.16	1.1 ± 0.200*

Values represent mean ± SEM; $n = 10$

* Significantly different from the previous value of each organ using unpaired Student's t-test ($p < 0.05$)

Table 7 Biodistribution of ^{99m}Tc-MTZL in ascites bearing mice

Organs and body fluids	Percent I.D./g organ			
	Time post injection			
	15 min	1 h	4 h	6 h
Blood	18.2 ± 1.00	12.0 ± 0.50*	5.90 ± 0.10*	4.0 ± 0.15*
Bone	3.10 ± 0.15	3.20 ± 0.150	2.30 ± 0.15*	1.6 ± 0.10*
Muscle	1.20 ± 0.09	2.3 ± 0.020*	1.6 ± 0.01*	0.9 ± 0.04*
Liver	6.40 ± 0.25	6.10 ± 0.20	4.60 ± 0.06*	3.3 ± 0.07*
Lung	5.20 ± 0.10	5.40 ± 0.04	2.20 ± 0.10*	1.5 ± 0.01*
Heart	8.00 ± 0.30	5.40 ± 0.30*	4.80 ± 0.10*	2.1 ± 0.02*
Stomach	12. 0 ± 0.30	13.4 ± 0.60*	10.7 ± 0.60*	7.1 ± 0.50*
Intestine	6.40 ± 0.50	5.60 ± 0.07*	4.20 ± 0.10*	2.1 ± 0.20*
Kidney	7.00 ± 0.40	5.20 ± 0.10*	4.01 ± 0.10*	2.1 ± 0.06*
Spleen	3.30 ± 0.10	2.35 ± 0.01*	1.70 ± 0.01*	0.9 ± 0.01*
Ascitic fluid	2.50 ± 0.30	4.50 ± 0.40*	6.20 ± 0.10*	5.9 ± 0.500

Values represent mean ± SEM; $n = 6$

* Significantly different from previous value of each organ using unpaired Student's t-test ($p < 0.05$)

also showed that some organs exhibit significant increase of uptake at 1 h post injection like stomach, ascitic fluid, bone and thyroid. On the other hand, significant decrease in ^{99m}Tc-MTZL uptake was observed in blood, heart, kidney and lung at the same time. At 12 h post injection, the majority of organs showed significant decrease in uptake of ^{99m}Tc-MTZL. Significant increase was only observed in ascitic fluid and thyroid at 12 h post injection. Similarly, at 24 h post injection, the majority of organs showed additional significant decrease in ^{99m}Tc-MTZL uptake. The results of biodistribution study of ^{99m}Tc-MTZL in ascites bearing animal revealed that ascites was one of the most site of uptake of ^{99m}Tc-MTZL and this was clear at l h and lasted to 24 h post injection. ^{99m}Tc-MTZL uptake in ascites was about 25% of the injected dose at 12 h post injection before reflecting the uptake per gram tissue. The uptake of each ml of ascites was 5.2, 6.5 and 6.3 at 1, 12 and 24 h, respectively. It was also observed that ascites was the site of highest uptake considering the average volume of ascites (8.2 ± 0.7). This result suggests the use ^{99m}Tc-MTZL in imaging of tumor. The high uptake of ^{99m}Tc-MTZL in kidney may reflect the excretion of the drug via urine [23].

In solid tumor bearing mice

Biodistribution of ^{99m}Tc-MTZL in solid tumor bearing mice was found to be greatest in blood, heart and stomach (22.8, 12 and 11.1, respectively) at 15 min post injection and lowest in left leg, bone and thyroid (0.8, 1.2 and 2, respectively) (Table 8). The biodistribution of ^{99m}Tc-MTZL in the right thigh (inoculated) was greater than that of left one. The uptake of ^{99m}Tc-MTZL in right thigh was significantly increased with time at 1 h and 12 h post injection, as it was 5.5 and 7% per g, respectively.

Liver showed significant increase in % ^{99m}Tc-MTZL uptake at 15 min, 1 h and 12 h post injection, when compared to ascetic bearing animals. In addition, ^{99m}Tc-MTZL uptake in the stomach of solid tumor mice was significantly

Table 8 Biodistribution of ^{99m}Tc-MTZL in solid tumor bearing mice

Organs and body fluids	Percent I.D./g organ			
	Time post injection			
	15 min	1 h	4 h	6 h
Blood	17.3 ± 1.80	12.10 ± 0.6*	6.7 ± 0.2*	4.2 ± 0.4 *
Bone	2.00 ± 0.15	2.3 ± 0.10	1.6 ± 0.1*	1.4 ± 0.10
Liver	6.50 ± 0.40	5.70 ± 0.2	3.1 ± 0.1*	2.7 ± 0.10
Lung	5.50 ± 0.50	6.50 ± 0.30*	4.4 ± 0.2*	2.2 ± 0.15*
Heart	8.00 ± 0.40	5.21 ± 0.10*	3.7 ± 0.05*	2.5 ± 0.200
Stomach	11.0 ± 0.70	14.6 ± 1.0*	8.5 ± 0.5*	6.2 ± 0.26*
Intestine	6.10 ± 0.20	7.70 ± 0.1*	6.1 ± 0.3*	4.6 ± 0.250
Kidney	3.50 ± 0.70	6.60 ± 0.2*	3.5 ± 0.3*	2.5 ± 0.20*
Spleen	6.00 ± 0.50	8.2 ± 0.4*	5.0 ± 0.5*	3.0 ± 0.25*
Left leg	1.40 ± 0.05	1.60 ± 0.10	1.5 ± 0.10	1.2 ± 0.03*
Right leg	2.70 ± 0.30	4.20 ± 0.5*	5.8 ± 0.8*	5.2 ± 1.10*

Values represent mean ± SEM; $n = 6$

* Significantly different from previous value of each organ using unpaired Student's t-test ($p < 0.05$)

increased at 15 min, 1 h and 24 h post injection when compared to ascetic bearing mice.

In the present study, the increase in % of ^{99m}Tc-MTZL in the blood of solid tumor bearing mice may be due to the large volume of ascetic fluid that are formed in ascetic bearing animals [25]. Significant increase in ^{99m}Tc-MTZL uptake in bone of ascites bearing mice may be due to high vascularities to ascetic fluid that may lead to destruction of blood cells. This may activate bone marrow and increase uptake of ^{99m}Tc-MTZL in the bone [24].

Conclusion

Incorporation of ^{99m}Tc-MTZL to a tumor site was achieved by labeling of MTZL with ^{99m}Tc. The appropriate conditions for labeling of ^{99m}Tc-MTZL (94% yield) were l00 µg tin as reducing agent, 25 µg MTZL as substrate, at pH 7, at room temperature and 15–30 min reaction time. The great incorporation of ^{99m}Tc-MTZL in tumor sites (asites or solid tumor) also facilitates tumor imaging. ^{99m}Tc-MTZL was found to be highly localized in tumor sites which considered an ideal victor to carry iodine-125 to the nucleus of tumor cells [25]. Also, increasing the dose (radioactivity) of ^{99m}Tc-MTX produced significant increase in the % non-viable cells revealed radiotoxicity of ^{99m}Tc-MTX on tumor cells. In conclusion, this study demonstrates a hopeful approach for cancer imaging.

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