Preparation and pharmacological evaluation of a new radiopharmaceutical, technetium-99m-5-fluorouracil, for tumor scintigraphy

Santosh Kumar[1],
Anil Kumar Mishra[2],
Bhupender S. Chhikara[2],
Krishna Chuttani[2],
Rakesh Kumar Sharma[3]

1. *Department of Pharmaceutical Sciences, Birla Institute of Technology, Mesra, Ranchi- 835215*
2. *Department of Radiopharmaceuticals, Institute of Nuclear Medicine and Allied Sciences, Delhi - 110054*
3. *Defense Research Laboratory, Post Bag No. 02, Tejpur, Assam- 784001.*

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Correspondence address:
Anil Kumar Mishra, Sci- 'E'
Department of Radiopharmaceuticals Institute of Nuclear Medicine and Allied Sciences, Brig. SK Mazumdar Marg,
Timarpur, Delhi-110054
E-mail: anilmishra99@sify.com
Phone No.: +91-11-23905177

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Abstract

5-fluorouracil (5-FU) has been used for cancer chemotherapy since more than four decades. There are reports of use of ^{18}F and ^{19}F analogues of 5-FU for tumor studies using positron emission tomography (PET) and nuclear magnetic imaging (NMR), respectively. However, study pertaining to its use in g-scintigraphy is still lacking. In the present study, we have optimized the methodology to radiolabel it with technetium-99m (^{99m}Tc) efficiently and evaluated its physicochemical and biological properties. *Methods: 5*-FU was radiolabeled with ^{99m}Tc and evaluated for physicochemical properties. Blood kinetics were studied in rabbits and biodistribution was carried out in normal as well as tumor bearing mice. *In vivo* and *in vitro* tumor uptake of the radiocomplex was evaluated in Ehrlich Ascites Tumor (EAT) bearing mice and human breast cancer cell line (MDA-MB-468). *Results:* The resultant radiopharmaceutical (^{99m}Tc-5-FU) has been found to be stable up to 24 h in both in vitro normal and physiological conditions. The blood clearance of the ^{99m}Tc-5-FU showed a bi-phasic pattern. High extraction of ^{99m}Tc-5-FU by the liver (36.41±2.79 % of injected dose/g tissue) has been observed in mice, along with time dependent increase in the solid tumor to muscle ratio (2:1) measured at 4 h. Incubation of the radiocomplex with human breast cancer cells lines also showed time dependent increase in the uptake of the tracer. *Conclusion:* It can be concluded that the ^{99m}Tc-5-FU possesses selectivity towards solid tumor tissue.

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Introduction

5-fluorouracil (5-FU), a thymidylate synthase inhibitor and its combinations with folinic acid/cisplatin/irinotecan in different schedules, comprise the first line treatment module for colorectal cancer [1-2]. The combination of 5-FU has also been found useful as an adjuvant, in radiotherapy [3-5]. Besides these studies some noninvasive techniques like position emission tomography (PET) and nuclear magnetic resonance (NMR) have been used with ^{18}F and ^{19}F analogues of 5-FU respectively, to explore the prognostic utility of the agent against 5-FU chemotherapy and patient responsiveness [6-8]. Studies have also been carried out to use 5-FU as a proliferation marker, lebelled with ^{18}F, which has shown a special feature of 5-FU described as 'entrapment' inside the tumor cell [9-11]. In spite of all these diverse and potentially useful applications of the different analogues of 5-FU there is no report till now regarding labeling this molecule with a g-emitting radionuclide. Donor groups (hydroxyl and nitrogen) present as structural units of 5-FU could be utilized to coordinate bond formation with radioactive metals. Radiometal complexes thus formed, by exploiting the combined properties of radioactive metal and pharmacological properties of 5-FU, would be used for radioimaging of tumors. ^{99m}Tc is an excellent radionuclide as shown by many studies, due to its excellent radiochemical properties: It posses a 6 h half life which is optimum for radiolabeling, good quality control and gives quality imaging studies. It emits a single energy gamma radiation of 141 keV which is suited to γ-camera imaging requirements and avoids unnecessary exposure of tested men or animals to secondary energy radiations. Considering its importance, we have attempted to radiolabel the 5-FU with ^{99m}Tc. We report here the radiolabeling of 5-FU with ^{99m}Tc and the evaluation of its physicochemical and biological properties for its use as a tumor scintigraphic agent.

Materials and methods

^{99m}Tc-pertechnetate was supplied as sodium pertechnetate by Regional Center for Radiopharmaceutical (Northern Region), Board of Radiation and Isotope Technology, Delhi, India.

^{99m}Tc was separated from molybdenum-99 by a solvent extraction method 5-fluorouracil was a kind gift from Biological E. Limited, Hyderabad, India and Biochem Pharma Industries, Mumbai, India. Monolayer culture of human breast carcinoma cells, MDA-MB-468 (kindly provided by Dr. Normando Iznaga-Escobar, CIMAB SA, Havana, Cuba) was maintained at 37°C in a humidified CO_2 incubator (5% CO_2, 95% air) in Dulbecco's Modified Eagle's Medium (DMEM; Sigma, USA) supplemented with 10% fetal calf serum (Biological Industries, Israel), 50 U/ml penicillin, 50 mg/ml streptomycin sulfate and 2 mg/ml nystatin. Cells were routinely subcultured twice a week using 0.05% trypsin (Sigma, USA) in 0.02% EDTA. Appropriate amount of stannous chloride dihydrate (Qualigens Fine Chemicals, Mumbai) was dissolved in sufficient volume of 10% acetic acid (Glaxo Laboratories India Ltd.) to produce final concentration of 1 mg/ml. Sodium bicarbonate (0.05M) (S. D. Fine Chemicals, Mumbai) was used to adjust the pH. Chromatographic analyses were carried out by using 0.5X10 cm Instant Thin Layer Chromatography-Silica Gel (ITLC-SG) strips (Gellman Sciences, USA). The solvents used for chromatography viz. acetone (Merck India Ltd. Mumbai), ethanol and ammonia (Qualigens Fine Chemicals, Mumbai), were of an analytical reagent grade. Phosphate buffer saline (pH- 7.4) was prepared with sodium dihydrogen phosphate and disodium hydrogen phosphate (Glaxo Laboratories India Ltd.) and sodium chloride (Merck India Ltd.). Membrane filters (Millipore, Germany) of 0.22 mm pore size, were used to filter the drug after radiolabeling. Haemocytometer was used for counting the tumor cells. Well type gamma counter (K2700B, ECA) was used for measuring the radioactivity. Scintigraphic studies were carried out on a single head gamma camera (Diacam, Siemens, USA) with parallel collimator adjusted to the energy level of ^{99m}Tc. All other chemicals were of analytical reagent grade or above.

Radiolabeling and radiochemical purity analysis

^{99m}Tc-5-fluorouracil (^{99m}Tc-5-FU) was prepared by dissolving 2 mg of 5-FU in 1ml distilled water, followed by addition of 75 mg stannous chloride and pH was adjusted to 7- 7.5 with 0.1N NaOH using pH-paper. Approximately, 185 MBq ^{99m}Tc pertechnetate was added to it, mixed and incubated for 5-10 min at room temperature. The contents were filtered through 0.22 mm membrane filter into a sterile vial.

Radiochemical purity of the labeled complex was determined by ascending ITLC using 100% acetone and 0.9% sodium chloride as mobile phase and amount of reduced/hydrolyzed ^{99m}Tc using Pyridine: Acetic acid:Water (3:5:1.5 v/v) as mobile phase on the ITLC-SG strip.

Stability of radiocomplex in serum

The *in vitro* stability of the complex was estimated in serum by incubating 100 ml of the ^{99m}Tc-5-FU with 900 ml of human serum at 37°C up to 24 h. Aliquots at different time intervals were applied on ITLC-SG paper and developed in 100% acetone to check for any dissociation/degradation of the labeled complex.

Plasma protein binding and lipophilicity

In vitro protein binding study of ^{99m}Tc-5-FU was carried out in human plasma by protein precipitation. To 0.9 ml fresh human plasma, 0.1 ml of the labeled complex was mixed and incubated for 1 h at 37°C. After mixing approximately equal volume of 10% trichloroacetic acid (TCA), the mixture was centrifuged at 500 g for 5 min. The supernatant was collected in a different tube, and the pellet was resuspended in 1 ml of 5% TCA, and centrifuged as before. The supernatant was collected in the same tube having the previous supernatant fraction, during each washing. Radioactivity was measured in both the precipitate and the supernatant fractions in a well type gamma counter. Protein binding of the complex was expressed as the fraction of radioactivity bound to protein, in percentage of the total radioactivity.

The lipophilicity was measured by measuring the organic/aqueous partition–coefficient. An aliquot of 0.1 ml of the labeled complex was mixed with 1.9 ml of saline and 2 ml of n-octanol. The contents were shaken and the two layers were allowed to separate by leaving the contents undisturbed for 15 min at room temperature. Both the fractions were collected separately and the radioactivity was measured in known aliquots of each fraction.

Blood kinetics and biodistribution

All animal experiments were carried out with the approval of the Institute Animal Ethics Committee. Blood clearance was studied in 4 New Zealand rabbits weighing about 2.5±0.3 kg after administering approximately 10 MBq of the labeled product into the marginal ear vein. At different time intervals blood samples were withdrawn from the marginal vein of the other ear of the animal. Radioactivity was measured, using well type gamma counter calibrated for ^{99m}Tc energy. The data was expressed as the percent administered dose per ml of blood at each time point.

Biodistribution of ^{99m}Tc-5-FU was carried out in normal as well as tumor bearing Balb/C mice weighing about 25-30 g. Ehrlich Ascites Tumor (EAT) cell line was maintained in the ascites form by serial weekly passage in mice. Exponentially growing EAT cells were harvested and resuspended in phosphate buffer saline. A fixed no. of EAT cells (10-15 million) were injected subcutaneously in the thigh region of the right hind limb of mice and the mice were used for study after 10 days when the tumor had grown to about 1cm in diameter. Each mouse was injected with 3.7 MBq of the complex into the tail vein. The mice were sacrificed by cervical dislocation at 1, 2, 4 and 24 h post administration of the complex. Tissue samples were collected from various organs, made free from adhering tissues, washed, dried on paper towel and weighed. The radioactivity was measured in each tissue sample and expressed as percent injected dose per gram of tissue.

Scintigraphy in EAT bearing mice

For scintigraphy in mice, EAT cells were implanted in Balb/C mice weighing approximately 25-30 g as described in previ-

ous section. The mice were administered 100 ml (3.7 MBq) of the labeled complex through the tail vein and images were recorded using single head gamma camera (Diacam, Siemens, USA) with a parallel hole collimator at different time intervals (1 h, 2 h, 4 h, and 6 h).

Cell-uptake study of the radioligand

The cell uptake of ^{99m}Tc-5-FU conjugate was studied in human breast carcinoma cell line (MDA-MB-468), grown in Leibovitz's L-15 medium supplemented with 1% antibiotic/antimycotic and 10% fetal bovine serum. Monolayer cell line cultures were incubated with fixed concentration of complex (1 mM) in HBSS at 37°C for different time intervals (0.5 h-6 h). The uptake was terminated by addition of 1 ml of ice-cold saline. Supernatant was collected after the stipulated duration and cells were harvested with 1 ml saline. The suspension was washed with saline for 7 times by centrifugation of cell suspension for 10 s at 800 g. The pellet was resuspended in saline and the radioactivity was determined in supernatant, washings, and pellet, by well type gamma counter. The counts were subtracted with the non-specific binding of the technetium obtained by incubating the equivalent amount of ^{99m}Tc in cell culture plates. The cell uptake was expressed as percent of radioactivity present in the cell pellet to the total radioactivity in the medium.

Results

The radiocomplex (^{99m}Tc-5-FU) exhibited more than 98% radiolabeling efficiency with less than 2% colloid formation when subjected to ITLC at different time intervals. The complex was sufficiently stable (only 2%-3% degradation) in *in vitro* normal and physiological conditions as observed up to 24 hr at room temperature and in human serum respectively. The protein binding assessed in human serum was found to be 28.8%±1.12%. Lipophilicity, estimated in terms of n-octanol: water partitioning value, was found to be 3.52±0.68. The blood kinetics of the ^{99m}Tc-5-FU was carried out in 4 rabbits and the blood level at each time interval was the mean of individual values and the data was treated using the graphical method of analysis. The blood clearance of the radiolabel in rabbits revealed bi-phasic pattern (Fig. 1). Half-life for fast-phase was found to be 0.22 h and for slow-phase 3.42 h. Biodistribution studies in normal as well as tumor bearing mice (Table 1) showed almost similar pattern of distribution. The major accumulation of the activity in terms of the percentage of injected dose per gram tissue was in the liver (36.41±2.79 % of injected dose/g tissue) followed by spleen (13.21±2.78 % of injected dose/g tissue) at 2 h time interval. There was moderate decrease in liver activity, observed at 4 h time interval (20.88±0.62 % of injected dose/g tissue) which again increased at 24 h (32.31±4.62 % of injected dose/g tissue). However, in all other organs there was a decrease in the activity after 2 h time interval except in the tumor tissue. Time dependent increase in tumor: muscle activity was observed after 2 h which remained almost constant at 4 h and at 24 h (1.96:1 and 2.08:1 respectively). Inverse relationship was observed between muscle and tumor concentration of the labeled moiety (Fig. 2). Scintigraphic study showed increase in the activity of the tumor tissue, with maximum tumor activity at 4 h post injection of the complex (Fig. 3). The cell uptake study carried out in human breast carcinoma cell line showed continuous increase in the cell associated activity achieving maximum at 4 h (9.17±0.89 %) leading to plateau phase (9.14±0.58 %) at 6 h (Fig. 4).

Table 1. *Biodistribution data of ^{99m}Tc-5-FU in tumor bearing mice, expressed as percent of injected dose per gram of tissue (±SD).*

Tissues	1 h	2 h	4 h	24 h
Blood	0.97±0.26	1.00±0.14	0.76±0.03	0.33±0.02
Heart	0.98±0.22	0.96±0.11	0.68±0.05	0.20±0.04
Lungs	2.95±0.52	3.93±0.60	2.19±0.15	0.85±0.01
Liver	36.34±3.84	22.41±2.79	20.88±0.62	12.31±4.62
Spleen	11.03±0.42	13.21±2.78	10.84±0.30	5.09±0.99
Kidney	5.09±0.21	6.08±0.93	3.86±0.57	3.52±0.28
Stomach	1.39±0.58	1.01±0.29	0.66±0.01	0.46±0.11
Intestine	1.70±0.22	2.66±0.83	2.68±0.83	1.72±0.21
Muscle	0.54±0.04	0.50±0.02	0.26±0.05	0.23±0.09
Tumor	0.36±0.08	0.34±0.01	0.51±0.07	0.48±0.06

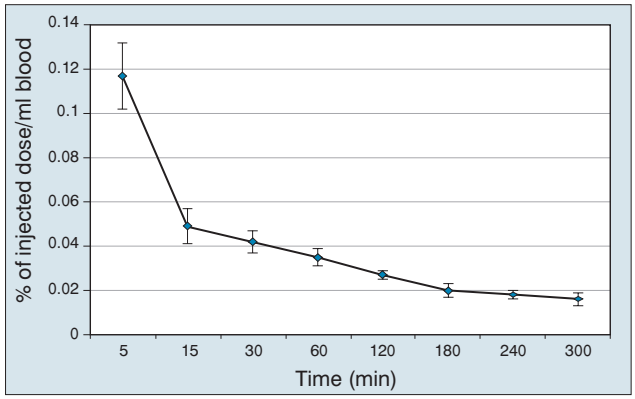

Figure 1. Blood kinetics of ^{99m}Tc-5-FU following intravenous injection in rabbit (10 MBq), expressed as percent of injected dose per ml of blood (±SD).

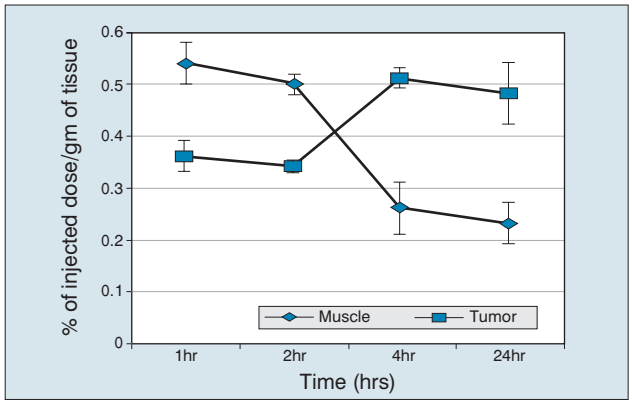

Figure 2. Distribution of ^{99m}Tc-5-FU in Muscle and Tumor in EAT bearing mice following intravenous injection, expressed as percent of injected dose per gram of tissue (±SD).

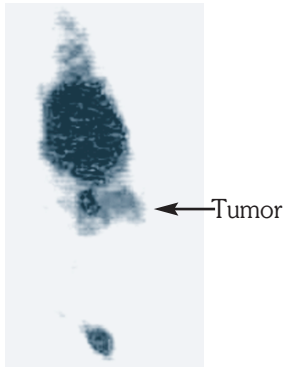

Figure 3. γ-scintigraphic image of EAT bearing mice (implanted in right hind limb) after 4 h of intravenous injection of ^{99m}Tc-5-FU (±SD).

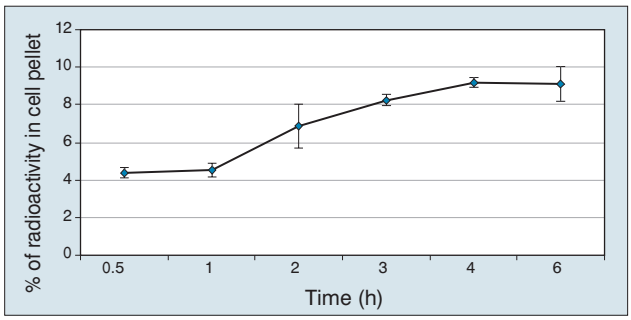

Figure 4. Uptake study of ^{99m}Tc-5-FU in human breast carcinoma cell line (MDA-MB-468) (±SD).

Discussion

The present study shows the γ-scintigraphic potential of ^{99m}Tc-5-FU for tissues expressing tumor. Increase in the accumulation of the tracer in target tissue with time and simultaneous decrease in non-target activity, is the important finding with respect to scintigraphic behavior of the radiocomplex.

Labeling of 5-FU with ^{99m}Tc showed >98% radiolabeling, which was sufficiently stable for 24 hours at room temperature and in *in vitro* physiological conditions too. Though further confirmative studies are required, a preliminary presumption for the complex formation between 5-FU and ^{99m}Tc could be chalked out from the various studies reported for ^{99m}Tc complexation with active pharmacophores. Among the conventional methods of labeling, the reduced ^{99m}Tc (V) (most probable oxidation state present in Tc complexes), monooxo (in some cases dioxo) with tetradentate to hexadentate system, form stable complexes with various donor groups explicitly with nitrogen, sulfur and oxygen ligands. The tetradentate system with N_2O_2 donor, present as amine-phenol ligands are known to form stable complexes as evaluated by others [12-13]. In case of 5-FU, the requisite geometry (tetradentate or octadentate system) of final ^{99m}Tc complex, would be achieved by the coordination from at least two molecules of 5-FU (Fig. 5). Among the possible coordination, any of the 1N and 2-hydroxy resonance, 2-hydroxyl, 2-hydroxy and 3N resonance, 3N and 4-hydroxy resonance or 4-hydroxyl groups, might be involved in from 5-FU. Considering the acidic nature of hydroxyl group (phenolic OH) of the uracil

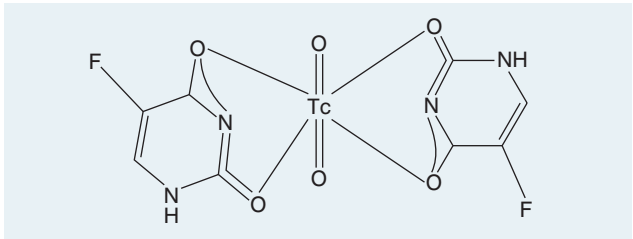

Figure 5. Putative Structure of ^{99m}Tc-5-FU with site of ^{99m}Tc attachment.

unit, it would exist as anion in our experimental conditions (pH 7.4), so leading to resonance in 5-FU for effective coordination to Tc metal. With a quite possibility of resonance structures in experimental conditions, the coordination from 5-FU may be through 3-center monodentate (N-C-O resonance) or through localized anion monodentate (O^- anion) ligation, with a further possibility of bidentate chelation (N-C-O resonance and O^- donor). In either case, there would be more then one pharmacophore units of 5-FU in one molecule of the complex, which suggests that the radiocomplex may behave like a new chemical entity.

The n-octanol: water partition coefficient of the ligand, is very low confirming that the complex is hydrophilic in nature, however, the plasma protein binding of 5-FU is little higher than (28.8±1.12) that has been reported to be (10% - 20%) for ^{18}F-5-FU [14]. Blood kinetics of the complex assessed as clearance of the tracer from blood pool, shows a biphasic pattern with fast and slow clearance component which is confirming previous reports [15]. Fast clearance observed in the first phase suggests that the radiocomplex has either very large volume of distribution or faster elimination. However, the large volume of distribution is mainly observed with molecules lipophilic in nature. As ^{99m}Tc-5-FU has been found to be hydrophilic so the faster elimination could be the more probable reason. The slow clearance of tracer observed in the second phase of blood kinetics could be due to the release of tracer from different organs in to the blood pool [14]. The biodistribution study of the radiocomplex carried out in tumor bearing as well as normal mice, reflected an almost similar pattern of distribution in both groups. Maximum tissue activity was found in the liver, which further got increased at 2 h and was not significantly reduced after a 24 h interval. Earlier studies with 5-FU have reported a high intracellular catabolite gradient, which has been proposed to be due to the slow transfer of charged metabolite formed across the cell membrane. Although in the present study there is no data regarding the nature of deposited activity in the liver tissue, it can be proposed that the present radiocomplex might be taken inside the lives cells and that its degradation/catabolism leads to the formation of the ^{99m}Tc-complex which is not being easily eliminated out of the cell. In other tissues, two different trends of change in activity have been observed. An increase in the tissue activity was found in lungs, spleen, kidney, and the intestine, at 2 h after the injection of the radiolabel, followed by a continuous decrease in activity. However, there was continuous decrease in activity in other tissues, except in tumor

tissue. The redistribution of tracer among organs could be one possible reason for the increase in tissue activity observed here at 2 h interval. Since no such increase was observed in the blood pool, it can be expected to be due either to the extractive nature of the tissue or to the metabolism of the radiocomplex getting accumulated there in blood. Another significant observation is the high uptake of activity by the spleen, which has not been reported in earlier studies, conducted by using ^{18}F-5-FU [15]. However, the amount of radioactivity in the target tissue in these studies was low (< 1% of the injected dose/g tissue).

The tumor uptake of the radiotracer is dependent on different factors, like the nature of the complex, pH, blood flow, plasma concentration etc. In the present murine model, a solid tumor was developed having poor angiogenesis. So, the low plasma concentration and the poor vasculature of the target tissue may also contribute towards the lesser uptake of the radiotracer. However, time dependent increase in activity was observed in the tumor tissue with simultaneous decrease in the contralateral muscle mass in the same animal, alleviating any inter-animal variation. This increase in uptake of the tracer with time, in target tissue is contrary to the nature of 5-FU uptake in tumor tissue, as reported in a study conducted earlier with ^{18}F-5-FU where continuous decrease in the tumor activity has been reported [15]. So, it can be proposed that the radiocomplex is not being treated like 5-FU by cellular transport system but is being transported by some different mechanism. Studies carried out in tumor bearing mice to assess the scintigraphic utility of the radiolabeled complex, showed relatively higher distribution of radioactivity in the peritoneal area replicating the result of the biodistribution study carried out in tumor bearing mice. The flank region had more activity than the contra lateral tumor bearing flank region as measured at an earlier time point which and progressed inversely. There was almost twice the radioactivity found in the tumor bearing flank as compared to the other flank at 4 h after the injection of radiolabeled complex. The specific nature of the EAT cell line could be one of the possible reasons for this selective uptake of radioactivity in tumor tissue. To alleviate this possibility the radiolabeled complex was further evaluated in the human cancer cell line, which showed a time dependent increase in the uptake of ^{99m}Tc-5-FU till 4 h, which was almost constant till 6 h interval. The plateau exhibited by the uptake curve after 4 h up to 6 h, is corroborating the findings of biodistribution studies where the amount of radioactivity in tumor tissue after 4 h, is decreasing very slowly. This finding suggests that the pattern of uptake and retention of the molecule towards the tumor tissue is almost similar in both the human and murine cell line as well as in *in vivo* and in *in vitro* conditions.

In conclusion with the support of the present findings it can be concluded that the ^{99m}Tc-5-FU possess selectivity towards the solid tumor tissue. Further studies are warranted to increase the radiolabel uptake in the target tissue with an attempt to minimize the background radiation by reducing the tissue activity in visceral organs like liver and spleen.

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