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NUCLEAR MEDICINE AND MOLECULAR IMAGING

ORIGINAL ARTICLE

Tc-99m-tamoxifen: A novel diagnostic imaging agent for estrogen receptor-expressing breast cancer patients

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PURPOSE
The aim of the study was to radiolabel, characterize, and perform *in vitro* and *in vivo* assessment of Technetium-99m (Tc-99m) tamoxifen for screening ER expressing lesions in breast cancer patients.

METHODS
In this study, tamoxifen has been radiolabeled with Tc-99m via Tc-99m-tricarbonyl core. The characterization and quality control tests of Tc-99m-tamoxifen were performed. *In vitro* receptor binding and blocking studies were performed in both positive control (MCF-7) and negative control cell lines (MDA-MB-231). Normal biodistribution studies were performed in female Wistar albino rats. The pilot clinical studies were performed in 4 ER-expressing breast cancer patients. Of the 4 patients, 1 was on tamoxifen therapy. All 4 patients had also undergone Fluorine-18 fluorodeoxyglucose (F-18-FDG) positron emission tomography/computed tomography.

RESULTS
Tamoxifen was radiolabeled with Tc-99m via Tc-99m-tricarbonyl core with more than 95% radiochemical yield. Mass spectra showed a peak corresponding to the molecular weight of Tc-99m-tricarbonyl and Tc-99m-tamoxifen. The site of binding of Tc-99m-tricarbonyl with tamoxifen was determined by proton nuclear magnetic resonance. The Tc-99m-tamoxifen showed 30% binding with MCF-7 and only 1%-2% receptor binding with MDA-MB-231 cell lines. Also, the percentage of receptor binding was drastically reduced (up to 72%) when ER was saturated with 50 times the excess molar ratio of unlabeled tamoxifen. In a pilot patient study, Tc-99m-tamoxifen uptake was observed in primary and metastatic lesions. However, no uptake was observed in a patient who was on tamoxifen therapy. The uptake of F-18-FDG was noted in all the patients.

CONCLUSION
Tamoxifen was radiolabeled with an in-house-synthesized Tc-99m-tricarbonyl core. The radiolabeled complex has been characterized and evaluated for receptor specificity in *in vitro* and *in vivo* studies. Also, this is the first clinical study using Tc-99m-tamoxifen for imaging ER. More patients need to be evaluated to further explore the role of Tc-99m-tamoxifen in ER-expressing lesions.

According to the recent report of the International Agency for Cancer Research, World Health Organization (2020), breast cancer has the highest age-standardized incidence rate of 47.8 per 100 000 worldwide. In addition, breast cancer is generally detected at advanced stages, particularly in developing countries. This drastically affects the survival rate and management of treatment options for the patients.[1]

Early detection and localization is the key to better management of breast cancer. Various diagnostic imaging modalities are available that can help in the detection of breast cancer such as ultrasound, mammography, computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET), and single photon emission computed tomography (SPECT). Among various existing molecular imaging modalities, nuclear medicine plays a vital role in diagnosis. Several PET and SPECT tracers like (F-18-FDG), F-18-fluorothymidine, F-18 choline, Gallium-68 RGD, and Tc-99m sestamibi have been

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used for imaging breast cancers. Nuclear medicine scans provide detailed information regarding alteration in physiological function throughout the body. In addition, detection can be done at early stages even before the occurrence of symptoms or anatomical changes. F-18-FDG is a widely used tracer for the staging, restaging, treatment response evaluation, and management of various cancers. The uptake of F-18-FDG is, however, nonspecific for cancer cells and can also be observed in inflammatory conditions, which leads to false-positive F-18-FDG interpretation.[2,3]

About two-thirds of breast cancers express ER, and it is considered as an important prognostic and predictive indicator of breast cancer. Patients with ER-expressing breast tumors have a better prognosis when compared to patients with non-ER-expressing tumors in terms of responsiveness to anti-estrogen treatment.

Currently, F-18 fluoroestradiol (F-18 FES) is the most commonly used radiopharmaceutical for ER imaging. However, the radiosynthesis of F-18 FES is a tedious and costly procedure. It requires an onsite medical cyclotron facility for F-18 production and a dedicated cassette for F-18 FES synthesis. Also, the yield is low, that is, 15%-40%. Due to all these factors, the availability of F-18 FES is limited. Hence, there is a need to explore an alternative and cost-effective radiopharmaceutical.[4]

Tamoxifen is one such receptor-specific FDA-approved chemotherapeutic drug used for the treatment of ER-expressing breast cancers. The principal mechanism of action is mediated by binding to the ER, resulting in inhibition of ER multiplication. Tamoxifen is a drug of choice for patients diagnosed with all stages of ER-expressing breast cancer.[5]

In various past studies, tamoxifen has been chemically modified for radiolabeling with various radioisotopes such as F-18, Tc-99m, Indium-111, Iodine-131, Iodine-123, Bromine-77, Bromine-75.[6-10]

Main points

- Tc-99m-tamoxifen is a cost-effective and in-house-synthesized radiopharmaceutical for estrogen receptor targeting.
- It is suitable for patient administration.
- Tc-99m-tamoxifen is highly specific for estrogen receptor.

However, none of these tracers are in clinical use.

Keeping in mind the economic factors and the limited availability of on-site cyclotron, the most commonly used and readily available Tc-99m was chosen. The ideal characteristics of Tc-99m include a wide range of oxidation states, that is, −1 to +7, favorable gamma energy of 140 keV for imaging, and optimum half-life ($t_{1/2}$) of 6 h. The radiolabeling of tamoxifen with Tc-99m will help in screening ER as well as the spread of disease throughout the body.

In the present study, tamoxifen has been radiolabeled with Tc-99m-tricarbonyl core. Using this metal core, small biomolecules can be radiolabeled with exceptionally high specific activity and with minimum alteration of the bioactivity of the ligand molecule. Also, any monodentate, bidentate, or tridentate donor ligands can be used. The Tc-99m-tricarbonyl cores are smaller metal complexes and are less likely to interfere with the biological activity of a targeting moiety in radiopharmaceutical development.[11]

In the present study, Tc-99m-tamoxifen was developed via metal tricarbonyl core. The efficacy of radiolabeled tamoxifen was studied *in vitro* and *in vivo*. In addition, a clinical study was performed to localize the ER-expressing primary and metastatic lesions.

Methods

Materials

The chemicals and materials used for the study were listed as follows: Mo-99/Tc-99m generator (PARS Isotope), tamoxifen (Sigma), methanol, HCl, Whatman paper (GE), Radio TLC scanner (Comecer), 24- and 96-well plates (HiMedia), RPMI-1640 medium (HiMedia), CO_2 incubator, trypsin (HiMedia), plastic tubes (Tarson), centrifuge (REMI), DMSO (Thermo Fisher), MTT (HiMedia), HPLC water (Merck), dissection kit, SPECT/CT (Discovery 670 DR, GE Healthcare), and PET/CT (Discovery 710 scanner, GE Healthcare). All chemicals were of high (99%) purity and were purchased from Sigma.

Synthesis of Tc-99m-tricarbonyl core and radiolabeling of tamoxifen

Tricarbonyls were prepared using a single component as described by Alberto and Winterthur.[12] The Tc-99m-tricarbonyl core was synthesized using sodium boranocarbonate to radiolabel tamoxifen. The reaction parameters such as the amount of tamoxifen, reaction volume, reaction pH, temperature, and incubation timings were optimized to achieve maximum radiolabeling yield (detailed radiolabeling protocol is provided as supplementary data).

Quality control and characterization of Tc-99m-tamoxifen

The radiochemical purity and stability of Tc-99m-tamoxifen were confirmed by paper chromatography using a scanner equipped with a radioactive detector. The Tc-99m-tamoxifen was subjected to various physiochemical and biological tests to ensure the safety of drugs for intravenous administration[13,14] (for detailed quality control study protocol, please refer to supplementary data).

Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF)

MALDI-TOF is a highly sensitive analytical technique used for determining the molecular weight of the samples on the basis of their mass (m) to charge (z) m/z ratio. About 2-3 µL of the sample was mixed uniformly with α-cyano-4-hydroxycinnamic acid matrix and was spotted onto the ground steel plate. After drying, the samples were irradiated with a nitrogen laser (2000 Hz). The analysis was carried out in linear detection mode, positive polarity, 20 000 V acceleration voltage, and 100 ns delayed extraction time. The acquisition mass range used was 0-1000 Da. Mass spectra of drug and radiolabeled compounds were compared to ensure the radiolabeling ability and stability of the labeled peptide.

^{1}H-NMR spectroscopy of tamoxifen and Tc-99m-tamoxifen

The lyophilized powder of tamoxifen and Tc-99m-tamoxifen were dissolved separately in 600 µL of deuterated chloroform and transferred to 5 mm nuclear magnetic resonance (NMR) tubes for experiments. The proton nuclear magnetic resonance (^{1}H-NMR) spectra were acquired on a narrow bore NMR spectrometer operating at 700 MHz equipped with a broadband probe. One-dimensional (1D) ^{1}H-NMR spectra were recorded with a 90° pulse. The parameters used for the 1D experiment were as follows: spectral width = 7716 Hz, data points = 32 K, number of scans = 64,

and relaxation delay = 2 s. Two-dimensional (2D) correlation spectroscopy (COSY) was carried out for the assignment of various resonances of tamoxifen. The typical parameters used for 2D COSY were as follows: 2K data points in the F_2 dimension, a relaxation delay of 2.5 s, 400 time-domain points (number of experiments) collected in t_1 with 16 scans.

The 1D and 2D NMR data were processed on a Dell 390N, PC, Red Hat Enterprise Linux workstation using Vnmrj 2.3 A. For the processing of 1D NMR data, the free induction decays were multiplied by an exponentially decaying function prior to Fourier transformation leading to a line broadening of 0.3 Hz. Spectra were manually phase corrected using zero- and first-order corrections. The 2D NMR data were processed using automated processing with Fourier Transform data size of 2K × 2K and Gaussian weighting function in both F2/F1 dimensions.

Cell culture studies

The MCF-7 cells were cultured in complete (working) RPMI-1640 medium and were grown as a monolayer. The cell monolayer was de-adhered by incubating 750 µL of 0.25% trypsin-EDTA solution for 5-10 min at 37°C and washed with 2 mL PBS to remove the residual serum and inactivate trypsin. The pellet was collected after centrifugation at 200 *g* for 10 min and resuspended in growth media. Cells were counted and plated in 24-well plates at a density of 3-5 × 10^3 cells/100 µL/well. Cells were plated in triplets for all the experiments. Plates were kept in a humidified CO_2 (5%) chamber, and after overnight incubation, cells were examined under a light microscope.

Immunocytochemistry

The expression of ER on MCF-7 cell lines was quantified by immunocytochemistry (for detailed steps, see supplementary data).

Receptor binding

Cells were grown in culture media, harvested, and transferred to a T25 flask. All binding assays were done in 24-well plates. The receptor binding of the Tc-99m-tamoxifen for ER was assessed in the MCF-7 (positive control) and MDA-MB-231 (negative control) cell lines. Fifty thousand cells were seeded in each well of the 24-well plate having RPMI-1640 medium. The cells were then incubated in a CO_2 incubator at 37°C with 5% CO_2 for 24 h. When cells were 60%-70% confluent, Tc-99m (100 µCi and 200 µCi), Tc-99m-tricarbonyl (100 µCi and 200 µCi), increasing doses of Tc-99m-tamoxifen (50 µCi, 100 µCi, 200 µCi, and 400 µCi) were added in triplicates, followed by incubation for 1 h in a CO_2 incubator. The supernatant of every well was collected in a separate tube for counting in a gamma well counter. Cells were washed and de-adhered using trypsin and neutralized with media and collected in tubes. The tubes were then centrifuged at 200 *g* for 5 min to obtain the cell pellet. Counts of supernatant and pellet were noted, and % binding was calculated as follows:

$$\%\,\text{receptor binding} = \frac{\text{counts of pellet}}{\text{total counts}(\text{counts of supernatant} + \text{pellet})} \times 100.$$

Blocking studies

To test the specificity of Tc-99m tamoxifen for ER, ER of MCF-7 cells was saturated with increasing amounts of tamoxifen (0.5 mM, 5 mM, and 50 mM), in triplicates, and kept for incubation for 1 h in a CO_2 incubator. After 1 h, increasing amount of Tc-99m-tamoxifen (50 µCi, 100 µCi, 200 µCi, and 400 µCi) was added in wells containing each concentration of tamoxifen and was incubated for 1 h in a CO_2 incubator. Counts were noted in pellet and supernatant as described in the previous section.

Preclinical study

Animal experiments were performed after obtaining ethical clearance from the Institutional Animal Ethics Committee (Ref no. 95/93/IAEC/652). Female Wistar albino rats (n = 15) were weighed and marked appropriately. The rats were then anesthetized with an intraperitoneal injection of sodium pentobarbitone (30-35 mg/kg). Each rat was injected ~100 µCi of Tc-99m-tamoxifen intravenously (i.v.) through the tail vein. Biodistribution was observed by sacrificing rats at 30 min, 1 h, 2 h, 4 h, 6 h post-injection taking 3 rats per time point. Blood samples were collected by cardiac puncture. Various organs such as heart, lung, liver, spleen, kidney, urinary bladder, femur bone, and thigh muscle were removed and washed with ice-cold saline. The tissue samples (2-3 mm) of each organ were weighed and counted in a gamma well counter in Tc-99m energy window (140 keV ± 15%), and percentage injected activity per gram (%ID/g) was calculated. The Tc-99m standard was prepared, and appropriate decay corrections were applied to all the samples.

Animal dosimetry

An animal dosimetry study was performed to study critical organs. Critical organs are those that are most susceptible to radiation damage resulting from the specific exposure conditions under consideration, taking into account the dose the various parts of the body receive under the exposure conditions. The residence time was calculated by values of %ID/g at different time intervals of various organs. The residence time obtained was used as an input in the dose calculations by Organ Level INternal Dose Assessment/EXponential Modeling version 2.0 (OLINDA/EXM 2.0).

Pilot patient study

The Tc-99m-tamoxifen scan was performed after obtaining clearance from the Institutional Ethics Committee (Ref NoINT/IEC/2017/83) and written informed consent from each patient. The F-18-FDG-PET/CT scan was performed in each patient. The patients with histopathologically proven ER-expressing breast cancer were recruited for imaging. However, claustrophobic, pregnant, and lactating patients were excluded from the study.

Immunohistochemistry

Immunohistochemistry was performed to find the receptor expression of the patients (detailed protocol in supplementary data).

Acquisition protocol

Technetium-99m tamoxifen (8-15 mCi) was injected intravenously. Serial images at 0 h, 1 h, 2 h, 3 h, 4 h up to 24 h post-injection were acquired. The SPECT/CT was acquired at 4 h after acquiring a whole-body (WB) image.

Imaging parameters

WB images (head-to-toe) were acquired using a dual-head gamma camera with LEHR collimator. Patient demographics were entered, and the images were acquired using both detectors in auto contour with energy window centered at 140 ± 10% keV, matrix size of 256 × 256 with zoom1, with table speed of 14 cm/min. The SPECT/CT images were obtained with step-and-shoot mode. The CT was acquired with

current of 2.5 mA, voltage of 140 keV, and total exposure time of 329 s.

All the recruited patients underwent F-18-FDG PET-CT imaging. Both the scans were performed on 2 separate days, within 1 week of each other and in random order. The F-18-FDG WB images were acquired from the base of the skull to mid-thigh 45 min after intravenous administration of radiotracer.

Image-based dosimetry studies

Five-planar WB images were acquired under gamma camera at 1-h intervals up to 4 h and an image within 24 h. A standard source placed at the level of feet was included with the acquisition at all the time points. The WB SPECT-CT was acquired at 4 h after WB image. Processing of 5 planar WB images, as well as WB SPECT-CT, was done using the Dosimetry Toolkit software. Five WB and WB SPECT/CT images were reconstructed. Patients' demographics, camera sensitivity parameters, and injected activity were entered. Organ volume was defined by constructing a voxel of interest for the selected organs for which dosimetry calculation was performed. For organ volume, both SPECT and CT images were used. The body contour of the patient was defined, and the standard was measured along with the WB scan. The software generated the residence time–activity curve. The values of the residence time of organs were entered in OLINDA/EXM 2.0, and the estimated radiation dose to each organ was obtained as mSv/MBq units.

Results

Tc-99m-tricarbonyls and Tc-99m-tamoxifen were synthesized with >95% radiolabeling yield. For optimization parameters and quality control, please refer to Supplementary data.

The molecular weights obtained from MALDI-TOF of Tc-99m-tricarbonyl, tamoxifen, and Tc-99m-tamoxifen were 234 Da, 371 Da, and 568 Da, respectively (Figure 1).

One-dimensional and 2D ^{1}H-NMR spectroscopy experiments were carried out to find out the binding site of Tc-99m-tricarbonyl with tamoxifen. Figure 2 shows the ^{1}H-NMR spectrum of tamoxifen alone (Figure 2a) and Tc-99m-tamoxifen (Figure 2b). Figure 3 shows the expanded region of 2D COSY of tamoxifen alone.

The resonances of various protons of tamoxifen were assigned using 1D and 2D COSY and were also compared with those reported in the literature. The β-CH_3 was observed as a triplet at 0.93 ppm that shows a cross peak with α-CH_2 (2.45 ppm) in 2D COSY (Figure 3b). The α-CH_2 (2.45 ppm) was

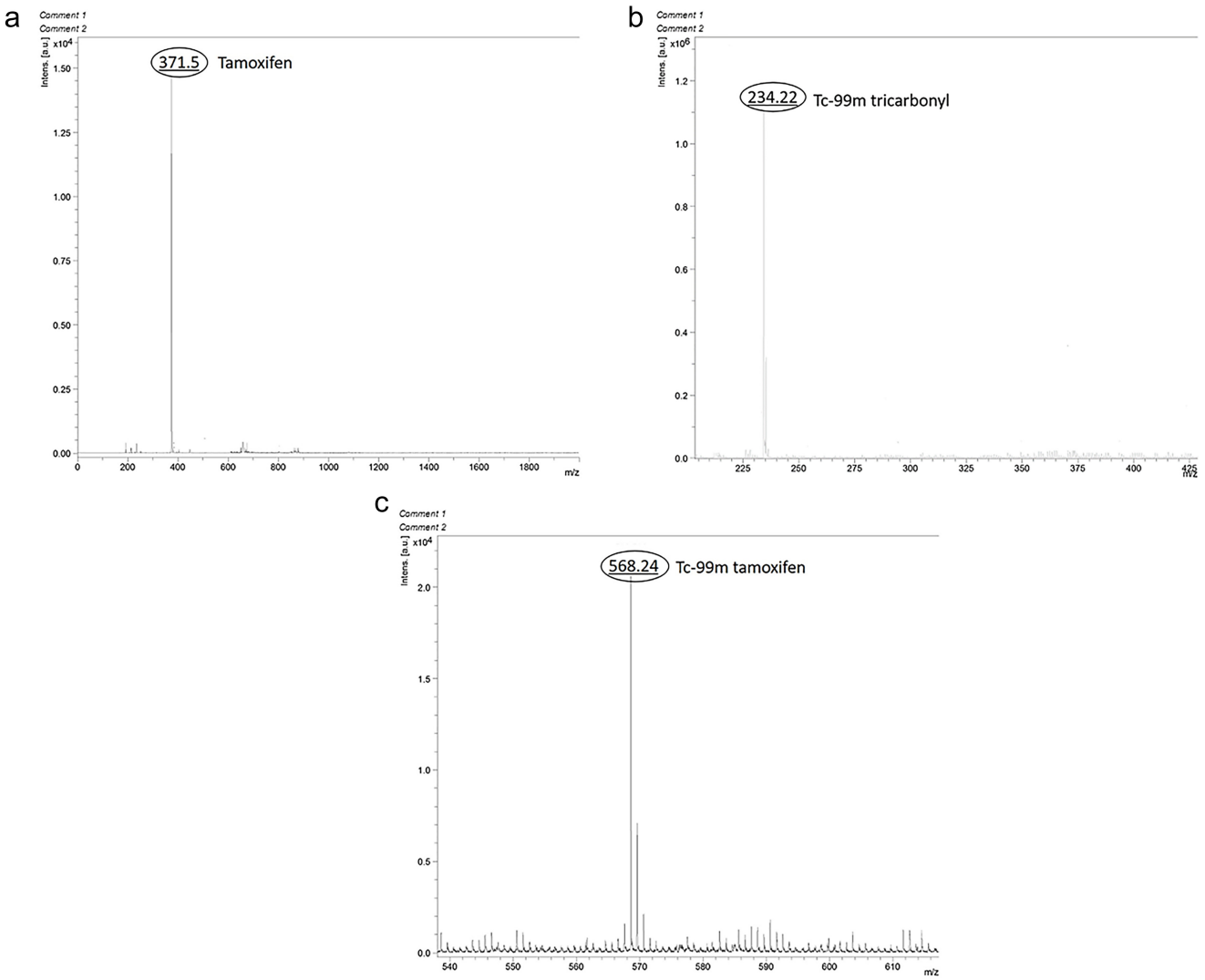

Figure 1. a-c. Mass spectra of **(a)** tamoxifen (MW = 371 Da), **(b)** Tc-99m-tricarbonyl (MW = 234 Da), and **(c)** Tc-99m-tamoxifen (MW = 568 Da). MW, molecular weight.

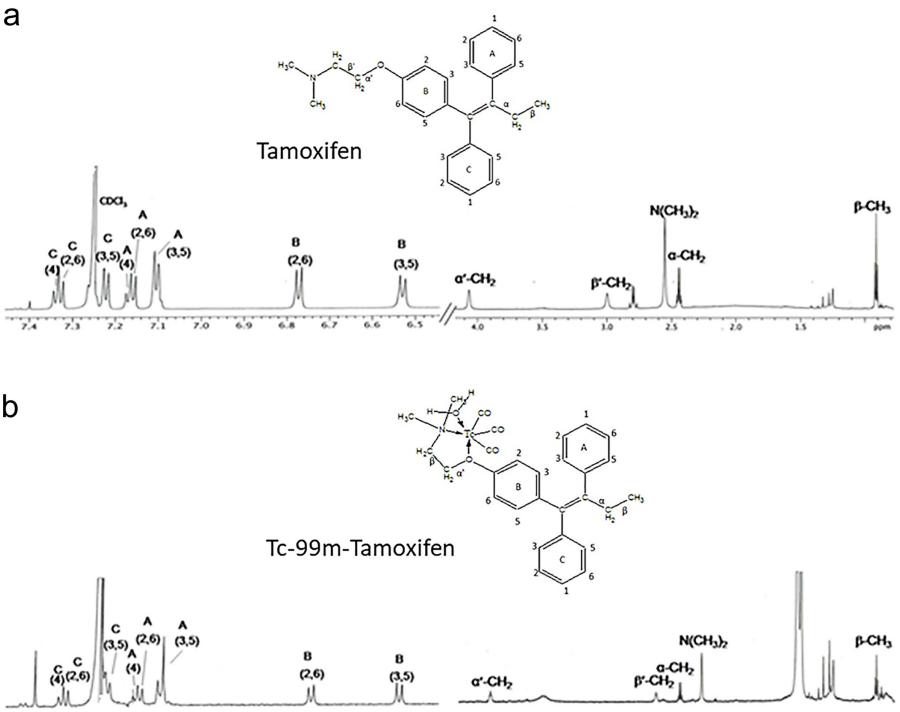

Figure 2. a, b. Expanded regions of one-dimensional proton NMR spectrum of tamoxifen alone **(a)** and Tc-99m-tamoxifen complex **(b)** acquired at 700 MHz in $CDCl_3$ at 25°C. NMR, nuclear magnetic resonance.

seen as a quartet due to its coupling with β-CH_3 protons (Figure 2a). The α′-CH_2 protons were observed at 4.07 ppm in the spectrum of tamoxifen alone (Figure 2a). A 2D COSY cross peak between α′-CH_2 protons and β′-CH_2 protons (2.64 ppm) confirms the assignment of these resonances (Figure 3b). The various aromatic protons of phenyl rings (A, B, and C) were observed in the downfield region. The ring B protons (2H, 6H) and (3H, 5H) were observed as a doublet at 6.54 ppm and 6.78 ppm, respectively. It may be noted that the resonances from 2H and 6H were overlapped in addition to overlap between resonances from 3H and 5H protons, due to the similar chemical environment. A COSY cross peak seen between (2H, 6H) and (3H, 5H) confirms their assignments. Further, a coupling constant of 7 Hz was calculated between these resonances, confirming their assignments. Resonances from phenyl A ring (3H, 5H) were observed at 7.11 ppm. It shows COSY cross peaks with both phenyl A ring (2H, 6H) and (4H) (Figure 3a). Similarly, resonances from phenyl C ring were assigned following the COSY cross peaks between them (Figure 3a). The chemical shifts of all the resonances are listed in Table 1.

The assignment of resonances from tamoxifen was carried out in Tc-99m-tamoxifen complex (Figure 2b), and chemical shift positions were compared to that seen for tamoxifen alone (Figure 2a). The chemical shift positions of various protons of tamoxifen upon complexation with Tc-99m are shown in Table 1. The resonances of α-CH_2 and β-CH_3 of tamoxifen were observed at 2.45 ppm and 0.93 ppm, respectively, and there was no change in their chemical shift positions when compared to those of tamoxifen alone after the formation of a complex with Tc-99m. The β'-CH_2 protons were assigned at 2.64 ppm, while α'-CH_2 protons were assigned at 3.92 ppm (Figure 2b). It may be noted that upfield shifts of 0.14 ppm and 0.37 ppm were observed in protons of α'-CH_2 and β'-CH_2, respectively, in Tc-99m-tamoxifen complex compared to that seen for tamoxifen, indicating shielding effect due to complex formation with Tc-99m. Further, a singlet due to 6 protons of $N(CH_3)_2$ was observed at 2.28 ppm in the spectrum of Tc-99m-tamoxifen complex (Figure 2b). The $N(CH_3)_2$ protons also showed an upfield shift of 0.28 ppm, indicating the shielding effect due to complex formation with Tc-99m of Tc-99m-tricarbonyl core.

The protons from phenyl B ring (3H, 5H) were observed at 6.55 ppm, while H2, H6 were assigned at 6.76 ppm (Figure 2b) following their COSY cross peaks. An upfield shift of 0.02 ppm was observed in H2, H6 protons of ring B, while a downfield shift of 0.01 ppm was observed in the H3, H5 protons, indicating the effect of complex formation. An upfield shift of 0.01 ppm was also observed in the H2, H6, and H4 protons of ring A, while there was no change in the chemical shift positions of ring C protons upon complexation with Tc-99m of Tc-99m-tricarbonyl core.

The ER expression on MCF-7 cell lines was 94%, which was suitable for receptor binding studies. The percentage receptor binding (% mean) of Tc-99m and Tc-99m-tricarbonyl (controls) with MCF-7 cell lines was 1%-3%. However, the percentage binding of increasing concentrations of Tc-99m-tamoxifen (50 μCi, 100 μCi, 200 μCi, and 400 μCi) with MCF-7 cell lines was 10% ± 0.24%, 12% ± 18%, 25% ± 39%, 30% ± 127.5%, respectively (Figure 4). The mean percentage binding of Tc-99m, Tc-99m-tricarbonyl, and Tc-99m-tamoxifen with non-ER expressing MDA-MB-231 cell line was 1%-4% under the same experimental conditions.

The receptors of MCF-7 cells were pretreated with various concentrations of tamoxifen (unlabeled) to study the specificity of Tc-99m-tamoxifen and compare the affinity of tamoxifen toward the ER. The percentage receptor binding of

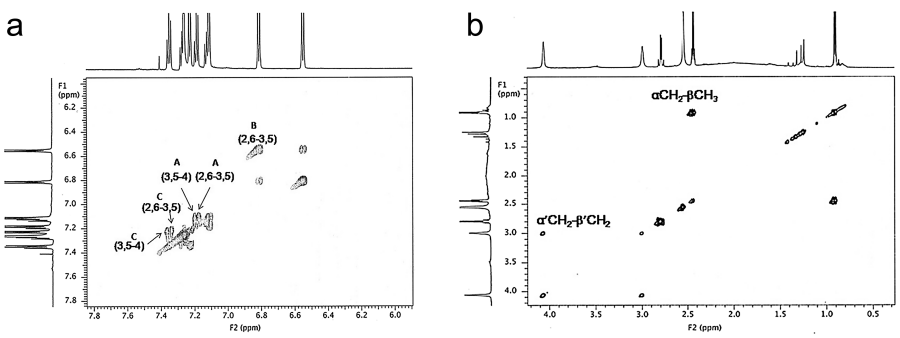

Figure 3. a, b. Expanded aromatic region **(a)** aliphatic region **(b)** of two-dimensional (^1H–^1H) correlation spectroscopy of tamoxifen alone acquired at 700 MHz in $CDCl_3$ at 25°C.

Table 1. Chemical shifts (δ) of tamoxifen protons alone and in Tc-99m-tamoxifen complex and changes in chemical shifts (Δδ) of tamoxifen protons in the complex

Protons	Tamoxifen (δ ppm)	Tc-99m-tamoxifen (δ ppm)	Δδ ppm
α-CH_2	2.45	2.45	0.00
β-CH_3	0.93	0.93	0.00
α'-CH_2	4.07	3.92	−0.14
β'-CH_2	3.01	2.64	−0.37
Phenyl A (H3, H5)	7.11	7.11	0.00
Phenyl A (H2, H6)	7.17	7.16	−0.01
Phenyl A (H4)	7.18	7.17	−0.01
Phenyl B (H3, H5)	6.54	6.55	0.01
Phenyl B (H2, H6)	6.78	6.76	−0.02
Phenyl C (H3, H5)	7.23	7.23	0.00
Phenyl C (H2, H6)	7.34	7.33	0.00
Phenyl C (H4)	7.35	7.35	0.00
$N(CH_3)_2$	2.56	2.28	−0.28

δ, chemical shift; Δδ, chemical shift alteration.

Tc-99m-tamoxifen was reduced as the ratio of tamoxifen to Tc-99m-tamoxifen (mentioned as cold : hot tamoxifen) was increased from 0.5 to 200 (Figure 4). The inhibition of Tc-99m-tamoxifen binding up to 72% was observed with MCF-7 cells when tamoxifen and Tc-99m-tamoxifen were present in the molar ratio of 50 : 1.

Special care is required while observing the organs that show receptor expression in normal subjects and the organs that are responsible for the elimination of the radiopharmaceutical from the body. For this purpose, animal studies have been undertaken. The intravenous administration of Tc-99m-tamoxifen in rats demonstrated its uptake in the liver, kidney, heart, and lungs. In female Wistar albino rats, increased counts in the breast were noted and remained high up to study duration of 6 h. The %ID/g values at 2 h in lungs, liver, spleen, heart, and blood were 5.79%, 17.32%, 8.35%, 6.9%, and 7.45%, respectively, which were decreased to 2.30%, 7.45%, 5.53%, and 0.58%, respectively, at 6 h. In addition, the blood activity remained high up to 2 h but was reduced at 4 h (Figure 5). The study in normal rats demonstrated the physiological uptake of Tc-99m-tamoxifen in liver, lung, and spleen.

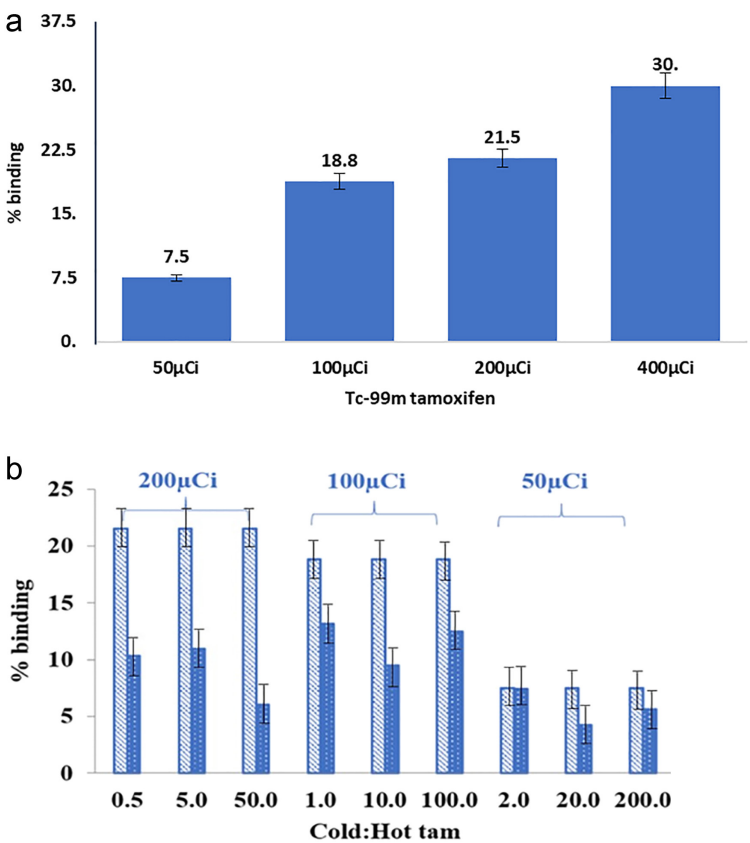

Figure 4. a, b. *In vitro* receptor binding and inhibition studies. **(a)** Increase in % receptor binding as the amount of Tc-99m-tamoxifen increases. **(b)** *In vitro* blocking study—Decrease in receptor binding of Tc-99m-tamoxifen in cells pre-treated with various concentrations of unlabeled tamoxifen (series 1: receptor binding of Tc-99m-tamoxifen with MCF-7 cell line and series 2: receptor binding after blocking of ER with cold tamoxifen).

Dosimetry study in rats showed high exposure to the urinary bladder (80.8 µSv/MBq) (Table 2). It was observed that the urine was retained in the bladder in 1 rat that resulted in overall high counts at this time point. In the rest of the study time points, bladder activity remained low (Figure 5).

Four patients were enrolled in the clinical study, having a mean age of 53 years. All the patients had histologically confirmed ER expression. The F-18-FDG-PET/CT showed the presence of breast and/or metastatic lesions in these patients. Demographics, clinical features, and scan findings of the patients are described in Table 3. Lesions at the primary site (breast) and axillary lymph nodes were visualized on Tc-99m-tamoxifen SPECT/CT in patients 3 and 4 (Figure 6 and Table 3). In patient 1, metastatic lesions in the lung and supraclavicular lymph nodes were also noted in addition to breast and axillary lymph nodes. The Tc-99m-tamoxifen SPECT/CT results were in concordance with F-18-FDG PET/CT scan results. In patient 2, no uptake of Tc-99m-tamoxifen was observed in the WB and SPECT/CT. This patient was on tamoxifen therapy for the last 6 months. However, lesions were visualized in F-18-FDG PET/CT (Figure 7). All patients well tolerated the Tc-99m-tamoxifen, and no adverse reactions were observed or reported by any of these patients.

Total exposure to various organs was calculated with OLINDA/EXM 2.0 using data extracted from images, represented in nSv/MBq, and was normalized according to ICRP-103 ED guidelines (Table 4). Similar to

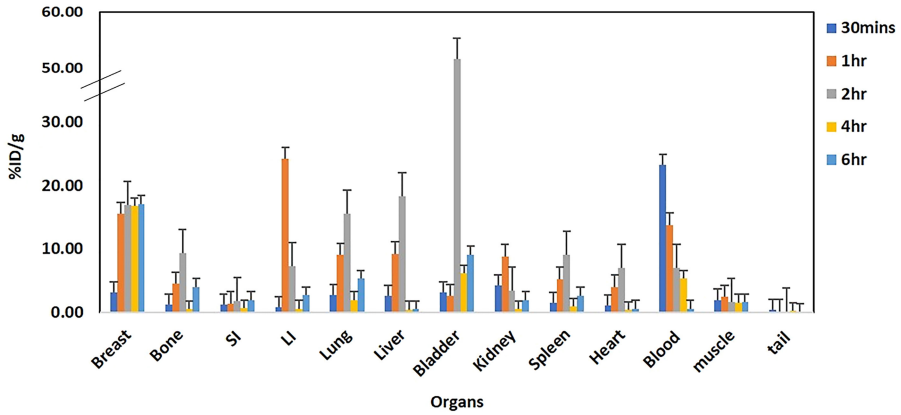

Figure 5. Percentage ID/g of Tc-99m-tamoxifen in various rat organs at different time intervals. ID/g = percent injected activity per gram.

animal study, uptake of Tc-99m-tamoxifen was high in the liver (696 nSv/MBq) and lungs (445 nSv/MBq). However, kidney exposure was reduced due to hydration and frequent void.

Discussion

Metal carbonyls are organometallic compounds having metal in their center with oxidation state +1. Tricarbonyl technology is helpful in radiolabeling of several peptides and antibodies. Various non-peptide molecules having molecular mass below 500 Da can also be radiolabeled.[15] Radiolabeling with Tc-99m is advantageous because Tc-99m and Re-188 belong to the same group (VII B) of the periodic table and share similar chemistry. Tc-99m is a gamma emitter used for imaging and Re-188 is a therapeutic radionuclide. Re-188 emits both beta and gamma rays (15%) which can be explored for theranostic application.[16,17]

Table 2. Doses in μSv/MBq to various organs (Wistar rat) by injection of 100 μCi of Tc-99m-tamoxifen

Organ	Mean dose (μSv/MBq)
Urinary bladder	80.8
Lungs	26.5
Skeleton	11.7
Spleen	11.0
Large intestine	8.68
Kidneys	6.54
Heart	6.17
Pancreas	5.45
Small intestine	5.06
Stomach wall	4.87
Liver	3.91
Thyroid	3.71

In the present study, Tc-99m-tricarbonyls were synthesized by a single precursor method. Sodium boranocarbonate was used as an *in situ* carbon monoxide-producing source. The results obtained in the study were similar, as reported by Alberto et al. using potassium boranocarbonate.[12] The quality control parameters of formulated Tc-99m-tamoxifen were found suitable for intravenous administration to animals as well as to patients.

The Tc-99m-tricarbonyl peak at 234 g/mol corresponds to the molecular formula $[M(CO)_3(H_2O)_3]^+$ (M = Tc-99m), hence confirming the formation of Tc-99m-tricarbonyl. The molecular weight obtained in the mass spectra of Tc-99m-tamoxifen spectra corresponds to the molecular formula $[M(CO)_3XL_2]$, where X is water and L_2 represents the bidentate tamoxifen.[15] The proposed structure indicated the binding of one tamoxifen with Tc-99m-tricarbonyl.

The large chemical shift changes in $N(CH_3)_2$, α'-CH_2, and β'-CH_2 suggested the formation of a Tc-99m-tamoxifen complex. Further, changes in these protons suggested that complex formation would have occurred due to the formation of co-ordinate bonds between Tc-99m and N and O atoms of oxy-N,N-dimethylethanamine group that was very well reflected in the changes in the chemical shift positions of these groups. The small changes in chemical shifts of ring A, B protons and no change in chemical shift position of ring C protons also supported that the site of complex formation is oxy-N,N-dimethylethanamine of tamoxifen which is away from these rings and therefore has resulted only in minor shifts in protons of A and B rings. Further, there was no change in the chemical shift position of α-CH_2 and β-CH_3 protons of tamoxifen on complexation with Tc-99m, supporting the site of complex formation is at oxy-N,N-dimethylethanamine, that has probably not affected the chemical environments of these groups.

The chemical shift positions of protons of tamoxifen alone were in agreement with those reported in the literature.[18] Our results showed that $N(CH_3)_2$, α'-CH_2, and β'-CH_2 protons of oxy-N,N-dimethylethanamine of tamoxifen shifted upfield on the formation of a Tc-99m-tamoxifen complex. The shielding of these resonances demonstrated the interaction of Tc-99m-tricarbonyl with oxy-N,N-dimethylethanamine of tamoxifen. It may be noted that if lone pairs of electrons on oxygen or nitrogen atoms are anti to C–H bonds, then it leads to upfield shifts in protons because of conformational dependence of chemical shifts of protons. Thus, the complex formation would have occurred due to the formation of co-ordinate bonds between Tc-99m and O and N atoms of oxy-N,N-dimethylethanamine group (of tamoxifen), resulting in observed upfield shifts of $N(CH_3)_2$, protons in Tc-99m-tamoxifen complex. It is known that the technetium with the atomic number of 43 possesses rich coordination chemistry.[19] Further, our findings are in agreement with Kyprianidou et al[20] wherein they reported the formation of complex of Tc-99m-tricarbonyl with ciprofloxacin and norfloxacin through coordination bonds with oxygen and nitrogen atoms of these drugs.

The molecular weight obtained from MALDI-TOF spectra of Tc-99m-tamoxifen was 568 Da. The mass and ^{1}H-NMR spectra information demonstrated the interaction of Tc-99m-tricarbonyl with oxy-N,N-dimethylethanamine of tamoxifen and supported the molecular formula $[M(CO)_3XL_2]$, where X is H_2O and L_2 represents the bidentate tamoxifen. It is pertinent to mention that formulations for MALDI-TOF and ^{1}H-NMR were prepared in HPLC water to rule out unwanted interference. However, for *in vivo* administration, sterile normal saline (0.9%) is preferred, and chloride of normal saline may replace the labile water molecule to form a more stable structure.

The three phenyl rings (A, B, and C) of tamoxifen have been reported for the binding with ER.[21] The ^{1}H-NMR spectrum of Tc-99m-tamoxifen revealed a negligible shift in phenyl rings A, B, and C of tamoxifen,

Table 3. Details of the patients enrolled in the study

	Patient 1	Patient 2	Patient 3	Patient 4
History	Initial staging	On tamoxifen for last 6 months	After 1 cycle of chemotherapy	Initial staging
Age (years)/sex	62/F	48/F	45/F	59/F
Dose (mCi)	10	13.5	8.85	12.5
IDC grade	III	II	II	II
ER	1+, 30%, 4/8	3+, 80%, 8/8	2+, 60%, 6/8	3+, 90%, 8/8
PR	Negative	3+, 5%, 5/8	2+, 40%, 5/8	3+, 70%, 8/8
HER2	Negative	Equivocal membrane, 2+,10%	Negative	Negative
TNM stage	T4N1Mn	T4N1M0	T4bN1Mx	T4bN0M0
Tc-99m-tamoxifen uptake	Breast, lung, supraclavicular lymph nodes, axillary lymph nodes	No uptake	Breast, axillary lymph node	Breast, lung
F-18-FDG uptake	Breast, lung, supraclavicular lymph nodes, axillary lymph nodes	Breast, liver, multiple bone lesions	Breast, supraclavicular lymph nodes, axillary lymph nodes	Breast, lung

F, female; IDC, invasive ductal carcinoma; ER, estrogen receptor; PR, progesterone receptor; HER-2, human epidermal growth factor receptor-2; TNM, tumor-node-metastasis stage F-18-FDG, 18-fluoro-2-deoxy-D-glucose.

which indicated that the binding site of tamoxifen is preserved (unaltered) after radiolabeling with Tc-99m-tricarbonyl core (Figures 2 and 3, Table 1) and suitable for targeting the ER.

The high binding efficiency of Tc-99m-tamoxifen with ER-expressing MCF-7 cells demonstrated affinity of Tc-99m-tamoxifen with ER. In addition, Tc-99m-tamoxifen showed negligible binding with MDA-MB-231 cells that do not express ER (negative control), which confirmed the specificity of Tc-99m-tamoxifen for ER. The results suggested that the receptor-binding site was occupied by pre-treatment of tamoxifen and blocked the binding of Tc-99m-tamoxifen. It could be inferred that the binding property of tamoxifen remained intact when labeled with Tc-99m using tricarbonyl core.

There are several reported mechanisms of binding of tamoxifen to cells. The principal mechanism of concern in this study is its binding with ER and inhibition of binding affinity in the presence of unlabeled tamoxifen. It has also been reported that binding of tamoxifen with ER induces autocrine production of TGF-β that has an inhibitory action on estrogen production.[22]

The blood pool activity remained high up to 4 h. The uptake in breast tissue was due to the presence of the ER. The F-18- and I-131-labeled tamoxifen analogs were used in the past. However, radiosynthesis with iodine is complicated and time-consuming.[23] As observed in this study, F-18- and I-131-labeled tamoxifen analogs show high uptake in the liver. Other radiolabeled steroids (F-18 FES) also showed high liver and lung uptake.[24] In a study, halogen was placed at the phenyl ring C of tamoxifen which showed increased ER binding affinity and increased anti-estrogen potency.[24] Conversely, placing an iodine

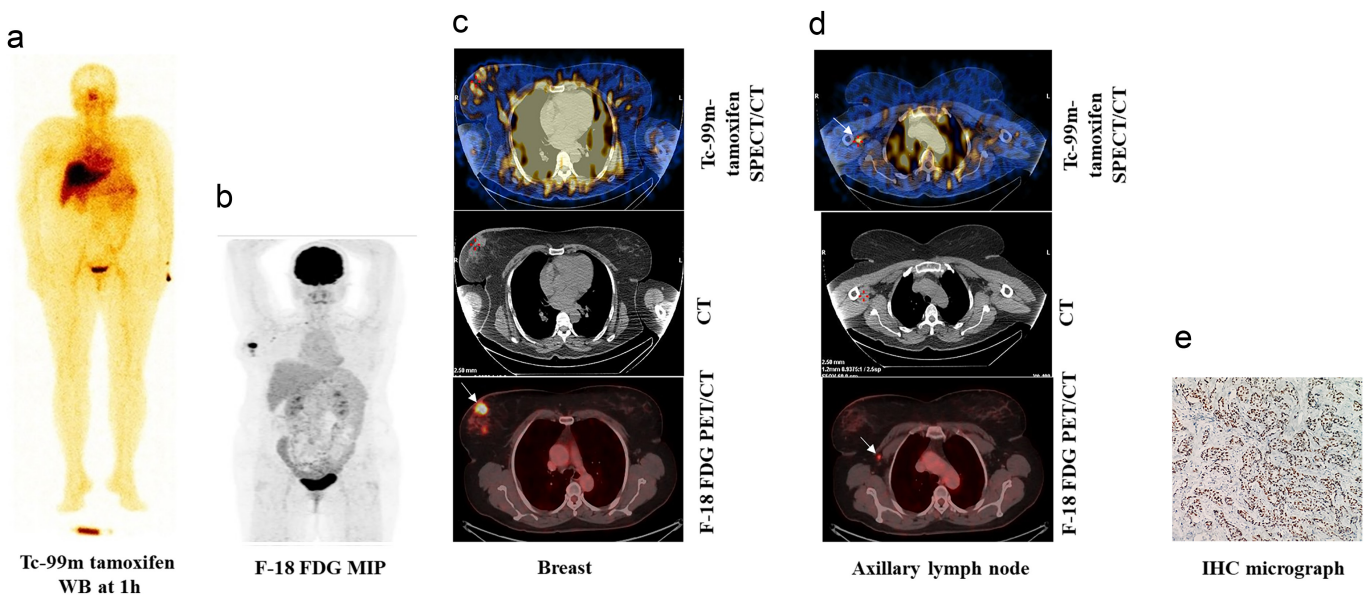

Figure 6. a-e. Patient 3, **(a)** WB Tc-99m-tamoxifen scintigraphy at 1 h; **(b)** F-18-FDG maximum intensity projection PET image; **(c)** transaxial SPECT/CT, CT, and PET/CT images show uptake in breast lesion; **(d)** transaxial SPECT/CT, CT, and PET/CT images show uptake in axillary lymph node; **(e)** IHC micrograph shows nuclear positivity for ER at ×100. ER, estrogen receptor; F-18-FDG, 18-fluoro-2-deoxy-D-glucose; PET, positron emission tomography; SPECT, single photon emission computed tomography; CT, computed tomography; IHC, immunohistochemistry; WB, whole body.

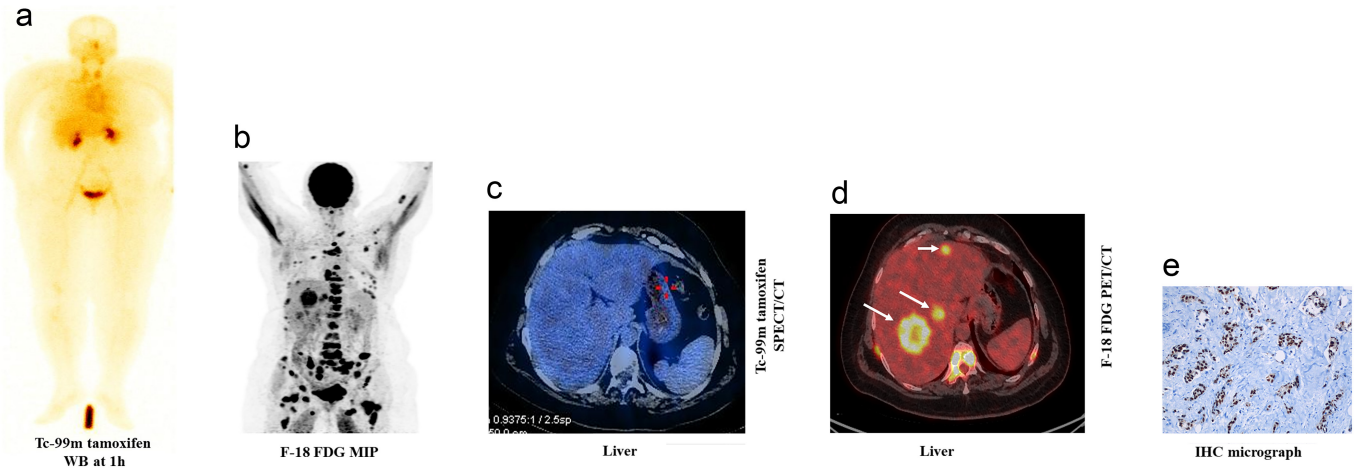

Figure 7. a-e. Patient 2, **(a)** WB Tc-99m-tamoxifen scintigraphy shows physiological uptake in kidneys, bladder, liver, and heart but no uptake in liver and bone lesions; **(b)** MIP F-18-FDG image shows multiple liver and bone lesions all over the body; **(c)** no uptake was observed in liver on Tc-99m-tamoxifen SPECT-CT image; **(d)** F-18-FDG PET/CT image shows multiple lesions in the liver; **(e)** IHC micrograph shows nuclear positivity for ER at ×200.

atom on the aromatic ring reportedly lowered the affinity.[25]

Proper hydration and frequent urination may reduce bladder activity in humans. It has been reported that the minimum value of gamma-ray exposure to cause any biological damage is 50-100 mGy. Several studies in rats indicated chromosomal aberrations with gamma-ray exposure of 50-100 mGy.[26-28] However, in the current study, exposure to different organs of the rat was at microgray level. Hence, it is well within the safe limit.

Table 4. Exposure to different patient organs from Tc-99m-tamoxifen in nSv/MBq

Target organ	ICRP-103 estimated dose (nSv/MBq)
Liver	696
Lungs	445
Stomach wall	228
Breasts	173
Red marrow	112
Esophagus	102
Spleen	58.2
Gallbladder wall	43.2
Pancreas	35.6
Osteogenic cells	29.1
Thyroid	22.0
Heart wall	15.6
Thymus	11.7
Ovaries	12.1
Urinary bladder wall	5.05
Rectum	4.07
Salivary glands	1.20
Uterus	1.11
Brain	0.937
Eyes	0

Similar to the animal study, the physiological activity of Tc-99m-tamoxifen was observed in the liver and lungs. Tamoxifen has high plasma protein binding which resulted in high blood pool activity, reduced target uptake, and poor contrast in all patients. The lesions were noted in the breast, lymph nodes, and lung on Tc-99m-tamoxifen SPECT/CT in 3 patients. The F-18-FDG PET/CT scan demonstrated uptake in primary and metastatic lesions in all patients (Table 3, Figure 6). One patient was on tamoxifen therapy for the last 6 months, only physiological activity of Tc-99m-tamoxifen in the liver and kidney was observed in that patient (Figure 7). However, F-18-FDG PET/CT scan showed both breast and metastatic lesions in this patient. The result clearly indicated the saturation of ER by tamoxifen given for treatment. Further, it proved the specificity of Tc-99m-tamoxifen used in this study for the ER.

Currently, F-18 estradiol is used in PET/CT imaging of ER. Similar to Tc-99m-tamoxifen, a high blood pool and the physiological uptake are reported.[4] The F-18 FES chemistry is complicated, and production is tedious. The requirement of an onsite cyclotron, expensive cassette, and low production yield make the availability of F-18 estradiol expensive and limited. The Tc-99m-tamoxifen is cost-effective, can be prepared easily using commercially available tamoxifen, and generator produced Tc-99m for imaging using a conventional gamma camera. Further studies with nanoformulation of tamoxifen are undergoing to reduce plasma protein binding and make tamoxifen more useful for clinical application.

This study has some limitations. The preclinical study in a xenograft model and the animal imaging were not performed in this study due to the non-availability of nude mice facility and animal SPECT/CT scanner in the institute.

In conclusion, Tc-99m-tamoxifen is an in-house synthesized radiopharmaceutical, highly specific for ER targeting.

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Conflict of interest disclosure

The authors declared no conflicts of interest.

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Supplementary data

Synthesis of Tc-99m-tricarbonyl core and radiolabeling of tamoxifen

Freshly eluted Tc-99m (5-30 mCi) was incubated with 1 mg sodium boranocarbonate in 0.1 M PBS (pH=7.5) at 80°C for 20 mins in a water bath to prepare Tc-99m-tricarbonyl core. Radio TLC was performed to confirm the formation of Tc-99m-tricarbonyl.

Tamoxifen 5-30 µg (increment of 5 µg) was used to optimize the amount of drug. The molar concentration varied from 13.4 to 80.4 mM. The reaction volume varied from 0.5 to 2 mL. Radiolabeling was performed at various pH (3.0-10.0) to determine the optimum pH of the reaction mixture. The radiolabeling reactions were carried out at 60°C, 75°C, 80°C and 100 °C. Incubation timings were varied from 20 to 45 mins.

A single parameter was changed in a reaction while keeping all other labelling parameters constant. The trials were repeated five times for each reaction parameter. Radio-paper chromatography (PC) was performed to calculate the radiolabeling yield.

Quality control of Tc-99m-tamoxifen

Paper chromatography was performed on Whatman paper #3 using Methanol: HCl (99:1) as mobile phase.[1] The radiolabeling yield was calculated by radio TLC scanner by selecting area under the peak. In case of low radiochemical yield (<90%), *in vitro* (cell culture) and *in vivo* studies, radiolabeled tamoxifen was purified using pre-conditioned C-18 cartridge.

The Tc-99m-tamoxifen was subjected to various physio-chemical, biological, and stability tests to ensure the safety of drugs before intravenous administration.[2,3]

Sterility testing was done using tryptic soy broth and fluid thioglycollate medium. Sample aliquots (0.5-1 mL) were inoculated and incubated at 37°C for sterility check. Turbidity in the broth was observed up to seven days for the presence of any microbial growth. Pyrogenicity was checked by colorimetric test on an automated cartridge-based point-of-use portable test system (PTS). To each well of the PTS cassette, 25 µL sample was added, and incubation was done for 15-20 mins at 38°C for the reaction to take place. All observations were noted with 50%-200% spike recovery. The sensitivity of the test is up to 0.01 Endotoxin Unit (EU)/mL.

The polarity of Tc-99m-tamoxifen was assessed by calculated Log P-value. The Tc-99m tamoxifen (1 mL) was dissolved in an equal volume of organic solvent (octanol) vortexed for 30 s and centrifuged at 3000 rpm for 10 mins. The activity was measured in the aqueous and organic phase.

Stability of Tc-99m-tamoxifen was tested in PBS for up to 6 h. Tc-99m-tamoxifen (0.5 mL) was incubated with PBS (0.5 mL). Radio PC was performed in methanol: HCl (99:1) at various time points up to 6 h.

Immunocytochemistry

The MCF-7 cells were fixed on a glass slide using 10% formalin (400 µL) for 10 mins. Washing was done two times with 1X PBS (500 µL) and 1% Triton X100 for 5 mins. Again, the slide was washed with 1X PBS (500 µL). Primary antibody was used in 1:50 dilution for 30 mins - 1 h at room temperature or 4°C. Washing was done with 1X PBS for three times. The secondary antibody was added, and the slide was incubated for 30 mins. Washing was done with PBS, and DAB was added in 1:100 dilution and incubated for 30 mins. Washing was done with water, and the slide was counterstained with hematoxylin.

Immunohistochemistry

The paraffin blocks were prepared, and 4 to 5 µm thick sections were mounted on 0.01% poly-l-lysine coated slides. Immunohistochemistry for ER was done using an anti-ER antibody (Dako, 1:50 dilution). The sections were de-waxed in xylene and rehydrated in a series of graded alcohol followed by washes in PBS. The activity of endogenous peroxidase was blocked by freshly prepared 0.03% hydrogen peroxide in methanol for 20 mins. Antigen retrieval was done by heat retrieval method using post link in Tris buffer (pH 9.0). The primary antibody was then applied to the sections for 1 h, followed by incubation with secondary antibody (Dako Envision) for 40 mins. The colour reaction was developed by diaminobenzidine, and counterstaining was done with hematoxylin. The sections were dehydrated and mounted with dextrene phthalate xylene.

Allred scoring system was used to analyze ER expression. Proportion and intensity score was calculated and added to obtain Allred score.

Supplementary results

Tricarbonyls were synthesized with >95% radiolabeling yield.

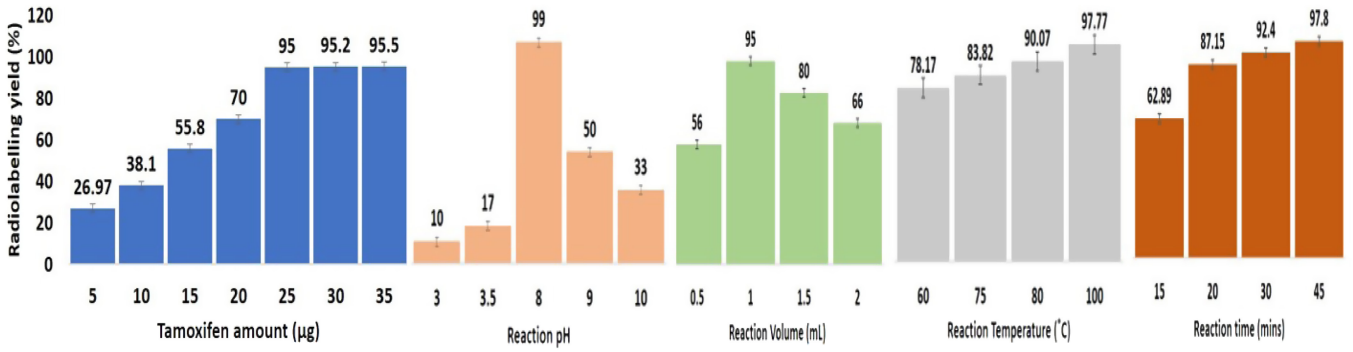
The 25-30 µg of tamoxifen was found to be sufficient to radiolabel 5-30 mCi of Tc-99m with >90% radiolabeling yield. Ninety-nine per cent radiolabeling yield was observed at reaction pH 7.5-8. For greater than 90% radiolabeling yield, 1 mL reaction volume was found to be optimal. Up to 97 % radiolabeling yield was observed after incubation at 100 °C for 45 mins (Figure S1).

Radiochemical purity (RCP) of Tc-99m-tricarbonyl was tested and found to be >95%. The R_f of Tc-99m and Tc-99m tricarbonyl was 0.9 and 0.3, respectively. The RCP of Tc-99m tamoxifen was >95%, and R_f of Tc-99m tamoxifen was 0.4 (Figure S2). There was no turbidity observed in broth up to seven days of incubation at 37°C. The endotoxin content of the samples was in the range of 3.17-4.32 EU/V, with spike recovery of 50%-200%. The log P value was -2.6. Labeling efficiency was 99%, 96.5, 95%, 94.7%, 92.1, 91.4% at 1, 2, 3, 4, 5, 6 h respectively (Figure S3). In case of low radiolabeling yield <90%, Tc-99m tamoxifen was purified using C-18 cartridge (Figure S4).

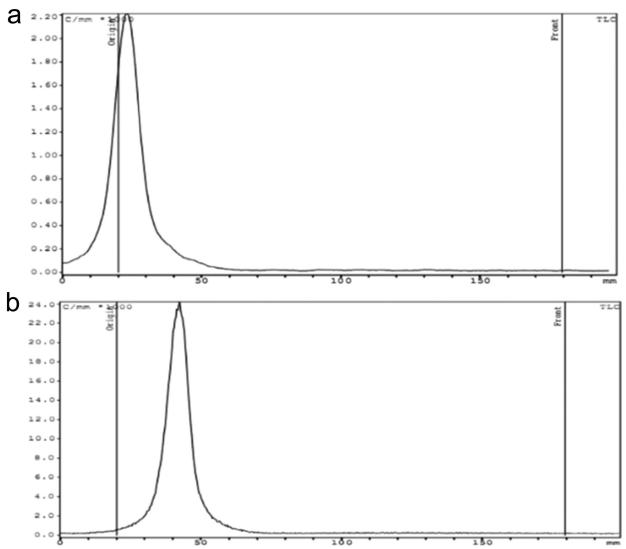
Supplementary discussion

Metal carbonyls are organometallic compounds having metal in its centre having oxidation state +1. Tricarbonyl technology is helpful in radiolabeling of several peptide and antibodies. Various non-peptide molecules having molecular mass below 500 Daltons can also be radiolabeled.[1] In the present study, Tc-99m-tricarbonyls were synthesized by single precursor method. Tc-99m-tricarbonyls have been synthesized by a number of methods described by different authors. These methods include using direct source, i.e., CO cylinders, ready-made kits and using different precursors containing a source of CO production in situ for the formation of Tc-99m tricarbonyls.[1,4-6] Carbon monoxide gas is hazardous and needs special precautions during handling and storage. Therefore, the use of CO cylinder was avoided in the study.

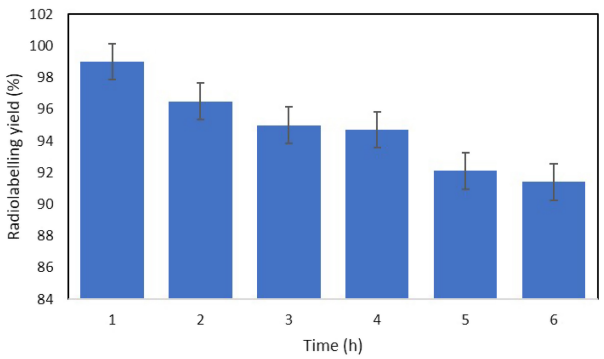
Alberto et al. synthesized Tc-99m-tricarbonyls using potassium boranocarbonate



Supplementary Figure 1. Bar graph showing the effect of tamoxifen amount, reaction pH, reaction volume, reaction temperature, incubation time on radiolabeling yield.



Supplementary Figure 2. a, b. Paper chromatogram showing formulation of **(a)** Tc-99m tricarbonyl core, R_f=0.3 and **(b)** Tc-99mtamoxifen using tricarbonyl core, R_f=0.4.



Supplementary Figure 3. Stability of Tc-99m tamoxifen in PBS.

as an in situ CO producing source and reported 1 mL reaction volume to be optimum. In the present study also, it was observed that reaction yield drops significantly as the volume is increased or decreased from 1 mL. The synthesis of in-house tricarbonyl core by the method used in this study is feasible and non-hazardous.

In our study, single component sodium boranocarbonate was used as an in situ CO producing source. The results obtained in the study were similar, as reported by Alberto et al., using potassium boranocarbonate.[1]

In the present study, pyrogenicity was checked by cartridge-based PTS (point-of-use, portable test system) which work on the principle of kinetic chromogenic technique. The limulus amebocyte lysate (LAL) reagent interact with the endotoxins present in the samples and initiate a cascade of reactions that results in the formation of the colored substrate.[7] The endotoxin levels were much below the permissible limit, i.e., 175 EU/V under our experimental conditions.

Log P value suggested the hydrophilic nature of Tc-99m tamoxifen. The Tc-99m tamoxifen was found to be reasonably stable (~95%) up to 6 h of incubation. When Tc-99m was obtained in diluted form from the generator; reaction volume exceeds 1 mL. In these cases, low radiolabeling yield <90% was obtained. In order to achieve high radiochemical purity, Tc-99m tamoxifen was purified using C-18 cartridge (Figure S4). The sterility was achieved by passing the labeled product through 0.22 micron filter.

The quality control parameters of formulated Tc-99m-tamoxifen was found suitable for intravenous administration to animals as well as in patients.

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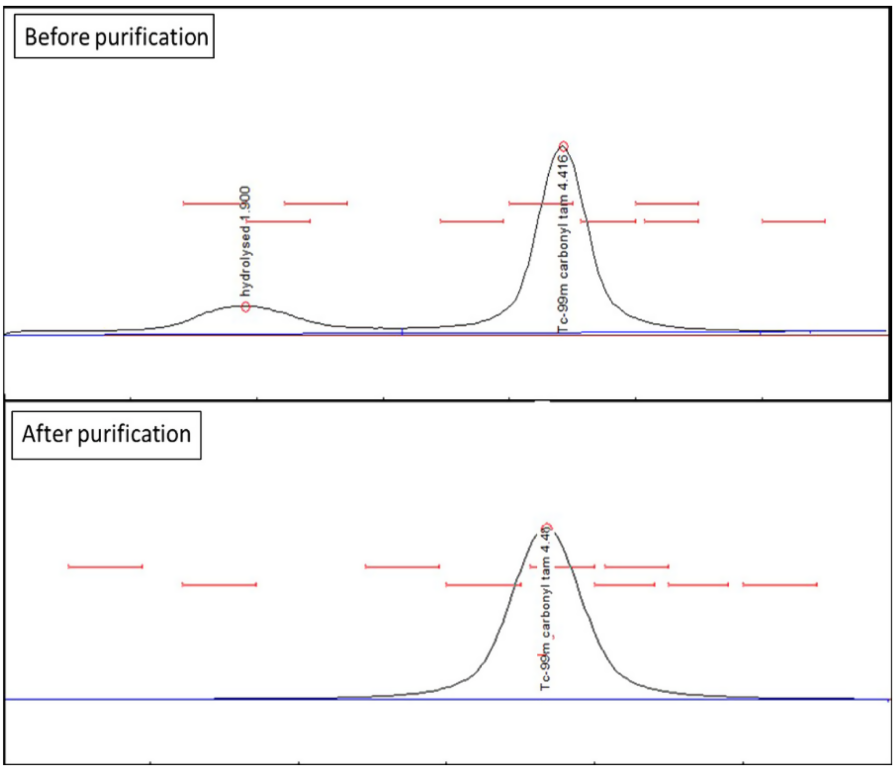

Supplementary Figure 4. Paper chromatography showing two peaks of the hydrolyzed fraction (near base) and Tc-99m tamoxifen before C-18 purification and single product peak post-C-18 purification.

Table 2. Receptor binding with MCF-7 and MDA-MB-231

S.No	Component	Activity (μCi)	Amount (μg)	% Binding in MCF-7 (Mean)	% Binding in MDA-MB-231
1	Tc-99m	100		1-3	
2	Tc-99m	200			
3	Tc-99m-tricarbonyl	100			
4	Tc-99m-tricarbonyl	200			
5	Tc-99m-tamoxifen	50	0.2	7.5±0.24	1-4 (average)
6	Tc-99m-tamoxifen	100	0.3	18.8±0.18	
7	Tc-99m-tamoxifen	200	0.4	21.5±0.39	
8	Tc-99m-tamoxifen	400	0.8	30±0.12	

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