International Journal of Research in Pharmacy and Life Sciences

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Research Article **Open Access**

Tumor Imaging in Experimental Animal Models of Breast Carcinoma with Routinely Available ^{99m}Tc-Sestamibi

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A B S T R A C T

The arise of dedicated imaging equipment and techniques facilitated the emergence of translational research paradigm. In this sense, the follow up and tracing of biological processes, pathologic or not, as well as the outcomes of therapy strategies in tumor bearing animals is expected to shorten the bridge between laboratory and patient bed. Thus the aim of this work was to evaluate ^{99m}Tc-sestamibi as a tracer to follow up chemical or clinical interventions in xenografted tumors of MDA MB-231 human breast cancer cell line and N-Nitroso-N-methyl urea (NMU) induced mammary tumors. Tumor bearing animals received ^{99m}Tc-sestamibi intravenously. Images were acquired with a gamma camera. 10min and 60min acquisitions were performed and regions of interest were created for semiquantitative analysis. ^{99m}Tc-sestamibi tumor uptake for xeno graft model (X) is poor at 10min biodistribution (T/NT_{10min}: 0.5). Delayed acquisition showed a tumor accumulation even lower (T/NT_{60min}: not possible to calculate). Chemically induced model (C) showed an important accumulation of ^{99m}Tc-sestamibi in the tumors at the 10min (T/NT_{10min}: 3.2) and 60min (T/NT_{60min}:1.7) of biodistribution. Histological analysis showed a 60% of necrosis in X model and a 10% of necrosis in C model. ^{99m}Tc-sestamibi proved not to be useful for X model but it is a suitable tumor tracer for C model.

Keywords: ^{99m}Tc-sestamibi, breast cancer, animal models, imaging, MDA-MB-231, N-Nitroso-N-methylurea

A R T I C L E I N F O

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Article History: Received 09 October 2014, **Accepted** 2 December 2014, **Available Online** 24 May 2015

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Manuscript ID: IJRPLS2344

PAPER-QR CODE

Citation: Fiorella Carla Tesán, et al. Tumor Imaging in Experimental Animal Models of Breast Carcinoma with Routinely Available ^{99m}Tc-Sestamibi. *Int. J. Res. Pharm, L. Sci*., 2015, 3(1): 254-259.

1. Introduction

Experimental animal models allow signaling pathways, cell migration and protein expression studies as well as evaluation of genetic modifications and development of new treatment drugs to be studied in an in vivo environment. Moreover the arising development of dedicated imaging equipment and techniques facilitated the emergence of translational research paradigm (1, 2). In this sense, the follow up and tracing of biological processes, pathologic or not, as well as the outcomes of therapy strategies in tumor bearing animals is expected to shorten the bridge between laboratory and patient bed. Compounds radiolabeled with single photon or positron emitters, are some of the available probes for small animal imaging and their use has taken off by high resolution and sensitivity preclinical molecular imaging devices equipped with appropriate detection and in some cases combined detection modalities. Furthermore, some of the clinically used and routinely available SPECT (single photon emission computed tomography) radiopharmaceuticals, like ^{99m}Tc-sestamibi, are being evaluated for their use as radioactive traceable probes in animal models (3- 8) with the advantage that its already approved for human use. ^{99m}Tc-sestamibi is the most popular nuclear medicine radiopharmaceutical for myocardial perfusion examination [9, 10]. Its use in oncology has also been shown; ^{99m}Tc-sestamibi proved to be useful particularly for imaging of human breast suspicious lesions at first and it was eventually evaluated and found helpful in some cases of primary tumor diagnosis and staging of other kinds of malignancies as bone and soft tissue sarcomas, lymphomas, lung and brain tumor [11,12]. Additionally ^{99m}Tc-sestamibi has a powerful potential in predicting chemotherapy success or failure given that it is a P Glycoprotein ligand which is associated with resistance to that kind of treatment [13-18]. Too many factors influence ^{99m}Tc-sestamibi tumor uptake: perfusion, membrane electrical gradient, ongoing apoptosis, necrosis, mitochondrial proteins abnormalities or their deregulated expression [16].

Consequently, information about cellular metabolism and status can be obtained by tracing this probe or evaluating its uptake. This makes it a potential functional tracer and those accumulation factors define altogether its usefulness in actually tracing malignant lesions. Moreover ^{99m}Tc-sestamibi uptake can be assessed under controlled conditions by in vitro assays (19-21). However, they do not necessarily replicate in vivo accumulation since many uptake factors can change or manifest differently, placing animal models assays as a very necessary and interesting alternative. Setting the appropriate experimental conditions and choosing the optimal model, could help the basic research draw near and mimic real disease and clinical diagnostic methods. Thus we consider a real challenge to evaluate ^{99m}Tc-sestamibi tumor uptake in vivo. Our laboratory understands breast cancer investigation as a priority being that it is the most common cause of cancer and most frequently diagnosed among women in 140 of 184 countries all over the world (22). We have experience in using two experimental animal models for research in this area: xenograft tumors of MDA MB-231 human breast cancer cell line (X) (23) and NMU induced mammary tumors (C) [24]. Thus the aim of this work was to evaluate the potential use of ^{99m}Tc-sestamibi as a diagnostic agent in preclinical studies as well as a tracer to follow up chemical or clinical interventions in the above mentioned Experimental Animal models of breast cancer.

2. Materials and Methods

Experimental Animal models of breast cancer

Xenograft tumors of MDA-MB-231 human breast cancer cell line

Female athymic nude mice (NIH nu/nu, 20-25 g) were purchased from the Division of Laboratory Animal Production, School of Veterinary Sciences, University of La Plata, Buenos Aires (Argentina). Animals were kept in stainless steel cages in sterile isolated conditions with food and water ad libitum and 12h light cycle. MDA-MB-231 human breast cancer cells (American Type Tissue Culture Collection, VA, USA) were cultured in RPMI 1640 supplemented with 10% v/v FBS, 0.3 g/L glutamine and 0.04 g/L gentamicin (Gibco BRL, NY, USA). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. The subcutaneous (s.c.) tumor model was established as described previously (23). Briefly, MDA-MB-231 cells were collected by centrifugation and resuspended in 100 µL RPMI-1640. Tumors were induced by s.c. injection of the cells (1×10^7) into the right flank of two nude mice. Tumor volume was calculated as $[4/3\pi \times (\text{large diameter} + \text{small diameter})/4]^3$. Diameters were measured with a caliper. When tumors developed and reached a volume of 500mm^3 they were excised, cut into 25-30 mm^3 pieces and grafted into the right flank of other nude mice. ^{99m}Tc-sestamibi images were taken when the graft volumes reached 100-150 mm^3.

NMU induced mammary tumors

Chemo-induced animal model consisted in Sprague Dawley rats 250-300 g bearing mammary tumors induced by the chemical carcinogen N-Nitroso-N-methylurea (NMU). The animal model was also obtained as previously described (24). Briefly, tumors were induced by three intraperitoneal (i.p.) injections (50 mg/Kg body weight) of NMU (10mg/mL in physiological solution) at 50, 80 and 110 days after rats birth. Tumors were detected from 90 days after the first administration of NMU. The development of breast

tumors was examined by palpation, three times a week. Tumor size was measured using a calipter. Female Sprague–Dawley rats were purchased from National University of La Plata, Division of Animal Production, Argentina. Animals were kept in stainless steel cages with food and water ad libitum and 12 h light cycle. Animal procedures were in accordance with international recommendations (Guide for the Care and Use of Laboratory Animals of the Research Council, USA, 1996; Guidelines for the welfare and use of animals in cancer research British Journal of Cancer, 2010), and protocols were approved by Ethical Committee for the Use and Care of Laboratory Animals of the School of Pharmacy and Biochemistry (EXP-FYB N° 53406/2013).

Probe radiolabelling and quality control

^{99m}Tc-sestamibi (Sestamibi, Laboratorios Bacon SAIC, Argentina) was radiolabelled according to the manufacturer instructions. Briefly, 60 mCi of a pertechnetate solution eluted from a ^{99}Mo–^{99m}Tc generator (Laboratorios Bacon SAIC, Argentina) were aseptically added to the 0.5mg freeze-dried sestamibi kit. Saline solution was added to the preparation to a final activity concentration of 20 mCi/mL. Finally, vial was heated for 10 min at 100ºC and then left at room temperature for 15min until thermal equilibrium. Radiochemical purity was assessed according to USP instructions and all requirements were met (25).

^{99m}Tc-sestamibi animal imaging

In vivo uptake of ^{99m}Tc-sestamibi was evaluated by intravenous (i.v.) administration of 0.05-0.1 mL of ^{99m}Tc-sestamibi (20 mCi/mL) to tumor bearing animals. Previously animals were anesthetized with a ketamine-xilazine i.p. injection (100-10 mg/Kg for mice and 90-10 mg/Kg for rats) (26). Images were acquired with a small field of view gamma camera (OHIONUCLEAR, software: IM512P, ALFANUCLEAR, Argentina) equipped with a high resolution parallel hole collimator which was used for ventral imaging of rats (C model) and a pinhole collimator adapted with a small lead insert with a 2 mm of diameter hole which was used for right anterior oblique imaging of mice (X model). A 256 x 256 matrix was used with a 1.25 zoom for mice and 1.5 for rats. Acquisitions were performed during 15 minutes reaching at least 10^6 counts. Images were visually analyzed in a band scale to optimize structure differentiation and identification. Regions of interest (ROIs) were created for tumors and non tumor areas. ROIs of the non tumor areas were created by duplicating shape and size of tumor ROIs and placing them over areas where uptake was homogeneous and avoiding superposition with organs implicated in physiologic elimination of ^{99m}Tc-sestamibi. Results were expressed as tumor to non tumor ratio (T/NT). Early and delayed acquisitions were performed similarly to the human clinical protocols in Argentina: 10 and 60 min post inyection (p.i.) and wash out rate (WO) was calculated when possible ($WO=T/NT_{early}$ / $T/NT_{delaved}$). As heart is one of the main target tissue for this radiopharmaceutical, ROIs were created around it as well and semi quantitative analysis were made and expressed as heart to non heart ratio (H/NH).

Tumor histological analysis

After images were acquired animals were euthanized by anesthesia overdose and tumors were excised, fixed with neutral buffered formalin (10 %), paraffin embedded and cut into 4 µm thick serial section. Tumor histopathological characteristics were examined on tissue sections after haematoxylin-eosin staining as previously described (23).

3. Results and Discussion

^{99m}Tc-sestamibi animal imaging

^{99m}Tc-sestamibi tumor uptake in the X model is poor at early stages of biodistribution (Figure 1a). So as shown by visual analysis, it is very difficult to localize the tumor unless it is suspected in advance or its location is known. When T/NT ratio is calculated from data in ROIs the average value obtained was 0.5. A T/NT ratio lower than 1 indicates that the target (tumor) delimited by the ROI has a low uptake. Semi quantitative analysis of the heart rendered a H/NH ratio of 6.4. Mice heart was similar in size than the xenografts, so the ability to delimit the heart by a ROI and the high H/NH ratio only reaffirms the fact that the tumor does not accumulate ^{99m}Tc-sestamibi. In this way the possibility of resolution restricting tumor visualization was dismissed. Delayed acquisition showed a tumor accumulation even lower than that of early stages (Figure 1b).

The tumor was not detectable since uptake was extremely low, and it was not even possible to create a ROI for semicuantitative analysis. This agrees with the above mentioned early acquisition results and a structure with no ^{99m}Tc-sestamibi accumulation. C model rats showed a high accumulation of ^{99m}Tc-sestamibi in the tumors at the early stages of biodistribution (Figure 2a). Consequently palpable and non palpable tumors were both localized during visual analysis of the images. T/NT ratio calculated from created ROIs were similar for both palpable and non palpable tumors: 3, 2. Delayed phase image (Figure 2b) showed higher liver, bladder and gastrointestinal ^{99m}Tc-sestamibi accumulation, this latter placed at a lower part of the gut. T/NT ratio calculated from created ROIs were again similar for both palpable and non palpable tumors: [1,7.

Different T/NT ratios were obtained for each tumor at early and delayed phase acquisitions. Tumor accumulation represented as T/NT ratio resulted lower for the images acquired at 60 min p.i. compared to those acquired at 10 min p.i. Therefore estimated wash out rate resulted 2,2. Early and delayed phase images showed that distribution of ^{99m}Tc-sestamibi predominantly accumulates in the heart, liver, gastrointestinal tract and kidneys while bladder accumulation means it has renal elimination. This pattern is observed both in mice and rats (Figure 1 and 2). It can be noticed that no organs can be clearly identified by analyzing ^{99m}Tc-sestamibi uptake within the abdominal area given that it is a planar acquisition. It is also worth mentioning that ^{99m}Tc-sestamibi accumulation pattern in both evaluated animal models was the expected for this kind of detection technique and this radiopharmaceutical in small rodents as similar to other published results [3-8].

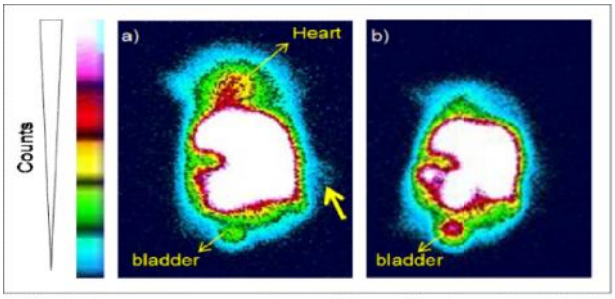

Fig 1 Gamma camera static acquisitions (duration: 15 min) of xenografted nude mice (MDA-MB-231 human cell line) after 1-2 mCi ^{99m}Tc sestamibi injection. a) early image (10min p.i.); b) delayed image (60min p.i). Tumor location is indicated by a thick yellow arrow. Band colors scale is shown at the left

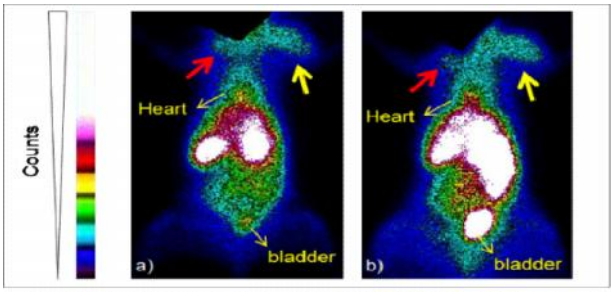

Fig 2 Gamma camera static acquisitions (duration: 15 min) of NMU induced tumor bearing rats after 1-2mCi ^{99m}Tc sestamibi injection. a) early image (10min p.i.); b) delayed image (60min p.i). Thick arrows indicate palpable (yellow) and non palpable (red) tumors. Band colors scale is shown at the left.

Tumor histopathological characteristics: Haematoxilin-eosine stained tumor sections of each animal model revealed their histopathological respective characteristics. Representative section images of each breast tumor model are shown (Figure 3 and 4).

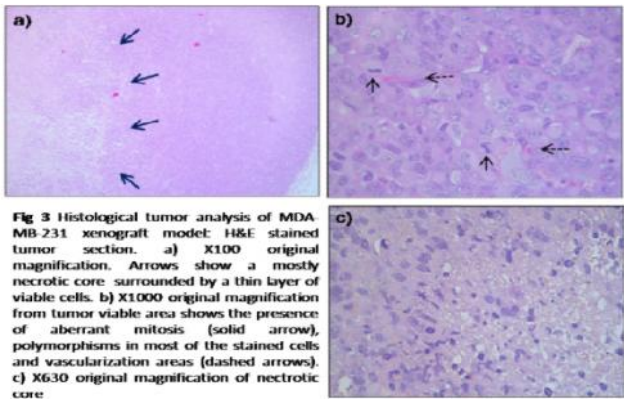

Fig 3 Histological tumor analysis of MDA-MB-231 xenograft model: H&E stained tumor section. a) X100 original magnification. Arrows show a mostly necrotic core surrounded by a thin layer of viable cells. b) X1000 original magnification from tumor viable area shows the presence of aberrant mitosis (solid arrow), polymorphisms in most of the stained cells and vascularization areas (dashed arrows). c) X630 original magnification of nectrotic core

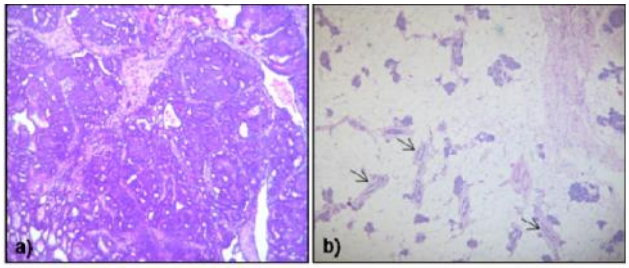

Fig 4 Histological tumor analysis of NMU chemoinduced model: a) H&E stained tumor section shows a clear hipercelullarity. Characteristic mammary tissue organization is slightly present and there are no necrotic areas, 400X original magnification. b) H&E stained non pathologic mammary gland, ducts are shown with arrows and white background corresponds to addipose tissue, 100X original magnification

Histological analysis of X model tumors showed highly invasive and undifferentiated adenocarcinoma with aberrant mitosis, nuclear polymorphism and high number of mitosis per field. Tumor sections showed a significant necrotic core surrounded by a thin viable layer of cells (Figure 3a). On the other hand, it can be noticed that almost all cells are viable in tumor section from C model and hipercelullarity is the main characteristic observed in tumor sections (Figure 4a). A clear difference in the percentage of necrotic areas was observed between the two breast tumor models. 60 % of necrosis was observed in X model whereas only a 10 % of necrosis was found in C model.

Discussion

Small animal imaging has grown tremendously in the last 10 years. Dedicated instruments and devices for small animal imaging have been developed as well as new probes taking advantage of the increasing knowledge in molecular biology field. All these together, have facilitated the emergence of the new paradigm of translational research in order to shorten the bridge between laboratory and clinic both in economical and regulatory terms. On the other hand, small animal imaging makes a great contribution to the welfare of laboratory animals needed for this kind of research, because it accompanies the fulfillment of the 3Rs concept [27]. Additionally, experimental designs and statistics analysis benefits from the possibility of carrying out longitudinal studies. One of the main beneficiaries of these arising technologies is oncology research. Many different phases of carcinogenesis as well as intervention outcomes may be imaged using the right probe. Particularly, for breast cancer investigation there are several experimental models which usefulness could be reconsidered or redefined by those molecular imaging techniques [28-32].

One of the most common animal models is human cell lines xenografted mice, it is useful to asses tumor growth, apoptosis, angiogenesis and metastases in vivo. This model can also be used to find new therapy drugs, adjuvant and protective agents against chemotherapy´s adverse effects (30, 32) among other interventions. Our findings suggest that ^{99m}Tc-sestamibi was unable to be used as a tracer for primary tumor in the MDA-MB-231 xenografted animals. This probe proved not to be useful since tumor uptake was not enough to allow an acquisition with diagnostic potential. A mostly necrotic tumor core can be responsible for this. In spite of the fact that tumor viable cells might have ^{99m}Tc-sestamibi uptake, that thin external layer cannot be resolved by this detection technique. Perhaps more sensitive and sophisticated equipment could solve this limitation. However, this in vivo imaging analysis agrees with some of our previous dissection results (unpublished data) that showed that at 1 h post injection of the radiotracer, the biodistribution was less than 0,1 % of the injected dose (ID) of ^{99m}Tc-sestamibi accumulated in the tumor and 1 % ID in the heart. In this sense similar results were replicated by dissection and image visualization. Unfortunately other popular tumor tracers showed the same limitations and would not be suitable either as ^{18}F-FDG

given that its uptake, as it occurs with ^{99m}Tc-sestamibi, depends on cellular viability (33). The above presented results should be considered as confined to this animal model and this particular cell line, other authors have demonstrated more optimistic results with this type of tumors and probe [4, 7].

In spite of the above mentioned, there is still hope for metastasis evaluation which is one of the mayor uses of this experimental breast carcinoma animal model. In this particular work images had not an adequate resolution to detect metastasized organs at the evaluated time points so further research is needed to solve this question. It must be mentioned that small animal dedicated equipment would more likely suit imaging of metastasis in mice. However, given developing countries limited resources, it is interesting whereas readily available equipment can be adapted into an efficient detector for small animal image acquisition favoring in this way, breast cancer research worldwide.

On the other hand chemo-induced models can be used for carcinogenesis research and tumor progression understanding as well as to evaluate potential tumor inducing agents. They have the advantage that the tumor develops in the mammary gland of the animal and this mimics the site of origin of human spontaneous breast cancer. Additionally hormone relevance in carcinogenesis process can be evaluated with these animal models by characterizing expression of hormone receptors and their regulation in tumors and more importantly in every different stages of tumor development (34). Our results suggest that ^{99m}Tc-sestamibi is a suitable tumor tracer for this animal model. Moreover early tumor detection could be done by analyzing ^{99m}Tc-sestamibi gamma camera images, even prior to tumor palpation, allowing very early stages of carcinogenesis to be evaluated.

Another future perspective relies in the possibility of new and old drugs resistance profiles screening or assessment by exploiting the fact that ^{99m}Tc-sestamibi is a functional tumor tracer that binds to P glycoprotein as mentioned earlier. Thus tumor wash-out rate may be another protocol tool for elucidating chemo-resistance mechanisms, treatment alternatives and outcomes using experimental animal models. In this work we demonstrated that ^{99m}Tc-sestamibi tumor accumulation dropped to half of the initial uptake after 50 minutes. More research is needed to correlate this radiopharmaceutical wash out to specific protein expression in NMU induced tumors.

4. Conclusion

In conclusion ^{99m}Tc-sestamibi proved not to be useful for X model but it is a suitable tumor tracer for C model. New perspectives in small animal imaging with available functional probes and commonly used detection equipment are being revealed as shown in this work, however further investigation in this area is still needed to redefine this old radiopharmaceutical for its use in basic research and other experimental animal models.

5. Acknowledgements

This work was supported by a grant from the University of Buenos Aires, UBACYT 20020100100489 (2011-2014).

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