Technetium-99m-Sestamibi Uptake by Human Benign and Malignant Breast Tumor Cells: Correlation with *mdr* Gene Expression

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Early diagnosis of multidrug-resistance (MDR) development is extremely important for the judicious choice of treatment protocols in breast cancer chemotherapy. In this study, the mechanism of ^{99m}Tc-sestamibi uptake by nine human breast tumor cell lines was analyzed as a function of P-glycoprotein (PgP) expression. **Methods:** Technetium-99m-sestamibi radioactivity incorporation into the cells was determined after different times of incubation at 37°C. We analyzed the mechanism of ^{99m}Tc-sestamibi uptake as follows: (a) effect of temperature (4°C); (b) influence of extracellular ^{99m}Tc-sestamibi concentration; and (c) competitive inhibition of cell uptake with cold ^{99m}Tc-sestamibi. Technetium-99m-sestamibi uptake was compared to the level of PgP determined by Western blotting. The PgP reversing effect of verapamil was evaluated at different drug concentrations (50, 200, 500 μM). **Results:** Technetium-99m-sestamibi uptake plateaued at 60 min, which was 14 times lower at 4°C than at 37°C and was directly proportional to the extracellular concentration between 0.3 and 10 nM. Technetium-99m-sestamibi percentage uptake by cells expressing nonimmunodetectable levels of PgP was significantly higher (7.3% ± 0.6% (s.d.) to 14.9% ± 1.9%) than that by cells expressing high PgP levels (0.7% ± 0.4%, $p < 0.001$). In the presence of verapamil, a known reverser of PgP functions, ^{99m}Tc-sestamibi uptake was increased by a factor of 2 in cells expressing no detectable levels of PgP and by a factor of 12 in cells with high PgP levels. **Conclusion:** Technetium-99m-sestamibi uptake by these breast tumor cells is energy-dependent but not specific. These data suggest that ^{99m}Tc-sestamibi imaging may be used as a noninvasive technique to diagnose the presence of MDR in breast tumors in vivo.

Key Words: multidrug resistance; technetium-99m-sestamibi; human breast tumor; P-glycoprotein

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Drug resistance is a major problem with cancer treatment. One of the reasons for this resistance is the overexpression of the multidrug-resistance gene (*mdr*) (*1*) before and after induction of chemotherapy with certain drugs. This gene encodes a 170-kDa membrane glycoprotein named P-glycoprotein (PgP) (*2,3*), which is an energy-dependent drug-efflux pump (*4*). In vivo, PgP-170 is expressed intrinsically in many but not in all normal tissues, for example, epithelial cells and kidney (*5*), and in untreated tumors, for example, non small-cell lung cancer (*6*). PgP recognizes diverse lipophilic compounds (*7*). It was recently reported that ^{99m}Tc-sestamibi could also be a substrate for PgP and could be extruded like drugs from cells (*8,9*).

Technetium-99m-sestamibi, a member of the isonitrile class of coordination compounds (*10*), is a lipophilic cation used for myocardial perfusion imaging (*11*). Clinically, ^{99m}Tc-sestamibi uptake by several kinds of tumors has been reported (*12*). Preliminary reports indicate that this compound may also be incorporated in vitro into several cancer cell lines (*13,14*), but the radiotracer does not penetrate into the cells through the potassium pathway using the ATPase-dependent Na^+/K^+ pump (*15*). It was demonstrated that neither the lipophilicity nor the cation charge alone was sufficient to predict myocardial uptake (*16*). It appears that ^{99m}Tc-sestamibi is driven into the mitochondria by the electron gradient between plasma and mitochondrial membrane potentials, which plays an important role (*17,18*).

The aim of this study was to characterize ^{99m}Tc-sestamibi uptake in nine human breast tumor cell lines as a function of temperature and the extracellular concentration of ^{99m}Tc-sestamibi.

To explore the potential use of ^{99m}Tc-sestamibi in breast tumor imaging, we evaluated its uptake in these human breast tumor cells lines in comparison to the latters' PgP expression and examined the effect of verapamil (a calcium inhibitor), a drug known to reverse PgP functions (*8*).

MATERIALS AND METHODS

Tracer Preparation

Technetium-99m-sestamibi was prepared according to the manufacturer's instructions. We used generator equilibrium equations to calculate the absolute concentration of total ^{99m}Tc-sestamibi (radioactive and cold) in the solutions. Molarity was expressed in terms of total ^{99m}Tc-sestamibi. Radiochemical purity was always greater than 96%. To obtain 2 n*M* solutions, ^{99m}Tc-sestamibi was diluted in RPMI (Gibco, Grand Island, NY) or Dulbecco's modified Eagle's medium (DMEM, Gibco).

Cell Line Culture

Nine human breast cell lines were used. Three lines were benign: HBL100, NPM14T and NPM21T4. Six lines were malignant: MDAMB231 (ATTC, Rockville, MD); MCF7 (ATTC); MCF7*ras* (Georgetown University Medical Center, Washington, D.C.); HBL100*ras* (Institut Curie, Paris, France); HH9; and MCF7*mdr*+ Hopital Henri-Mondor, Creteil, France). Cell lines were grown in 75-cm^2 tissue-culture flasks in DMEM supplemented with L-glutamine (2 mmole/liter), penicillin/streptomycin (50 IU/liter, Gibco) and 10% fetal calf serum. For MCF7*mdr*+, the medium was RPMI supplemented with doxorubicin (5.8 μg/ml).

Technetium-99m-Sestamibi Uptake

Adherent cells were detached with 0.05% trypsin, harvested and washed twice with RPMI or DMEM. The cells were counted in a hemocytometer and suspended in the appropriate medium at a concentration of 1×10^7 cells/ml. To minimize nonspecific ^{99m}Tc-sestamibi binding to plastic tubes, the tubes were presaturated for 1 hr with a phosphate-buffered saline (PBS) solution containing 1% fetal calf serum, followed by three washes in PBS. Uptake was initiated in plastic tubes by adding an equal volume of

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the ^{99m}Tc-sestamibi stock solution to the cell suspension. At various time intervals, 50-μl aliquots were transferred to 400-μl microfuge tubes containing 200-μl of fetal calf serum and centrifuged for 1 min in an Ependorf microfuge (15,000 × g). After freezing the tubes, their tips were cut off and the pellet and the supernatant were separated. Radioactivity was determined in a gamma counter. Nonspecific binding was determined with the same method but without cells.

The results are expressed as an accumulation ratio calculated by dividing the total specific cell uptake (after subtracting nonspecific binding) by the total radioactivity (pellet and the supernatant).

The influence of the extracellular ^{99m}Tc-sestamibi concentration was studied by using different radiotracer concentrations (0.3, 1, 3 and 10 n*M*). Competitive inhibition was assessed with 40-n*M* stock solution of cold ^{99m}Tc-sestamibi over a period of 1.5 hr (0, 15, 30, 60 and 90 min). All data points represent the means of two independent experiments performed in triplicate.

Effect of Verapamil

We explored whether verapamil, a known PgP-reversing agent was able to change ^{99m}Tc-sestamibi uptake into MDAMB231 (*mdr*-line) and MCF7*mdr*+ cell lines. A verapamil stock solution was prepared in dimethyl sulfoxide; the final concentration of the latter was <0.5%. This concentration was reported to have no effect on ^{99m}Tc-sestamibi uptake (*8*). Aliquots of the cell suspension were pretreated with different verapamil concentrations (50, 200 and 500 μM) at 37°C for 10 min prior to incubation with the radiotracer as described above.

PgP Detection by Western Blotting

Membrane fractions were extracted from the tumor cells before being loaded onto 6% sodium dodecyl sulfate-polyacrylamide gels and electrophoresed for 1 hr 50 min at 125 V. Gels were then equilibrated in transfer buffer (4.16 g/l Tris-base, 19.8 g/l glycine, 360 ml methanol, 180 ml H_2O) and proteins were transferred onto nitrocellulose sheets using a blotting apparatus (40 V for 1 hr 45 min). Blots were blocked for 1 hr at 37°C in TBST (2 n*M* Tris-base, 17.5 g NaCl, 1 ml Tween 20, 2000 ml H_2O) and 3% bovine milk powder, followed by incubation with the primary antibody at 1 μg/ml (polyclonal rabbit anti-PgP antibody) overnight at 4°C. Blots were washed five times for 10 min in buffer (0.9% NaCl, 10 m*M* Tris, pH 7.5), incubated with horse anti-rabbit antibody at 0.2 mg/ml conjugated to alkaline phosphatase for 30 min at 37°C, then washed five times for 10 min in TBST. Specific antigen-antibody complexes were revealed by incubation with 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt. A solution containing several proteins of known molecular masses was used as the internal standard.

Values given are means ± s.d. Statistical significance was determined using an unpaired Student's t-test; $p < 0.05$ was considered to be significant.

RESULTS

HBL100 Studies

Technetium-99m-sestamibi binding to plastic tubes without cells was always less than 2% of the total radioactivity for incubation periods up to 2 hr.

Technetium-99m-sestamibi uptake reached a plateau at 60 min and its accumulation was 15.7% and 1.1% of the radioactivity added at 37°C and 4°C, respectively (Fig. 1). Technetium-99m-sestamibi accumulation by HBL100 over 2 hr was proportional to the extracellular concentration of ^{99m}Tc-sestamibi. Saturation was not obtained at the concentrations used (Fig. 2). Technetium-99m-sestamibi uptake decreased from 15.7% to 3.2% when 40 n*M* ^{99m}Tc-sestamibi (cold) were added to the incubation mixture for 90 min. At this concentration, ^{99m}Tc-sestamibi was toxic to the cells (>80%). Therefore, it was impossible to run competition experiments with concentrations higher than 40 n*M*.

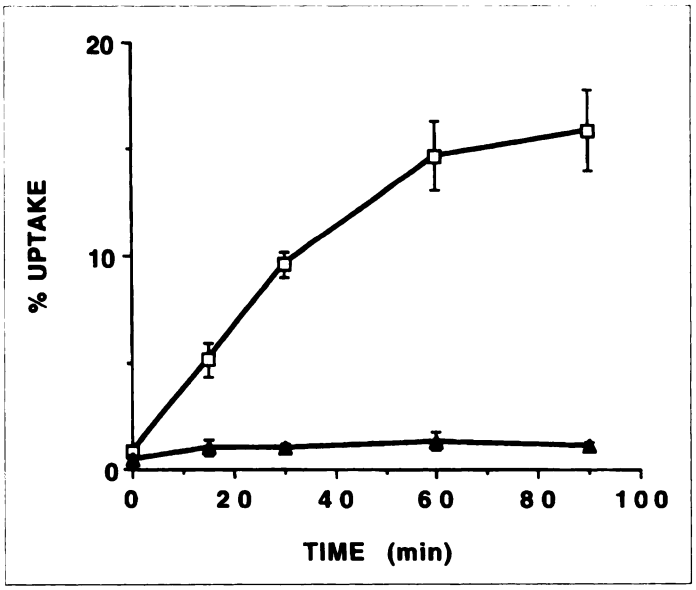

FIGURE 1. Curves of ^{99m}Tc-sestamibi cellular uptake by HBL100 cells expressed as a percent of the total dose added at 37°C (-□-) and 4°C (-▲-). Error bars not shown are within the symbol.

Other Cell Lines

Curves of ^{99m}Tc-sestamibi cellular uptake by nine breast cell lines are shown in Figure 3. Kinetic studies showed that radiotracer incorporation plateaued at 60 min for every cell line. Although the uptake curves were all asymptotic, the plateau levels reached were different and occurred at different times. Uptake varied widely among the different cell lines (Fig. 4).

Detection of PgP-170

Expression of human PgP-170 in MCF7*mdr*+ cells was demonstrated by the presence of a large band corresponding to a molecular mass of 170 kDa. PgP-170 was not detectable in the other eight cell lines.

Verapamil Effect

Verapamil increased the ^{99m}Tc-sestamibi accumulation in the two cell lines examined (MDAMB231 (*mdr*−) and MCF7*mdr*+).

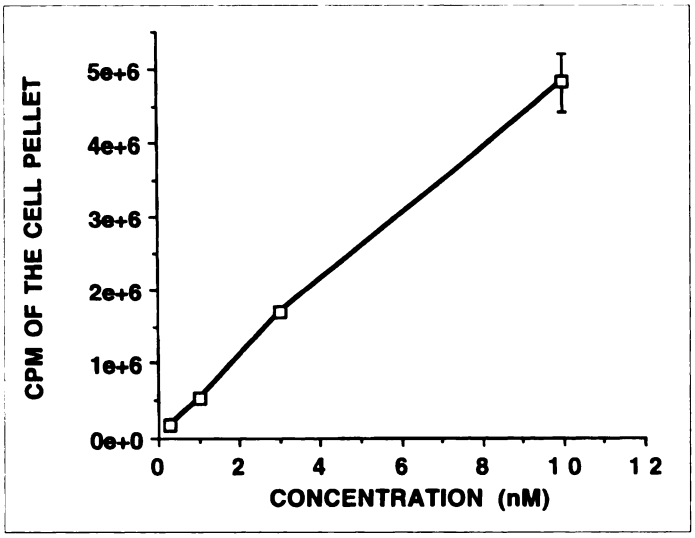

FIGURE 2. Effect of the extracellular concentration of ^{99m}Tc-sestamibi (0.3, 1, 3, 10 nM) on HBL100-cell uptake of the radiotracer expressed in cpm. Each point represents the mean ± s.d. of three experiences. Error bars not shown are within the symbol.

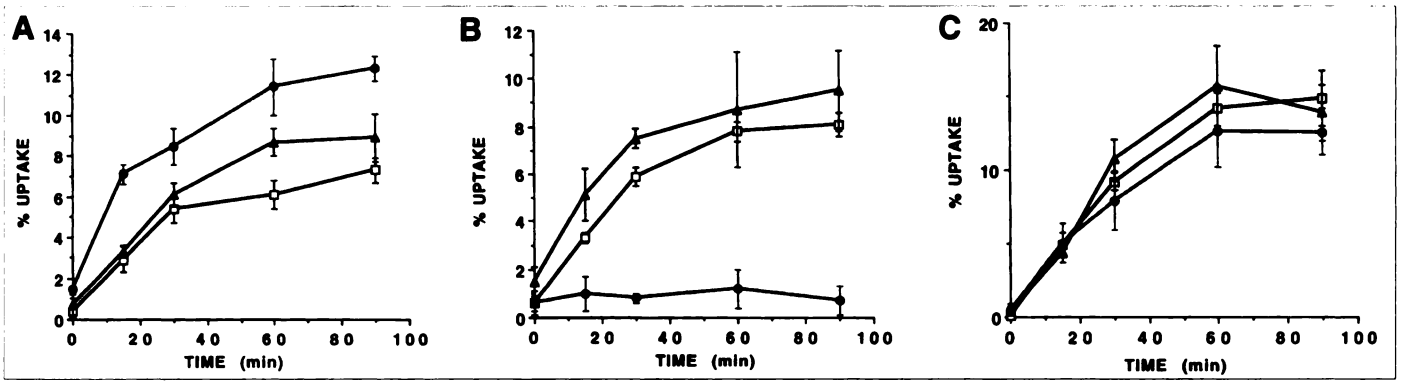

FIGURE 3. Technetium-99m-sestamibi uptake at 37°C by MDAMB231 (-□-), NPM14T (-▲-) and NPM21T4 (-●-) cells (A); MCF7 *mdr*+ (-●-), HH9 (-□-) and HBL100*ras* (-▲-) cells (B); HBL100 (-□-), MCF7 (-▲-), MCF7ras (-●-) cells (C). Radiotracer cell uptake is expressed as a percent of the total dose added.

Maximal verapamil enhancement was observed at the concentration of 200 μM (Fig. 5A,B). Uptake was negligible when 500-μM verapamil was incubated with each cell line (results not shown). At the two other concentrations used the ratios of uptake percentages (with/without verapamil) were higher by a factor of two in MDAMB231 (*mdr*−) cells and by factors of 5 and 12, at 50 μ*M* and 200 μ*M*, respectively, in MCF7*mdr*+ cells.

DISCUSSION

Uptake of ^{99m}Tc-sestamibi was studied in nine breast cancer cell lines as compared to their PgP expression. To determine the mechanism of radiotracer incorporation, we studied the effects of two different temperatures and different extracellular ^{99m}Tc-sestamibi concentrations.

Characterization of Sestamibi Uptake

Our data confirm preliminary observations demonstrating that ^{99m}Tc-sestamibi uptake plateaus at 60 min and that a low temperature (4°C) restricted incorporation (*13*). Technetium-99m-sestamibi uptake is driven by a negative transmembrane potential and 90% of the tracer activity is concentrated in the mitochondria. Because these potentials are directly under the control of energy-consuming biochemical reactions, the data indicate that ^{99m}Tc-sestamibi uptake is energy-dependent. The use of various extracellular ^{99m}Tc-sestamibi concentrations showed that uptake was directly proportional to the extracellular concentration. These results indicated that there was no saturation with the concentrations used and no receptor for the radiopharmaceutical. Similar results were obtained by Delmon-Moingeon et al. with the same molecule (*13*) and by Piwnica-Worms et al. who used hexakis (carbomethoxyisopropylisonitrile) technetium(I) (*15*). Unfortunately, it was not possible to interpret the attempted competition experiments because of the very high cytotoxicity of the test molecule.

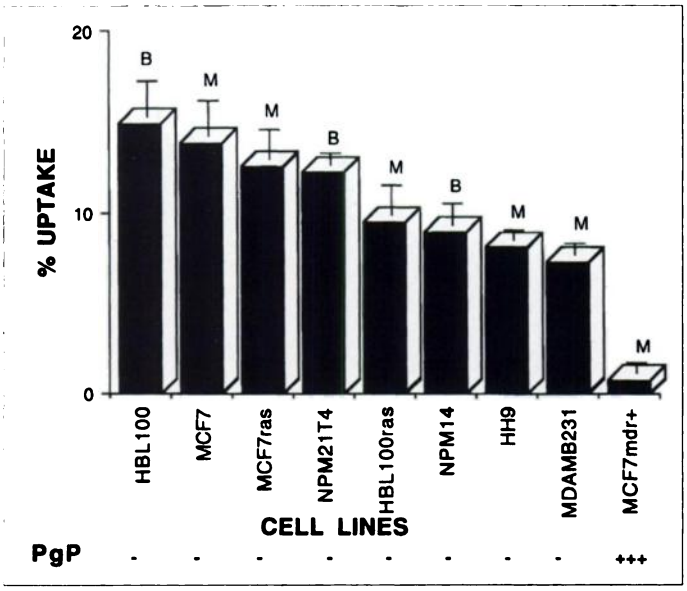

FIGURE 4. Technetium-99m-sestamibi uptake at 37°C after 90 min of incubation in three benign (B) cell lines and in 6 malignant (M) cell lines. PgP expression is indicated at the bottom of the figure. Radiotracer cell uptake is expressed as a percent of the total dose added.

Technetium-99m-sestamibi uptake after 90 min of incubation varied widely among the cell lines. Nevertheless, two categories could be discerned: cell lines with a percentage uptake between 7.3%–14.9% and a cell line with only 0.7%. This last cell line was the only one to express immunodetectable PgP. A preliminary report suggested that a high PgP level restricted ^{99m}Tc-sestamibi accumulation (*8*) by Chinese hamster V79 lung fibroblasts and derivative resistant cells. We also found that a high quantity of PgP limited ^{99m}Tc-sestamibi accumulation in our breast tumor cell lines. We found no significant difference between ^{99m}Tc-sestamibi uptake by benign and malignant cells. The known high-level uptake of this radiotracer by tumors has been attributed to their greater intrinsic electron-gradient potentials (*19*) and/or linked to their high numbers of mitochondria. The latter were not evaluated in our cell populations. The uptake probably reflects the level of metabolic activity which we did not determined and which can be high in benign and malignant cells.

Effect of Verapamil

To study the verapamil reversing effect, we used two cell lines: one expressing a high PgP level (MCF7*mdr*+) and the other expressing no PgP (MDAMB231 *mdr*-). We hypothesized that calcium accumulation could lead to a modification of the permeability which could explain the small increase of ^{99m}Tc-sestamibi uptake in the *mdr*− cells. Verapamil increased radiotracer uptake much more in the cells expressing high levels of PgP compared to cells with no PgP expression. Similar results were obtained by Rao et al. (*9*) and Piwnica-Worms et al. (*8*). Verapamil (500 μ*M*) produced unexpectedly small ^{99m}Tc-sestamibi uptake in both cell lines. In light of a preliminary report describing a verapamil-induced increase of ^{99m}Tc-sestamibi uptake, even up to a concentration of 500 μ*M* when Chinese hamster lung fibroblasts and derivative cell lines were used (*8*), these data may indicate that cell sensitivity to calcium flux inhibition by verapamil differs markedly from one cell to another.

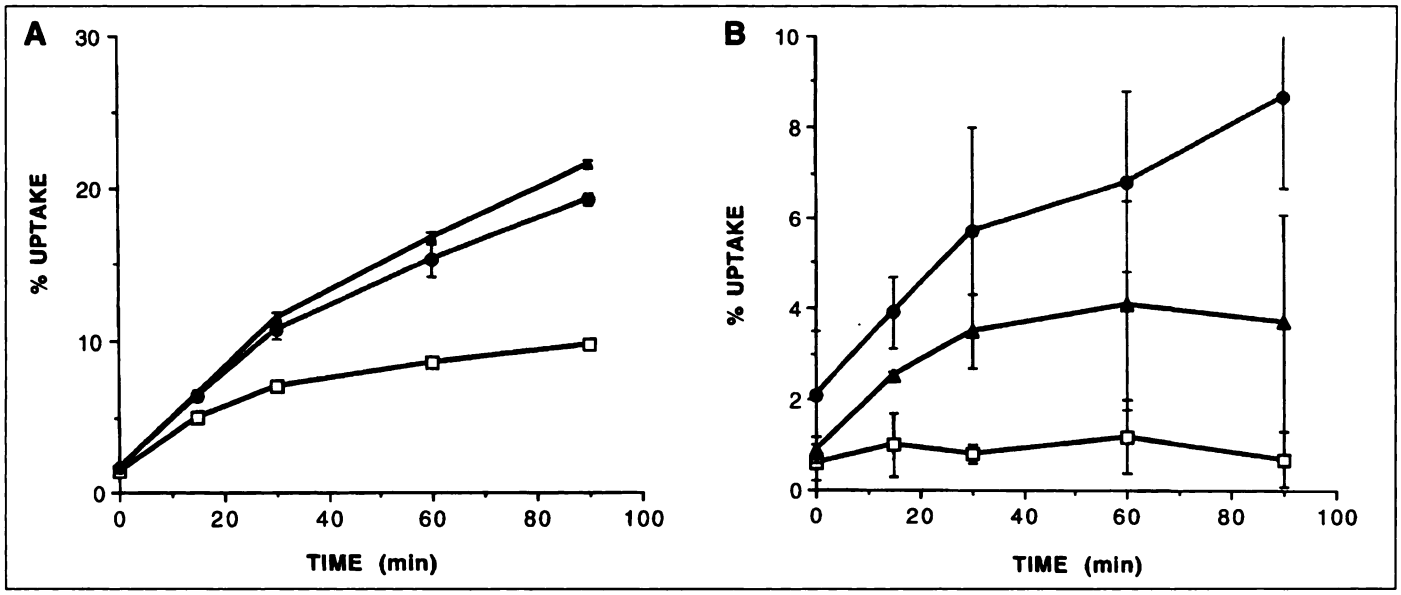

FIGURE 5. Effect of three concentrations of Verapamil 0(-□-), 50 (-▲-) and 200 (-●-) μM on ^{99m}Tc-sestamibi uptake at 37°C by *mdr*− (MDAMB231) (A) and *mdr*+ cells (MCF7) (B). Radiotracer cell uptake is expressed as a percent of the total dose added. Error bars not shown are within the symbol.

CONCLUSION

Different human breast tumor cell lines took up ^{99m}Tc-sestamibi and the different uptakes varied according to cell line and PgP status. We found that:

1. Low temperature could inhibit ^{99m}Tc-sestamibi uptake.
2. Technetium-99m-sestamibi uptake was directly proportional to the extracellular concentration of the tracer.
3. Verapamil increased ^{99m}Tc-sestamibi uptake much more in *mdr*+ cells, which express high levels of PgP than in *mdr*− cells.

These data suggest that ^{99m}Tc-sestamibi imaging could be a good noninvasive technique for the in vivo diagnosis of the presence of the MDR protein (PgP-170 kDa) in breast tumors. Since our results were obtained in vitro, however, clinical studies are needed to validate our hypothesis. We postulate that a chemoresistant tumor will not accumulate ^{99m}Tc-sestamibi and that this approach could be used to scintigraphically monitor therapeutic protocols.

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