Comparative ^{99m}Tc-Sestamibi and ^{3}H-Daunomycin Uptake in Human Carcinoma Cells: Relation to the MDR Phenotype and Effects of Reversing Agents

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Because ^{99m}Tc-sestamibi (MIBI) appears to be a potent candidate for multidrug resistance (MDR) evaluation in tumors, its cellular uptake should be similar to that of ^{3}H-daunomycin in a variety of conditions of expression and inhibition of MDR activity. **Methods:** We used a human rhinopharyngeal carcinoma cell line (KB-3–1) and its MDR variant (KB-A1). Cells were incubated 2 h with ^{99m}Tc-MIBI and ^{3}H-daunomycin under control conditions or in the presence of a reversing agent such as verapamil (10 μmol/L), PSC833 (1 μmol/L) or S9788 (5 μmol/L). **Results:** Relative to the KB-3–1-sensitive cells, accumulations of ^{99m}Tc-MIBI and ^{3}H-daunomycin were reduced to 31% ± 5% and 36% ± 11% ($P <$ 0.001 for both) in KB-A1-resistant cells. In sensitive cells, accumulation of both agents was increased by verapamil and PSC833 (range 115%–140%; $P < 0.05$) but not by S9788. In KB-A1 cells, only S9788 significantly increased the cellular uptake of ^{99m}Tc-MIBI (138% ± 25%; $P < 0.01$), whereas the intracellular uptake of ^{3}H-daunomycin was markedly increased with the three reversing agents (up to 311% ± 37% with S9788; $P < 0.001$). With this last treatment, uptake of ^{3}H-daunomycin in KB-A1 cells nearly returned to its basal level in sensitive cells. **Conclusion:** ^{99m}Tc-MIBI monitors the MDR phenotype of tumor cells effectively but responds to reversing agents differently than ^{3}H-daunomycin.

Key Words: multidrug resistance phenotype; ^{99m}Tc-sestamibi; ^{3}H-daunomycin; multidrug resistance reversing agents

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Development of resistance to multiple drugs is a common clinical problem in the treatment of cancer. The hallmark of the multidrug resistance (MDR) phenotype is a cross-resistance to multiple drugs (vinca alkaloids, epipodophillotoxins, anthracyclines and other xenobiotics) that lack homology in structure and mechanism of cytotoxic action (*1*). P-glycoprotein (Pgp)-mediated MDR is the most studied form of anthracycline resistance, although other MDR-related proteins, such as the MDR-associated protein (MRP), lung-resistance protein (LRP), glutathione-S-transferase (GST) and topoisomerase II, may contribute to the MDR phenotype (*2,3*). Pgp is a 170-kDa membrane glycoprotein that acts putatively as an energy-dependent, adenosine triphosphate-consuming, efflux pump to decrease the intracellular content of cytotoxic drugs (*4*).

Several compounds, called MDR inhibitors or reversing agents, can inhibit Pgp function and restore chemotherapeutic sensitivity to resistant tumor cells (*5*). Combined therapy with MDR-related drugs and MDR inhibitors can decrease tumor size and prolong life span in some animal models (*6*). Several new potent reversing agents are undergoing clinical trials (*7,8*).

Increased uptake of ^{99m}Tc-sestamibi (MIBI), a lipophilic cationic radiopharmaceutical commonly used in nuclear cardiology, has been reported in several kinds of tumors, including breast, lung, thyroid, solid tumors of the bones and soft tissues, brain, head and neck, gastrointestinal tract tumors and lymphomas (*9*). ^{99m}Tc-MIBI has been validated as a substrate of Pgp, and its net cellular accumulation is inversely proportional to the level of Pgp expression (*10*). Increased uptake of this tracer is observed after exposure to MDR reversing agents (*11*). Functional in vivo imaging with ^{99m}Tc-MIBI could enable rapid characterization of Pgp expression in tumors, assessment of the efficacy of reversing agents and selection of optimized chemotherapy regimens.

In a previous study, we reported the presence of *mdr1a* gene expression in rat cardiac cells (*12*). Accumulations of ^{3}H-daunomycin, a common chemotherapeutic agent, and ^{99m}Tc-MIBI, a documented Pgp substrate, were then compared in the presence of various reversing agents (*13*). The behavior of the two radiotracers was different in the presence of some reversers. The purpose of this study was to conduct the same comparison in a chemosensitive human tumor cell line and its multidrug resistant variant.

MATERIALS AND METHODS

Cell Lines

Cell Culture. We used human drug-sensitive rhinopharyngeal carcinoma cell line KB-3–1 and its resistant variant KB-A1, originally isolated in Gottesman's laboratory from the KB-3–1 parental cell line by successive stepwise selections in adriamycin (*14*). Both cell lines were grown as monolayers in Dulbecco's modified Eagle's medium (Seromed; Biochrom AG, Berlin, Germany) supplemented with 10% fetal bovine serum (Seromed), 1% L-glutamine (200 mmol/L) and 0.1% gentamycin (20 mg/mL) at

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37°C in a 5% CO_2 humidified atmosphere. Drug-resistant KB-A1 cells were cultured in the presence of adriamycin (1 μg/mL) until experiments. Cells were passaged every 3 or 4 d to ensure exponential growth in 75-cm^2 flasks. Four days before experiments, cells were plated in 35-mm-diameter culture dishes (5×10^5 cells in 2 mL complete medium).

MDR Phenotype Expression. Total ribonucleic acid was extracted from KB-A1 and KB-3-1 cell pellets (*13*), and a reverse transcription polymerase chain reaction (RT-PCR) was used to evaluate *mdr1* gene expression with a standardized technique (*15*). To confirm the sensitive and resistant phenotypes of KB-3-1 and KB-A1 cell lines, respectively, cells were grown simultaneously in the presence and absence of adriamycin (1 μg/mL) and counted before plating as well as 4 d later. KB-3-1 cells did not survive adriamycin treatment, whereas KB-A1 cells were not affected.

Chemicals

^{99m}Tc-Sestamibi. Hexakis (2-methoxyisobutyl isonitrile) ^{99m}Tc-MIBI was prepared from a commercially available kit (Cardiolite; Du Pont de Nemours, North Billerica, MA) as previously described (*12*).

^{3}H-Daunomycin. ^{3}H-daunomycin with a specific activity of 163 GBq/mmol was purchased from Du Pont de Nemours and diluted to 100 × in ethanol for experiments.

Reversing Agents. Verapamil was obtained from Sigma (St. Quentin Fallavier, France), PSC833 from Novartis Pharma AG (Basel, Switzerland) and S9788 from Servier (Courbevoie, France). All compounds were solubilized in sterile water except for PSC833, which was first solubilized in ethanol and then diluted in sterile water.

Intracellular Accumulation of Tracers

Thirty minutes before the experiment, the incubation medium was replaced by 2 mL fresh medium. To all dishes were added 37 kBq (1 μCi) ^{99m}Tc-MIBI in 20 μL and 10 kBq (0.27 μCi) ^{3}H-daunomycin (50 nmol/L) in 20 μL. A subset of dishes received a reversing agent, either the calcium channel blocker verapamil 10 μmol/L, the non-immunosuppressive hydrophobic peptide PSC833 1 μmol/L or the uncoupler of oxidative phosphorylation S9788 5 μmol/L. After 2 h of incubation at 37°C, the dishes were rapidly washed three times with isotope-free cold (4°C) saline to block all membrane exchanges. The 2-h incubation time has been chosen because the accumulations of ^{99m}Tc-MIBI and ^{3}H-daunomycin are known to plateau after 1 h and 15–30 min, respectively (*16,17*). The cells were then scraped, withdrawn in 2 mL phosphate buffered saline solution and transferred into a sodium iodide gamma well counter for ^{99m}Tc-MIBI activity counting (1 min per sample). The radioactivity of ^{3}H-daunomycin was determined 4 d later in a 1-mL aliquot after sonication and addition of Ultima-Gold scintillation cocktail (Packard Instruments, Meriden, CT), using a Winspectral Wallac 1414 scintillation spectrophotometer (Wallac, Turku, Finland). The amount of proteins was determined by the Coomassie Protein Assay Reagent (Pierce, Rockford, IL). The net accumulation of the two tracers in dishes treated with verapamil, PSC833 and S9788 was expressed relative to the total radioactivity dose added per dish and per milligram of proteins. Finally, the value was expressed relative to that under control conditions on the same day, using the same batch of cells and the same tracer preparation. Five independent experiments, including 24 dishes each, have been performed. All data points were determined in triplicate with cells obtained from the same culture preparation. Results are expressed as mean ± SD. Comparisons between groups were made using the non-parametric Kruskal-Wallis H test followed by the a posteriori Steel test.

RESULTS

RT-PCR performed from total RNA of KB-3-1 and KB-A1 cells showed, after gel electrophoresis and staining with ethidium bromide, a 165-nucleotide *mdr1*-specific band in KB-A1 not perceptible in KB-3-1 cells (Fig. 1). These results confirm the strong MDR phenotype of KB-A1 cells. Under basal conditions, the intracellular accumulation of ^{99m}Tc-MIBI and ^{3}H-daunomycin in KB-A1 cells, compared with KB-3-1 cells, was 36% ± 11% and 31% ± 5% (n = 15), respectively.

The effects of the three tested reversing agents are presented in Table 1. In KB-A1-resistant cells, uptake of ^{99m}Tc-MIBI was significantly modified only by S9788, which produced a 38% increase. For ^{3}H-daunomycin, all three agents significantly increased cellular accumulation, the strongest effect was produced by S9788, which nearly tripled the uptake. In KB-3-1-sensitive cells, S9788 had no detectable effect on either ^{99m}Tc-MIBI or ^{3}H-daunomycin accumulation. However, verapamil and PSC833 moderately increased cellular uptake of both radiotracers. In Figure 2, these results are presented with the uptake value in KB-3-1

FIGURE 1. Image shows *mdr1* gene expression in human KB-3-1 and KB-A1 cells. Polymerase chain reaction products (20 μL) were loaded next to mol wt deoxyribonucleic acid marker (Gibco/BRL, Cergy, Pontoise, France) (line 3). The 165-base pair (bp) *mdr1* fragment (arrow) is present only in KB-A1 cells (line 2), whereas 120-bp β2 microglobuline fragment (arrow) is present in both KB-3-1 (line 1) and KB-A1 (line 2) cells.

TABLE 1
Effects of Reversing Agents on Cellular Accumulation of ^{99m}Tc-sestamibi and ^{3}H-daunomycin in KB-A1 and KB-3-1 Cells

Treatment	KB-A1		KB-3-1	
	^{99m}Tc-sestamibi	^{3}H-daunomycin	^{99m}Tc-sestamibi	^{3}H-daunomycin
Controls	100 ± 12	100 ± 13	100 ± 10	100 ± 5
Verapamil 10 μmol/L	94 ± 15	188 ± 33*	140 ± 14*	116 ± 11
S9788 5 μmol/L	138 ± 25*	311 ± 37*	93 ± 12	102 ± 7
PSC 833 1 μmol/L	118 ± 27	126 ± 27†	116 ± 12 *	115 ± 19†

*$P < 0.01$ and †$P < 0.05$ compared with controls.
Results are given in percent of controls, mean ± SD, n = 15 samples for each group.

in control conditions as a reference. KB-A1 cellular uptake of ^{3}H-daunomycin in the presence of S9788 is close to that obtained under control conditions in sensitive cells, suggesting a near complete reversion of MDR phenotype (Fig. 2B). However, such a reversal is not observed with ^{99m}Tc-MIBI (Fig. 2A).

DISCUSSION

The accumulation of tracers in drug-resistant KB-A1 cells was nearly three times lower than in drug-sensitive KB-3–1 cells. Because KB-A1 overexpressed Pgp compared with KB-3–1 cells (Fig.1), these results support the fact that ^{99m}Tc-MIBI, as well as ^{3}H-daunomycin, reflects the MDR phenotype. As expected, the reversing agents clearly enhanced cellular uptake of ^{3}H-daunomycin in the KB-A1 cell line. A near complete return to the control level of the sensitive line was attained with S9788. This is in accordance with the results of Merlin et al. (*18*), who found S9788 at 2 and 5 μmol/L to be more potent than verapamil and cyclosporin A in several human tumor cell lines.

However, the effects of the reversing agents on ^{3}H-daunomycin uptake do not match those observed on ^{99m}Tc-MIBI uptake, because only one agent, S9788, was able to increase its cellular accumulation in the resistant cells, and only with a reduced magnitude. These results would suggest that ^{99m}Tc-MIBI and ^{3}H-daunomycin cellular concentrations behave similarly as a function of Pgp expression in basal conditions but behave differently in the presence of reversing agents. In fact, Bosch et al. (*19*) have reported that a higher concentration of reversers is necessary to restore the accumulation of ^{99m}Tc-MIBI than for ^{3}H-daunomycin in resistant tumor cell lines. Nevertheless, this conclusion should be considered carefully because the accumulation of radiotracers and the effects of reversing agents seem to vary widely according to the concentrations and cell lines. Ballinger et al. (*20*) observed an increased accumulation of ^{99m}Tc-MIBI using our concentrations of verapamil and PSC833, but in different cell lines, namely transplantable rat breast adenocarcinoma *Matb* sensitive and doxorubicin-resistant cells. Using higher concentrations of verapamil (100–500 μmol/L), Piwnica-Worms et al. (*10*) and Rao et al. (*21*) showed increased ^{99m}Tc-MIBI accumulation in both wild-type and MDR1 baculoviral infected insect Sf9 cells. In nine human breast tumor cell lines, not including KB-A1 or KB-3–1 and with high concentration of verapamil (50–500 μmol/L), Cordobes et al. (*22*) found that the effect of reversing agents on ^{99m}Tc-MIBI accumulation was very different between cell lines, varying between a factor of 2–12. In our study, the reversing agent concentrations were determined in preliminary experiments (Cayre et al., unpublished data, 1997). For each reversing agent, we tested a panel of concentrations known to be without any toxic effect (*10,20,22–24*). To select the concentration that did not modify, in these experimental conditions, the amount of adherent cells, we performed a cytotoxicity sulforhodamine B test after a 2-h incubation time with verapamil (5–100 μmol/L), PSC833 (0.1–10 μmol/L) or S9788 (2.5–20 μmol/L) (*25*). Each reversing agent was used alone or with 50 nmol/L ^{3}H-daunomycin. This led to the concentrations used in this study, i.e., verapamil 10 μmol/L, PSC833 1 μmol/L and S9788 5 μmol/L.

The results are partly at variance with a previous study in

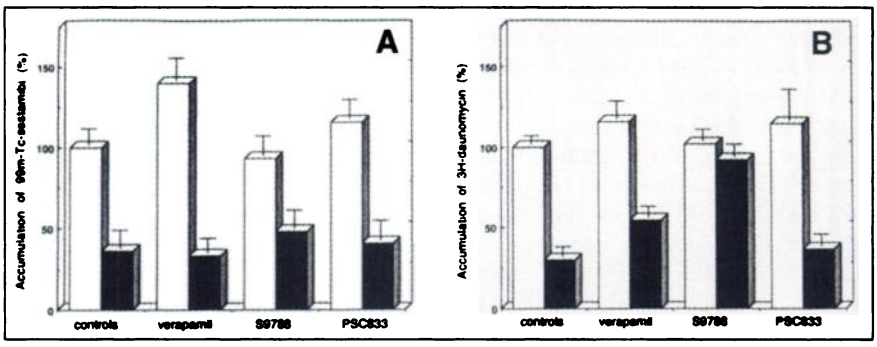

FIGURE 2. Relative accumulation of ^{99m}Tc-MIBI (A) and ^{3}H-daunomycin (B) after 2 h in KB-3–1 (open bar) and MDR KB-A1 (solid bar) cells. Values are expressed relative to accumulation in sensitive cells under control conditions. Reversing agent concentrations were verapamil 10 μmol/L, S9788 5 μmol/L and PSC833 1 μmol/L. (n = 15 per group, mean ± 1 SD).

which we reported a marked decrease of ^{99m}Tc-MIBI concentration in rat cultured myocardial cells treated with S9788 (*12*). An explanation may be related to the properties of S9788 as an uncoupler of oxidative phosphorylation (*26*). The uptake of ^{99m}Tc-MIBI is membrane potential-dependent and so decreases with depolarizing agents (*27*). In S9788-treated KB-A1 cells, the reduced enhancement of ^{99m}Tc-MIBI uptake compared with ^{3}H-daunomycin may be a result of a reduction in uptake due to the uncoupler properties of S9788, whereas the accumulation of ^{3}H-daunomycin in the same cells, being independent of membrane potentials, could reflect primarily the MDR-reversing properties of S9788 (*28*).

Although S9788 proved to be the most effective in KB-A1-resistant cells, it failed to show any effect in KB-3–1-sensitive cells, whereas verapamil and PSC833 increased ^{99m}Tc-MIBI and ^{3}H-daunomycin accumulations in these sensitive cells. A possible explanation could be that S9788 is the most specific for Pgp among the tested reversing agents. It would therefore indicate that S9877 is most likely to revert Pgp activity, and conversely it has no effect when Pgp is not expressed, as in KB-3–1 cells. Supporting this hypothesis is the ability of the S9788-treated resistant cells to concentrate ^{3}H-daunomycin at nearly the control level of the sensitive lines.

Several clinical studies have demonstrated the value of ^{99m}Tc-MIBI in tumor imaging (*9*) and, in particular, the good sensitivity and specificity of scintimammography for the detection of malignant breast tumors (*29*). Some researchers have already used ^{99m}Tc-MIBI scintigraphy to evaluate the MDR status of human tumors (*30–33*).

These results suggest that ^{99m}Tc-MIBI uptake does not always parallel MDR phenotype when ^{3}H-daunomycin accumulation is used as a reference probe. First, some difficulties could arise because Pgp is not the only protein involved in MDR. The MDR phenotype can also be related to overexpression of the MRP gene, encoding for an MRP 190-kDa protein, and of the GST gene, which regulates the intracellular level of glutathione through the GST enzyme activity (*34–36*). Preliminary results suggest that ^{99m}Tc-MIBI is also a substrate for MRP but not for GST (*37,38*). By RT-PCR, an MRP gene expression has been reported in KB-3–1 cells (*39*). These results are in accordance with the increased accumulation of ^{99m}Tc-MIBI and ^{3}H-daunomycin in KB-3–1 cells observed in the presence of reversing agents reported to block MRP protein dependent efflux weakly (*35*). It would certainly be of interest to study the status of these sensitive cells for these other resistance-related proteins.

CONCLUSION

^{99m}Tc-MIBI uptake parallels that of ^{3}H-daunomycin in tumor cells in vitro, but its behavior is less subject to variation than ^{3}H-daunomycin under treatment by reversing agents. The MDR phenotype appears to be a complex multifactorial phenomenon, and only double-blind clinical studies comparing the initial tumor ^{99m}Tc-MIBI uptake or washout rates with response to chemotherapy will be able to demonstrate the predictive value of tumor ^{99m}Tc-MIBI scintigraphy.

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