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Uptake of the Cation Hexakis(2-methoxyisobutylisonitrile)-Technetium-99m by Human Carcinoma Cell Lines *in Vitro*[1]

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ABSTRACT

Uptake of the cationic compound hexakis(2-methoxyisobutylisonitrile)-technetium-99m ([^{99m}Tc]MIBI) was examined in nine human tumor cell lines. The concentration of [^{99m}Tc]MIBI after a 1-h incubation with the compound varies from 5 to 28% of the activity in the external medium. In contrast, normal V79 cells (Chinese hamster lung fibroblasts) and human peripheral blood mononuclear cells exhibit a minimal uptake of less than 2% of the activity in the medium. Kinetic experiments with SW-13 cells indicate a rapid uptake over time ($t_{1/2}$ of 10 min) until a steady state is approached whose concentration appears directly correlated with the extracellular concentration of [^{99m}Tc]MIBI with no evidence of saturation over the range tested (10^{-12}–10^{-9} M). [^{99m}Tc]MIBI is taken up by a temperature dependent process that is restricted to living cells. Microautoradiography demonstrates that [^{99m}Tc]MIBI is clustered in the cytoplasm around the nucleus. Given that depolarizing the plasma membrane potential in high K^+ buffer results in lowering the uptake of [^{99m}Tc]-MIBI and that alteration of the mitochondrial membrane potential with valinomycin or nigericin induces, respectively, a significant decrease or increase of [^{99m}Tc]MIBI uptake, we propose that the plasma and mitochondrial membrane potentials play a major role in the uptake. These data suggest that the γ emitter [^{99m}Tc]MIBI exhibits interesting tumor cell interaction characteristics with promise for *in vivo* tumor imaging.

INTRODUCTION

[^{99m}Tc]MIBI,[3] a member of the isonitrile class of coordination compounds (1, 2), is a lipophilic cation presently under investigation for clinical use as a myocardial perfusion imaging agent (3–5). Preliminary reports indicate that this compound may also localize *in vivo* in primary malignant tumors and metastatic deposits from thyroid, lung, and bronchial carcinoma (6–8). Since other lipophilic cationic compounds, such as rhodamine 123 and tetraphenylphosphonium, have demonstrated high affinity for both myocytes and carcinoma cells (9–11), we evaluated the uptake of [^{99m}Tc]MIBI *in vitro* with a panel of human carcinoma cell lines and compared it with that of [^{3}H]TPP. We have observed that some tumor cell lines exhibit a very high uptake of [^{99m}Tc]MIBI, and we propose as a possible mechanism of interaction the participation of the plasma and mitochondrial membrane potentials.

MATERIALS AND METHODS

Chemicals. Hexakis(2-methoxyisobutylisonitrile)-technetium-99m was synthesized as described (12). Radiochemical purity was always greater than 95%. Equilibrium equations were used to evaluate total moles of both [^{99m}Tc]MIBI and [^{99}Tc]MIBI (13) and molarity was expressed in terms of total technetium-MIBI (Tc-MIBI). Specific activity varied from 1.4 to 4.6 TBq/μmol.

Tritiated tetraphenylphosphonium ([^{3}H]TPP) was obtained as the bromide salt with a specific activity of 1.3 TBq/mmol, 37.0 MBq/ml (Du Pont, Billerica, MA).

The ionophores nigericin and valinomycin were purchased from Sigma (St. Louis, MO).

To minimize nonspecific binding of [^{99m}Tc]MIBI to plastic tubes, we presaturated them with a freshly prepared solution of 1% bovine serum albumin in 0.1 M phosphate buffered saline (Sigma), pH 7.4, for 1 h followed by three washes in PBS. Control experiments showed that Tc-MIBI binding to plastic tubes was always less than 2% of total radioactivity for incubation periods up to 3 h.

Cells. The human carcinoma cell lines used in this study are listed in Table 1. Cell lines were grown to confluence in 75-cm^2 tissue culture flasks (Costar, Cambridge, MA) in RPMI 1640 medium (Gibco, Grand Island, NY), supplemented with 10% heat-inactivated FCS, 50 units/ml penicillin, 50 μg streptomycin, 4 mM L-glutamine, and 1 mM sodium pyruvate, at 37°C in 5% CO_2/95% air and 100% relative humidity. Since substantial numbers of cells were necessary to standardize and reproduce the experiments, cells were subcultured and cryopreserved in RPMI 1640 medium supplemented with 20% FCS and 10% dimethyl sulfoxide (Sigma) in multiple identical aliquots. Prior to each experiment, cells were thawed, washed twice in ice-cold RPMI 1640 medium containing 2.5% FCS, and incubated for 24 to 48 h in culture medium at 37°C in 5% CO_2/95% air and 100% relative humidity.

The normal cell types used as controls were V79 Chinese hamster lung fibroblasts and human PBMC. The V79 cells were grown in culture as described above. PBMC were isolated from the blood of normal human volunteers by Ficoll-Hypaque density gradient centrifugation and represent more than 80% of resting T-lymphocytes.

Cell viability was examined before and during all the experiments. The cells excluded trypan blue dye for at least 3 h under all experimental conditions, and viability was always greater than 85%.

For some experiments, dead cells were produced by anoxic incubation of highly concentrated cell suspensions (5×10^7 cells/ml) for 24 h in RPMI 1640 medium at room temperature in sealed vials. Viability was assessed by trypan blue dye exclusion and was always 0% following this procedure.

Cell Volume Determination. Cell volume was measured on a Coulter Counter Model Z_F connected to a Coulter Channelyzer 256 (Coulter Electronics, Inc., Hialeah, FL). Values were determined on 2 to 16 × 10^3 cells, depending on the cell line. Mean cell volume was expressed in pl.

Uptake of [^{99m}Tc]MIBI. Adherent cells were harvested with 0.05% trypsin/0.53 mM EDTA (Gibco) and washed twice with ice-cold RPMI 1640 medium. The cell suspension was then incubated at 37°C in 5% CO_2/95% air in culture medium for 2 h prior to use. After this recovery period, cells were washed once in RPMI 1640 medium, counted on a hemocytometer, and suspended in the same serum-free medium at a concentration of 1×10^7 cells/ml. For some experiments, the cells were washed in low K^+ buffer (135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 5.5 mM dextrose, pH 7.4) and suspended either in low or high K^+ buffer (135 mM KCl, 5 mM NaCl, other components identical to the low K^+ buffer). Both media were 285 mosm/l in salt. [^{99m}Tc]MIBI solutions were prepared in either RPMI 1640 medium or high K^+ or low K^+ buffer as indicated. Both cell suspensions and [^{99m}Tc]MIBI solutions were equilibrated for 10 min at 37°C before use.

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[3] The abbreviations used are: MIBI, hexakis(2-methoxyisobutylisonitrile); PBMC, human peripheral blood mononuclear cells; PBS, phosphate-buffered saline (pH 7.4); FCS, fetal calf serum; TPP, tetraphenylphosphonium.

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[99mTc]MIBI uptake was initiated by adding to the cell suspension an equal volume of [99mTc]MIBI solution (final Tc-MIBI concentration 0.3 to 3.3 nM, ethanol concentration always less than 1%). At various time intervals 200-μl aliquots were transferred to 400-μl microfuge tubes (Fisher Scientific, Pittsburgh, PA) containing 150 μl of FCS and centrifuged for 1 min in an Eppendorf microfuge (approximately 15,000 × g). After freezing the tubes in dry ice, their tips were cut off and the radioactivity of the pellets was determined in a gamma counter (Packard Auto-Gamma Counter 500). All activities were corrected for decay. At each determination, an identical volume of cell suspension was also directly assayed for radioactivity (total radioactivity). Radioactivity retained by the serum interface was determined in each experiment and was always less than 3% of the total activity placed in the tube. Assuming that about 10 μl of this serum are associated with the cell pellet, corrected cell activity was obtained by subtracting serum contamination from the cell activity. [99mTc]MIBI uptake was expressed (*a*) as an accumulation ratio calculated by dividing the total cell uptake in cpm by the cpm in a volume of external medium equal to the volume of the cells, or (*b*) as fmol/10^6 cells calculated as:

$$\frac{\text{Corrected cell activity (cpm)}}{\text{Total radioactivity (cpm)}} \times \frac{\text{Molarity of Tc-MIBI}}{\text{Cell concentration}}$$

Uptake of [3H]TPP. Measurement of [3H]TPP uptake was performed as described for [99mTc]MIBI. [3H]TPP was used at a final concentration of 0.29 μM, 370 kBq/ml, in either low or high K^+ buffer. Radioactivity was determined with a Beckman LS 800 liquid scintillation counter.

Autoradiography. Cells grown on 75- x 25-mm glass microscope slides (Fisher Scientific) were rinsed once with RPMI 1640 medium and labeled for 1 h at 37°C in 5% CO_2/95% air with [99mTc]MIBI solution (2.4 nM, 3.8 TBq/μmol) in RPMI 1640 serum-free medium. The slides were rinsed in three changes of ice-cold PBS and fixed in 10% buffered formaldehyde solution for 10 min, rinsed in PBS, and dried. They were then dipped in NTB-2 emulsion (Eastman Kodak, Rochester, NY). A few slides were prepared with unlabeled cells as background controls. The slides were exposed for 8 days at 4°C in complete darkness, developed for 3 min in Kodak developer D-19, and fixed for 5 min in Kodak fixer. The slides were then stained in Harris hematoxylin for 5 min, followed by 0.5% alcoholic eosin for 1 to 3 min.

RESULTS

Uptake of [99mTc]MIBI. The concentration of [99mTc]MIBI in several human tumor cells lines (Table 1) after a 1-h incubation with the compound varies from 5 to 28% of the activity in the external medium (Fig. 1*A*). In contrast, normal V79 cells (Chinese hamster lung fibroblasts) and human PBMC exhibit a minimal uptake of less than 2% of the activity in the medium.

Kinetic studies (Fig. 1*B*) show that [99mTc]MIBI uptake into intact SW-13 cells approaches a plateau by 30 min with a half-time of 10 min. A maximum level of uptake is reached in about 1 h at 37°C and is maintained for at least 3 h thereafter if the compound is not removed. Similar results were obtained with the A-549 and HT-1376 cell lines (data not shown). As opposed to intact cells, uptake in dead SW-13 cells is minimal (15-fold less than that of intact cells) and constant over time.

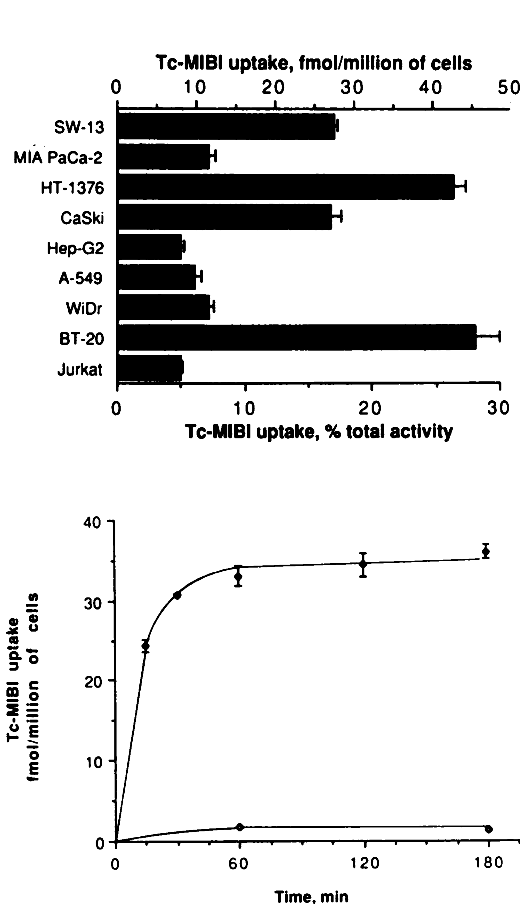

Fig. 1. Characterization of [99mTc]MIBI uptake by human tumor cells. (*A*) Uptake of [99mTc]MIBI (0.8 nM) by panel of human carcinoma cell lines (1-h incubation). ■, mean of six determinations; *bars*, SD. Uptake by V79 (Chinese hamster lung fibroblast) and human PBMC is less than 2% of total activity. (*B*) [99mTc]MIBI uptake kinetics. Living (◆) and dead (◇) SW-13 cells were incubated with 1.2 nM [99mTc]MIBI for various periods of time. *Points*, mean of three determinations; *bars*, SD.

Table 1 *Human carcinoma cell lines used for the evaluation of [99mTc]MIBI uptake in vitro*

Histology cell lines	Name	Mean cell volume (pl)[a]
Small cell adenocarcinoma of the adrenal cortex	SW-13	2.47
Undifferentiated pancreatic carcinoma	MIA PaCa-2	1.22
Poorly differentiated bladder carcinoma	HT-1376	2.26
Epidermoid cervical carcinoma	CaSki	1.16
Differentiated hepatocellular carcinoma	Hep G2	0.69
Non-small cell lung adenocarcinoma	A-549	1.02
Colon adenocarcinoma	WiDr	1.16
Breast adenocarcinoma	BT-20	1.05
T-cell leukemia	Jurkat	0.19

[a] Mean cell volume was determined with a Coulter Counter Model Z_F connected with a Coulter Channelyzer 256 (Coulter Electronics, Inc., Hialeah, FL). Number of cells used, 2,000 to 16,000.

[99mTc]MIBI accumulation by SW-13 cells after 1 h is linearly related to cell number from 1 to 8 × 10^6 cells/ml and is proportional to the extracellular concentration of Tc-MIBI over the entire range of 4 to 2800 pM. Compared to the uptake at 37°C, that at 4°C is inhibited by 97% (data not shown).

Given that some lipophilic cations, such as rhodamine 123 and TPP, were shown previously to accumulate passively within cells driven in part by the negative plasma membrane potential, we further analyzed the uptake of [99mTc]MIBI under conditions that change the plasma membrane potential. SW-13 cells suspended in low K^+ medium (*i.e.*, 5 mM K^+/135 mM Na^+) take up [99mTc]MIBI rapidly (Fig. 2*A*) for about 30 min and achieve a steady state level of accumulation after 1 h. When cells are suspended in high K^+ medium (135 mM K^+/5 mM Na^+) to depolarize the plasma membrane, the uptake of [99mTc]MIBI is reduced by 60%. Uptake experiments with [3H]TPP performed in parallel under identical conditions show a 66% decrease in high K^+ buffer after a 1-h incubation (Fig. 2*B*, middle). The same experiments were carried out with two other cell lines, BT-20 and A-549, which have higher and lower [99mTc]MIBI uptake, respectively, than SW-13 (Fig. 1*A*). Depolarizing the plasma membrane potential in high K^+ buffer results in all cases in lowering the uptake of [99mTc]MIBI in a manner similar to that of [3H]TPP (Fig. 2*B*).

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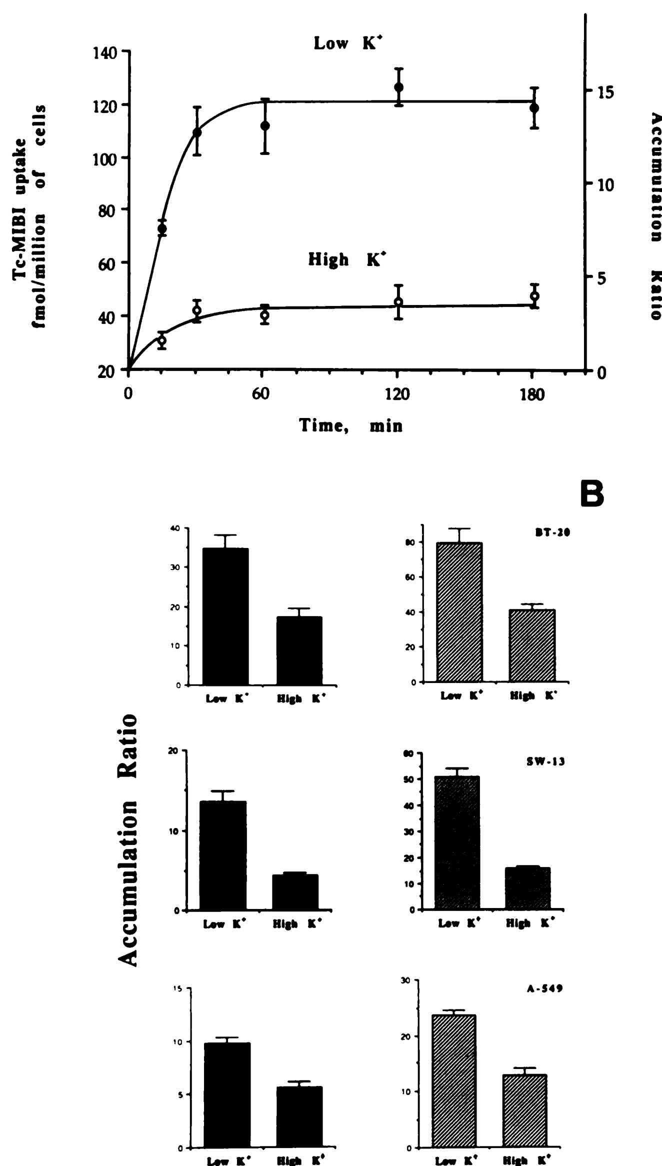

Fig. 2. Effect of depolarization of plasma membrane potential on accumulation of [^{99m}Tc]MIBI by several tumor cell lines. (*A*) Uptake kinetics in SW-13 cells of [^{99m}Tc]MIBI (3.3 nM) in low or high K^+ medium. *Points,* mean of three determinations; *bars,* SD. (*B*) Comparison of [^{99m}Tc]MIBI (3.3 nM) (■) and [^{3}H]-TPP (0.29 µM) (▨) accumulation by three carcinoma cell lines in low or high K^+ medium following 1-h incubation. *Columns,* mean of three determinations; *bars,* SD.

In order to evaluate the contribution of the mitochondrial membrane potential, we examined the effect of two ionophores, valinomycin and nigericin, which have been shown to disrupt the metabolism of mitochondria. As shown in Fig. 3, valinomycin, which dissipates the mitochondrial membrane potential, eliminates 80 to 85% of Tc-MIBI uptake by A-589 and BT-20 cells, respectively, in high K^+ medium (depolarization of the plasma membrane potential). By contrast, treatment with nigericin, known to increase the mitochondrial membrane potential, induces (Fig. 3) a dramatic increase in [^{99m}Tc]MIBI uptake, when compared to low K^+ medium control values.

Cell Volume Determination. In order to determine whether uptake in the panel of human tumor cells reflects variation in cell size, the mean volume of each type of human tumor cell was measured (Table 1). While cells with larger volumes generally accumulate more [^{99m}Tc]MIBI, some human tumor cells (*e.g.,* MIA PaCa-2, WiDr, CaSki, and BT-20) have major differences in [^{99m}Tc]MIBI uptake despite similar volumes. This suggests the absence of a strict correlation between incorporation and the size of a cell.

Autoradiography. To further characterize the interaction of [^{99m}Tc]MIBI with human tumor cells, microautoradiographs were analyzed. The microautoradiographs obtained with SW-13 cells after a 1-h incubation with [^{99m}Tc]MIBI (Fig. 4) indicate that almost all the radioactivity is located inside the cell, clustered in the cytoplasm around the nucleus, while the nucleus

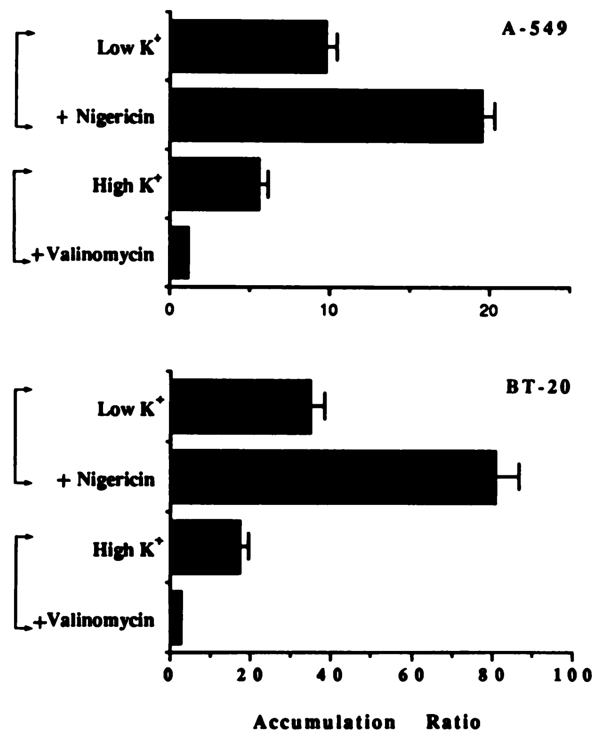

Fig. 3. Effect of mitochondrial membrane potential alterations on uptake of [^{99m}Tc]MIBI by A-549 and BT-20 cells. Accumulation of [^{99m}Tc]MIBI (0.7 nM) in low K^+ medium in the absence or presence of nigericin (5 µg/ml) and in high K^+ medium in the absence or presence of valinomycin (1 µg/ml). One-h incubation. ■, mean of three determinations; *bars,* SD.

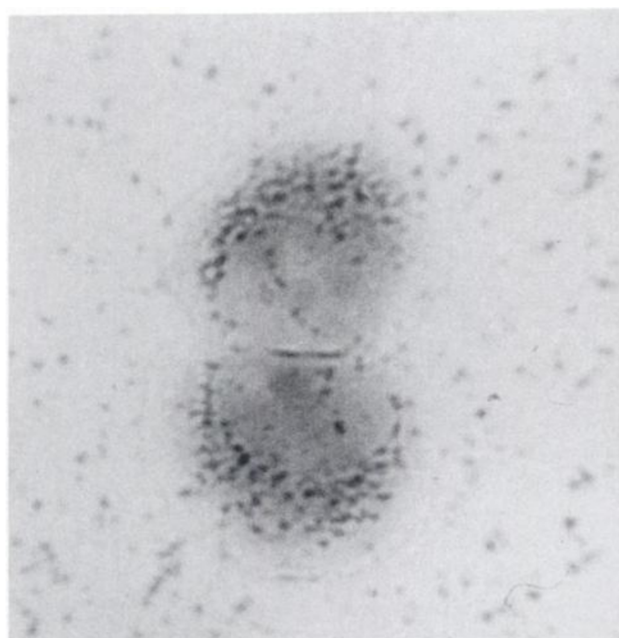

Fig. 4. Microautoradiography of SW-13 human tumor cell line, × 950.

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remains free of radioactivity. Furthermore, no radioactivity is detectable on the plasma membrane and the nuclear envelope.

DISCUSSION

In order to examine [^{99m}Tc]MIBI uptake in human carcinoma cells, we selected a panel of tumor cell lines including eight solid tumor types of epithelial origin and one leukemia cell line (Table 1). A wide diversity in the 1-h uptake of [^{99m}Tc]MIBI by these cell lines was seen (Fig. 1*A*). In contrast to these tumor cells, we observed a low accumulation of the compound in the nontransformed V79 cells and PBMC. Furthermore, preliminary reports of *in vivo* human tumor imaging with [^{99m}Tc]MIBI have demonstrated a significant difference between uptake in tumor *versus* normal tissues (more than 50% higher in tumors) (6-8). No MIBI uptake was observed in patients with benign lesions, and a significant decrease in uptake was seen in tumor tissues following radiotherapy (8). Analysis of the *in vitro* uptake reveals that [^{99m}Tc]MIBI is accumulated in living cells, by a temperature dependent, nonsaturable (over the range of [^{99m}Tc]MIBI concentrations tested) process that is very likely diffusion. In fact, we observed less than 20% inhibition of tracer [^{99m}Tc]MIBI uptake by carrier [^{99}Tc]MIBI, even using a 10^5-fold excess of carrier (data not shown).

One of the potential explanations for the mechanism by which [^{99m}Tc]MIBI accumulates in cells is that the compound binds to cell components (*e.g.*, lipid components of membrane) relatively nondifferentially. Recent studies have explored this possibility for cellular accumulation of hexakis isonitrile technetium complexes (12, 14). This explanation is invalidated by two observations: the poor correlation of cell size with [^{99m}Tc]-MIBI uptake and the decreased accumulation of the compound in dead cells. Furthermore, microautoradiographs of SW-13 cells (Fig. 4) show that the compound is localized mainly in the cytoplasm and that no labeling of the nuclear envelope or of the plasma membrane is detectable, suggesting more strongly that [^{99m}Tc]MIBI does not react nonspecifically with biological membranes.

TPP and rhodamine 123 are other lipophilic cationic compounds whose uptake mechanism in various cell types, including neonatal rat cardiac muscle and human carcinoma cells, is known to be dependent on the plasma and mitochondrial membrane potentials, with resultant accumulation within the mitochondria of living cells (9, 15-17). We tested whether such a mechanism could also be implicated in the uptake and accumulation of the cation [^{99m}Tc]MIBI. Using [^{3}H]TPP, which has been widely used for monitoring changes in cell membrane potential (15, 18-20), we confirmed that the plasma membrane potential of SW-13 cells is primarily a potassium diffusion potential, as expected from data obtained with other epithelial cells (21), and that incubating cells in a high K^+ medium induces plasma membrane depolarization (Fig. 2*B*, *middle*). By comparing [^{99m}Tc]MIBI uptake in high K^+ and low K^+ media in various tumor cells, we showed that [^{99m}Tc]MIBI uptake was significantly decreased in the absence of the plasma membrane potential. This diminution was consistent with that obtained with [^{3}H]TPP for each cell line tested (Fig. 2*B*). Given that temperature modification also affects the membrane potential of cells (15), the decreased uptake of [^{99m}Tc]MIBI by SW-13 cells observed at 4°C could, therefore, also be the result of a decreased plasma membrane potential. While the exact cytoplasmic structure labeled with [^{99m}Tc]MIBI remains to be determined, the microautoradiographs obtained with SW-13 cells exhibit a pattern of [^{99m}Tc]MIBI distribution with clusters around the nucleus, resembling the distribution of mitochondria in human adrenal cortex carcinoma (17). Furthermore, alterations of the mitochondrial membrane potential induce significant variations of [^{99m}Tc]MIBI uptake, suggesting that mitochondrial membrane potential could also be a contributing factor.

[^{99m}Tc]MIBI is a member of the class of coordination compounds which have a key atom of radioactive technetium in their structures. Given its physical properties (*e.g.*, energy, half-life), the metastable isotope represents the radionuclide of choice for *in vivo* diagnosis in nuclear medicine. While we do not exclude the contribution of other factors in explaining the variability in the accumulation of this compound, such as the existence of multidrug resistance involving ATP-binding proteins that pump drugs out of cells (22-24), the data are consistent with the hypothesis that the plasma and mitochondrial membrane potentials are involved in [^{99m}Tc]MIBI uptake by living tumor cells. Given that abnormalities of membrane potential have been reported for many tumor cell types (17, 20, 25, 26), [^{99m}Tc]MIBI could be of interest for scintigraphic *in vivo* tumor diagnosis.

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