\( ^{99m}\text{Tc}(\text{N})\text{-DBODC(5)}, \) a potential radiolabeled probe for SPECT of multidrug resistance: in vitro study

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Abstract \(^{99m}\text{Tc}(\text{N})(\text{DBODC})(\text{PNP5})\)\(^{+}\) [DBODC is bis(N-ethoxyethyl)dithiocarbamate; PNP5 is bis(dimethoxypropylphosphinoethyl)ethoxyethylamine], abbreviated as \(^{99m}\text{Tc}(\text{N})\text{-DBODC(5)}, \) is a lipophilic cationic mixed compound investigated as a myocardial imaging agent. The findings that this tracer accumulates in mitochondrial structures through a mechanism mediated by the negative mitochondrial membrane potential and that the rapid efflux of \(^{99m}\text{Tc}(\text{N})\text{-DBODC(5)}\) from nontarget tissues seems to be associated with the multidrug resistance (MDR) P-glycoprotein (P-gp) transport function open up the possibility to extend its clinical applications to tumor imaging and noninvasive MDR studies. The rate of uptake at 4 and 37 °C of \(^{99m}\text{Tc}(\text{N})\text{-DBODC(5)}\) was evaluated in vitro in selected human cancer cell lines and in the corresponding sublines before and after P-gp and/or MDR-associated protein (MRP) modulator/inhibitor treatment using \(^{99m}\text{Tc}\text{-sestamibi} as a reference. The results indicated that (1) the uptake of both \(^{99m}\text{Tc}(\text{N})\text{-DBODC(5)}\) and \(^{99m}\text{Tc}\text{-sestamibi} is correlated to metabolic activity of the cells and (2) the cellular accumulation is connected to the level of P-gp/MDR expression; in fact, an enhancement of uptake in resistant cells was observed after treatment with opportune MDR inhibitor/modulator, indicating that the selective blockade of P-gp/MDR prevented efflux of the tracers. This study provides a preliminary indication of the applicability of \(^{99m}\text{Tc}(\text{N})\text{-DBODC(5)}\) in tumor imaging and in detecting P-gp/MDR-mediated drug resistance in human cancer. In addition, the possibility to control the hydrophobicity and pharmacological activity of this heterocomplex through the variation of the substituents on the ligands backbone without affecting the \(\text{P}_2\text{S}_2\) coordinating sphere makes \(^{99m}\text{Tc}(\text{N})\text{-DBODC(5)}\) a suitable scaffold for the preparation of a molecular probe for single photon emission computed tomography of MDR.

Keywords Technetium · P-glycoprotein · Multidrug resistance · MK571 · Myocardial

Introduction

The in vivo evaluation of complex cellular processes such as proliferation, apoptosis, receptor–ligand interactions, transport of substrates, and metabolism of nutrients in human cancers is a wide and continuing evolving area of investigation in nuclear medicine. A major goal in this area is the noninvasive detection of well-known biochemical, molecular, and histological markers of tumor aggressiveness, invasiveness, and resistance to therapy, which may provide rational criteria for fine-tuning of therapeutic strategies in individual patients [1].

In recent years, increasing knowledge of the cause and physiological basis of cancer has permitted the identification of biomarkers potentially useful for diagnosis and/or
therapy [2, 3]. In this context, nuclear medicine and radiopharmaceutical science have explored some of these substances as potential targets for in vivo molecular imaging and/or targeted radionuclide therapy for cancer, but despite the efforts, the development of specific tools and the successful application of new targeted strategies are still limited [4].

Consequently, chemotherapy remains one of the major therapeutic options for treatment of many malignant tumors and multidrug resistance (MDR) is one of the primary causes of failure of this treatment [5]. This phenomenon is mediated by plasma membrane ATP-binding cassette (ABC) transporters such as P-glycoprotein (P-gp) and MDR-associated protein (MRP) 1 (MRP1) and MRP2 [5–7]. P-gp function is responsible for diminished drug cellular accumulation. This effect has been, in part, explained through a “flipase model” [8], in which P-gp acts as a transporter of drugs from the inner leaflet of the lipid bilayer to the outer leaflet or the external medium, allowing the agents to diffuse away, or acts by altering the membrane permeability, thus reducing the intracellular concentration of drugs. Besides this, in cells overexpressing P-gp, effects associated with variation of the intracellular pH and the membrane potential may alter indirectly the intracellular distribution of substrates [7]. Like P-gp, MRP1 and MRP2 are involved in MDR, but their specific role needs to be clarified.

P-gp proteins, in addition to their overexpression in tumors, are normally expressed in normal tissues involved in excretory function and in barriers such as the blood–brain barrier, blood cerebrospinal fluid barrier, and blood–testis barrier. These strategic locations give P-gp a crucial role exerting control functions in absorption and excretion of xenobiotics across these barriers. Recent studies reported a potential correlation between P-gp activity/expression in the central nervous system and the onset of neurodegenerative disorders such as Alzheimer’s disease, Parkinson’s disease, and epilepsy [7–10]. Hence, measurement of P-gp activity and expression is one of the important goals in planning systemic therapy not only for cancer but also for neurodegenerative diseases [11, 12].

Expression of MDR P-gp, as detected at the messenger RNA or protein level, does not always correlate with the functional assessment of P-gp-mediated transport activity. This is because P-gp transport activity is affected by specific mutations as well as the phosphorylation state of the protein. Altered or less-active forms of P-gp may be detected by polymerase chain reactions or immunohistochemistry, which do not accurately reflect the status of tumor cell resistance. Thus, noninvasive methods to functionally interrogate P-gp transport activity in vivo are required [11–14]. Imaging with radiolabeled transport substrates, may help to noninvasively identify those tumors and tissues in which ABC transporters are not only expressed but also functional.

Over the past years, different radionuclides suitable for noninvasive single photon emission computed tomography (SPECT) and positron emission tomography (PET) techniques have been used in the design of molecular probes for functional imaging of MDR.

The positron emitters $^{11}$C and $^{18}$F have been used to label small molecules such as MDR cytotoxic drugs or classic MDR modulators/inhibitors [15–17]. In spite of the encouraging preliminary in vitro and in vivo results, they present some limitations such as modest radiochemical yields (RCYs) and fast in vivo metabolism. In addition, owing to the short half-life of $^{11}$C ($t_{1/2} = 20$ min) and $^{18}$F ($t_{1/2} = 110$ min), the preparation of such molecules needs a cyclotron nearby, limiting their access and widespread distribution [18, 19].

Conversely radiometals such as $^{99m}$Tc, $^{67/68}$Ga, and $^{64}$Cu, owing to their half-life, chemistry, cost, availability, and in vivo stability of the corresponding compounds, present advantages over $^{11}$C and $^{18}$F and have been investigated for the in vivo evaluation of MDR [4–14].

Nowadays, $^{99m}$Tc is, because of its optimal nuclear properties ($t_{1/2} = 6.02$ h; $E_c = 141$ keV), low cost, and easy availability, the radioisotope of election in nuclear medicine, being used in different chemical forms in more than 80% of clinical SPECT practices. $^{99m}$Tc is indispensable for the estimated 70,000 medical imaging procedures that take place daily around the world, primarily in myocardial imaging. The current global interruption of $^{99}$Mo supply has made the situation particularly problematic from a medical standpoint, underlining the importance of $^{99m}$Tc in nuclear medicine practices [18, 19], and the need for a reliable supply network comprising alternative production options [20]. Nevertheless, the $^{99m}$Mo shortage should not be used as an argument to switch large research programs to other radionuclides [19]. Although the development of PET and associated fluorinated agents has led to an almost complete interruption of SPECT research programs and the development of new Tc-labeled molecules is quite rare, this can refocus attention on technetium, also in view of the arrival on the market of new high-quality SPECT cameras that can compete with PET technology [19].

In the last decade, different $^{99m}$Tc-based agents have been characterized as transport substrates for MDR P-gp. Among these, lipophilic cations such as $^{99m}$Tc-sestamibi followed by $^{99m}$Tc-tetrofosmin, originally developed as myocardial perfusion agents and subsequently used as tumor-seeking agents in a variety of human neoplasms [21], emerged as suitable tools to explore specific cellular processes and functions in malignant tumors [1, 21–24].

From the clinical point of view, the in vivo examination of the rate of uptake of these $^{99m}$Tc-based compounds in
tumors is relevant for tumor response to treatment. The evaluation of the mitochondrial status in untreated tumors as well as the evaluation of the MDR-P-gp expression and function are hot topics in clinical oncology. Indeed, these assessments allow one to select patients likely to benefit from therapeutic treatments or to distinguish if the onset of the resistance is P-gp/MPR-mediated or not; hence, to select patients likely to benefit from treatment with P-gp/MPR inhibitors [1, 10]. Moreover, functional measures revealing the MDR P-gp expression, obtained by noninvasive imaging techniques such as SPECT/PET, have the advantage that they do not require any surgical biopsy in contrast to the convectional in vitro evaluation techniques.

Although $^{99m}$Tc-sestamibi was used as an in vivo marker of P-gp function, it is not the ideal radiolabeled substrate [10], and its diagnostic and prognostic value is often limited, owing to insufficient tumor localization and high liver and muscle uptake followed by slow washout. Thus, compared with the magnitude of the signal produced by other radiolabeled substrates, that produced by $^{99m}$Tc-sestamibi is smaller. In addition, its substrate activity for both P-gp and MRP1 decreases its utility to image the function of only P-gp [7, 10]; nevertheless, this feature allows $^{99m}$Tc-sestamibi to be a more general probe of MDR transporters in some cancers.

Thus, there is an unmet medical need for radiotracers able to monitor noninvasively the MDR transport function in tumors [4, 10, 14]. Such need has promoted research on chemistry and biology, with the ultimate goal of identifying a good-performing radiopharmaceutical for MDR functional assessment.

$$[^{99m}Tc(N)(DBODC)(PNP5)]^+$$  
[DBODC is bis(N-ethoxyethyl)dithiocarbamato; PNP5 is bis(dimethoxypropylphosphinoethyl)ethoxyethylamine], abbreviated as $^{99m}$Tc-DBODC(5), is a lipophilic cationic mixed compound [25] (Fig. 1) currently under investigation as a potential myocardial imaging agent [26] owing to its favorable biodistribution profile characterized by rapid blood clearance, high and persistent myocardial accumulation, and low lung and liver uptakes followed by a rapid and quantitative washout [27–29]. The findings that this complex, analogously to $^{99m}$Tc-sestamibi and $^{99m}$Tc-tetrofosmin, accumulates in mitochondrial structures through a mechanism mediated by the negative mitochondrial membrane potential and that the remarkable rapid efflux of $^{99m}$Tc-NDBODC(5) from nontarget tissues seems strongly correlated to the P-gp/MPR transport functions open up the possibility to extend the applications of this agent and its derivatives to tumor imaging and noninvasive MDR studies [30].

With this background, this work aimed to validate through in vitro studies $^{99m}$Tc(N)-DBODC(5) as a substrate of the P-gps/MPR proteins and to identify in this complex a possible “scaffold” suitable for the design of $^{99m}$Tc-based molecular probes capable of imaging noninvasively P-gp and closely related transporter activities in tissues as well as tumors.

Basic parameters such as the rate of uptake (time course 5–120 min), at 4 and 37 °C, of $^{99m}$Tc(N)-DBODC(5) were evaluated in vitro in selected cell lines by using $^{99m}$Tc-sestamibi as a reference. The rate of uptake of the radiocomplex was assessed in the corresponding resistant cell lines before and after treatment with various P-gp/and or MRP modulators/inhibitors, e.g., verapamil (Vrp), a calcium channel blocker; cyclosporin A (CsA), an immunosuppressive agent with broad-spectrum MDR modulator activity; butionine sulfoximine (BSO), a glutathione (GSH) synthesis inhibitor, which especially inhibits the MRP activity indirectly through the depletion of GSH storage; 5-(3-(2-(7-chloroquinolin-2-yl)ethenyl)phenyl)-8-dimethylcarbamoyl-4,6-dithiaoctanoic acid sodium salt hydrate (MK571), a selective MRP family inhibitor; and 6,7-dimethoxy-2-[3-[4-methoxy-3,4-dihydro-2H-naphthalen-1(1E)-ylidene]propyl]-1,2,3,4-tetrahydroisoquinoline (MC18), a potent in vitro and in vivo P-gp inhibitor [31–33].

To determine the contribution of the mitochondrial membrane potential, the effect of carbonyl cyanide m-chlorophenyl hydrazone (CCCP), affecting the mitochondrial membrane potential, on the cellular uptake of the tracers was evaluated.

The cell lines, ABC transporters overexpressed in the different cell sublines, and the corresponding inhibitors used in our study are reported in Table 1.

To assess the suitability of $^{99m}$Tc(N)-DBODC5 in detecting MRP-mediated drug resistance, the rate of uptake of the complex was studied in the C13* ovarian adenocarcinoma cell line expressing MRP1 and MRP2 transporters, in the A2780/ADR ovarian adenocarcinoma cell line expressing MR2 and MRP1 transporters, and in Madin–Darby canine kidney (MDCK) cells transfected with human MRP1 (MDCK/MPR1) in the presence or absence of MK571.

**Materials and methods**

**Materials**

All chemicals were purchased from Sigma-Aldrich (Milan, Italy). Solvents were reagent grade and were used without further purification. PNP5 was prepared according to published procedures [41]. The DBODC ligand was purchased from Alchemy (Altedo, Italy). 2-Methoxyisobutylisonitrile (MIBI) as the copper salt was a gift from R. Pasqualini (IBA Cis Bio) Vrp, CsA, BSO, MK571 and CCCP were purchased from Sigma-Aldrich (Milan, Italy). Technetium-
Fig. 1 Chemical structure of the $^{99m}$Tc compounds with their corresponding log $p$ values and molecular volume. Raw estimation of the shape of the $^{99m}$Tc complexes indicated that the molecular volume of the molecules is comparable. Planar imaging of the complexes collected 60 min after injection with a PRISM 3000 XP gamma camera is also reported.

Table 1 Cell lines, ATP-binding cassette (ABC) transporters overexpressed in the different cell sublines, and the corresponding inhibitors used in our experiments

<table>
<thead>
<tr>
<th>Cell wild type</th>
<th>Cell subline</th>
<th>ABC transporters</th>
<th>Inhibitors</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>MCF7 (breast cancer)</td>
<td>MCF7/ADR (breast cancer; doxorubicin-resistant)</td>
<td>P-gp, MRP1</td>
<td>Verapamil, cyclosporin A, MC18</td>
<td>[34–36]</td>
</tr>
<tr>
<td>A2780 (ovarian cancer)</td>
<td>A2780/ADR (ovarian cancer; doxorubicin-resistant)</td>
<td>MRP1, MRP2</td>
<td>Cyclosporin A, MK571</td>
<td>[37]</td>
</tr>
<tr>
<td>2008 (ovarian cancer)</td>
<td>2008 (ovarian cancer)</td>
<td>P-gp, MRP1</td>
<td>Verapamil, cyclosporin A, MC18</td>
<td>[37]</td>
</tr>
<tr>
<td>MDCK</td>
<td>MDCK/MRP1 (MRP1-transfected)</td>
<td>MRP1, MRP2</td>
<td>Cyclosporin A, MK571</td>
<td>[38–40]</td>
</tr>
</tbody>
</table>

$^{99m}$Tc(II)-DBODC(5)  
$log P = 1.61; 830.1 \text{ Å}^3$

$^{99m}$Tc-Sesta-MIBI    
$log P = 0.87; 813.2 \text{ Å}^3$

99m as Na$^{99m}$TcO$_4$ was eluted from a Elumatic III $^{99m}$Mo/$^{99m}$Tc generator purchased from IBA Cis Bio. Sep-Pak RP-C18 and Sep-Pak CM cation-exchange cartridges were purchased from Waters (Milford, MA, USA).

A2780 and the corresponding doxorubicin-resistant A2780/ADR human ovarian cancer cells were kindly provided by A. Rosato (Istituto Oncologico Veneto, Padua, Italy). The 2008 and the corresponding cisplatin-resistant
C13* human ovarian cancer cells were kindly provided by G. Marverti (Department of Biochemical Science, University of Modena, Italy). MCF-7 human breast cancer cells were purchased from ATCC (Rockville, MD, USA). MCF-7/ADR, MDCK, and the corresponding transfected MDCK/MRP1 cells were provided by N.A. Colabufo. RPMI-1640 culture medium was purchased from EuroClone (Milan, Italy).

Analysis

Thin-layer chromatography (TLC) and/or high-performance liquid chromatography (HPLC) analyses were used to evaluate the radiochemical yield (RCY) and stability of the compounds as radiochemical purity (RCP) over time.

TLC analysis was performed on SiO$_2$ F$_{254}$ plates (Merck) using a mixture of 0.5 M EtOH/CHCl$_3$/toluene/ NH$_4$Ac (5:3:3:1). The activity on the plates was detected using a Cyclone phosphor imaging instrument (Packard Instruments, Meriden, CT, USA). Under these analysis conditions, the Rf values for $^{99m}$Tc(N)-DBODC(5) and $^{99m}$Tc-sestamibi were 0.78 and 0.85, respectively.

HPLC analysis was performed using a Beckman System Gold instrument equipped with a model 126 programmable solvent module, a model 210A sample injection valve, a model 166 scanning detector module, and a Bioscan B-FC-3200 radioisotope detector. HPLC analysis was done using a reversed-phase Vydac 218TP C$_{18}$ precolumn (4.6 mm × 45 mm, 5 µm) and a reversed-phase Vydac 218TP C$_{18}$ column (4.6 mm × 250 mm, 5 µm). Solvent A was water (0.1 % trifluoroacetic acid) and solvent B was acetonitrile (0.1 % trifluoroacetic acid). The gradient was as follows: 0–2 min, 20 % solvent B; 2–38 min, 80 % solvent B; 38–40 min, 20 % solvent B. The flow rate was 1 mL/min for 30 min. UV detection was at 215 nm. Under these analysis conditions the $R_t$ values for $^{99m}$Tc(N)-DBODC(5) and $^{99m}$Tc-sestamibi were 23.69 and 24.19, respectively.

Alternatively, a different HPLC analysis can be applied. This condition used a reversed-phase Beckman octadeclisilane precolumn (4.6 mm × 45 mm, 5 µm), and a reversed-phase Beckman octadeclisilane column (4.6 mm × 250 mm, 5 µm). For isocratic elution the solvents were as follows: 0.02 M phosphate buffer pH 7.4 (solvent A) and MeOH (solvent B) (20:80). The flow rate was 1 mL/min for 30 min. UV detection was at 215 nm. Under these analysis conditions the $R_t$ values for $^{99m}$Tc(N)-DBODC(5) and $^{99m}$Tc-sestamibi were 18.13 and 9.15, respectively.

Methods

Radiopharmaceutical preparation

$^{99m}$Tc(N)-DBODC(5) Na($^{99m}$TcO$_4$) (1 mL, 200 MBq) was added to a vial containing 5.0 mg succinic dihydrazide, 5.0 mg ethylenediaminetetraacetic acid, and 0.1 mg SnCl$_2$ suspended in 0.1 mL saline. The vial was kept at room temperature for 15 min, giving a mixture of $^{99m}$Tc-nitro precursors. Then, 1.0 mg PNP5 ligand (dissolved in 0.5 mL saline containing 2 mg/mL γ-cyclodextrin) and 2.0 mg DBODC (dissolved in 0.2 mL saline) were simultaneously added to the reaction vial. RCY determined by TLC and HPLC after 30 min at 80 °C was greater than 96 %.

$^{99m}$Tc-sestamibi $^{99m}$Tc-sestamibi was prepared following the manufacturer’s instructions. A stock solution of [Cu(MIBI)$_4$]BF$_4$ was prepared by adding 1 mL saline to a vial containing [Cu(MIBI)$_4$]BF$_4$ (1 mg), cysteine (1 mg), sodium citrate (2.6 mg), and mannitol (20 mg). Na($^{99m}$TcO$_4$) (1 mL, 200 MBq) was added to a vial containing 0.2 mL of stock solution and 0.1 mg SnCl$_2$ suspended in 0.1 mL saline. The vial was heated at 100 °C for 15 min. RCY determined by TLC and HPLC was greater than 97 %.

Dose preparation for in vitro studies Before the experiments the $^{99m}$Tc compounds were purified from the reagents using the following procedure.

A two-step procedure was employed for $^{99m}$Tc(N)-DBODC(5) purification. A Sep-Pak C$_{18}$ cartridge was activated with EtOH (5 mL) and equalized with deionized water (5 mL). Then, the reaction solution containing the $^{99m}$Tc(N)-DBODC(5) complex was diluted with deionized water (8.0 mL) and loaded on the cartridge. All the activity was retained. After the cartridge had been washed with water (20.0 mL) and 35 % EtOH (5.0 mL), the complex was eluted using a mixture of 90:10 EtOH–saline (1.0 mL). Ninety percent of the starting activity was collected. Subsequently a Sep-Pak CM cation-exchange cartridge was preconditioned with deionized water (10.0 mL). The $^{99m}$Tc(N)-DBODC(5) solution collected from the first purification step was diluted with deionized water (8 mL), withdrawn with a syringe (10.0 mL), and loaded on the cartridge. All the activity was retained. After the cartridge had been washed with water (10.0 mL) and 70 % EtOH (5.0 mL), the complex was eluted using a mixture of 90:10 EtOH–saline (0.250 mL × 1, 0.750 mL × 1). Approximately 90 % of the starting activity was collected in the second fraction.

$^{99m}$Tc-sestamibi was purified on a Sep-Pak CM cation-exchange cartridge following the procedure described for $^{99m}$Tc(N)-DBODC(5).

In both cases the elution solvents were completely evaporated under a dinitrogen stream; the complex was dissolved in dimethyl sulfoxide (DMSO), diluted with water to obtain an isotonic saline solution containing less than 0.5 % (v/v) DMSO, and used for in vitro studies. After dilution, the activity of the $^{99m}$Tc complex was 6.5 μCi/
5 μL. The RCP of the purified compounds determined by TLC and HPLC was 99 %.

Cell studies

Cellular uptake of 99mTc(N)-DBODC(5) was evaluated in suspension in suitable drug-sensitive cell lines and in the corresponding resistant sublines (Table 1) before and after MDR modulator/inhibitor treatment using 99mTc-sestamibi as a reference. Experiments were conducted according to the published procedures [42–45].

Stability in RPMI-1640 medium Before the uptake experiments, stability studies were conducted on the purified 99mTc complexes. To a glass test tube containing 450 μL of RPMI-1640 cell culture medium was added 50 μL of the purified 99mTc complexes. The mixture was vortexed and incubated at 37 °C for 2 h. At 30 min, 1 h, and 2 h, aliquots of the reaction mixture were withdrawn and analyzed by TLC and HPLC. The RCP of the complexes was greater than 95 %.

Uptake in baseline conditions Cell lines were maintained in the logarithmic phase at 37 °C in 5 % CO2 atmosphere, using RPMI-1640 medium supplemented with 10 % heat-inactivated fetal bovine serum (FBS; EuroClone), 25 mM N-(2-hydroxyethyl)piperazine-N’-ethanesulfonic acid buffer, 4 mM l-glutamine, 50 U/mL penicillin, and 50 mg/mL streptomycin. Cells adhering to the culture flask were harvested with 0.05 % trypsin–0.53 mM ethylenediaminetetraacetic acid (EuroClone), washed, and suspended in the appropriate medium at a concentration of 5 × 10⁶ cells per milliliter for the uptake studies.

Preliminary studies were performed in order to set the experimental conditions to avoid cell damage and/or death during uptake experiments. With use of these results, cell suspensions were preincubated in sterile glass tubes at 4 and 37 °C for 30 min. MCF7 and MCF7/ADR cells were preincubated in RPMI-1640 medium added to 10 % FBS; all other cell lines were serum-starved for 30 min and then resuspended in serum-free RPMI-1640 medium. Afterward, 350 μL of the cell suspension (5 × 10⁶ cells per milliliter) was incubated with intermittent agitation with 5 μL (6.5 μCi) 99mTc agents. At select time points (5, 15, 30, 60, 90, and 120 min), the tubes were vortexed and triplicate samples of the suspension (8 μL) were layered on 350 μL cold FBS in a 500-μL Eppendorf microcentrifuge tube. After centrifugation at 14,000 g for 2 min, the tubes were frozen in a dry ice–EtOH bath. The bottom tip containing the cell pellet was cut off and placed in a counting tube. The remaining portion of the tube with the supernatant was placed in a separate counting tube. The activity of both fractions was determined using a gamma counter (Cobra II, Packard Instruments). The amount of supernatant in the cell pellet was determined to be less than 1 % in separate experiments. Uptake of 99mTc complexes expressed as percentage cellular uptake of the total activity was calculated according to the literature [42, 43, 45–47] as follows:

\[
\text{Cell uptake} = \frac{\text{cpm (pellet)}}{\text{cpm (pellet)} + \text{cpm (supernatant)}} \times 100
\]

where cpm is counts per minute.

All assessments were conducted in duplicate for three experiments.

Uptake after exposure to MDR modulator/inhibitor To identify the maximal accumulation of 99mTc agents after modulator/inhibitor treatment, pilot experiments were done by preincubating selected cell lines (MCF7/ADR and 2008) with different concentrations (20, 50, 80 μM) of modulator/inhibitor in order to identify the best concentration. The concentration of Vrp and MK571 was fixed at 50 μM and the concentration of CsA and MC18 was set to 80 μM.

All MDR modulators/inhibitors were prepared in DMSO. The final concentration of DMSO in the experimental buffers was less than 0.5 %, which has been found to have no effect on the cell viability and on the net uptake of the 99mTc agents in cultured cells [48]. Before the addition of the 99mTc tracer, the cells were treated with 3.5 μL of the selected MDR modulator/inhibitor (50 mM/80 mM) and preincubated at 37 °C for 30 min.

The experiments were conducted as described in the previous section. The uptake of the 99mTc complexes was expressed as the percentage cellular uptake of the total activity. All assessments were conducted in duplicate for three experiments.

Determination of intracellular GSH levels Cells (1 × 10⁶) were washed with cold phosphate-buffered saline, treated with 6 % metaphosphoric acid, kept on ice for 15 min, and then centrifuged (2,000 g for 15 min). The pellet was dissolved in radioimmunoprecipitation assay buffer and used for protein determination according to the method of Lowry et al. [49]. Aliquots of supernatant were neutralized with Na2PO4 and assayed for total GSH following the procedure of Tietze [50]. To deplete intracellular GSH, cells were pretreated with BSO (25 μM) added to the culture medium for 24 h prior to analysis and uptake experiments.

Cell viability Cell viability was determined by trypan blue dye exclusion before and at the end of each experiment [51].

Evaluation of CCCP effects After 120 min of incubation with the radiotracers, in baseline conditions, the cells were treated with CCCP (10 μM final concentration) and maintained at 37 °C for an additional 5 min. Then,
triplicate samples of suspension (8 μL) were layered on 350 μL of cold FBS in a 500-μL Eppendorf microcentrifuge tube and treated as described earlier.

Data analysis

The results are presented as the mean ± the standard deviation. The calculation of the significance was performed by a Student t test; p < 0.05 was considered significant.

Western blots

Western blot and cytofluorimetric analyses for the detection of P-gp, MRP1, and MRP2 were performed as previously described [36–40].

For Western blot experiments, equal amounts of total proteins were resolved by gel electrophoresis, transferred to nitrocellulose membranes, and subjected to immunoblotting using the following primary antibodies: mouse anti-MDR, D-11 (Santa Cruz); rat anti-MRP1, MRPr1 (Abcam) [38–40]; mouse anti-MRP2, M2III-5 (Santa Cruz). Following incubation with the appropriate anti-mouse horseradish peroxidase linked secondary antibodies, chemiluminescence images were obtained using a Fujifilm LAS-3000 intelligent dark box and the LAS-3000 Lite Image Reader software program.

As examples, Western blot analyses of MCF7/ADR and transfected MDCK/MRP1 cell lines are reported in Fig. 2.

Results

Each experiment compares the uptake of 99mTc(N)-DBODC(5) with that of the commercially available complex 99mTc-sestamibi. The experiments were conducted at 37 °C to measure the net accumulation and at 4 °C to measure membrane-potential-independent (non-specific) uptake; at this temperature, the plasma membrane potential is depolarized and other energy-dependent processes, such as acidification of cell lysosomes, are abrogated [52]. Hence, the lysosomal trapping component of cellular accumulation was eliminated.

Figure 3 shows the time-dependent variation in cellular uptake, in baseline conditions at 4 and 37 °C of 99mTc(N)-DBODC(5) and 99mTc-sestamibi in human breast and human ovarian cancer drug-sensitive cell lines and in the corresponding drug-resistant sublines.

It is noteworthy to note that both 99mTc agents are retained by human breast and human ovarian cancer cells.

In general, in terms of cellular uptake, the different lipophilicity and solubility of the two compounds affects their permeability and relative affinity for the MDR transporters, resulting in the difference in their initial accumulations. Variations of percentage cellular uptake of the complexes into the different cell lines were strictly correlated to the intrinsic characteristics of the cells, in particular to the diverse mitochondrial density and over-expression of ABC transporters. In C13* and 2008 cell lines, the high level of accumulation of the tracers may be attributed to their greater intrinsic electron-gradient potential or may be linked to their high number of mitochondria. Thus, the tracer uptake could reflect the high level of metabolic activity of these cell lines [53–55].

The complexes display an almost similar accumulation profile. The uptake values of each tracer rapidly increased within the first 5 min and the plateau level reached was different for the cell lines and occurred at different times (30–60 min).

For the drug-sensitive cell lines, the percentage cellular accumulation of the 99mTc compounds was higher in human ovarian cancer cell lines than in human breast cell cancer lines and no variation in cellular efflux between 99mTc(N)-DBODC(5) and 99mTc-sestamibi was found. At the steady-state level, the percentage cellular uptake in MCF7 cells was 6.64 ± 0.83 and 8.64 ± 1.44 for 99mTc(N)-DBODC(5) and 99mTc-sestamibi, respectively (p < 0.001), and in A2780 cells, the percentage cellular accumulation was 17.67 ± 1.35 and 18.98 ± 0.99 (p ≤ 0.1). In the 2008 cell line (cisplatin-sensitive), the percentage cellular accumulation was 10.68 ± 1.50 and 10.53 ± 1.85 for 99mTc(N)-DBODC(5) and 99mTc-sestamibi, respectively (p ≥ 0.1).

At 4 °C a significant reduction of cellular accumulation was observed for both 99mTc agents. In particular, the nonspecific uptake was assessed as around 2% after 60 min (p < 0.001). A slight increase was detected for both tracers with time (3% at 120 min). A reduction of the net

![Fig. 2 Western blot analyses of MCF7, MCF7/ADR, Madin–Darby canine kidney (MDCK), and MDCK/multidrug-resistance-associated protein (MRP) 1 cell lines. MDCK and MDCK/MRP1 cell lines were obtained from Netherlands Cancer Institute-Antoni van Leeuwenhoek Hospital (NKI-AVL), with an Material Transfer Agreement between NKI-AVL and N.A. Colabufo. P-gp P-glycoprotein, WT wild type](image-url)
cellular uptake between drug-sensitive cells and drug-resistant tumor cells was observed \( p < 0.001 \) for
\[ \text{99mTc(N)-DBODC(5) and 99mTc-sestamibi in all cell lines} \]
(Fig. 3).

The ratio of the percentage cellular accumulation of the
\[ \text{99mTc complexes in drug-sensitive and drug-resistant cell lines} \]
was used as a measure of the P-gp, MRP1, and MRP2 activities. Data are reported in Tables S1–S3.

Under similar experimental conditions, the change in
\[ \text{99mTc(N)-DBODC(5) cellular uptake produced by MRP1} \]
pump activity was found to be higher than that produced by P-gp.

MDR inhibitor/modulator effects

To evaluate the effect of the different MDR reversal agents, drug-sensitive and drug-resistant cell lines were preincubated at 4 and 37 °C with various P-gp and/or MRP modulators/inhibitors before the addition of \[ \text{99mTc agents} \].
As documented by cytofluorimetric [36] and Western blot analyses, cells express good levels of P-gp, MRP1, and MRP2 transporters, confirming the literature data [34–40]. Thus, the mediated efflux is expected to result in decreased cellular accumulation with respect to the corresponding drug-sensitive cell lines.

Figures 4 and 5 display the percentage cellular uptake after 30 min at 37 °C of the complexes in drug-resistant cell lines.

Fig. 4 Percentage cellular uptake of 99mTc(N)-DBODC(5) (DBODC5) and 99mTc-sestamibi (MIBI) in human drug-resistant cell lines evaluated at 37 °C after 30 min of incubation in the presence or absence of the selected multidrug resistance (MDR) inhibitor/modulator. CSA cyclosporin A, MC18 6,7-dimethoxy-2-{3-[4-

metoxy-3,4-dihydro-2H-naphthalen-1(E)-ylidene|propyl]-1,2,3,4-
tetrahydroisoquinoline, MK571 5-(3-{2-(7-chloroquinolin-2-yl)eth-

enyl}phenyl)-8-dimethylcarbamyl-4,6-dithiaoctanoic acid sodium salt hydrate, VRP verapamil
used as an assessment of the modulator-induced effect; data are reported in Tables S1–S3.

Vrp is a calcium channel blocker capable of reversing P-gp function. At the cellular level the effects of Vrp are responsible for modification of the cell permeability, which consequently may induce alteration of the uptake ratio of the $^{99m}$Tc agents [42]. This could explain the small increase of the $^{99m}$Tc cell-associated activity found in the drug-sensitive cell lines (data not shown).

In general, the addition of Vrp (P-gp substrate) was responsible for an increase of the cellular uptake of both radiotracers in all cell sublines [$p < 0.001$ for $^{99m}$Tc(N)-DBODC(5) and $^{99m}$Tc-sestamibi]; however, for $^{99m}$Tc(N)-DBODC(5) its effect was much more evident in cells expressing a high level of P-gp compared with drug-resistant cell lines with no expression of P-gp such as A2780/ADR and C13* (Fig. 4). Indeed, the $^{99m}$Tc(N)-DBODC(5) uptake was increased by a factor of 5 in MCF7/ADR cells and by a factor of 3/3.5 in 2008 cells, which express high P-gp levels; conversely, its accumulation was increased twofold in A2780/ADR and C13* cell lines, which do not express detectable P-gp levels [35]. These differences were less marked for uptake of $^{99m}$Tc-sestamibi: its accumulation was increased by a factor of 15 in A2780/ADR and C13* cells; however, for $^{99m}$Tc-sestamibi uptake was increased by a factor of 3 in 2008 cells, by a factor of 5 in A2780/ADR cells, and by a factor of 2.5 in C13* cells.

The addition of CsA (a broad-spectrum MDR modulator) resulted in an increase of $^{99m}$Tc(N)-DBODC(5) and $^{99m}$Tc-sestamibi accumulation in MCF7/ADR cells [$p < 0.001$ for $^{99m}$Tc(N)-DBODC(5) and $^{99m}$Tc-sestamibi], which was quantified to be two times and six times the basal value, respectively.

A slight enhancement of the $^{99m}$Tc(N)-DBODC(5) uptake (1.5-fold) was observed in 2008 cells and A2780/ADR cells ($p < 0.001$), whereas no differences in cell-associated activity of $^{99m}$Tc-sestamibi were detected (0.05 ≤ $p < 0.1$). A reduction of the uptake levels of both $^{99m}$Tc agents was detected in the C13* cell line, which expresses MRP1 and MRP2 transporters. This may be attributable to the effects of CsA on the mitochondrial energetics [56].

Modulation by MC18, a P-gp inhibitor which has a pharmacological profile similar to that of CsA [31, 32], resulted in an increase of $^{99m}$Tc-sestamibi accumulation in cells expressing a high level of P-gp ($p < 0.001$), whereas no variation of the $^{99m}$Tc(N)-DBODC(5) uptake was observed in the same cell line ($p \geq 0.1$). Minimal or no effects on cellular accumulation of the radiotracers were found in drug-resistant cell lines with no expression of P-gp transporters such as A2780/ADR and C13* cells [$0.05 \leq p < 0.1$ for $^{99m}$Tc(N)-DBODC(5) and $^{99m}$Tc-sestamibi].

MRP1 proteins may act as a GSH–drug cotransporter. To examine the contribution of intracellular GSH to $^{99m}$Tc(N)-DBODC(5) transport, some uptake experiments were performed by pretreating cells with BSO (25 μM). The effect of cellular GSH depletion on net accumulation of the complex in C13* (MRP1, MRP2) and 2008 (P-gp, MRP1) cells was evaluated and compared with that of $^{99m}$Tc-sestamibi (Fig. 5, Table S3). In both cases, BSO induced a slight enhancement of $^{99m}$Tc(N)-DBODC(5) and $^{99m}$Tc-sestamibi uptake [$p < 0.001$ for $^{99m}$Tc(N)-DBODC(5) and $^{99m}$Tc-sestamibi in both cell lines], suggesting a possible recognition of $^{99m}$Tc(N)-DBODC(5) as a substrate of MRP1-mediated transport. This hypothesis was strongly supported by the data collected after pretreatment of cells with MK571. In fact, the addition of MK571, a potent and selective MRP1 inhibitor, was responsible for a considerable increase in uptake of complexes in MRP1-expressing cell lines, which was mostly evident in the C13* overexpressing subline [$p < 0.001$ for $^{99m}$Tc(N)-DBODC(5) and $^{99m}$Tc-sestamibi]. After 60 min of incubation, the ratio of the percentage cellular accumulation in the presence of MK571 to the percentage cellular accumulation in the absence of the drug was 6.76 for $^{99m}$Tc(N)-DBODC(5) and 4.40 for $^{99m}$Tc-sestamibi (Tables S1–S3).

Complete inhibition of the P-gp and MRP1 activity was obtained by incubating the different drug-resistant cell
To test the specificity of $^{99m}$Tc(N)-DBODC(5) for MRP1 transporter, uptake assays were performed on the transfected MDCK cell line overexpressing MRP1 transporter (MDCK/MRP1). This cell line provides a good model system for analysis of MRP1 [38–40]. The cellular uptake was compared with that of the corresponding wild type (MDCK). $^{99m}$Tc-sestamibi was used as a reference.

The rate of uptake of the two tracers evaluated in baseline conditions at 4 and 37 °C in MDCK and MDCK/MRP1 cell lines is reported in Fig. 6.

Cell-associated activity increased with time and reached a plateau after 60 min of incubation. For $^{99m}$Tc(N)-DBODC(5) a lower cellular uptake was found with respect to $^{99m}$Tc-sestamibi in the MDCK and MDCK/MRP1 cell lines. Steady-state levels of $^{99m}$Tc(N)-DBODC(5) were 8.53 ± 0.87 in MDCK cells and 3.21 ± 0.88 in MDCK/MRP-1 cells versus 11.01 ± 1.28 and 4.68 ± 0.49 for $^{99m}$Tc-sestamibi in the same cultured cells.

The ratio of the percentage accumulation (at 60 min) for the $^{99m}$Tc agents in MDCK and MDCK/MRP-1 cells was used as a measure of the function of MRP1 proteins. This value was comparable for the two $^{99m}$Tc agents [2.65 for $^{99m}$Tc(N)-DBODC(5); 2.26 for $^{99m}$Tc-sestamibi], suggesting a similar recognition of the complexes by the MRP1 transporters.

The rate of uptake of $^{99m}$Tc(N)-DBODC(5) and $^{99m}$Tc-sestamibi after MDR modulator/inhibitor treatment is reported in Table S4. Figure 7 shows the enhancement of the percentage cellular uptake of the two agents in MDCK/MRP1 cells induced by the selected MDR modulator/inhibitor after 60 min of incubation at 37 °C.

Incubation of transfected MDCK/MRP1 cells with $^{99m}$Tc-enhanced the cellular accumulation of $^{99m}$Tc(N)-DBODC(5) by 3.2 times with respect to the baseline condition. For $^{99m}$Tc-sestamibi, this improvement was only 1.7 times ($p < 0.001$). Treatment with the other MDR modulators/inhibitors which do not possess specific and selective activity for MRP1 transporters did not result in a significant effect on the accumulation of $^{99m}$Tc(N)-DBODC(5) and $^{99m}$Tc-sestamibi ($p > 0.1$). Exposure to Vrp induced a partial increase of cellular accumulation of both $^{99m}$Tc agents. Since transfected MDCK/MRP1 cells do not express P-gp proteins (Fig. 2) [38–40], this effect can be attributed to an overall modification of the cell permeability.

Cell viability

Cell viability, determined before and during all uptake experiments by the trypan blue dye exclusion technique, was greater than 90 % in each the experiments reported. No differences between preincubation and postincubation with the $^{99m}$Tc agents were detected, indicating that incubation with the $^{99m}$Tc compounds did not affect cell viability.
Evaluation of CCCP effects

The influence of CCCP, which affects the mitochondrial membrane potential, on cellular uptake of the $^{99m}$Tc complexes was evaluated for drug-sensitive and drug-resistant cell lines after treatment with P-gp and/or MRP modulators/inhibitors. The addition of CCCP released approximately 70% of the accumulated activity of both $^{99m}$Tc(N)-DBODC(5) and $^{99m}$Tc-sestamibi.

For drug-resistant cell lines, these findings suggest that the enhanced uptake of $^{99m}$Tc(N)-DBODC(5) generated by the addition of a modulator/inhibitor was sequestered into the structure of mitochondria.

Discussion

MDR mediated by overexpression of P-gp is one of the best characterized transporter-mediated barriers limiting the success of chemotherapy in cancer patients and is also a rapidly emerging target in the progression of neurodegenerative disorders, such as Alzheimer’s disease, Parkinson’s disease, and epilepsy [10, 14]. Thus, molecular probes capable of imaging noninvasively P-gp and closely related transporter activities in tissues as well as tumors would be expected to contribute to personalized medicine. Interrogation of P-gp-mediated transport activity in vivo via noninvasive SPECT could be helpful for stratification of patient populations likely to benefit from a given therapeutic treatment, assist in the management of chemotherapy, and aid the study of neurodegenerative diseases [14].

In the past decade, $^{99m}$Tc-sestamibi has been characterized as a transport substrate for MDR P-gp and MRP1 (although to a lesser degree) and used in functional imaging of MDR [7]. Cross-reactivity with MRP1 may reduce the specificity of the tracer for functional imaging of P-gp in tumors; nevertheless, SPECT scans with $^{99m}$Tc-sestamibi have accurately predicted chemotherapy response in a range of cancers, even though $^{99m}$Tc-sestamibi is also a substrate for MRP1 [7, 23]. In spite of this, the extensive
hepatobiliary excretion of this radiopharmaceutical and its uptake in heart, liver, kidneys, and gastrointestinal tract make imaging in the abdomen difficult.

To explore the potential use of $^{99m}$Tc(N)-DBODC(5) in tumor imaging and in detecting the P-gp/MDR-mediated drug resistance in cancer and in neurodegenerative diseases, the rate of uptake of this agent was evaluated in vitro in selected drug-sensitive human tumor cell lines and in the corresponding drug-resistant sublines before and after treatment with a suitable P-gp and/or MRP modulator/inhibitor. For this purpose some pharmacological properties and experimental criteria must be fulfilled: (1) low nonspecific binding to cell membranes and hydrophobic regions in biological preparations; (2) high distinction in net uptake levels between drug-sensitive cells and P-gp-expressing multidrug-resistant cells; (3) high enhancement of uptake in resistant cells after addition of an MDR modulator/inhibitor.

In general, the percentage cell accumulation of $^{99m}$Tc(N)-DBODC(5) was found to decrease in drug-resistant cell lines in proportion to the P-gp expression levels, and an enhancement of the tracer has been observed upon treatment with an MDR modulator/inhibitor. Addition of MDR modulator/inhibitor (CsA and MC18), which blocks the function of P-gp, was responsible for enhanced cellular accumulation of the $^{99m}$Tc(N)-DBODC(5) tracer, indicating a possible recognition of $^{99m}$Tc(N)-DBODC(5) by this transporter. This finding supports a possible use of this agent in the functional assessment of MDR P-gp even though the data collected for $^{99m}$Tc-sestamibi show that this latter compound is better recognized by P-gp pumps than is $^{99m}$Tc(N)-DBODC(5). Hence, $^{99m}$Tc-sestamibi probably remains the most appropriate compound for the detection of P-gp activity. It is interesting to note that for both $^{99m}$Tc complexes the uptake was significantly lower in cells expressing MRP1 or MRP2 with respect to those expressing P-gp/MDR ($p < 0.001$).

The data reported here suggest that $^{99m}$Tc(N)-DBODC(5) is better recognized by MRP1 than by P-gp. This finding is supported by the fact that the accumulation of $^{99m}$Tc(N)–DBODC(5) in the drug-resistant cell line was the inverse of the expression of MRP1 and MRP2. Thus, (1) the percentage cellular accumulation of $^{99m}$Tc(N)-DBODC(5) was lower in the A2780/ADR and C13* cell lines, which are characterized by high expression of MRP1 and MRP2 with respect to the MCF7/ADR and 2008 cell lines, which express both P-gp and MRP1 transporter; (2) the net accumulation of $^{99m}$Tc(N)-DBODC(5) was the inverse of the expression of MRP1 in the transfected MDCK/MDRP1 cell line; (3) depletion of GSH in MRP1-expressing cells (2008 and C13*) resulted in enhancement of the cellular uptake; (4) a potent reverse effect was observed by inhibition with MK571, which is selective for MRP1 transporter; and (5) the reverse effect induced by MK571 was significantly greater than that generated by P-gp MDR inhibitor/modulator.

Similarly to P-gp, the MRP family of transporters is also a member of the ABC transporter superfamily [5]. Among this superfamily, MRP1 transporter is widely expressed in normal tissues such as lung, testes, kidneys, skeletal tissues, cardiac muscles, and placenta. Overexpression of MRP1 and sister proteins, such as MRP2, has subsequently been shown for a number of drug-resistant cancer cell lines, including lung, gastric, and colorectal cancer cell lines, and their expression levels are thought to confer resistance to many antineoplastic agents and chemotherapeutic drugs as well as vincristine, doxorubicin, and cisplatin [5].

MRP1 transporter is involved in GSH homeostasis and is responsible for its export. In addition, it is also involved in inflammatory process mediated by leukotriene $\Delta_4$ (LTD$_4$), which constitutes the highest-affinity substrate for this transporter [5]. Whereas P-gp is primarily involved in the active transport of organic cations [5], MRP1 typically transports compounds that have been GSH-, glucuronide-, or sulfate-conjugated, or compounds that are amphiphatic and anionic, such as LTD$_4$ [57]. However, studies have shown that cytotoxic natural products that are neutral or cationic in character can be also transported by MRP1. Furthermore, these compounds appear to be cotransported with GSH but not conjugated to it. More than one mechanism has been proposed to mediate the resistance by MRP1 [58, 59], the mechanism by which the resistance is associated with the intracellular GSH levels and entails the conjugation of the compounds to GSH via glutathione S-transferases; and the mechanism by which the efflux of unmodified drug is mediated by direct interaction/binding of the compound with MRP1.

$^{99m}$Tc(N)-DBODC(5) is a lipophilic compound characterized by a single positive charge. Biological studies revealed for this compound a remarkable in vitro and in vivo stability: no evidence of biotransformation and metabolization was detected after incubation and extraction of $^{99m}$Tc(N)-DBODC(5) from biological fluids and tissues [27–30]. Challenge experiments conducted in presence of high excess of GSH showed a high inertness of the complex toward transchelation and the formation of GSH adducts was never observed.

To reverse the efflux of $^{99m}$Tc(N)-DBODC(5) mediated by MRP1, the effects of BSO (a GSH synthesis inhibitor) and MK571 (an LTD$_4$ receptor antagonist) were investigated (Fig. 4). GSH depletion resulted in a slight increase of $^{99m}$Tc(N)-DBODC(5) accumulation in C13* cells (1.5 times the basal level at the steady state). This value was drastically lower compared with that induced by MK571 inhibition. Under the same conditions, the percentage cellular accumulation in MK571-pretreated cells...
was approximately six times higher than the basal uptake value [30 min after incubation the percentage cellular uptake was 49.23 ± 2.23 for $^{99m}$Tc(N)-DBODC(5) versus 42.45 ± 5.13 for $^{99m}$Tc-sestamibi].

These results seem to indicate that $^{99m}$Tc(N)-DBODC(5) is only partially effluxed as GSH-conjugated from the cells and that the effects of MK571 are mostly due to the blocking of $^{99m}$Tc(N)-DBODC(5) efflux. The data obtained with MK571 suggest a different mechanism of recognition of the complex by MRPI, by which the resistance might involve the direct interaction/binding of the unmodified $^{99m}$Tc(N)-DBODC(5) with MRPI.

The collected data indicate that $^{99m}$Tc(N)-DBODC(5) has almost the same transporter profile as $^{99m}$Tc-sestamibi. Actually, $^{99m}$Tc(N)-DBODC(5) is recognized as a transport substrate for both P-gp and MRPI, but findings from inhibition experiments revealed a better recognition of $^{99m}$Tc(N)-DBODC(5) by MRPI than by P-gp transporters. Resembling $^{99m}$Tc-sestamibi, cross-reactivity with MRPI may reduce the specificity of the tracer for functional imaging of P-gp in tumors, but alternatively this property may cause $^{99m}$Tc(N)-DBODC(5) to be a more general probe of transporter-mediated MDR in some cancers and neurodegenerative diseases [7].

These in vitro studies confirm what we previously observed from gender-related pharmacokinetic studies, in which the influence of P-gp and MRPI on the remarkable rapid elimination of $^{99m}$Tc(N)-DBODC(5) from critical nontarget tissues was investigated by evaluating the effect of CsA on the biodistribution profile of the complex [30]. When CsA was administered before intravenous injection of the radiocomplex, a significant reduction of lung, liver, and kidney washout was observed along with a considerable variation in the activity distribution in the intestinal tract and in myocardial uptake, thus indicating a role of P-gp and MRPI transporters in determining the pharmacokinetic behavior of $^{99m}$Tc(N)-DBODC(5).

Therefore, it is realistic to believe that $^{99m}$Tc(N)-DBODC(5) is recognized both in vitro and in vivo as a transport substrate for both P-gp and MRPI. These findings provide indications concerning the possibility to extend the diagnostic application of $^{99m}$Tc(N)-DBODC(5) in tumor and in noninvasive MDR studies, even if further in vivo studies need to be performed in more suitable animal models to evaluate the diagnostic value of $^{99m}$Tc(N)-DBODC(5) as well as to identify with respect to $^{99m}$Tc-sestamibi the optimal in vivo imaging agent. For in vivo application, not only is the accumulation of radioactivity in target organ or tissue critical, also critical is the ratio of radioactivity in the target versus the background. $^{99m}$Tc(N)-DBODC(5) has a more favorable pharmacokinetic profile than $^{99m}$Tc-sestamibi [25–27]. Therefore, the advantages of in vivo pharmacokinetics of $^{99m}$Tc(N)-DBODC(5) for myocardial imaging may also be applied to tumor imaging. For $^{99m}$Tc(N)-DBODC(5), hydropathicity and pharmacological activity can be controlled through the independent variation of the phosphine substituents (at the phosphorous and nitrogen atoms) and the dithiocarbamate ligand without impacting the coordinating sphere and consequently the remarkable stability properties of this compound [60]. This fact makes $^{99m}$Tc(N)-DBODC(5) a suitable platform for the preparation of a molecular probe for SPECT of MDR.

Furthermore, different $^{99m}$Tc mixed compounds characterized by the presence of an alcoxyalkyl aminodiphosphine ligand (PNP) such as $[^{99m}$Tc(N)(L)(PNP)]$^+$ (L is a linear-chain, alicylic, or crown-ether-containing dithiocarbamate or thiomaltol derivative) [27, 60–64], $[^{99m}$Tc(NNPh)(L)(PNP)]$^+$ (L is crown-ether-containing dithiocarbamate) [65], and $[^{99m}$Tc(CO)$_3$(PNP)]$^+$ have recently been reported [66]. The evidence that all these compounds maintain similar biodistribution patterns, characterized by a high and persistent myocardial uptake and an extremely rapid elimination of the activity from nontarget tissues such as lung, liver, kidney, and intestine, regardless of the presence of the terminal technetium–nitrogen multiple bond and regardless of the nature of the dithiocarbamate, malathol, or CO co-ligands, suggests that this behavior may be mainly correlated to the [Tc(PNP)]$^{n+}$ scaffold and, in particular, to the chemical structure of the PNP ligand as well as to the presence of alcoxyalkyl pendant groups. Animal studies aimed at elucidating the mechanisms of distribution, retention, and elimination of some of these [Tc(PNP)]$^{n+}$ complexes demonstrated that the remarkably rapid efflux of these compounds from nontarget tissues seems strongly correlated to the action of P-gp and MRPI transporters [30, 64]. This behavior suggests that the [Tc(PNP)]$^{n+}$ and [Tc(N)(PNP)]$^{2+}$ scaffolds can be viewed as a substrate for P-gp and sister proteins. Consequently, it should be possible to conjugate, through a suitable chelating system, these moieties to conventional cytotoxic compounds and MDR modulator and/or substrates in the development of more specific molecular probes for use in assessing the activity, the expression, and the function of P-gp and sister proteins in cancer and neurodegenerative diseases.

$^{99m}$Tc(N)-DBODC(5) uptake

The application of $^{99m}$Tc-sestamibi as a tumor-seeking agent is made possible by irreversible trapping of the tracer in the mitochondrial structures driven by a passive diffusion process also supported by an electric gradient due to a high mitochondrial transmembrane potential [21–24]. For this technetium-based compound, the net cellular content is a function of both passive potential-dependent influx and transport-mediated efflux.
The mechanism of uptake of $^{99m}$Tc(N)-DBODC(5) is unknown. Subcellular distribution studies conducted on isolated rat myocardial tissue indicated that $^{99m}$Tc(N)-DBODC(5), analogously to $^{99m}$Tc-sestamibi and $^{99m}$Tc-tetrofosmin, is localized in the mitochondrial fraction (86.3 %) of the myocardial cells, suggesting for this monocationic agent a mechanism of myocardial accumulation similar to that observed for $^{99m}$Tc-sestamibi [30].

To add some information to the mechanism of $^{99m}$Tc(N)-DBODC(5) cellular incorporation, we studied the effects of two different temperatures (4 and 37 °C) and the addition of CCCP on the cellular uptake. We found that the tracer cellular incorporation was correlated to the metabolic activity of the cells, with a cellular accumulation profile that was temperature-dependent. In fact, low temperature restricts the cellular uptake under all conditions investigated, whereas a considerable increase of the cell-associated radioactivity was detected at 37 °C.

Likewise, the addition of CCCP, which depolarizes the mitochondrial membrane potential and may affect the cell membrane potential, caused approximately 70 % release of accumulated $^{99m}$Tc(N)-DBODC(5) from the cells, indicating that the percentage released was connected to mitochondrial accumulation. The residual fraction of $^{99m}$Tc activity may be attributed to the effect of cell membrane potential, which could not have been totally diminished after addition CCCP, on the passive influx of the tracers into the cells [67].

Moreover, the effects induced by CCCP on drug-resistant cell lines pretreated with P-gp and/or MRP modulator/ inhibitor, combined with the results obtained at 4 °C, confirmed that blockade of P-gp/MPR allowed $^{99m}$Tc(N)-DBODC(5) to permeate into mitochondria in a membrane-potential-dependent manner and provide evidence that $^{99m}$Tc(N)-DBODC(5) was only minimally absorbed onto membrane lipids and hydrophobic compartments.

Concluding remarks

The results from this preliminary report clearly indicate that the uptake of $^{99m}$Tc(N)-DBODC(5) was correlated to metabolic activity of the cells. In fact, low temperature inhibited the cellular uptake of the $^{99m}$Tc tracers, indicating that there was not binding to the cell membranes or to hydrophobic regions of the cell membrane protein. The cellular accumulation is correlated to the level of P-gp/MPR expression, and an enhancement of uptake in resistant cells was observed after treatment with an MDR inhibitor/ modulator, indicating that the selective blockade of P-gp/MPR prevents efflux of the tracer. From the evaluation and comparison of the usefulness of the two tracers, it seems that $^{99m}$Tc(N)-DBODC(5) may have an advantage over $^{99m}$Tc-sestamibi in assessing MRP-mediated drug resistance. The collected data provide indications concerning the possibility to extend the diagnostic application of $^{99m}$Tc(N)-DBODC(5) in tumor and in noninvasive MDR studies. In vivo studies on appropriate animal models are in progress.

For this mixed compound, the pharmacological activity and the pharmacokinetic profile can be controlled by variation of the substituents at the periphery of the molecule without affecting the P$_2$S$_2$ coordination sphere. Hence, $^{99m}$Tc(N)-DBODC(5) might be a suitable “scaffold” for the design of new $^{99m}$Tc-based molecular probes capable of imaging noninvasively P-gp and closely related transporter activities.

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References
