Malignant Gliomas Display Altered Plasma Membrane Potential and pH Regulation – Interaction with Tc-99m-MIBI and Tc-99m-Tetrofosmin Uptakes

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Abstract. Two radiopharmaceuticals, Tc-99m-MIBI (MIBI) and Tc-99m-Tetrofosmin (Tfos), are currently used for *in vivo* non-invasive monitoring of the MultiDrug Resistant (MDR) status of tumours. As gliomas are highly multidrug resistant, it is expected that the tracers would be poorly retained in those cells, but the *in vivo* and *in vitro* studies to date have shown that Tfos was highly retained in malignant gliomas. The high degree of malignancy of tumour cells is linked to alterations of physiological parameters as plasma membrane potential and intracellular pH. In order to elucidate the contribution of those parameters to Tfos and MIBI uptakes in malignant gliomas, we used several glioma cell lines – G111, G5, G152, and 42 MG-BA. These cells showed to be chemoresistant with a high level of expression and activity of the Multidrug Resistant associated Protein 1 (MRP1). They also had an alkaline intracellular pH (pHi) related to the Na^+/H^+ antiporter (NHE-1) expression and depolarised plasma membranes (−45 to −55 mV). In spite of their chemoresistance, we have found a high accumulation of both radiotracers in gliomas, more important for Tfos than MIBI, related to the presence and activity of NHE-1. In conjunction, the uptakes of the tracers were only partially dependent upon the plasma membrane potential of the glioma cell lines, again Tfos uptake being less dependent on this parameter than MIBI uptake. In conclusion, the evidence accumulated in this study suggests that Tfos could be a suitable glioma marker *in vivo*.

Key words: Na^+/H^+ exchanger — Membrane potentials — Glioma cell lines — Tc-99m-Tetrofosmin — Tc-99m-MIBI

Abbreviations: BCECF-AM, 2',7'-bis-(2-carboxyeth yl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester; Calcein-AM, Calcein acetoxymethyl ester; diBA-C4

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(3), Bis(1,3-dibutylbarbituric acid (5)) trimethine oxonol; EIPA, 5-(N-Ethyl)-N-Isopropyl Amiloride; LD50, lethal dose 50; MDR, multidrug resistance; MIBI, Tc-99m-MIBI = Tc-99m-Sestamibi; MRP1, multidrug resistance protein 1; NHE-1, Na^+/H^+ exchanger isoform 1; P-gp, P-glycoprotein; Tfos, Tc-99m-Tetrofosmin; VP-16, etoposide.

Introduction

The resistance of human malignancy to multiple chemotherapeutic agents remains a major obstacle in cancer therapy. This resistance phenomenon is called "multidrug resistance (MDR)" because the neoplastic cells fail to respond to a broad range of structurally unrelated anticancer agents. This leads to a complete ineffectiveness of any treatment and has dramatic consequences for the patients (Kvačkajová-Kišucká et al. 2001). Malignant gliomas are generally considered to be among the most multidrug resistant type of tumour (Chinot 1995). Thus, numerous undertaken chemotherapy trials have proved to be rather inefficient (Hosli et al. 1998). P-glycoprotein (P-gp), the main ABC transporter involved in the multidrug resistance (MDR) phenotype has not been associated with high multidrug resistance of malignant gliomas (Bae and Piwnica-Worms 1997; Baggetto 1997; Demeule et al. 2001). Indeed, several studies have shown that rather than P-gp, the multidrug resistance related protein 1 (MRP1) is often expressed in those cells (Abe et al. 1994, 1995; Zaman et al. 1994; Barrand et al. 1997; Borst et al. 1999). Two technetium lipophilic cationic complexes Tc-99m-MIBI (MIBI) and Tc-99m-Tetrofosmin (Tfos) are used for non-invasive monitoring of the MDR and it has been demonstrated that they are substrates for P-gp and MRP1 (Piwnica-Worms et al. 1993; Ballinger et al. 1995; Ballinger et al. 1996, 1998; Bae and Piwnica-Worms 1997; Mansi et al. 1997; Hendrikse et al. 1998, 1999; Bergmann et al. 2000; Muzzammil et al. 2000; Schomacker and Schicha 2000). Gliomas are highly multidrug resistant and it is expected that these two tracers would be poorly retained in those cells (Andrews et al. 1997; Yokogami et al. 1998). On the contrary, *in vivo* studies have shown that MIBI (Soler et al. 1998; Beauchesne et al. 2000) and especially Tfos were good tumour markers for malignant gliomas (Choi et al. 2000). The authors also suggested that MIBI and Tfos may accumulate in brain tumours by a similar mechanism and possibly in relation to the tumour cell proliferation. One of the main reasons could be that the chemoresistance of malignant gliomas might be mediated by other mechanisms in priority than by increased drug efflux through the MDR-ABC transporters (Choi et al. 2000). Our previous work on glioma cell lines confirmed those findings (Perek et al. 2000). We found a better accumulation of Tfos than MIBI in the glioma cell lines studied, and that could not be solely explained by interactions of those tracers with the MDR mechanisms. This suggested that other mechanisms contribute to their uptake.

The high degree of malignancy of tumour cells is linked to alterations of many physiological parameters, which have been associated to their increased metabolic activity, among them are the intracellular pH (pHi) and the plasma membrane

potential (Stern et al. 1999). Biochemical and morphological changes, including alterations of membrane ion transport and intracellular pH, are thought to play a role in drug resistance and accompany the expression of the drug efflux pumps (Hoffman et al. 1996). Studies, carried out on this subject, have shown that MDR cells have a higher pHi than sensitive cells (Roepe et al. 1993; Simon and Schindler 1994; Hoffman et al. 1996), and this has been associated with a decrease of the plasma membrane potential ($\Delta\psi$) in the resistant cells. The acidic pHi of tumour cells is believed to help the retention of the cytotoxic agents inside the cells, as most of them are weak bases. In contrast, resistant cells have a more alkaline pHi and, therefore, anticancer drugs would be less trapped in the cell. In addition, low transmembrane potential has been functionally related to rapid cell cycling, changes in membrane structure, and increased proliferation (Stern et al. 1999). In gliomas, a recent study has shown that the alkaline steady state pHi was highly dependent upon increased activity of the ubiquitous Na^+/H^+ exchanger isoform 1 (NHE-1), the primary membrane transporter used by cells to regulate the pHi (McLean et al. 2000).

Tc-99m-Sestamibi (or Tc-99m-MIBI or MIBI) uptake is known to be dependent on plasma membrane potential (Piwnica-Worms et al. 1990a,b). Tc-99m-Tetrofosmin has also been shown to possess significant membrane potential-dependent uptake properties, but did not respond in the same manner as MIBI (Arbab et al. 1996, 1997). In addition, these studies have shown a possible implication of the Na^+/H^+ antiporter in MIBI and Tfos uptakes in tumour cells. We have therefore explored how pHi and variations in cellular membrane potential, factors that contribute to the high malignancy, may influence the uptake of these two tracers in cells, and could explain the great affinity of glioma cells for Tfos.

Materials and Methods

Reagents

The radioactive tracers used were prepared using the one step Kit formulations: Cardiolite from Dupont Pharma for Tc-99m-Sestamibi, from Amersham International for Tc-99m-Tetrofosmin and from Mallickrodt (Petten, Netherlands) for Tc-99m-Annexin V. The fluorescent dyes Bis(1,3-dibutylbarbituric acid (5)) trimethine oxonol (diBA-C4(3)), ester 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM) and Calcein acetoxymethyl ester (Calcein-AM) were supplied by Molecular Probes (Interchim, France). The stock solutions of the fluorescent dyes used were prepared in DMSO and stored in the dark at $-20\,℃$. All the culture media and supplements were provided by Biowhittaker (Europe).

Cell culture

MCF-7 is an established cell line derived from breast tumour (American Type Tissue Culture Collection). The derived cell line MCF-7MDR (a cell line transfected with Hu MDR1 cDNA) was a gift from Mrs. S. Bertrand (INSERM U453 Lyon)

and MCF-7 VP-16 cell line was obtained as described before (Schneider et al. 1994). Briefly, selection of MCF-7 VP16 resistant cell line was performed by exposure of the parental MCF-7 cell line to etoposide (VP-16) at an initial concentration of 2×10^{-8} mol/l. Fresh drug was added two times *per* week. As allowed by cell growth, the concentration of drug was slowly increased in a multiple step procedure over a period of one year to reach a maximum concentration of 2×10^{-6} mol/l, representing a 100-fold increase. These cells are chronically cultured in media containing the highest indicated concentration of VP-16.

Several human cell lines originating from patients with gliomas were used in this study: G111 low grade II astrocytomas, G5 grade III anaplastic astrocytomas, G152 glioblastomas multiforma, (Beauchesne et al. 1998). All the cell lines used were kindly given by INSERM U453 Centre Léon Bérard (Lyon, France). An established cell line (DSMZ, Germany) 42 MG-BA glioblastomas multiforma was also used. All cells, except 42 MG-BA, were cultured in RPMI 1640 medium supplemented with 10% foetal calf serum (FCS), 2 mmol/l L-glutamine, 100 units *per* ml of penicillin, 100 mg/ml of streptomycin and 1% of amphotericin (Biowhittaker) at 37℃, in a 5% CO_2 humidified atmosphere.

42 MG-BA was cultured in a 1 : 1 mixture of RPMI 1640 and MEM (with Earle's salts) supplemented with 20% FCS and L-glutamine and antibiotics at the same concentration as above.

Preparation of radioactive tracers

Tc-99m-MIBI (Cardiolite, E. I. Du Pont Pharma, N. Billerica, MA, USA), Tc-99m-Tetrofosmin (Amersham International, Aylesbury, Buckinghamshire, UK), and Tc-99m-Annexin V (Mallinckrodt, Petten, Netherlands) were prepared according to the manufacturer's instructions. Pertechnetate (99m TcO_4^-) was obtained from a molybdenum/technetium generator (CIS BIO International, Gif sur Yvette, France) and a 370 MBq dose was used for the labelling of each kit. To determine the absolute concentration of total Tc-99m-MIBI or Tc-99m-Tetrofosmin (radioactive and cold) in solution, the generator equilibrium equation was used and the molarity was expressed in terms of total Tc-99m-MIBI or Tc-99m-Tetrofosmin as described previously (Cordobes et al. 1996).

Annexin V kit (Mallinckrodt, Petten, Netherlands) containing lyophilised 2-iminothiolane modified annexin V was prepared as followed. Annexin V kit was dissolved in 0.5 ml (370 MBq) pertechnetate eluate and the mixture was incubated for at least 20 minutes at room temperature.

The radiochemical purity of Tc-99m-MIBI was determined by thin-layer chromatography using Baker Flex Al_2O_3 IB-F plates and absolute ethanol as the mobile phase. The radiochemical purity of Tc-99m-Tetrofosmin was checked by thin-layer chromatography on silica plates (Silica Gel type G, Sigma) using a mixture of acetone-dichloromethane as the mobile phase, according to the manufacturer's directions. For each tracer used, the radiochemical purity was higher than 95%.

Radiochemical purity of Tc-99m-Annexin V was measured by column chromatography with a sephadex PD-10 column (Pharmacia, France) and 1% serum

bovine albumin (Sigma) in saline solution. For Tc-99m-Annexin V, the radiochemical purity was almost 90%.

Radiotracer experiments

One day before radiotracer experiments, cells were harvested (1% trypsin – EDTA solution, Biowhittaker) and then plated in 24-well plates (Falcon) at a density of 150,000 to 200,000 cells *per* well and were used in the exponential phase of growth. Cells were equilibrated in FCS-free RPMI-HEPES medium (Biowhittaker) for 30 minutes before adding radioactive tracers. MIBI and Tfos were added to the medium at a concentration of 0.5–1 nmol/l. After 60 minutes of incubation with the tracer, the medium was discarded.

The cells were rapidly washed three times with phosphate-buffered saline (PBS) at 4°C so as to eliminate the free tracer present in the extracellular spaces. The cells were then solubilized with a 1% sodium dodecyl sulfate solution in 10 mmol/l sodium borate (Sigma, France). The radioactivity in the cellular lysate was counted with a scintillation gamma counter (Packard Cobra 5002). All the experiments were carried out in quadruplicate and repeated five times. Results were expressed as the percentage of the dose administered *per* μg of protein (% AD/μg protein). Protein content was determined with the micro BCA (bicinchoninic acid) protein assay kit (Pierce).

The effect of plasma and mitochondrial membrane depolarisation on tracer uptake was studied by incubating cells with MIBI or Tfos during one hour in either high K^+ medium (130 mmol/l K^+), or in RPMI-HEPES containing the K^+ ionophore valinomycin (10 μmol/l). High K^+ medium with low Cl^- was made by equimolar substitution of sodium chloride by potassium methane sulfonate. Potassium methane sulfonate was made by titration of methanesulfonic acid with potassium hydroxide before addition to serum-free RPMI 1640 and adjusted to pH 7.4 with methanesulfonic acid. To test Na^+/H^+ activity, cells were incubated simultaneously with MIBI or Tfos and 100 μmol/l 5-(N-ethyl)-N-isopropyl amiloride (EIPA). Acidification was obtained by incubating cells in RPMI-HEPES, 20 mmol/l NH_4Cl, pH 7.4 (Sigma) for one hour.

Apoptosis measurements

After 45 minutes incubation of the cells in serum-free medium, a 5–10 μCi dose of Tc-99m-Annexin V was added *per* well. After 15 minutes of incubation at 37°C the medium was discarded and the cells processed as described above for the Radiotracer Experiments. Apoptosis was quantified as percentage relative to the untreated sample.

Evaluation of spontaneous apoptosis in the control cells has been performed as described by Wride and Sanders (1998) with some modifications. Cells were cultivated in growth media as before on chamber slides (Becton Dickinson, France), until they reached sub-confluence. Annexin V conjugated to biotin (Roche Biomoleculars, France) was diluted immediately before use 1 : 50 in pre-warmed (37°C) incubation buffer (10 mmol/l HEPES, pH 7.4, 140 mmol/l NaCl, 5 mmol/l KCl,

5 mmol/l $CaCl_2$). The growth medium was removed from the chamber slides, and the diluted Annexin V conjugate was added to each chamber and incubated for 15 min at 37℃ in a humidified atmosphere in the dark. The chamber slides were then rinsed twice with fresh incubation buffer. Cells were further incubated with streptavidin coupled to peroxidase (Roche Biomoleculars) in incubation buffer at a final concentration of 500 U/ml. Apoptotic cells were revealed with diaminobenzidine solution (Dako, France) for 15 minutes. Cells were counterstained with a hematoxylin solution (Sigma) for 5 minutes, rinsed twice in distilled water and incubated in phosphate buffer saline, to stain the nuclei in blue. Cells were then fixed in 1% paraformaldehyde before reading using a Leitz DMIRB microscope (Leica). The number of apoptotic cells was evaluated by using Oncor Image 2.0 imaging software (Oncor). Using this technique in all the cell lines studied, we found no more than 10% spontaneous apoptotic cells.

MDR functionality test

To assess MRP1 functional activity, we measured the cellular accumulation of the fluorescent probe Calcein-AM. Cells exposed to Calcein-AM become fluorescent after the cleavage of Calcein-AM to calcein by cellular esterases. P-gp and MRP1 actively extrude Calcein-AM but not the fluorescent calcein. The confluent cells monolayers were exposed to Calcein-AM (250 nmol/l) for 60 minutes at 37℃ as previously described (Hollo et al. 1994). Cells were incubated alone or in the presence of verapamil (25 μmol/l), a MDR protein blocker. After incubation, the Calcein-AM was removed and the cells were quickly washed and solubilized in 1% SDS, 10 mmol/l sodium borate solution. All the experiments were carried out in triplicate and repeated three times. Fluorescence was determined using a spectrofluorometer (Kontron Instrument) with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. Protein content was also determined with the micro BCA protein assay kit (Pierce). Results were expressed as percentage relative to the untreated sample considered as 100%, reflecting the cellular net accumulation of Calcein-AM in presence of the MDR blocker verapamil.

Determination of absolute plasma membrane potential

Absolute plasma membrane potential of cells was determined by the method described by Kraznai et al. (1995) using the oxonol diBA-C4(3) as follows. Owing to their net negative charge, diBA-C4(3) molecules distribute across the plasma membrane according to the Nernst law:

$$\psi = \frac{RT}{zF} \ln \left(\frac{D_\mathrm{i}}{D_\mathrm{e}} \right)$$

where D_i and D_e are the intracellular and extracellular concentrations of the free fluorescent dye respectively. The mean fluorescence intensity (MFI) measured from a stained cell is proportional to the amount of dye incorporated and/or bound to the cell. D_e and D_i were determined from a fixed cell sample and an unfixed cell

sample respectively incubated at the same diBA-C4(3) concentration i.e. 150 nmol/l in RPMI-HEPES. The MFI of the fixed cell sample gave us the external diBA-C4(3) concentration D_e and corresponded to the completely depolarised sample with $\psi = 0$, where $D_e = D_i$. The MFI of the unfixed cell sample corresponds to the internal diBA-C4(3) concentration D_i. For cell fixation, 500 μl of ice cold 2% formaldehyde was slowly added to 500,000 cells kept at 4°C for 30 minutes with continuous mild stirring. Before measurement, fixed and unfixed controls were washed two times in 5 volumes of RPMI-HEPES and incubated in 150 nmol/l of diBA-C4(3) in RPMI-HEPES. The correspondence between MFI and diBA-C4(3) concentration was obtained with a logarithmic calibration curve. The standard curve was prepared in RPMI-HEPES containing several concentrations of diBA-C4(3): 75, 150, 300, 600, 900, 1200 and 1500 nmol/l in a final volume of 1 ml, and incubated for 10 minutes at room temperature.

Spectrofluorometric analysis was performed immediately thereafter. Results were expressed as MFI. The calibration curve MFI = $f(\text{diBA-C4(3)}_e)$ was plotted after measuring the MFI for each concentration of diBA-C4(3) of the standard curve. Absolute plasma membrane potential was calculated according to the Nernst equation and D_i and D_e were determined for each sample with the exponential calibration curve. Finally, we obtained a direct measure of plasma membrane potential in mV. To determine absolute membrane potential in High K^+ + 10 μmol/l valinomycin medium, another calibration curve was performed in RPMI-HEPES containing 10 μmol/l valinomycin to avoid effects of non-expected valinomycin fluorescence. With this curve we could obtain directly absolute plasma membrane potential in high K^+-valinomycin medium in mV.

Intracellular pH determination

Intracellular pH was measured spectrofluorimetrically at 37°C with the fluorescent pH-sensitive probe, BCECF-AM as described previously (Boyer and Hedley 1994).

After harvesting, 1×10^5 cells *per* sample were loaded with 3 μmol/l BCECF-AM in sodium-HEPES buffer, pH 7.4 (25 mmol/l HEPES, 140 mmol/l NaCl, 5 mmol/l KCl, 0.8 mmol/l $MgCl_2$, 1.8 mmol/l $CaCl_2$, 5.5 mmol/l glucose) for 30 minutes at 37°C. Samples were centrifuged (300 × g, 7 minutes) and the supernatant discarded. The pellets were resuspended either in sodium-HEPES buffer pH 7.4 or in high K^+ medium (25 mmol/l HEPES, 145 mmol/l KCl, 0.8 mmol/l $MgCl_2$, 1.8 mmol/l $CaCl_2$, 5.5 mmol/l glucose, buffered at different pH values: 6.4; 6.8; 7.2; 7.6) supplemented with the K^+/H^+ ionophore nigericin (10 μg/ml) for 10 minutes.

Fluorescence was monitored using a spectrofluorometer (Kontron) using alternatively 440 nm (pH insensitive) and 490 nm (pH sensitive) as excitation wavelengths. Emission was measured at 530 nm. pHi was calculated from the fluorescence emission ratio of the two excitation wavelengths using a standard calibration procedure based on the use of the ionophore nigericin in high K^+ medium buffered at different pH values (Boyer and Hedley 1994; Hoffman et al. 1996).

Determination of cellular multidrug resistance

Cells were incubated for 1 hour with increasing concentrations of etoposide (Sandoz, France), cisplatin or paclitaxel (Sigma) and then cultured in 96-well plates (1000 cells/well) for 4 days. Lethal dose 50 (LD50) was determined with the cell proliferation kit MTT (Roche) according to the manufacturer's instructions and calculated as follows: optical density (OD) of treated cells/OD of untreated cells. Experiments were performed in quintuplet and repeated three times.

Western blots

Total proteins were extracted in M-Per Mammalian kit (Pierce). Protein concentration was determined with the BCA Protein Assay kit (Pierce). Protein fractions (40 μg) were loaded on a 7.5 % polyacrylamide-SDS gel, size-fractionated by electrophoresis and transferred to a nitro-cellulose membrane. The nitrocellulose membranes were incubated in blocking buffer (1X Tris Buffered Saline (TBS 1X) 0.5 % Tween 20, 5 % non-fat dry milk) for 1 hour. Proteins were revealed by overnight incubation at 4 ℃ with either primary rabbit polyclonal antibody to NHE-1 clone AB3081 (1 : 20, Chemicon) and primary mouse monoclonal antibody to MRP1 clone MPRm6 (1 : 100, Sanbio) or P-gp clone MAB 4114 (1 : 100, Chemicon). For detection of actin 1-hour incubation with a 1 : 2000 dilution of rabbit polyclonal or mouse monoclonal (clone AC-40) antibodies against actin (Sigma) has been performed. After three 15-min washes with washing buffer (TBS 1X, 0.05 % Tween 20), membranes were incubated for one hour with horse radish peroxidase conjugated anti-rabbit or anti-mouse IgG (1 : 3000, Sigma) according to primary antibody used. The blots were revealed using enhanced chemiluminescence (ECL) detection system (Amersham) using X-ray film (Biorad). Films were scanned and analysed for quantification with IMAGE software (NIH). The relative amounts of NHE-1 protein were normalised to actin for each sample. The ratios of NHE-1, MRP1 and P-gp were used to compare relative amounts of NHE-1, MRP1 and P-gp between the different cell lines used.

Statistical analysis

Data were expressed as mean ± S.D. unless otherwise stated. Statistical significance in all experiments was determined by unpaired two-tailed Student's t-test, $p < 0.05$ considered significant.

Results

Cellular characterisation

Determination of the MDR protein levels

P-gp and MRP1 expression levels were measured in the different cell lines by western blotting. Semi-quantitative analysis was performed by comparing P-gp or MRP1 signal with the actin one (Table 1, section II). Three cell lines were

Table 1. Biochemical and physiological characterisation of the cell lines and accumulation of Tc-99m-Sestamibi (MIBI) and Tc-99m-Tetrofosmin (Tfos). The different experiments were performed as described in Materials and Methods. Statistical tests were performed as follows: Section I – comparison of MIBI *vs.* Tfos uptakes in each cell line (* $p < 0.05$); Section II – comparison between untreated control (considered as 100 %) and verapamil treatment (*** $p < 0.001$); Section III – comparison of steady state pHi in MCF-7-sensitive control *vs.* the other cell lines (* $p < 0.05$)

	Section I		Section II			Section III		Section IV
Cell lines	Plateau heights (% AD/μg protein) Mean ± S.D. ($n = 20$)		MDR expression ($n = 4$)		Functionality test (% relative to untreated control) Mean ± S.D. ($n = 9$)	Steady state pHi Mean ± S.D. ($n = 12$)	NHE-1 expression ($n = 4$)	Absolute Plasma membrane potential Mean ± S.D. ($n = 12$)
	MIBI	Tfos	Ratio P-gp/actin	Ratio MRP1/actin	Calcein Fluorescence	pHi	Ratio NHE-1/actin	mV
MCF-7	0.71 ± 0.06	0.63 ± 0.05	–	0.08	105 ± 5.6	7.1 ± 0.2	2.7	−85.8 ± 5.7
MCF-7-MDR	0.35 ± 0.02	0.3 ± 0.03	1	0.05	190 ± 8.2***	7.54 ± 0.12*	3.4	−58.8 ± 3.8
MCF-7 VP-16	0.096 ± 0.008	0.13 ± 0.01	–	3	256 ± 12.2***	7.65 ± 0.14*	3.9	−65.1 ± 4.5
G111	0.71 ± 0.04	1.3 ± 0.06*	–	1.8	173 ± 9.1***	7.28 ± 0.06	3.8	−52.1 ± 2.8
G5	0.26 ± 0.02	0.55 ± 0.06*	–	2.2	183 ± 8.5***	7.37 ± 0.08*	3.9	−45.8 ± 1.7
G152	0.81 ± 0.08	1.1 ± 0.09*	–	1.7	167 ± 7.4***	7.6 ± 0.14*	4.3	−55.2 ± 2.4
42 MG-BA	0.63 ± 0.05	0.95 ± 0.08*	–	1.5	144 ± 5.3***	7.45 ± 0.14	3.4	−55.4 ± 2.5

used as sensitive and resistance controls respectively: MCF-7, MCF-7-MDR and MCF-7 VP-16. MCF-7-MDR is a clone derived from MCF-7 transfected with the MDR1 gene and overexpresses P-gp as expected. MCF-7 VP-16, a resistant cell line selected with low doses of VP-16, overexpresses the MRP1 protein. None of the glioma cell lines studied express the P-gp. In contrast, MRP1 was significantly overexpressed in all the glioma cell lines.

As multidrug resistance is defined by the functional transport capacity of these drug efflux pumps, the functionality of the MRP1 pump was tested using the fluorescent dye Calcein-AM. Results obtained with MCF-7-MDR and MCF-7 VP-16 showed an increase in fluorescence in the presence of verapamil, proving that in those cell lines the MDR proteins are functional (Table 1, section II). In the glioma cell lines, we also observed an increase in Calcein-AM accumulation after verapamil treatment, confirming that MRP1 is functional.

The multidrug resistance of the glioma cell lines was evaluated by testing their resistance to three anticancer drugs: etoposide, cisplatin and paclitaxel. The results from the MTT tests performed are presented in Table 2. They show that all the glioma cell lines used in this study were more resistant to etoposide, cisplatin and paclitaxel than the sensitive MCF-7 cells (5 to 20 fold higher LD50). Our results confirmed that those glioma cells have a multidrug resistant phenotype.

Table 2. Resistance to anticancer agents. Drug sensitivity to anticancer agents was examined by MTT assay. Resistance to etoposide, paclitaxel and cisplatin is presented with the 50% lethal dose (LD 50). Mean ± S.D., $n = 12$. Comparison of drug resistance in MCF-7-sensitive control *vs.* the other cell lines (* $p < 0.05$)

	Drug resistance (LD50)				
Drugs	G5	G111	G152	42 MG-BA	MCF-7
Etoposide (μg/ml)	14 ± 1.1*	9.5 ± 0.8*	12 ± 1.1*	11 ± 1.2*	2 ± 0.15
Paclitaxel (μmol/l)	7 ± 0.8*	12 ± 1.3*	7.5 ± 0.09*	9 ± 0.8*	1.5 ± 0.2
Cisplatin (μmol/l)	10 ± 0.9*	7.5 ± 0.6*	9.5 ± 1.1*	10 ± 1.1*	0.5 ± 0.06

Determination of pHi and levels of NHE-1

Intracellular pH (pHi) was measured by spectrofluorimetry and differences were observed between MCF-7 and its two resistant homologues MCF-7-MDR and MCF-7 VP-16: the resistant controls had a more alkaline pHi than expected (0.4 to 0.5 pH unit difference) (Table 1, section III). Glioma cell lines showed pHi values varying from 7.3 to 7.6, which is consistent with the MDR phenotype of those cell lines.

In order to evaluate the contribution of the NHE-1 to the high pHi values measured, we determined the levels of expression of NHE-1 in the glioma cells compared to the MCF-7 cell lines. Western blots were performed and the level of NHE-1 expression was normalised to the actin content for each cell line (Table 1,

section III). MCF-7 cells showed lower level of expression of NHE-1 compared to the resistant control cells MCF-7-MDR and MCF-7 VP-16. The glioma cells showed a NHE-1 to actin ratio varying from 3.4 to 4.26 – equal to or higher than that of the resistant control cell lines, confirming the correlation between MDR and NHE-1 levels.

Determination of absolute plasma membrane potential

The method described by Krasznai et al. (1995) allows to determine the absolute membrane potential of the cells using the fluorescent dye diBA-C4(3). As shown in Table 1 section IV, in absence of any treatment, the absolute plasma membrane potential measured was -85.78 ± 5.67 mV in the control cell line MCF-7. In comparison, the resistant cell lines MCF-7-MDR and MCF-7 VP-16 showed lower transmembrane potential, just as it had been expected (-58.8 ± 2.8 mV and -65.1 ± 3.8 mV, respectively). We verified that there were no interactions between oxonol diBA-C4(3) and MRP1 or P-gp efflux pump. The fluorescence signal was stable during the 10 minutes of measurement in the MCF-7, MCF-7 VP-16 or MCF-7-MDR cell lines (data not shown). In the glioma cells, the membrane potentials measured were lower than those of resistant controls and varied between -55 mV and -45 mV. We could relate those lower plasma membrane potentials measured to the levels of expression of the MRP1 protein (Table 1, section IV): G5 cell line had the highest MRP1/actin ratio (2.2) and showed the most depolarized plasma membrane (-45 mV) whereas the other three cell lines G111, G152 and 42 MG-BA had ratios varying from 1.5 to 1.7 and their absolute plasma membrane potentials measured were between -52 mV and -55 mV.

Study of MIBI and Tfos uptake

Interactions with MDR proteins

MIBI and Tfos uptakes were measured after one-hour incubation of the radiotracers with the different cell lines used in this study (Table 1, section I). We observed lower accumulations of MIBI and Tfos in MCF-7-MDR and MCF-7 VP-16 cells than in the sensitive cell line MCF-7. The MIBI retention inside those cells was equal or slightly higher than Tfos uptake. In contrast, the accumulation of the two tracers in glioma cells was different: Tfos was retained inside the cells to a higher level than MIBI (Table 1, section I). Moreover, the amounts of Tfos and MIBI retained in the glioma cells were higher than in the control cell line MCF-7, which had not been expected regarding the MDR phenotype of those cells.

Influence of plasma membrane potential on MIBI and Tfos uptake

We investigated whether the differences in the plasma membrane potentials of the glioma cells could explain the higher retention of MIBI and Tfos observed. To perform that, we analysed the uptake of the radiolabelled compounds under conditions that modulate their membrane potentials. In parallel, we monitored the changes in the plasma membrane potential under the same conditions with the fluorescent dye diBA-C4(3).

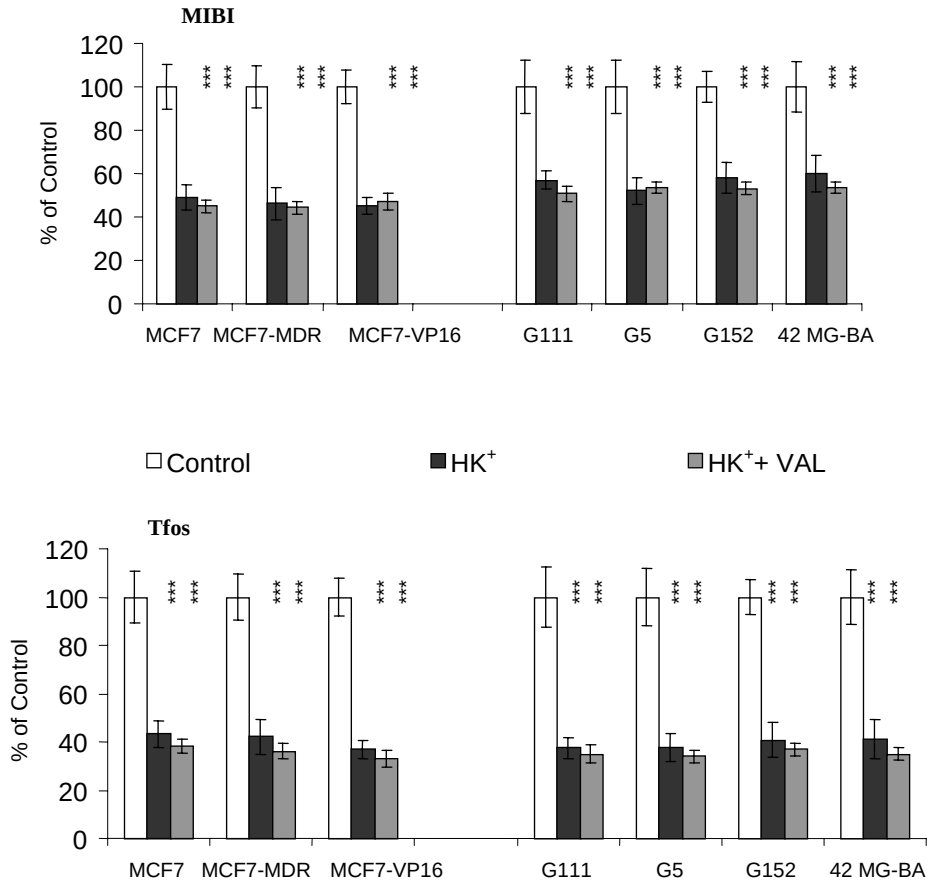

Figure 1. Effect of variation in plasma and mitochondrial membrane potentials on MIBI and Tfos uptake. Radiotracer uptake was measured after one hour incubation under several conditions: Control or Low K^+ (5 mmol/l of K^+); High K^+ medium (130 mmol/l K^+); High K^+ plus 10 μmol/l valinomycin (HK^++ VAL). Results are expressed in % of accumulation relative to the untreated control considered as 100 %, mean ± S.D., $n = 15$. Comparison: control *vs.* each treatment: *** $p < 0.001$.

Figure 1 summarises the results obtained for MIBI and Tfos uptakes when incubating the cells in different conditions: Low K^+ (5 mmol/l) or High K^+ medium (130 mmol/l) and in High K^+ medium in combination with the K^+ ionophore valinomycin.

When cells were incubated in High K^+ medium to depolarise the plasma membranes, the uptake of MIBI and Tfos in the MCF-7 and resistant derived cell line decreased by almost 45 %. When glioma cell lines were placed in the same conditions, a 60 % decrease in MIBI uptake and a 40 % decrease in Tfos uptake were observed. The plasma membrane potential measurements done in parallel with

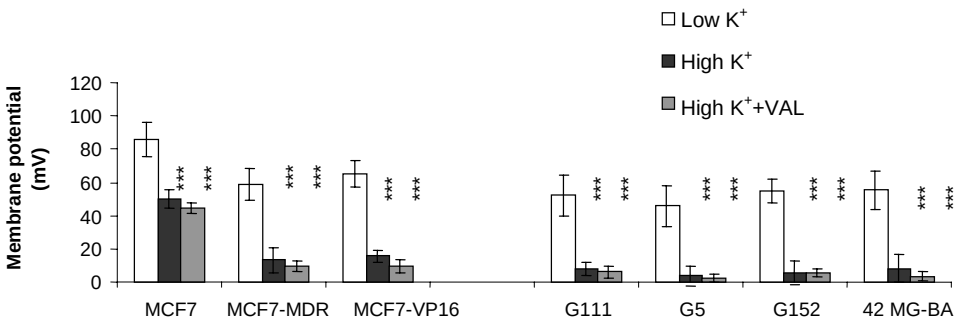

Figure 2. Absolute membrane potentials. Absolute plasma membrane potentials were determined with the fluorescent dye diBA-C(4)3 under several conditions: Control or Low K^+ (5 mmol/l K^+); High K^+ medium (130 mmol/l K^+); High K^+ and 10 μmol/l valinomycin (HK^++ VAL). Results were expressed in mV. Mean ± S.D., $n = 9$. Comparison of control *vs.* treatment: *** $p < 0.001$.

diBA-C4(3) showed a decrease in plasma membrane potentials when increasing the K^+ concentration (Fig. 2). The plasma membranes of the glioma cell lines were completely depolarised after 1-hour incubation in 130 mmol/l K^+ medium whereas the MCF-7 and variant cell lines were only partially depolarised. The influence of the mitochondrial membrane potential on MIBI and Tfos uptakes was also investigated by comparing tracer uptakes in High K^+ medium in presence or absence of valinomycin. We could not see any additional effect on the radiotracers' uptake in the presence of valinomycin in any of the cell lines studied (Fig. 1). The fact that even when the plasma membranes were almost completely depolarised, MIBI and Tfos uptakes were decreased only by 50 %, especially in the glioma cell lines, suggests that other cellular characteristics may play a role in the uptake of those tracers.

The apoptotic state of our different cell lines when incubated in High K^+ and High K^+ – valinomycin media was determined by measuring the binding of Tc-99m-Annexin V (Fig. 3). Treatment of the cells in High K^+ and High K^+ – valinomycin media did not induce more than 10 % apoptosis in any of the cell lines in comparison with the cells incubated in control conditions. In addition, we evaluated the amount of spontaneous apoptosis in each cell line in control conditions using a modification of the method described by Wride and Sanders (1998). No more than 10 % apoptosis was detected by this technique in the control cells. Therefore, in our experimental conditions apoptosis did not play a significant part in MIBI and Tfos accumulation and the decrease observed in presence of High K^+ medium was a true effect of the variation in plasma membrane potential.

Influence of NHE-1 on MIBI and Tfos uptakes

We studied the effect of pHi modulations on MIBI and Tfos uptake. To inhibit the Na^+/H^+ antiporter, we used an amiloride derivative – EIPA. To induce an

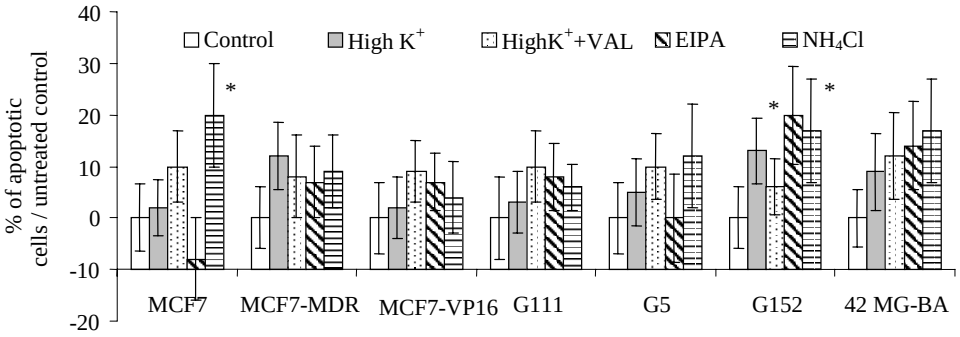

Figure 3. Measurement of apoptosis with Tc-99m-Annexin V. Tc-99m-Annexin V uptake was measured after one hour incubation under several conditions: Control or Low K^+ (5 mmol/l of K^+); High K^+ medium (130 mmol/l K^+); High K^+ and 10 μmol/l valinomycin (HK^++ VAL), EIPA (100 μmol/l) or RPMI 20 mmol/l NH_4Cl. Results are expressed in % of apoptotic cells relative to the untreated control considered as 0%, mean ± S.D., $n = 15$. Comparison of control *vs.* treatment: * $p < 0.05$.

intracellular acidosis mediated by NHE-1, the cells were also incubated in 20 mmol/l NH_4Cl solution for one hour.

We observed a dramatic decrease in MIBI and Tfos uptake after EIPA or NH_4Cl treatment (Fig. 4). Inhibition of the NHE-1 exchanger by EIPA provoked an almost 85% inhibition of MIBI uptake and an 82% inhibition of Tfos uptake. After NH_4Cl acidification, an 89% inhibition of Tfos uptake and a 95% inhibition of MIBI uptake were measured.

No effect of EIPA treatment on the pHi of the MCF-7 cell line was observed (Fig. 4). In all the other cell lines studied, NH_4Cl treatment had a more potent effect on pHi than EIPA. The pHi values measured after EIPA treatment showed a decrease of 0.2 to 0.4 unit of pH while after NH_4Cl incubation the drop in pHi measured was of 1 unit of pH. Only 15% to 20% apoptosis was estimated by Tc-99m-Annexin V binding after one-hour incubation with EIPA or NH_4Cl (Fig. 3). These results suggest that the nearly 85% inhibition of the accumulation of the two radiotracers observed in the presence of EIPA is not solely due to a decrease in the pHi value but to a direct interaction of MIBI and Tfos with the NHE-1 exchanger. In addition, the effect of EIPA on the activity of the MDR proteins was measured, but no modifications in Calcein-AM accumulation were observed (data not shown).

Discussion

Evidence is now accumulating to suggest that the two radiopharmaceuticals Tc-99m-MIBI (MIBI) and Tc-99m-Tetrofosmin (Tfos) are good markers of the multidrug resistant state of tumours (Ballinger et al. 1995, 1996; Hendrikse et al. 1998; Moretti et al. 1998; Perek et al. 2000). However, in malignant gliomas, known for

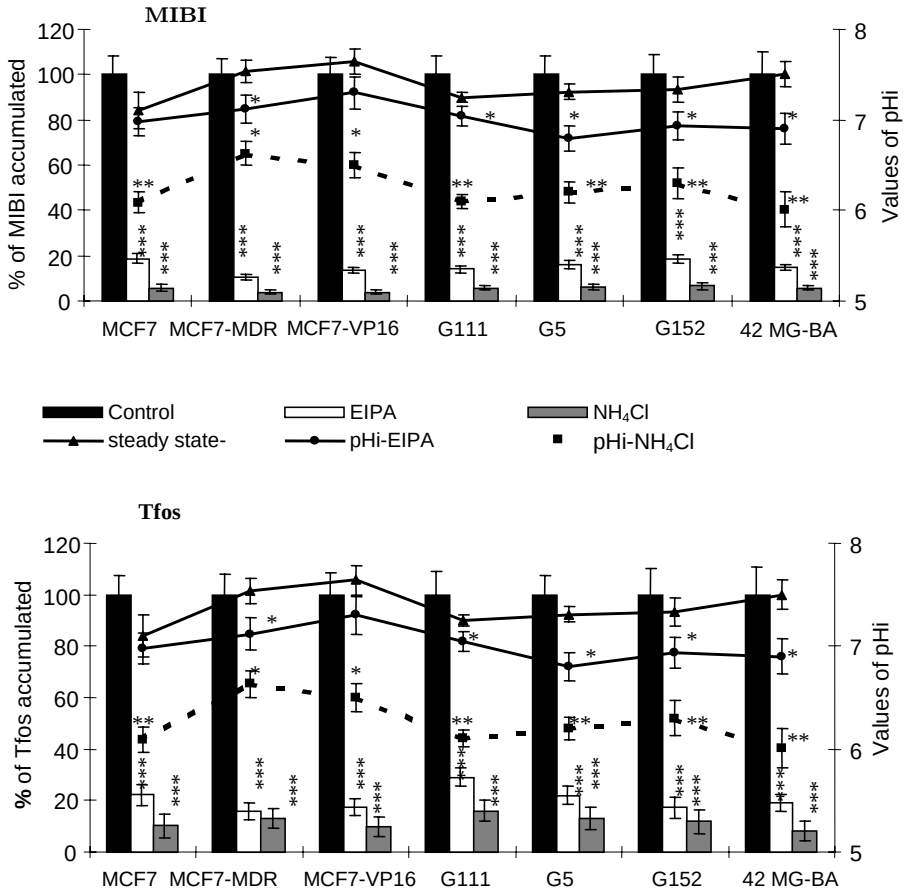

Figure 4. Modulation of pHi. MIBI and Tfos uptakes were measured after incubating cells with the NHE-1 inhibitor EIPA (100 μmol/l) or in RPMI – 20 mmol/l NH_4Cl medium for one hour. Results are expressed in % of accumulation relative to the untreated control considered as 100%, mean ± S.D., $n = 15$. Parallel measurements of pHi were performed with BCECF AM dye as described in Materials and Methods. Results are expressed in absolute pH units, mean ± S.D., $n = 12$. Comparisons of control *vs.* each treatment: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

their high degree of MDR, *in vitro* and *in vivo* data have demonstrated that these two radiopharmaceuticals are not behaving in the expected way (Choi et al. 2000), suggesting that other mechanisms are involved in their retention in this particular type of tumours.

We studied the links between pHi, plasma membrane potential, drug resistance and the uptake of MIBI and Tfos in malignant gliomas, in order to explain the greater affinity of these cells for Tfos. As a sensitive control we have chosen the breast carcinoma cell line MCF-7 commonly referred to in the literature (Ballinger

et al. 1995, 1996; Bernard et al. 1998). The two variant homologues, MCF-7-MDR, transfected and expressing the human MDR1 gene, and MCF-7 VP-16, expressing the MRP1 protein after chronic treatment with VP-16, were used as positive controls for cells expressing the MDR proteins. These cell lines have been extensively used for studying the biological properties of MIBI and Tfos (Ballinger et al. 1996; Cordobes et al. 1996; Kabasakal et al. 1996; Moretti et al. 1998).

Several cell lines originating from human gliomas with different grade of malignancy: G111, G5, G152, and 42 MG-BA, were used in this study. They have been previously characterised as cell lines with high degree of malignancy and radioresistance (Giollant et al. 1996; Beauchesne et al. 1998) and expressing a MDR phenotype (Perek et al. 2000). In this study, we confirmed this status by demonstrating their high level of expression of MRP1 as well as their resistance to three anticancer drugs – etoposide, cisplatin or paclitaxel. The data obtained showed that all the glioma cell lines used in this study were drug resistant compared to the MCF-7 cell line and expressed high levels of functionally active MRP1. This is in accordance with data published previously in the literature on the MDR phenotype of different glioma cell lines (Abe et al. 1994, 1995). However, we could not detect clear differences between low-grade (G111), intermediate (G5) and high-grade gliomas (G152, and 42 MG-BA) in the levels of expression of MRP1 and their resistance to anticancer drugs.

It has been described in the literature that alterations of the cellular physiological parameters are related to increased metabolic activity and cell proliferation. Among them are the pHi and the plasma membrane potential (Stern et al. 1999). We performed a series of experiments to verify whether this was the case in the glioma cell lines chosen for this study.

The pHi measurements carried out in the control and glioma cell lines have shown that the resistant cells, MCF-7-MDR and MCF-7 VP-16, exhibit a more alkaline pHi than the parental MCF-7 cell line directly linked to the P-gp- or MRP1-dependent multidrug resistant phenotype respective to those cells. Previous studies have reported that the MDR cells have a pHi around 0.4 pH unit higher than the cells sensitive to the tumour agents, with values very similar to those we have measured in this study (Roepe et al. 1993; Simon and Schindler 1994). Our results indicated that the malignant glioma cell lines also have an alkaline pHi. In addition, immunoblots' analysis demonstrated that MCF-7-MDR and MCF-7 VP-16 as well as the glioma cell lines expressed abundant levels of NHE-1 protein compared to the MCF-7 sensitive cells. Roepe et al. (1993) have reported higher levels of mRNA encoding the human NHE-1 in the resistant cell lines than in the sensitive ones but they could not correlate directly the level of overexpression of NHE-1 with the relative drug resistance or the steady state pHi. In glioma cells, Mc Lean et al. (2000) demonstrated that the NHE-1 was the main proton transporter responsible for the maintenance of the alkaline pHi, but also failed to find a direct correlation between NHE-1 expression levels and their pHi measurements. These findings suggest that it is an increased activity of the NHE-1, due probably to post-translational modifications of the NHE-1, that is responsible for the maintenance of

an alkaline pHi in these tumours (McLean et al. 2000). Our work tends to indirectly confirm this, as we could not correlate directly the levels of NHE-1 expression to the measured pHi values.

The MDR phenotype has also been associated to a decrease in membrane potential related to the P-gp expression (Roepe et al. 1993; Hoffman et al. 1996; Robinson and Roepe 1996). Our results show that the expression of MRP1 is also associated with a drop in the absolute plasma membrane potential of the cells as illustrated by the values obtained with the MCF-7 VP-16 cell line. The glioma cell lines had an even lower plasma membrane potential compared to MCF-7-MDR and MCF-7 VP-16. Several studies have reported that astrocytoma and glioblastoma cells have significantly lower membrane potential in comparison with astrocytes and neurons (Labrakakis et al. 1997; Silver et al. 1997; Bordey and Sontheimer 1998). This has been attributed to the particular physiological properties of the glioma cells, but as our glioma cells overexpress MRP1, the depolarised plasma membrane could also be associated to their multidrug resistance phenotype.

We have demonstrated that glioma cells exhibit criteria of malignancy such as MDR phenotype, an alkaline pHi and depolarised plasma membranes. We studied further how these factors could be involved in MIBI and Tfos uptakes. MIBI and Tfos are two radiopharmaceuticals used as MDR probes *in vivo*, and we first investigated their dependence upon MDR mechanisms in glioma cells. In accord with the literature (Ballinger et al. 1995, 1996; Bernard et al. 1998), we observed no difference between MIBI and Tfos accumulation in MCF-7 cells. In MCF-7-MDR and MCF-7 VP-16 cell lines, MIBI and Tfos accumulations were lower compared to MCF-7 directly linking the tracers' uptakes to the expression of the resistance protein P-gp and MRP1. We have found that MIBI uptake in glioma cells was less or equal to the sensitive cell line MCF-7. By contrast, Tfos uptake values were similar in all the glioma cell lines and more important than in the MCF-7. This suggests that the resistant phenotype in glioma cells is not playing the same role for Tfos as it does for MIBI and that other mechanisms are more likely to be the key characters in the process of Tfos uptake in gliomas.

As it has been reported in the literature that MIBI and Tfos may accumulate in glioma cells according to a process of tumour cell proliferation (Choi et al. 2000), and based on an alkaline pHi that has been associated with increased metabolic activity and cell proliferation (Rich et al. 2000), we investigated whether the alkaline pHi and the low plasma membrane potential of our glioma cell lines could explain the high accumulation of Tfos or not.

To assess this possibility, we used two modulators of the pHi: EIPA and NH_4Cl. Both treatments caused a dramatic decrease in MIBI and Tfos uptakes. Arbab et al. (1996, 1997) suggested that the inhibition of MIBI and Tfos uptake by dimethyl amiloride (DMA) was a sign of a probable interaction of those tracers with the Na^+/H^+ antiporter. We used EIPA, a more potent inhibitor than DMA (McLean et al. 2000), and our results confirm that MIBI and Tfos interact with the Na^+/H^+ exchanger. The EIPA treatment provoked a decrease of only 0.2 to 0.4 pH unit in the different cell lines (Lee and Tannock 1998; McLean et al. 2000; Rich et

al. 2000), except for the control cell lines MCF-7. This is in agreement with Lee and Tannock (1998), who found no modification of the pHi in MCF-7 cells upon EIPA treatment. The acidification after ammonium chloride treatment was more important (−1 pH unit) than after EIPA treatment. The fact that EIPA and NH_4Cl induced the same extent of inhibition of Tfos uptake despite the differences in the degree of acidification, suggests that the effect is rather due to an interaction of the radiotracer with the NHE-1 exchanger than to an effect of the intracellular pH. This interaction and the higher activity of the NHE-1, maintaining the alkaline pHi of the gliomas (McLean et al. 2000), may explain the higher level of accumulation of Tfos and to a lower extent that of MIBI in these cells.

We have checked whether the pHi acidification and the decrease in MIBI and Tfos uptakes did not reflect an apoptotic process induced by EIPA or NH_4Cl treatments. This was imposed by the fact that several reports using different tumour cell lines demonstrated that when Na^+/H^+ exchanger was inhibited by amiloride analogues, acidification of the cells occurred with a concomitant induction of apoptosis (Perez-Sala et al. 1995; Chen et al. 1997). In addition, in a recent study Vergote et al. (2001) have shown a possible implication of apoptosis on MIBI uptake in MCF-7 cell line treated with the antitumoral agent NaPa. For almost 20% of apoptosis, they observed only a 25% decrease in MIBI accumulation. Even if the acidification of our glioma cell lines induced a slight apoptosis (15 to 20%) this could not explain the 90% decrease in radiotracers' accumulation that we observed. Our results confirmed that in glioma cells MIBI and Tfos uptakes are strongly related to the NHE-1 activity.

We have shown that the glioma cell lines used in this study displayed low plasma membrane potentials and we have investigated how this can influence MIBI and Tfos uptakes. We compared values of absolute plasma membrane potentials and MIBI and Tfos uptake in High K^+ medium containing valinomycin or not. MIBI and Tfos uptakes were significantly decreased after incubation in High K^+ medium, which shows the importance of the plasma membrane potential.

In High K^+ medium, MCF-7 cells were not totally depolarised, which correlated with the 60% inhibition in MIBI accumulation and 50% inhibition in Tfos uptake measured. Additional studies performed in our laboratory indicated that the MCF-7 cell lines needed longer incubation time in High K^+ medium to achieve a total depolarisation of the plasma membrane, i.e. 90 min (data not shown). This difference in the time necessary for depolarisation of the plasma membrane of glioma and MCF-7 cell lines might be due to the physiological differences existing between those two cell types. To keep our experiments consistent all along and to be able to make comparison between them, we have kept depolarisation time of 60 min, time at which the glioma cells are almost completely depolarised, and at which we could not detect a significant amount of apoptosis.

In glioma cells, the High K^+ treatment almost completely depolarised the plasma membranes, but even then MIBI and Tfos uptakes were not completely abolished. Addition of valinomycin to depolarise the mitochondrial membrane potential (de Jong et al. 1996; Bernard et al. 1998) failed to further decrease MIBI

and Tfos uptakes. This indicates a lower level of contribution of the mitochondrial membrane potential to MIBI and Tfos retention in glioma cells (Arbab et al. 1997; Bernard et al. 1998). Chen et al. (2000) have hypothesised that when cells are totally depolarised the residual net accumulation of a radiopharmaceutical under isoelectric membrane potential is a measure of non specific adsorption of hydrophobic cationic complexes to lipid compartments within cells. Glioma plasma membranes are known to have a particular lipid composition (Sung et al. 1994; Yates et al. 1999; Kappel et al. 2000) and changes in the lipid content and composition of plasma membranes in brain tumours of different degree of malignancy are the subject of several studies (Dorszewska et al. 2000; Campanella 1992). It is therefore possible that MIBI and Tfos accumulation in gliomas may be to a certain extend also the result of tracer adsorption on lipid residues. We have measured the lipophilicity of these tracers and we found that Tfos was more lipophilic than MIBI (data not shown). This, in combination with a particular interaction of the tracer with the NHE-1 exchanger, might be a plausible explanation of its better accumulation in gliomas. Further analyses of the lipid composition of the plasma membrane bilayer of the glioma cells could give us additional explanations as to the importance of the radiotracers adsorption on the membranes.

Conclusion

We have shown that the malignant glioma cell lines used in this study exhibit several criteria of malignancy: depolarised plasma membranes, alkaline pHi, and a chemoresistant phenotype. We have tried to determine how these factors could interact with the uptake of MIBI and Tfos. Despite their chemoresistant phenotype, we failed to find a direct correlation between MDR phenotype and MIBI and Tfos uptakes in the glioma cells. A particular good affinity of these cells for Tfos has been demonstrated. We observed that maintenance of alkaline pHi is dependent upon NHE-1 and demonstrated that NHE-1 expression is correlated with MIBI and Tfos uptakes. Contribution of plasma membrane potential has been also investigated and showed that Tfos uptake was less dependent than MIBI upon plasma membrane potential in gliomas. A possible implication of hydrophobic interactions of the radiotracers with a particular lipid bilayer composition of glioma cells has been suggested.

Our findings indicate that in malignant gliomas, Tfos did not behave as a MDR probe but its uptake was rather linked to the biochemical and physiological characteristics of the cells. Preliminary *in vivo* studies carried out in our laboratory tend to confirm this. This can be an important issue for the application of Tfos as a specific *in vivo* radiotracer of malignant gliomas.

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