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CHARACTERIZATION OF MULTIDRUG RESISTANCE REVERSION IN HUMAN COLORECTAL ADENOCARCINOMA CELL LINES

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Abbreviation List

MDR – Multidrug resistance

Pgp / ABCB1 – P-glycoprotein / ABC transporter B1

MRP-1 / ABCC1 – Multidrug resistance-related protein 1 / ABC transporter C1

MVP / LRP – Major vault protein / Lung resistance-related protein

BSO – L-Buthionine-sulfoximine

ABC – ATP-binding cassette

DMEM – Dulbecco's Modified Eagle's medium

RPMI-1640 – Roswell Park Memorial Institute-1640

PBS – Phosphate buffer saline

BCA – Bicinchoninic acid

IC_{50} – Concentration that inhibits the culture cell proliferation in 50%

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

5-FU – 5-fluorouracil

Abstract

Introduction: Multidrug resistance (MDR) is a condition defined by the cross-resistance to several non-structurally related drugs, representing one of the major setbacks to the success of chemotherapy. One of the best studied MDR mechanisms is the overexpression of efflux pumps, such as P-glycoprotein (Pgp), multiple resistance-related protein 1 (MRP-1) and major vault protein (LRP). These proteins confer resistance to a large spectrum of similar substrates, despite their different extrusion mechanisms. Pharmacologic inhibition of MDR transporters is the major strategy to overcome this phenotype. Verapamil is an L-type calcium-channel blocker and a modulator for Pgp and MRP-1. L-buthionine-sulfoximine (BSO) is a γ-glutamylcysteine synthase inhibitor and can be used to functionally decrease MRP-1 activity.

Aim: In this study we aim to compare transport kinetics for human colorectal adenocarcinoma cell lines, one sensitive and another resistant to chemotherapy, in the presence and absence of MDR reversers, using ^{99m}Tc-Sestamibi.

Methods: MDR proteins expression was evaluated in sensitive (WiDr) and resistant (LS1034) human colorectal adenocarcinoma cell lines. Intracellular and plasma membrane Pgp and MRP1, and LRP expression was analyzed by flow-cytometry; Pgp expression was also analyzed by western blot. Cellular transport kinetics was analyzed in the presence and absence of MDR modulators, verapamil and BSO, using ^{99m}Tc-Sestamibi. Uptake and retention studies were performed using sensitive and resistant cells. MDR modulation was evaluated by performing retention studies in resistant cells after incubation with the referred drugs, for different time intervals (10 and 60 minutes) and concentrations (10, 25, 50 and 100 μM).

Results: Pgp and MRP-1 expression was significantly higher ($p<0.05$) in resistant cells when comparing with the sensitive ones, although LRP was also expressed. Western blot

studies confirmed flow-cytometry results. ^{99m}Tc-Sestamibi uptake and retention percentage were significantly higher ($p<0.05$) in the sensitive cell line, comparing with the resistant one for all time-points considered. In resistant cells incubated with MDR modulators there were no statistically significant differences ($p>0.05$) when considering the curves as a whole. However, for the first minutes after incubation with ^{99m}Tc-Sestamibi, there were differences among the MDR modulators used.

Conclusions: *In vitro* kinetic studies using ^{99m}Tc-Sestamibi could be an indicator of MDR phenotype in colorectal adenocarcinoma cells. As the modulators used showed a reversion of the retention profile only for the first minutes, their administration should occur immediately before the administration of cytotoxic drugs.

Key-words: Multidrug resistance, P-glycoprotein, MRP-1, LRP, ^{99m}Tc-Sestamibi

Resumo

Introdução: A multirresistência a fármacos (MDR) é definida pela resistência cruzada a diversos fármacos não relacionados estruturalmente, representando um dos principais factores de insucesso da quimioterapia. Um dos mecanismos mais estudados de MDR é a sobre-expressão de proteínas de efluxo, como a glicoproteína P (Pgp), a *multiple resistance-related protein-1* (MRP-1) e a *major vault protein* (LRP). A inibição farmacológica dos transportadores associados a MDR é a principal estratégia investigada para superar este fenótipo. O verapamil é um bloqueador dos canais de cálcio do tipo L e um modulador da Pgp e da MRP-1. A L-butionina-sulfoximina (BSO) é um inibidor da γ-glutamilcisteina sintetase e pode ser utilizada para diminuir a actividade funcional da MRP-1.

Objectivo: Neste estudo pretendemos comparar a cinética de transporte para linhas celulares de adenocarcinoma colorrectal, uma sensível (WiDr) e outra resistente (LS1034), na presença e ausência de reversores de MDR, utilizando ^{99m}Tc-Sestamibi.

Métodos: A expressão de proteínas MDR foi avaliada nas duas linhas celulares referidas. A expressão de Pgp e MRP-1 intracelular e membranar, e a expressão de LRP foram analisadas por citometria de fluxo; a expressão de Pgp foi também analisada por *western blot*. A cinética de transporte foi analisada na presença e ausência dos moduladores de MDR verapamil e BSO, usando ^{99m}Tc-Sestamibi. Foram efectuados estudos de captação e retenção utilizando células sensíveis e resistentes. A modulação de MDR foi avaliada pela realização de estudos de retenção em células resistentes após a incubação com os fármacos referidos, durante diferentes intervalos de tempo (10 e 60 minutos) e concentrações (10, 25, 50 e 100 μM).

Resultados: A expressão de Pgp e MRP-1 foi significativamente superior ($p<0,05$) nas células resistentes, embora a LRP também estivesse expressa. Os estudos de *western blot* confirmaram os resultados da citometria de fluxo. A captação e retenção de ^{99m}Tc-Sestamibi

foram significativamente superiores ($p<0,05$) na linha celular sensível para todos os tempos de amostra considerados. Nas células resistentes incubadas com moduladores de MDR não houve diferenças estatisticamente significativas ($p>0,05$) quando se consideraram as curvas como um todo. No entanto, para os primeiros minutos após a incubação com ^{99m}Tc-Sestamibi, houve diferenças entre as células incubadas com os moduladores.

Conclusões: Estudos cinéticos *in vitro* com ^{99m}Tc-sestamibi podem ser um indicador de fenótipo MDR em células de adenocarcinoma colorrectal. Como os moduladores usados apenas mostraram reversão do perfil de retenção para os primeiros minutos, a sua administração deverá ser feita imediatamente antes da administração de citotóxicos.

Palavras-chave: Multirresistência a fármacos, Glicoproteína-P, MRP-1, LRP, ^{99m}Tc-Sestamibi

Introduction:

Multidrug resistance (MDR) is a condition defined by the cross-resistance to several non-structurally related drugs. It represents one of the major setbacks to the success of chemotherapy, as drugs do not take effect on tumor cells. It was observed in several hematologic and solid tumors [1].

Several mechanisms have been described to explain this phenomenon: (1) changes in drug and plasma membrane interaction, (2) drug metabolism modifications, (3) intracellular drug sequestration, (4) development of DNA repair mechanisms, (5) evasion of drug-induced apoptosis, (6) expression of vault particles, (7) changes in tumor microenvironment and/or (8) expression of cancer stem cells, which acquire most of the previous mechanisms [2].

One of the best studied MDR mechanisms is the overexpression of efflux pumps, such as those belonging to the ATP-binding cassette (ABC) transporters superfamilly [2].

ABC-transporters are preferentially expressed in plasma membranes, although they are also expressed in several intracellular organelles. Seven ABC-subfamilies have been described in humans, labeled from ABCA to ABCG [2,3].

These proteins are present in blood-brain barrier, blood-testis barrier, placenta, gastrointestinal tract, liver and kidney, playing an important role in the protection against xenobiotics and endogenous metabolites [2,3,4].

Fifteen ABC transporters, belonging to ABCB, ABCC and ABCG subfamilies, have been associated with MDR up to date [2]. The first one described was ABCB1, also known as P-glycoprotein (Pgp), a 170 kDa glycoprotein that transports several substrates against concentration gradients [5,6]. These not structurally-related substrates are hydrophobic, have a molecular mass between 300 and 2000 kDa and most of them are cationic at physiologic pH [7].

Another ABC transporter associated with MDR, identified in 1992, is ABCC1, also known as multidrug resistance-related protein 1 (MRP-1) [8]. It has a molecular weight of 190 kDa and recognizes most of the cytotoxic substrates that are recognized by ABCB1, despite only presenting a 15% homology with this pump. It also transports lipophilic anions as glutathione, sulfate or glucuronate conjugates and by co-transport with glutathione without conjugation [6,9,10].

Vaults are ribonucleoprotein complexes that have been detected in several organisms and are composed by different proteins. One of them, the major vault protein (MVP), discovered in 1993 and named lung resistance-related protein (LRP), has been associated with MDR as it is overexpressed in several resistant cell lines [11,12,13].

Pharmacologic inhibition of MDR transporters is the major strategy to overcome this phenotype. They can either be a substrate of these transporters, modulating their activity by competitive inhibition, or they can induce conformational changes in these proteins, preventing substrate recognition or ATP hydrolysis. Verapamil, an L-type calcium-channel blocker and a first generation MDR modulator, modulates ABCB1 and ABCC1 activity [14,15]. Other MDR modulators were developed aiming to reduce toxicity and increase efficacy [14].

Another way to functionally inhibit MDR pumps is depleting the cell from substrates essential to its function. L-buthionine-sulfoximine (BSO) is an inhibitor of γ-glutamylcysteine synthase, an enzyme involved in *de novo* glutathione synthesis. Consequently, the glutathione reduced levels within the cell lead to a lower activity of the glutathione-dependent pump ABCC1 [16].

^{99m}Tc-Sestamibi is a radiotracer used in myocardial perfusion studies. Its uptake occurs by passive diffusion due to transmembrane electric potential, and it accumulates in mitochondria [17]. This radiotracer is cationic and mildly lipophilic, which are common

characteristics of several substrates of MDR efflux pumps. Therefore ^{99m}Tc-Sestamibi could be a possible probe for functional activity of this type of proteins [18].

In this study we aim to compare transport kinetics for two human colorectal adenocarcinoma cell lines, one sensitive and another resistant to chemotherapy, in the presence and absence of MDR reversers, using ^{99m}Tc-Sestamibi.

Materials and Methods

Cell Culture: Sensitive WiDr (CCL-218, ATCC) and resistant LS1034 (CRL-2158, ATCC) human colorectal adenocarcinoma cell lines were obtained from American Type Culture Collection (Rockville, MD, USA). These cell lines were cultured in 5% CO_2 atmosphere, at 37ºC using Dulbecco's Modified Eagle's medium (DMEM, Sigma) for WiDr cell line and Roswell Park Memorial Institute-1640 (RPMI-1640, Sigma) for LS1034 cell line, both supplemented with 10% fetal calf serum (Gibco) and 1% of antibiotic-antimycotic (Gibco 15240).

MDR Characterization

Flow Cytometry: In order to study/characterize the expression of MDR proteins, membrane and intracellular Pgp and MRP-1 and intracellular LRP were assessed by flow cytometry (Santa Cruz biotechnology, Inc). The analysis was performed using a six-parameter, four-color FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) equipped with a 15 nW argon laser. For each assay, 10^6 cells were used and data on at least 10.000 events was collected using Cell Quest software (Becton Dickinson) and analyzed using Paint-a-gate software (Becton Dickinson).

Western Blot: Both cell lines were cultured in 25mm Petri dishes in order to prepare the protein extracts. Cells were washed twice with cold phosphate buffer saline (PBS; in mM: 137 NaCl, 2.7 KCl, 10 Na_2HPO_4, and 1.8 KH_2PO_4 [pH 7.4]), and lysed in Ripa buffer (150 mM NaCl, 50 mM Tris, 5 mM EGTA, 1% Triton X-100, 0.5% DOC, 0.1% SDS) supplemented with complete miniprotease inhibitor cocktail tablets (cOmplete Mini, 11836153001, Roche).

Protein concentration was determined by bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, Rockford, IL, USA) and samples containing equal amount of protein

were used for immunoblots, after adding 6x concentrated sample buffer (0.5 M Tris, 30% glycerol, 10% SDS, 0.6 M dithiothreitol [DTT], 0.012% bromophenol blue) and heating the samples for 5 minutes at 95°C. Proteins were separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred electrophoretically to polyvivylidene fluoride membrane (Amersham Hybond^{TM}-P, GE Healthecare). Prestained molecular weight marker proteins were used as standards for the SDS–PAGE. A ponceau staining was performed to verify the quality of the transfer. The membranes were blocked for 1 hour at room temperature in PBS containing 0.1% Tween-20 (TBS-T) and 5% low-fat milk. Incubation with the primary antibodies (Anti-P-Glycoprotein Mouse monoclonal antibody, 517310 from Calbiochem) was performed overnight at 4°C. After washing for 1 hour in TBS-T with 0.5% low-fat milk, the membranes were incubated for 1 hour at room temperature with an alkaline phosphatase-linked secondary antibody (anti-mouse IgG+IgM, NIF1316 from Sigma, 1:20,000 in TBS-T with 1% low fat milk). Immunoblots were developed using the Enhanced Chemi-Fluorescence system (ECF; GE Healthcare) and a Storm device (Molecular Dynamics, GE Healthcare). The membranes were then reprobed and tested for β-actin immunoreactivity (Anti-β-Actin Mouse monoclonal antibody, A5441 from Sigma, 1:5,000) to prove that similar amounts of protein were applied in the gels.

Kinetic Studies

Preparation and Quality Control of ^{99m}Tc-Sestamibi: ^{99m}Tc-Sestamibi (Cardiolite®, Bristol-Meyers and Squibb) was prepared by reconstitution of a sterile lyophilized kit formulation. This kit was reconstituted with 1480 MBq of sterile sodium pertechnetate to a final radioactive concentration of 370 MBq/ml. The radiochemical purity was determined by ascending microchromatography using Baker-Flex #1 B-F (J.T. Baker, USA) as stationary phase and ethanol as mobile phase. The radiopharmaceutical was spotted

approximately 1 cm from the bottom of the strip after spotting first in the same application point with an ethanol drop.

Uptake Studies: To perform uptake cell studies, cells were washed with PBS, then harvested with a solution of 0.25% trypsin/EDTA and finally re-suspended in medium at 2×10^6 cells/ml in 25 cm^2 flasks. The flask was incubated at 37°C in 95% O_2 and 5% CO_2 during one hour.

Subsequently, ^{99m}Tc-Sestamibi was introduced in the medium at 2×10^6 cells/0.2 MBq/ml after achieving steady state conditions. Triplicate samples of 200 µL were removed to eppendorfs containing chilled PBS for determination of tracer uptake at 5, 15, 30, 60, 90 and 120 min. During tracer uptake studies, for every sample token, cells were re-suspended in order to ensure uniformity. Cell suspensions were then centrifuged at 10.000 rpm for 1 min, followed by aspiration of the supernatant. Radioactivity of cell pellets and supernatants were measured separately with a well-type gamma counter (DPC Gamma C12) to determine tracers' uptake percentage in the cells. Cell viability was assessed by trypan blue exclusion test at the conclusion of experiments.

Retention Studies: To perform retention studies the cells were submitted to the same procedures as described before. After achieving steady state conditions, the radiopharmaceutical was added so that cells could uptake ^{99m}Tc-Sestamibi during one hour. After this, cells were centrifuged at 1000 rpm for 5 min at 4ºC, followed by the aspiration of the supernatant that was replaced by radiopharmaceutical free medium. Triplicate samples of 200 µL were removed to eppendorfs containing chilled PBS for determination of tracer retention at 1, 2.5, 5, 10, 15, 30, 45, 60, 90 and 120 min. Cell suspensions were then centrifuged at 10.000 rpm for 1 min, followed by aspiration of the supernatant. Radioactivity of cell pellets and supernatants were measured separately with a well-type gamma counter

(DPC Gamma C12) to determine tracers' uptake percentage in the cells. Cell viability was assessed by trypan blue exclusion test at the conclusion of experiments.

Cell Proliferation Assays

To perform cytotoxicity studies, cells were grown to 90% confluence and harvested by treating with 0.25% trypsin with 0.02% EDTA. For each experiment, cells were plated in 24 multiwells (Corning Costar Corp), in a concentration of 50.000cells/mL and kept in the incubator overnight, in order to allow their attachment. Cell plates were then incubated with different concentrations of verapamil (Sigma V4629) and BSO (L-Buthionine-sulfoximine, Sigma B2515), ranging from 1 to 200 µM.

For every experiment two controls were performed, no treatment cells and cells incubated with the vehicle of administration of the drugs alone, which is dimethyl sulfoxide (DMSO, D8418) for verapamil and water for BSO. Each experiment was performed in duplicate and repeated in three sets of tests.

The sensitivity of the cell lines to the previously referred drugs was accessed using the MTT colorimetric assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma–Aldrich, Inc.; Sigma M2128) to measure cell proliferation. Yellow MTT is reduced to purple formazan in the mitochondria of living cells [19]. Therefore, the purple formazan products formation can be quantified measuring absorbance at the wavelength of 570 nm.

Cytotoxicity was expressed as the percentage of inhibition of cell proliferation correlated with the cultures treated with the vehicle of administration for each drug. This procedure allows the determination of the concentration that inhibits the culture cell proliferation in 50% (IC_{50}), which was determined using OriginPro, version 7.

MDR modulation

To evaluate MDR modulation, retention studies were performed in the resistant cell line (LS1034) in the presence of verapamil and BSO prior to incubation with ^{99m}Tc-Sestamibi. The procedure used was the same as described before; however, after achieving steady state conditions and before adding the radiopharmaceutical to the cells, they were incubated for different periods of time (10 and 60 minutes) with different concentrations of the referred drugs (ranging from 10 to 100µM). These different conditions are summarized in Table I.

Drug / **Period of incubation**	*Verapamil*	*BSO*
10 minutes	25µM 50µM	10µM 50µM
60 minutes	100µM	100µM

Table I: Concentrations and incubation periods for each drug used in retention studies for MDR modulation.

Statistical analysis

Results were analysed using Statistica package, version 7 and SPSS, version 16, at a significance level of 5%. For kinetic studies, time curves were compared as a whole by applying the general linear model (repeated measures ANOVA within time and one factor: incubation condition). For each time point, each condition was compared using one factor ANOVA test, with a post-hoc analysis using Tuckey's method. To compare flow cytometry data we used Student's t test for two independent samples, after confirming normal distribution by the Kolmogorov-Smirnov test.

Results

Flow Cytometry

Pgp expression analysed with flow cytometry was significantly higher ($p<0.05$) in the resistant cell line, when comparing to the sensitive one, both in the plasma membrane and intracellular organelles. MRP-1 expression in plasma membrane was also significantly higher in the resistant cell line ($p<0.05$). There were no statistical differences ($p>0.05$) between the two cell lines regarding to LRP and intracellular MRP-1 expression. Fluorescence intensity average (FIA) for each of the studied proteins is represented in Fig.1.

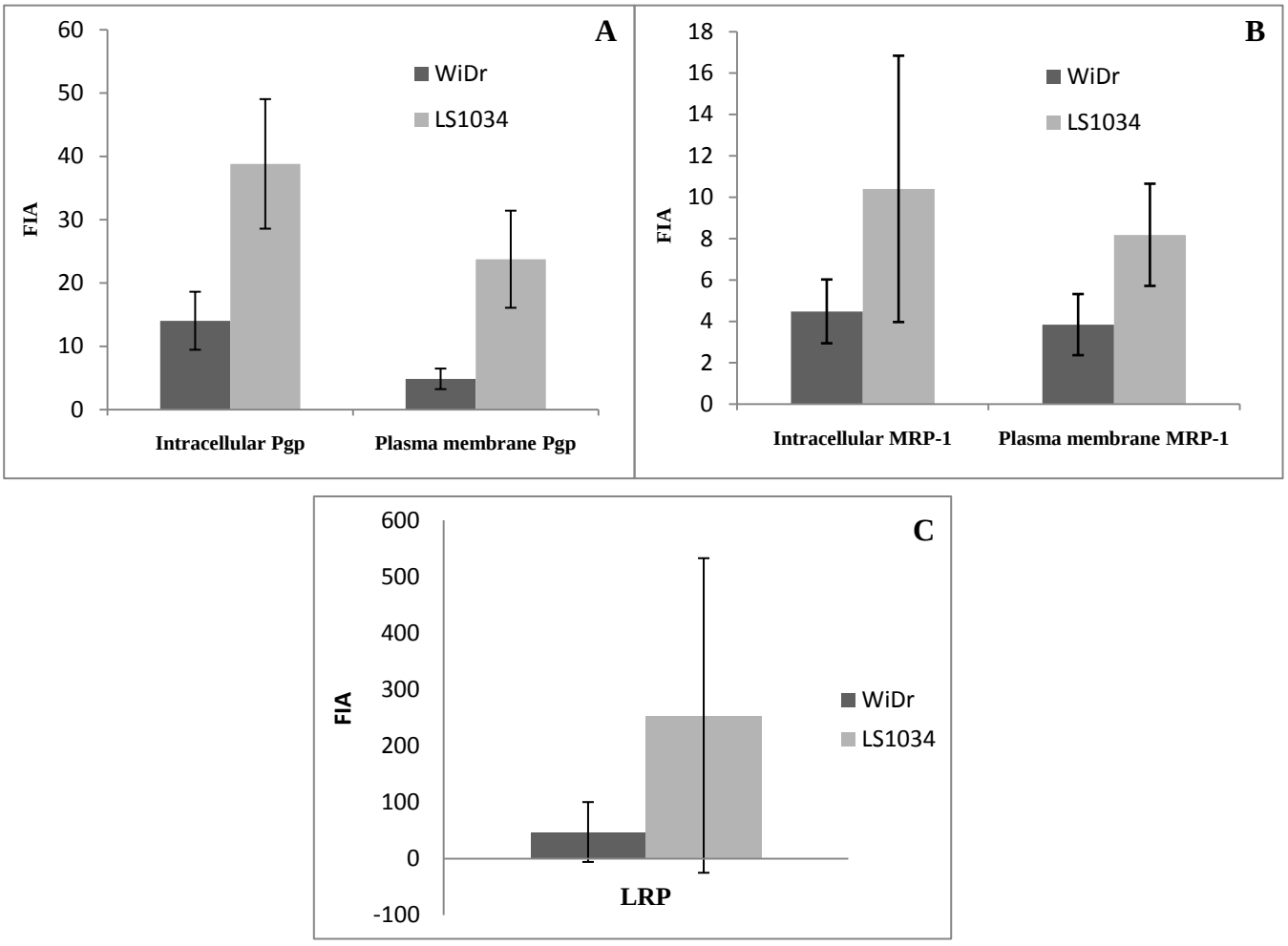

Fig. 1: Expression of Pgp (A), MRP-1 (B) and LRP (C) assessed by flow cytometry. Bars represent fluorescence intensity average (FIA). Error lines represent standard deviation.

Western Blot

Pgp expression was also analysed using Western Blot techniques. Equal amounts of protein extracts from the two colorectal adenocarcinoma cell lines were applied into the gel. Incubation with anti-Pgp antibody showed a higher intensity of staining in the resistant cell line for a molecular weight of 170 kDa, which is consistent with a higher expression of Pgp (Fig.2A). Incubation with anti-β-Actin antibody confirmed that similar amounts of protein were applied in the gel, as the staining intensity was similar in both cell lines (Fig.2B).

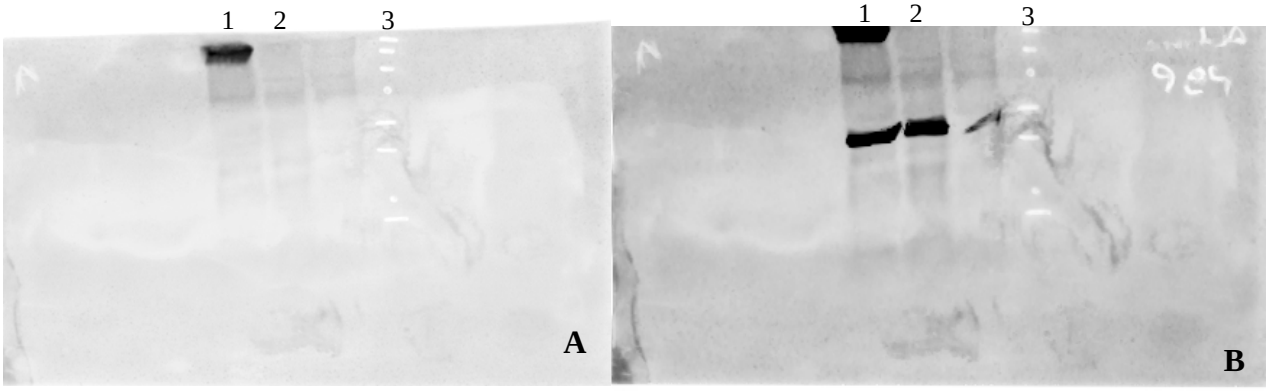

Fig. 2: Western blot membrane after incubation with anti-Pgp (A) and anti-actin (B) antibodies. In this membrane there are samples of WiDr and LS1034. Wells 1, 2 and 3 correspond to WiDr, LS1034 and weight control, respectively.

Kinetic studies without modulators

Uptake studies

Uptake time curves for WiDr and LS1034 cell lines are represented in Fig.3A. An uptake peak can be observed after 60 minutes of incubation of WiDr with ^{99m}Tc-Sestamibi, with an average uptake percentage of 18.86% and a standard deviation of 6.49%. For LS1034, uptake percentage was mostly constant, varying between 1.36% and 1.85%. When comparing the two cell lines there where observed statistically significant differences ($p<0.05$) for every time-point considered, as well as when considering the curve as a whole.

Cell viability, assessed by trypan-blue exclusion test, was higher than 85% for all performed assays.

Retention studies

Retention curves for both WiDr and LS1034 are represented in Fig.3B. There is a higher retention percentage for WiDr when comparing to LS1034 ($p<0.05$) for every time-point considered, as well when considering the curve as a whole. Five minutes after incubation with ^{99m}Tc-sestamibi, sensitive cells showed a mean retention percentage of 82.99%, with a standard deviation of 4.79% while the resistant ones showed a mean of 24.75%, with a standard deviation of 5.82%. After one hour, both curves almost overlap at approximately steady retention percentages, with mean values of 6.51% for WiDr and 3.42% for LS1034.

Cell viability, assessed by trypan-blue exclusion test, was higher than 85% for all performed assays.

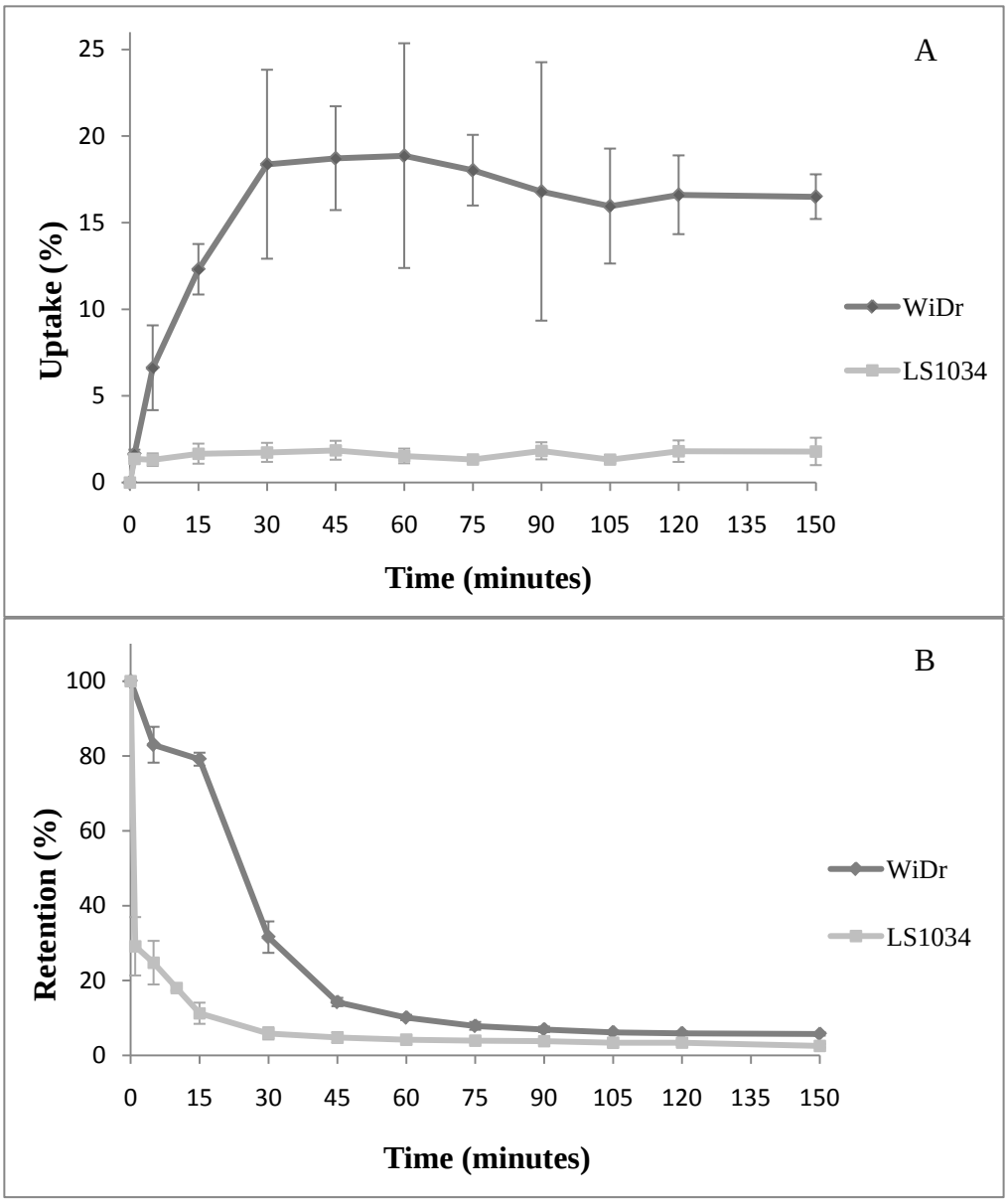

Fig.3: Uptake curves (A) and retention curves (B) obtained through ^{99m}Tc-Sestamibi for WiDr and LS1034 cell lines. Curves represent retention percentage through time. Error lines represent standard deviation for each time point considered.

Cell proliferation assays using MDR modulators

MTT assays were performed in LS1034 cell line using verapamil and BSO, in concentrations ranging from 1 to 200µM. Proliferation percentage correlated to control was determined at for 24h, 48h, 72h and 92h of incubation. Cytotoxicity curves were drawn and

IC_{50} was determined for each drug and period of incubation. IC_{50} values are shown in Table II.

Drug / **Incubation**	*Verapamil*	*BSO*
24h	90,0 ± 10,88 µM	>200 µM
48h	39,0 ± 6,76 µM	>200 µM
72h	28,4 ± 8,00 µM	>200 µM
96h	26,6 ± 4,20 µM	>200 µM

Table II: IC_{50} values obtained for LS1034 cells incubated with Verapamil and BSO for periods of time of 24h, 48h, 72h and 96h.

Proliferation obtained for all BSO concentrations tested was similar to that of the control well; therefore, IC_{50} for this drug was not determined for LS1034 cell line.

Retention studies using MDR modulators

Retention curves obtained are shown in Fig.4.

Incubation with verapamil (Fig.4A): Regarding the curves as a whole, there were no statistically significant differences ($p>0.05$) between the conditions studied. However, considering time-points of 1, 2.5 and 5 minutes, there were significant differences ($p<0.05$) between those conditions, that are due to a lower retention in cells that were not incubated with the modulator. There were no differences between different concentrations or periods of incubation for any of the time-points considered.

Incubation with BSO (Fig.4B): Considering all the time-points, there are no statistical differences ($p>0.05$) between the conditions studied. However, for time-points of 1, 2.5 and 5 min there were differences between the retention percentages of those conditions, which as lower for LS1034 cell line incubated without the modulator.

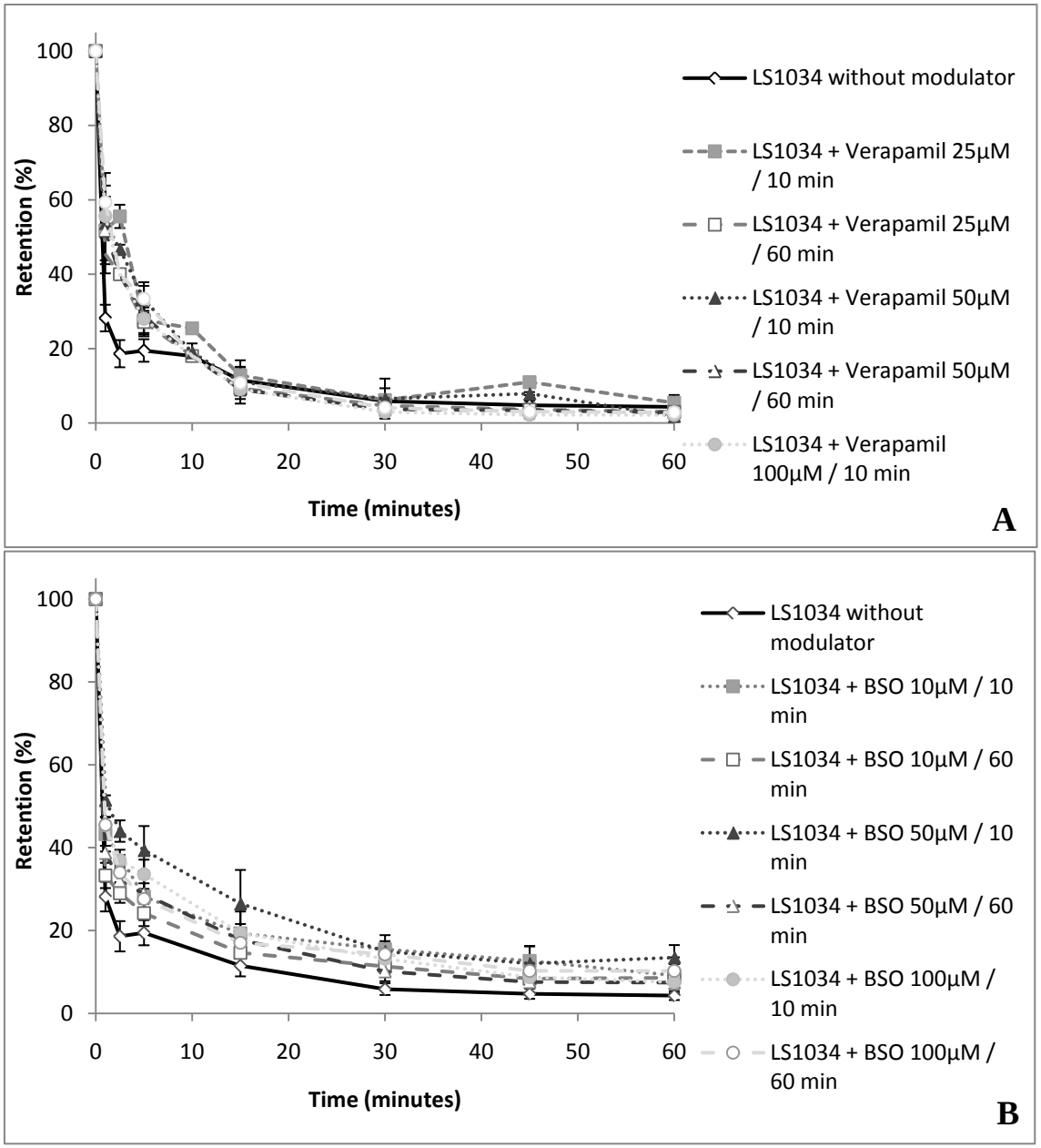

Fig. 4: Retention curves to study MDR modulation for LS1034, using verapamil (A) and BSO (B). Curves represent retention percentage through time. Error lines represent standard deviation for each time point considered.

Discussion

The main goal of this work was to evaluate the use of radioisotopic methods to identify resistance mechanisms mediated by efflux pumps, using ^{99m}Tc-Sestamibi. This radiotracer is cationic and hydrophobic, similar to the characteristics of ABC-transporters substrates [7]. Therefore the expression of these proteins in tumor cells may influence the uptake and efflux kinetics of ^{99m}Tc-Sestamibi and, in this case, this radiotracer could give information about their expression and become an important diagnosis tool.

In this study we first evaluated MDR proteins expression in two colorectal adenocarcinoma cell lines, one of them known to be sensitive (WiDr) and other resistant (LS1034) to chemotherapy. Resistance to treatment with 5-fluorouracil (5-FU), the first choice for colorectal adenocarcinoma chemotherapy, is common [20]. Resistance to oxaliplatin and irinotecan, two drugs also used in the treatment of this type of cancer, is also described, being the last one a known substrate for MRP-1 [21,22]. It is therefore useful to evaluate the types of resistance mechanisms that are present in these types of tumors. As ABC-transporters are expressed in normal intestinal epithelial cells [23], this could represent a resistance mechanism in colorectal adenocarcinoma.

After characterizing MDR proteins expression, we accessed if ^{99m}Tc-Sestamibi could be used as a probe for their expression in our cells lines. Its uptake and efflux kinetics could be influenced by the expression of these proteins but also by mitochondria density within the cell and physical properties of the membrane. We compared uptake and retention curves in both the sensitive and the resistant cell line in order to determine if ^{99m}Tc-Sestamibi is a good probe for MDR in colorectal adenocarcinoma cell lines. The same studies were performed using different modulators of ABC-transporters, in order to access MDR reversion in the resistant cell line.

MDR proteins expression

Flow cytometry analysis of Pgp, MRP-1 and LRP expression showed that the resistant cell line LS1034 overexpressed Pgp and MRP-1 in the plasma membrane, as well as Pgp in intracellular organelles. This finding confirms the resistant phenotype described for this cell line, the association of Pgp and MRP-1 overexpression with MDR as been well established by different studies using cell lines [3].

Western blot was used to confirm Pgp expression in cellular membranes for both cell lines. The comparison between different samples is done by observing staining intensity for the molecular weight of Pgp (170 kDa). Our results showed that LS1034 cells express Pgp. The staining intensity in the sensitive cell line, WiDr cells, was inexistent. β-actin staining confirmed that equal amounts of protein were applied into the gel, making the direct comparison between samples possible. These results are consistent with those obtained by flow cytometry, confirming the overexpression of Pgp in the resistant cell line and, therefore, its resistant phenotype.

Kinetic studies

Although Pgp and MRP-1 only have 15% of homology in aminoacid sequence [15] and have different transport mechanisms, it is known that these proteins transport a similar spectrum of substrates [9], being ^{99m}Tc-Sestamibi one of them [18,24].

Uptake kinetic studies using ^{99m}Tc-Sestamibi showed a higher uptake in WiDr when compared with that of LS1034. This radiopharmaceutical's uptake occurs by passive diffusion and due to transmembrane electric potential, accumulating also in mitochondria [17]. However, when cells are resistant, efflux occurs simultaneously with uptake, not allowing ^{99m}Tc-Sestamibi to diffuse into the mitochodria, which can lead to a lower uptake percentage of this radiotracer in cells which overexpress ABC-transporters. Therefore, the higher

expression of Pgp and MRP-1 in LS1034 cell line can justify the lower uptake percentage observed in kinetic studies. These results are consistent with those described in literature for other cell lines [18,25,26,27,28,29,30,31].

Retention studies using the same radiopharmaceutical showed a lower retention percentage for the resistant cell line, with a faster extrusion from the cell. This result is also correlated with the higher expression of ABC-transporters observed in LS1034 cell line, as their overexpression could lead to a faster and ATP-dependent efflux of ^{99m}Tc-Sestamibi. These results are consistent with those found on literature [18,25]. The faster extrusion observed for ^{99m}Tc-Sestamibi is related to the resistant phenotype described for these cells, as cytotoxic drugs could also be transported out of the cell before having the desired effect.

MDR modulation evaluation

The main strategy to overcome MDR is the use of modulators and/or inhibitors of efflux pumps. In this study we used a first generation MDR modulator, verapamil, a substrate for Pgp known to modulate its activity by competitive inhibition, as well as MRP-1 function [25,32]. We also used a γ–glutamylcysteine synthase inhibitor, BSO, in order to inhibit MRP-1 function, as it depends on glutathione to transport many substrates.

Retention studies using these modulators showed a difference in the retention percentage between the resistant cells incubated without any modulator and those incubated with one of the previously referred drugs, for the first five minutes after incubation with ^{99m}Tc-Sestamibi. However, retention curves tend to overlap, as the studies proceeds. This result could represent a reversion for the first minutes after incubation and does not seem to be dependent on time of incubation with the modulator or its concentration, for both drugs used.

Utsunomiya *et al.* performed retention studies in a nasopharyngeal carcinoma cell line, in the presence and absence of several MDR modulators, including verapamil and BSO, using ^{99m}Tc-Sestamibi. Incubation with both these modulators resulted in a lower efflux percentage at 20 min after incubation with the radiotracer, comparing to control cells [33]. Studies performed by Gomes *et al.* in osteosarcoma cell lines also showed higher ^{99m}Tc-Sestamibi retention through time in cells incubated with these modulators, when comparing to controls [25]. In our study, however, the reversion induced by these two modulators was only verified for a short period of time. This could be explained by the involvement of additional transporters in efflux of this radiotracer, leading to a lower reversion on the retention rate in LS1034 cells.

Another factor that may influence the results obtained is the period of incubation with BSO. Its mechanism of action is the depletion of glutathione, therefore inhibiting the function of MRP-1 [16]. A higher period of incubation could be necessary to deplete glutathione within the cell in a higher grade, so that a better reversion of MDR could be seen in these cells, as they express MRP-1. Meijer *et al.* described that after 24h of incubation with BSO, glutathione is no longer present within several different cells, without growth delay or loss of viability [34]. Studies using BSO as a MDR reverser used this drug for periods of incubation of 24h or incubation overnight [24,26,33]. Therefore, a similar period of incubation with BSO in our cell line could perhaps result in a more significant reversion of MDR.

Conclusions:

In this study we observed that MDR proteins Pgp and MRP-1 are overexpressed in the colorectal adenocarcinoma resistant cell line LS1034, when comparing to sensitive WiDr cells. This result was confirmed using two different techniques, flow cytometry and western blot.

Kinetic studies comparing both cell lines showed a lower uptake and retention percentages of ^{99m}Tc-Sestamibi in the resistant cell line LS1034, which are related to the higher expression of efflux pumps in this cell line, as these proteins can promote a fast extrusion of different substrates, including ^{99m}Tc-Sestamibi.

Regarding MDR modulation retention studies performed in resistant cells, we observed a higher retention percentage in cells incubated with verapamil and BSO for the initial minutes, when comparing to that observed in the same cells incubated without any of the modulators. Retention curves tended to overlap over time, indicating that these findings could represent a reversion for the initial minutes after incubation with the radiotracer, which does not seem to depend on time of incubation or concentration of any of the modulators used.

In summary, we established a correlation between efflux pumps expression and the retention of ^{99m}Tc-Sestamibi within colorectal adenocarcinoma cell lines. Therefore, *in vitro* kinetic studies could indicate MDR phenotype in this type of cells.

As the modulators used during these experiments showed a reversion of the retention profile only for the first minutes of the retention studies, their administration should occur immediately before the administration of cytotoxic drugs.

Future perspectives

In this study we compared two different cell lines, one sensitive and one resistant to chemotherapy. In future studies it would be important characterize MDR in resistant adenocarcinoma cells obtained by exposing the sensitive ones, WiDr cells, to increasing concentrations of 5-fluorouracil, oxaliplatin and irinotecan, three of the most used cytotoxic drugs in the treatment of these types of tumours. This would minimize the influence of other factors in the kinetics of ^{99m}Tc-Sestamibi uptake and efflux, such as plasma membrane fluidity, that could be different in our cell lines, giving their different origin. I would also be important to study the actual reversion of MDR using verapamil and BSO by performing cytotoxicity assays using the different chemotherapeutical agents referred before.

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