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Human Sarcoma Cell Lines MES-SA and MES-SA/Dx5 as a Model for Multidrug Resistance Modulators Screening

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Abstract. *The choice of cell lines for multidrug resistance (MDR) modulators screening may affect the results obtained. Screening is most often performed in model systems which employ cell lines derived from haematological malignancies. Cell lines originating from solid tumours are far less popular. In the present work, we aimed to test the usefulness of the drug-sensitive human sarcoma cell line MES-SA, and its multidrug-resistant counterpart MES-SA/Dx5, as a model system for modulators' anti-MDR potency evaluation. Overexpression of P-glycoprotein in the resistant but not in the sensitive cell line was confirmed by flow cytometry and confocal microscopy. Flow cytometry demonstrated that verapamil and trifluoperazine reduced MDR in MES-SA/Dx5 cells as assessed by the rhodamine 123 accumulation test. Both modulators also restored in MES-SA/Dx5 cells the drug accumulation pattern typical for sensitive cells, as judged by confocal microscopy. We conclude that the MES-SA and MES-SA/Dx5 cell line pair constitute a good model for MDR modulators study.*

Malignant cancers are among the most important causes of mortality in the western world and constitute a persistent challenge to medical science. Various forms of cancer cells resistance to chemotherapy are responsible for frequent therapy failures. Beyond a doubt, multidrug resistance (MDR) is the best known phenomenon in this respect. Overexpression of transmembrane proteins belonging to the ABC-transporter superfamily is typical for MDR cells. These transporters (*e.g.* P-glycoprotein, multidrug resistance associated proteins) act as ATP-dependent efflux pumps, characterized by extremely wide substrate specificity (1, 2). Due to transporter proteins overexpression, MDR cells are insensitive to a wide spectrum of chemotherapeutic agents. A great number of structurally divergent compounds, whose molecules are amphiphilic and neutral or positively-charged, belong to the P-glycoprotein (P-gp) substrates (3). P-gp transport activity leads to a decrease of anticancer drug concentration inside the tumour cell that enables it to survive. MDR can be reduced, at least partially, by MDR modulators that inhibit pumping of the drugs out of the cell. Simultaneous administration to patients of a chemotherapeutic drug and an MDR modulator may improve the results of cancer therapy. Although the first clinical trials were not very encouraging, mainly because of inherent modulators' toxicity (4), the search for novel MDR modulating agents remains a task of great importance. Regularly numerous papers report the discoveries of promising new MDR modifiers (5-7). The quest for other methods that could reduce multidrug resistance in cancer cells, like *e.g.* use of antisense oligonucleotides (8) or small interfering RNA molecules (9), is also on going.

There are several methods used for screening of putative MDR modulators. One of the approaches is based on the measurement of restoring the cytotoxicity of a given chemotherapeutic drug towards the multidrug-resistant cancer cells in the presence of a modulator (10,11). The other common method of anti-MDR activity assessment is based on flow cytometric measurement of the effect exerted by putative modulators on fluorescent anticancer drug (*e.g.*

Abbreviations: PHMs, phenothiazine maleates; MDR, multidrug resistance; TFP, trifluoperazine; P-gp, P-glycoprotein; FCS, fetal calf serum; PBS, phosphate buffered saline; FITC, fluorescein isothiocyanate; FAR, fluorescence activity ratio.

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Key Words: Multidrug resistance (MDR), MDR modulators, P-glycoprotein, sarcoma, flow cytometry, confocal microscopy.

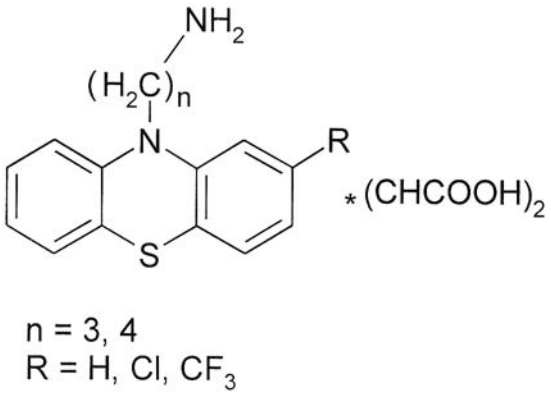

Figure 1. *Chemical structure of phenothiazine maleates. The compounds differ in the type of ring substituent (H– in 3HPhM and 4HPhM; Cl– in 4ClPhM and 3FPhM; CF_3– in 3FPhM and 4FPhM) and in the length of the alkyl bridge connecting the phenothiazine nucleus with side chain amino group (3 carbon atoms in 3HPhM, 3ClPhM, 3FPhM; and 4 carbon atoms in 4HPhM, 4ClPhM, 4FPhM).*

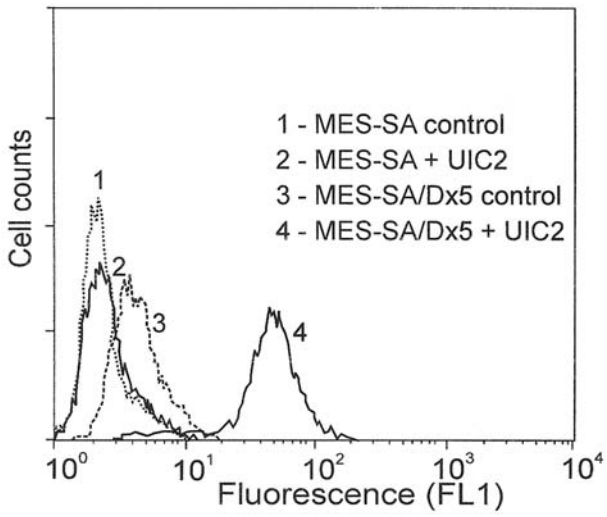

Figure 2. *P-glycoprotein expression in MES-SA and MES-SA/Dx5 cells assessed by anti-P-gp antibody UIC2.*

daunorubicin) accumulation inside resistant cells or its efflux out of MDR cells (12,13). Instead of anticancer agents, fluorescent substrates of MDR transporters, such as rhodamine 123 or other dyes, are commonly used (14,15). Rhodamine 123 has been shown to be a specific substrate of P-gp but not of multidrug resistance associated protein (MRP1) (16). The proper selection of a fluorescent substrate for drug accumulation or efflux studies is essential, though it should also be borne in mind that the choice of the pair of model cell lines, drug-sensitive and its multidrug-resistant counterpart, could influence the results obtained. The anti-MDR activity of different compounds assessed in different cell lines can rarely be compared directly, however, some common patterns have been noted (14). Multidrug-resistant cell lines derived from leukemias and lymphomas are often used as model systems (16-19), mainly due to their properties that make them a favourable object of flow cytometric analysis. Cell lines derived from solid tumours, that grow as monolayers and have to be detached from the surface before analysis by flow cytometry, are less popular.

To date, three pairs of sensitive and multidrug-resistant cell lines derived from sarcomas have been described. Reversal of MDR was studied in murine (20,21) and human (22,23) sarcomas and also in murine osteosarcoma cell lines (24). We decided to study the drug-sensitive human uterine sarcoma cell line MES-SA and its multidrug-resistant counterpart MES-SA/Dx5 as a model system for screening MDR modulators. MES-SA/Dx5 was derived from the sensitive cell line by prolonged *in vitro* exposure to doxorubicin (25). By means of flow cytometry and confocal microscopy, we demonstrated P-glycoprotein overexpression in the MES-SA/Dx5 cell line. MDR reversal in the MES-SA/Dx5 cell line by known modulators was shown by both methods. Additionally, some novel phenothiazine derivatives, that had been previously demonstrated to modulate multidrug resistance in mouse lymphoma cells (26), were tested.

Materials and Methods

Reagents. Trifluoperazine was purchased from ICN Biomedicals (Costa Mesa, USA). Verapamil, tamoxifen and rhodamine 123 were from Sigma (St. Louis, USA). Six phenothiazine maleates (3HPhM, 4HPhM, 3ClPhM, 4ClPhM, 3FPhM, 4FPhM) were synthesised as described in (27). Their chemical structures are shown in Figure 1.

Cell lines. The human uterine sarcoma cell line MES-SA (ATCC accession number CRL-1976) and its multidrug-resistant subline MES-SA/Dx5 (ATCC accession number CRL-1977), obtained by continuous *in vitro* exposure to doxorubicin (25), were purchased from the American Type Culture Collection (Manassas, USA). Both cell lines were grown in standard RPMI 1640 medium (Sigma), containing 10% FCS (Sigma) and antibiotics at 37˚C and 5% CO_2. Cells were detached from the culture flasks by treatment with a mixture of 0.25% trypsin and 0.05% EDTA (w/v) in PBS. The cells were washed and resuspended in RPMI 1640 medium without phenol red for drug accumulation assay or in PBS for immunotyping.

Flow cytometry. The fluorescence of the cell population was measured by flow cytometry using the Beckton Dickinson (Sunny Valley, USA) FACSCalibur equipped with a 488 nm argon laser. Five thousand events were registered and analysed with the use of Cell Quest® and PC Lysis software (Beckton Dickinson). The mean geometric channel of fluorescence was calculated for evaluation of the cell fluorescence intensity.

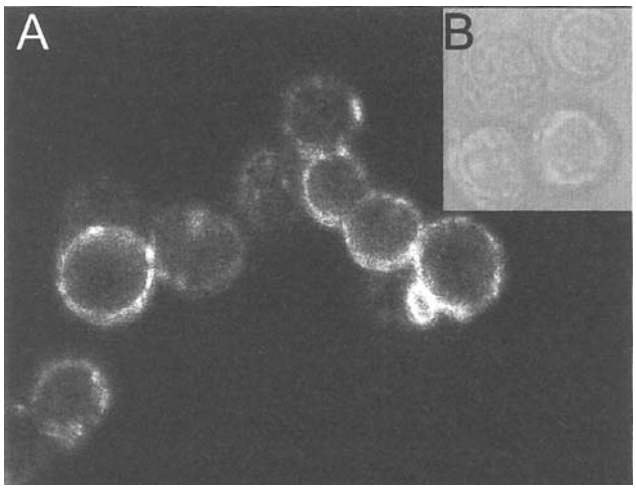

Figure 3. *Confocal microscopic images of MES-SA/Dx5 (A) incubated with anti-P-gp antibody UIC2. As MES-SA cells (B) showed no fluorescence, transmitted light image was presented for their visualisation. Final image magnification 800x.*

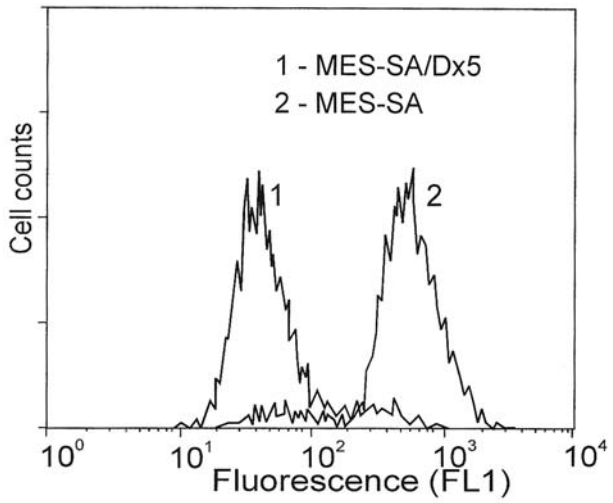

Figure 4. *Rhodamine 123 accumulation by MES-SA and MES-SA/Dx5 cells.*

P-glycoprotein immunodetection. Mouse UIC2 hybrydoma, producing UIC2 antibody (IgG 2a), was purchased from the American Type Culture Collection (accession number HB11027) and propagated *in vivo* in Balb/c mice. Peritoneal exudate was used to detect P-gp expression in indirect immunofluorescence assay. Cancer cells in PBS supplemented with 1% FCS ($1x10^5$ cells/sample) were incubated with UIC2 antibody (diluted 1:10 000 for flow cytometry and 1:2000 for confocal microscopy experiments) for 45 min at room temperature. The cells were then washed in PBS and incubated with secondary polyvalent goat anti-mouse immunoglobulins, FITC-conjugated antibody (Sigma), diluted 1:200, for 30 min at room temperature. After final washing, cells were resuspended in PBS for analysis. In the control experiments, cancer cells were incubated only with secondary FITC-labelled antibody or with non-related mouse IgG 2a immunoglobulin and secondary fluorescent antibody (isotypic control).

Drug accumulation studies. Both in the flow cytometric and confocal microscopy experiments, rhodamine 123 was used as a fluorescent P-gp substrate. Rhodamine 123, verapamil and trifluoperazine were dissolved in water, while PhMs and tamoxifen were dissolved in DMSO. Care was taken not to exceed 1% of DMSO concentration in the samples. Cells ($1x10^5$ cells/sample) in RPMI 1640 were incubated with MDR modulators (2 – 20 μg/ml) for 15 min at room temperature. Rhodamine 123 was then added (final concentration 2.6 μM) to the samples and incubation was continued for 60 min at 37°C. The cells were then washed twice and resuspended in PBS for analysis.

A fluorescence activity ratio (FAR) was calculated from the following equation (28) on the basis of measured fluorescence values (FL).

$$FAR = \frac{(FL_{mdr\ treated})/(FL_{mdr\ control})}{(FL_{parent\ treated})/(FL_{parent\ control})}$$

Confocal microscopy. The cells were prepared in the same way as for flow cytometry experiments. After final washing, 10 μl of cell suspension was deposited onto glass slides and mounted with coverslips. The edges were sealed and the slides were kept on ice until analysed. Transmitted light and fluorescent images were collected on a Bio-Rad Laboratories (USA) Confocal Imaging System (model MRC-1024ES) equipped with krypton-argon 488 nm laser. It was configured with a Zeiss Axiovert S100 microscope, equipped with a Plan NeoFluar oil 40x (1.30) objective. The emission filter 522DF35 employed allowed the collection of fluorescent light in the range 487–557 nm. LaserSharp (Bio-Rad Laboratories) software was used for image acquisition and analysis.

Results

P-glycoprotein expression. The human uterine sarcoma MES-SA cell line and its multidrug-resistant variant, MES-SA/Dx5, were chosen to test their usefulness as a model for screening MDR modulators. Expression of P-glycoprotein was demonstrated with the use of UIC2 mouse monoclonal antibody in the MES-SA/Dx5 line by means of flow cytometry and confirmed by confocal microscopy. In Figure 2, the histograms typical for both the sensitive and resistant line are presented. The histogram for parental MES-SA cells stained with anti-P-gp antibody was practically overlaid on that of the isotypic control. The fluorescence intensity of MES-SA/Dx5 cells incubated with UIC2 antibody was ten times higher than the fluorescence of control cells. The mean channel of fluorescence values were 41.3 and 4.2 for MES-SA/Dx5 and the isotypic control, respectively.

Expression of P-glycoprotein by the multidrug-resistant MES-SA/Dx5 cells was also demonstrated by means of

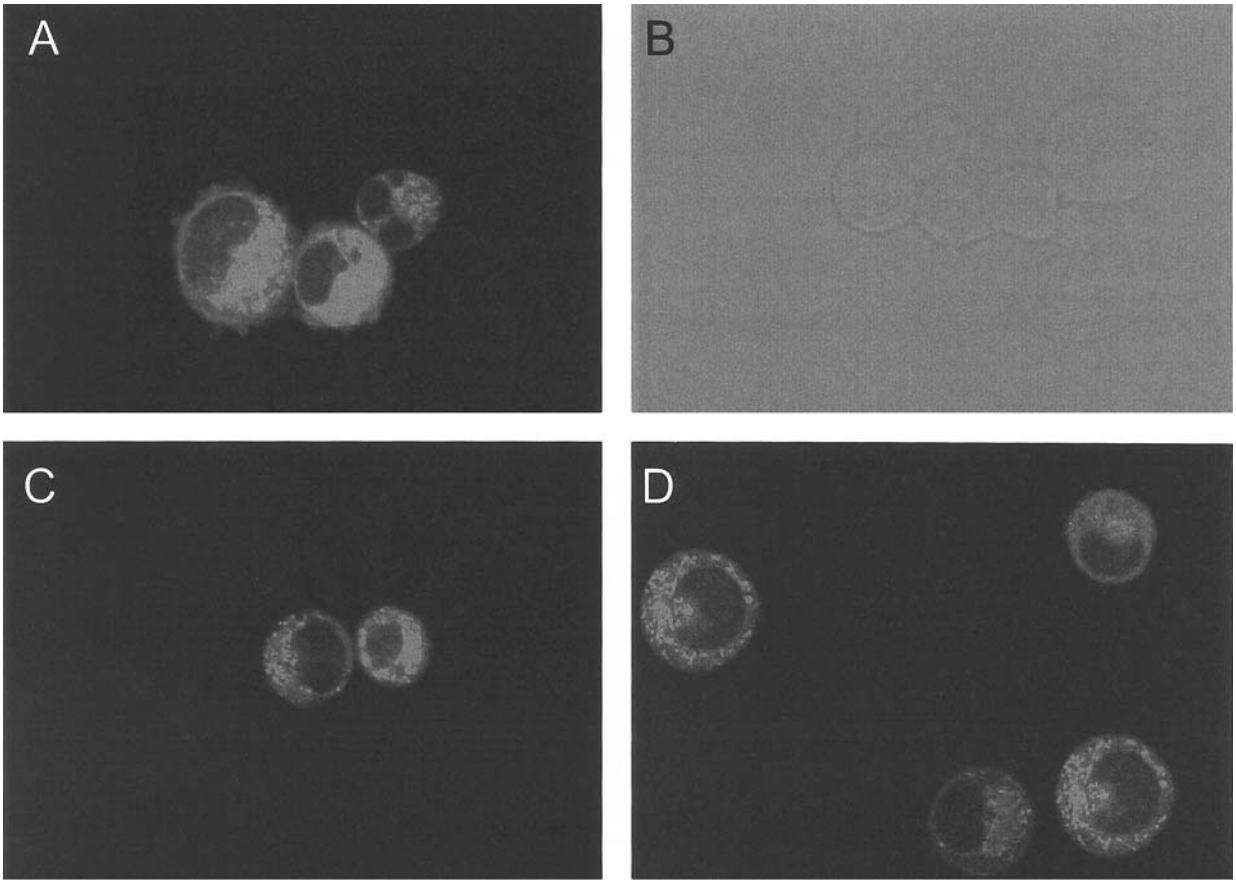

Figure 5. *Rhodamine 123 accumulation in MES-SA cells (A), MES-SA/Dx5 cells (B), MES-SA/Dx5 treated with 10 μg/ml of verapamil (C) and MES-SA/Dx5 treated with 10 μg/ml of trifluoperazine (D). As no fluorescence was observed in MES-SA/Dx5 cells, a transmitted light image was presented in order to visualise all cells. Final image magnification 800x.*

Table I. *The effect of MDR modulators on rhodamine 123 accumulation by multidrug-resistant MES-SA/Dx5 cells.*

Modulator [10 μg/ml]	Fluorescence activity ratio (FAR)
Verapamil	9.33
Trifluoperazine	5.24
3HPhM	1.83
4HPhM	1.87
3ClPhM	1.33
4ClPhM	1.71
3FPhM	1.71
4FPhM	1.32
Tamoxifen	2.20

confocal microscopy (Figure 3A). In the resistant subline, fluorescence was visible on the periphery of cells suggesting superficial localization of the target protein. Drug-sensitive MES-SA cells did not show any fluorescence under the experimental conditions (Figure 3B).

Drug accumulation studies. A drug accumulation assay was employed to study P-glycoprotein transport activity in cancer cells. Flow cytometric experiments showed that the drug-sensitive cell line MES-SA accumulated more rhodamine 123 than its multidrug-resistant derivative MES-SA/Dx5 (Figure 4). Mean channels of fluorescence recorded for the population of sensitive and resistant cells were 1139 and 109, respectively. Confocal microscopic images of MES-SA cells (Figure 5A) showed that the fluorescent probe was located mainly in the cytoplasm, whereas the nuclear areas of the cells seemed to be deprived of rhodamine 123. No fluorescent signal could be detected inside resistant MES-SA/Dx5 cells (Figure 5B).

Flow cytometry was also employed to study the ability of known MDR modulators to restore the sensitive cell-like pattern of rhodamine 123 accumulation in resistant MES-SA/Dx5 cells (Table I). Verapamil was found to be the most effective MDR modulator among all tested compounds. Trifluoperazine and tamoxifen were weaker inhibitors of P-glycoprotein, whereas the anti-MDR activity of newly

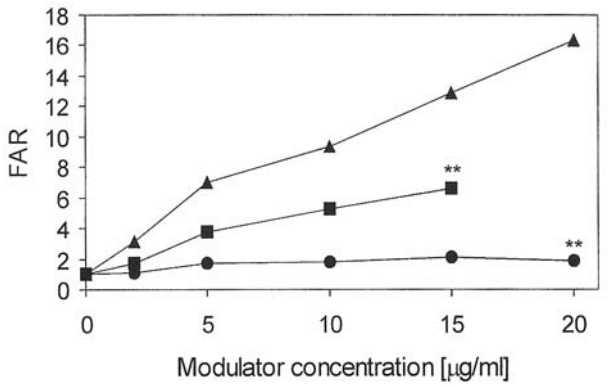

Figure 6. *The effect of verapamil (▲), trifluoperazine (■), and 3HPhM (●) on rhodamine 123 accumulation by MES-SA/Dx5 cells. Double asterisks mark the concentrations at which only 25% of cells remained alive.*

synthesised phenothiazine derivatives was only marginal. The most active compounds were 3HPhM and 4HPhM (derivatives without ring substituent) followed by chlorine-substituted 4ClPhM and trifluoromethyl-substituted 3FPhM. For verapamil and TFP, the extent of MDR reduction in MES-SA/Dx5 cells was directly proportional to the modulator concentration (Figure 6). It should also be noticed that phenothiazine derivatives in higher concentrations (TFP above 10 μg/ml and 3HPhM above 15 μg/ml) were toxic to resistant cancer cells after 60-min incubation.

The ability of modulators to restore the rhodamine 123 staining pattern typical for sensitive cells in the drug-resistant MES-SA/Dx5 line was confirmed by confocal microscopy. Verapamil and trifluoperazine at 10 μg/ml concentration made resistant cancer cells accumulate rhodamine 123 (Figure 5C and D). In the presence of modulator, the fluorescent probe stained resistant cells in the same pattern as for sensitive cells, *i.e.* rhodamine 123 was present in the cells' cytoplasm but not in the nucleus. The phenothiazine derivative 3HPhM did not influence rhodamine 123 accumulation in resistant cancer cells significantly (not shown).

Discussion

Using the anti-P-gp antibody UIC2, that recognizes an extracellular epitope of the protein (29), we demonstrated, by means of both flow cytometry and confocal microscopy, that the multidrug-resistant MES-SA/Dx5 cell line but not the drug-sensitive MES-SA express P-glycoprotein. Superficial P-gp localization in resistant MES-SA/Dx5 live cells was observed. Additionally, we found that the MES-SA/Dx5 subline expressed neither multidrug resistance associated protein (MRP1) nor lung resistance protein (LRP) (data not shown).

Accumulation of rhodamine 123, fluorescent P-glycoprotein substrate, in the drug-sensitive and resistant cell line was significantly different. The fluorescent probe diffused through the membrane and remained inside the MES-SA cells where it was evenly distributed in the cytoplasm (probably bound to cellular organelles *e.g.* mitochondria), but apparently did not enter the nucleus. On the contrary, no fluorescent probe was visible inside MES-SA/Dx5 cells. As rhodamine 123 is a P-gp substrate (16), it was probably recognized by this transporter while crossing resistant cells' plasma membranes and subsequently pumped out of the cancer cells.

The treatment of MES-SA/Dx5 cells with the known MDR modulators, verapamil and TFP (30,31), caused MDR reversal in these cells, recorded both by flow cytometry and confocal microscopy. Multidug resistance in MES-SA/Dx5 cells has been reported previously to be successfully reversed also by modulators such as cephalosporins and the acridonecarboxamide derivative GF120918 (22,32). By means of confocal microscopy, we were able to demonstrate that the action of the MDR modulators resulted not only in rhodamine intracellular accumulation increase in MES-SA/Dx5 cells, but also in restoration of a rhodamine 123 staining pattern typical for sensitive cells.

However, the newly synthesised modulators, phenothiazine maleates, that had been previously shown to reduce multidrug resistance in a mouse lymphoma cell line (26), turned out to be only marginally active in the human sarcoma cell line. Multiple factors could be responsible. First of all, the distinctions between the two species, mouse and human, should be taken into consideration. The physiological differences between tissues from which cancer has originated could also be significant. Lastly, the two types of malignancies, lymphoma and sarcoma, could develop slightly different mechanisms of drug resistance. Volm *et al.* (33) studied MDR reversal using a doxorubicin cytotoxicity restoration assay and they observed that trifluoperazine was able to reduce multidrug resistance in murine leukemia completely, but only partially in a murine sarcoma cell line. The authors concluded that, in spite of P-glycoprotein overexpression, the sarcoma cell line also exhibited an increased level of glutathione S-transferase. However, as we directly measured P-gp transport activity in MES-SA/Dx5 cells, multidrug resistance mechanisms other than P-gp overexpression were not likely to influence our results. Finally, the level of P-glycoprotein overexpression could also be dissimilar in cell lines derived from different types of malignancies. Moreover, the method that had been used to obtain the MDR variant of the tumour cell line could be important. The MES-SA/Dx5 cell line was selected by continuous *in vitro* exposure to doxorubicin (25), whereas the mouse T lymphoma cell line L5178 MDR, used previously (26), was obtained by *mdr1* gene transfection (34). Transfected cell lines are likely to display stronger P-gp

overexpression than selected ones. A high level of P-gp expression in resistant cell lines corresponds to a high fluorescence after incubation with rhodamine 123. The extent to which the fluorescence of parental and MDR cells differ influences the FAR values recorded for modulators in the rhodamine accumulation assay. FAR values, calculated as a ratio of fluorescence of modulator-treated to untreated MDR cells, are higher in cell line pairs in which the resistant subline is characterised by strong P-gp overexpression, *i.e.* the fluorescence of MDR cells population after incubation with the fluorescent probe is very low. In our opinion, this is the reason for the low FAR values of phenothiazine maleates recorded in the human sarcoma cell line as compared to the mouse T lymphoma cell line (26). In the present work, the FAR value obtained for 10 μg/ml of trifluoperazine (known MDR modulator) in the MES-SA/Dx5 cell line was 5.24, whereas the FAR value for 4 μg/ml of TFP measured by the same method in the mouse T lymphoma cell line was 49.2 [O. Wesolowska, unpublished observation]. Concluding, differences between the sarcoma MES-SA/Dx5 cell line used in the present work and the lymphoma L5178 MDR cell line used previously are likely to be the cause of different anti-MDR potencies of phenothiazine maleates in both lines.

Finally, we would like to point out that miscellaneous mechanisms of multidrug resistance of cancer cell lines, or even small differences between lines *e.g.* dissimilar level of P-gp expression, could result in different determination of the MDR reversing potency of modulators when assessed in different cell lines. Therefore, the proper choice of a model system, *i.e.* sensitive and resistant cell line pair, could influence the results of MDR modulators screen. As the activity of MDR modulators could differ between cell lines and/or types of malignancies, it might be important to have as many diverse cell lines at the scientists' disposal as possible. In our opinion, the MES-SA and MES-SA/Dx5 line pair is a good model to study MDR modulators, however, further studies on the mechanisms of multidrug resistance reversal in these lines are needed.

Acknowledgements

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