

# Assessment of Multidrug Resistance Reversal Using Dielectrophoresis and Flow Cytometry

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**ABSTRACT** In cancer, multidrug resistance (MDR) is the simultaneous resistance of tumor cells to different natural product anticancer drugs that have no common structure. This is an impediment to the successful treatment of many human cancers. A common correlate of MDR is the overexpression of a membrane protein, P-glycoprotein. Many studies have shown that MDR can be reversed after the use of substrate analogs, called MDR modulators. However, our understanding of MDR modulation is incomplete. In this article, we examine the electrical properties of the human leukemic cells (K562) and its MDR counterpart (K562AR) using dielectrophoresis and flow cytometry (with a membrane potential sensitive dye, DIOC5), both before and after treatment with XR9576 (a P-glycoprotein-specific MDR-reversal agent). The results show significant differences in the cytoplasmic conductivity between the cell lines themselves, but indicate no significant changes after modulation therapy. We conclude that the process of MDR modulation is not associated with changes in the electrical properties of cancer cells. Moreover, the results demonstrate that using the flow cytometry method alone, with MDR cells, may produce artifactual results—whereas in combination with dielectrophoresis, the results show the role of MDR modulators in preventing drug efflux in MDR cells.

## INTRODUCTION

Malignant cancers are a major cause of morbidity in the western world; they account for a quarter of all deaths. The most common methods of treatment include local excision or radiation, chemotherapy, immunotherapy, and biological-response modifiers. Chemotherapy has been considered as the most effective treatment for metastatic tumors. However, drug resistance is a serious impediment to the successful treatment of many human cancers and is responsible for many tens of thousands of deaths each year. One form of classical drug resistance is called multidrug resistance (MDR), characterized by cross-resistance to many anticancer drugs that have no common structure. A common feature of MDR is the overexpression of membrane glycoproteins, collectively termed as the ATP-binding-cassette (ABC) transporters. The *MDR1* gene product P-glycoprotein (P-gp) pump has been postulated to cause an increase in drug efflux, which gives rise to reduced and ineffective intracellular drug concentrations.

It has long been recognized that membrane transport of anticancer drugs can be blocked, with consequential reversal of MDR, by the use of pharmacological agents termed modulators or chemosensitizers. Modulation therapy is given in combination with anticancer drugs, as was first described using in vitro cell line models (Biedler and Riehm, 1970; Tsuruo et al., 1981), an approach subsequently used in many clinical trials (Bell et al., 1985; Cowie et al., 1995). The first-

generation MDR modulators such as verapamil and cyclosporin A (CsA), proved disappointing due to side effects and drug concentrations in the blood which were too low to bring about MDR reversal (Pennock et al., 1991; Kerr et al., 1986). The second-generation modulators (e.g., PSC-833) were more potent than their predecessors, and less toxic. However, PSC-833 has subsequently been shown to be a CNS toxin, a substrate for P-gp, and regarded as a partial antagonist. Third-generation modulators were developed to overcome the limitations of second-generation modulators and have been shown to specifically and potently inhibit P-gp function. An example of a third-generation modulator is the anthranilamide Tariquidar (XR9576; Xenova Ltd., Slough, UK), which has now reached phase III clinical trials.

Despite more than 25 years of research, the mechanisms underlying MDR reversal have not been fully clarified. Recent data describing binding affinity studies (Martin et al., 1999) showed that XR9576 interacted with P-gp with very high affinity and potently inhibited its function. The mechanism of action of XR9576 has been suggested to be via noncompetitive interaction at sites that are allosterically linked. P-gp can be considered as a multisite model with sites that either function to transport cytotoxic drugs or mediate inhibition of this process. Notwithstanding, there is a paucity of data regarding the biophysical character of drug-sensitive and -resistant cancer cells. An earlier study (Vayuvegula et al., 1988) used a flow cytometric-based assay with the membrane potential sensitive dye DIOC5 to compare the membrane potentials of drug-sensitive and MDR cancer cell lines before and after treatment with verapamil and CsA. The MDR cell lines were reported to have a lower membrane potential compared with their corresponding drug-sensitive parental cell lines. Subsequent incubation of the MDR cell lines with CsA or verapamil appeared to restore membrane

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potentials to that of the parent cell lines. These data suggested that alteration of membrane potential is a feature of MDR cancer cells and that modulation therapy acts to reverse this effect.

The AC-electrokinetic techniques such as dielectrophoresis (DEP) and electrorotation have been described as the motion of neutral matter caused by polarization in a non-uniform field (Pohl, 1978; Jones, 1995; Hughes, 2002). They have been extensively used both as characterization and separation techniques to study dielectric properties of both the membrane and cytoplasm for a number of biological particles (Wang et al., 1993, 1999; Washizu et al., 1994; Markx et al., 1994; Stephens et al., 1996; Gascoyne et al., 1997; Yang et al., 1999; Archer et al., 1999; Chan et al., 2000; Arnold, 2001; Hughes et al., 2002). DEP and electrorotation have also been employed both as an investigative tool and as the basis of a diagnostic method in cancer research (Hu et al., 1990; Burt et al., 1990; Gascoyne et al., 1992, 1993, 1997; Becker et al., 1994; Wang et al., 1997, 1999; Cristofanilli et al., 2002).

The study presented here has employed DEP to examine the differences in dielectric properties between drug-sensitive and MDR cancer cells before and after treatment with the P-gp specific modulator, XR9576. DEP measurements showed that the cytoplasmic conductivity of the doxorubicin human leukemic resistant cell line (K562AR) is significantly higher than the drug-sensitive parent cell line (K562), in the absence of any drug treatment. Subsequently, treatment with the MDR modulator (XR9576) had no significant effects on the biophysical properties of either cell line. In this article, we also demonstrate that by using DEP in combination with flow cytometry, modulating agents (such as XR9576) do not alter the membrane potential of MDR cells. Our data suggest that DIOC5 is a substrate for the ABC transporter P-gp and that using this technique with MDR cells gives rise to artifactual results.

## MATERIALS AND METHODS

### Chemicals and reagents

Doxorubicin (Sigma Aldrich, Poole, UK) was dissolved in sterile distilled water and stored as frozen stock solution in aliquots, which were thawed before use. XR9576 (kindly provided by Xenova Ltd., Slough, UK) was dissolved in DMSO and stored as frozen stock solutions and were thawed before use in experiments.

### Cell culture

Human chronic myelogenous leukemia (K562) and its doxorubicin-resistant counterpart (K562AR) were grown in modified RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS; Invitrogen, Paisley, UK), 2 mM L-glutamine, and 100 units  $\text{mL}^{-1}$  penicillin-streptomycin. All cell culture reagents were obtained from Sigma Aldrich (Poole, UK), unless stated otherwise. Cell lines were cultured in a humidified incubator with 5%  $\text{CO}_2/95\%$  air at 37°C. K562AR was maintained in the

presence of 100 nM doxorubicin, which was removed for at least one passage before use in experiments.

## Confirmation of MDR phenotype in K562AR

### Chemosensitivity testing

The colorimetric assay using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), was performed, as described by Mosmann (1983) to titrate cell viability after drug treatment. Leukemic cells at a density of  $1 \times 10^5$  cells  $\text{mL}^{-1}$  in RPMI-1640 containing 10% FCS were dispensed into 96-well plates in volumes of 200  $\mu\text{L}$  and left to equilibrate for 24 h. Freshly thawed drug solution was diluted in RPMI-1640 containing 10% FCS and added in a volume of 50  $\mu\text{L}$  in increasing concentrations. Control wells containing cell suspension were supplemented with a similar volume of medium. After 72 h incubation, cultured cells were treated with 20  $\mu\text{L}$  of 5 mg/ml MTT in phosphate-buffered saline (PBS) solution (Sigma Aldrich, UK) to each well. After 4 h of incubation, the plates were centrifuged at  $200 \times g$  and the medium was removed. The tetrazolium crystals were resuspended in 200  $\mu\text{L}$  of DMSO. Absorbance was read at 540 nm using an automated enzyme ELISA plate reader. The process was repeated when using the XR9576 at 100 nM final concentration. The results were expressed as  $IC_{50}$ , i.e., the concentration of cytotoxic drug that reduces cell viability by 50% relative to the control (untreated cells).

### Western immuno-blotting

Crude cell membrane preparations were made using hypotonic lysis buffer containing 1 mM PMSF, 1 mM  $\text{NaVO}_4$ , aprotinin, leupeptin, and 150 mM NaCl, in 10 mM Tris buffer (pH 7.4). The cells were left to lyse for 30 min, sonicated, and then centrifuged for 10 min (Helena Biosciences, Sunderland, UK) at  $450 \times g$ , followed by a further centrifugation (Centrikon-T2060) of  $60,000 \times g$  at 4°C. The final pellet was resuspended in lysis buffer containing 0.2% SDS. The protein content of the lysates was determined using a modified Bradford protein assay (Bio-Rad Laboratories, Hemel Hempstead, UK).

50  $\mu\text{g}$  of membrane protein was loaded onto a 10% acrylamide SDS and resolved by SDS-PAGE. The presence of P-gp was detected with anti-P-gp rabbit polyclonal antibody, H241 (Santa Cruz Biotechnology, Santa Cruz, CA). Visualization was carried out using HRP conjugated secondary antibody with chemiluminescence.

## Cell preparation

### Flow cytometry

The leukemic cell suspensions were resuspended in fresh 20 mM HEPES-modified RPMI-1640 containing 10% FCS (both from Sigma, Aldrich, UK) to obtain a cell density in the region of  $10^6$   $\text{mL}^{-1}$ . The chemosensitizer XR9576 was used at a final concentration of 100 nM, as for chemosensitivity testing. The membrane-potential-sensitive dye DIOC5 (3, 3'-Dipentyllox-acarbocyanine iodide) was prepared, as a stock, in PBS and added to the cell suspensions at a final concentration of 10  $\mu\text{M}$ . The dye was substituted with media in control samples (with and without XR9576). After incubating at 37°C for 30 min (with and without XR9576), membrane potentials were recorded using a FACS analyzer (Becton Dickinson, Oxford, UK). Resting membrane potentials from drug-sensitive cells were set on the fluorescence scale by adjusting the laser using the FL 1-PMT (525 nm) green fluorescence setting on the analyzer. The cells were gated on volume vs. scatter for uniform size distribution. The membrane potentials were compared according to the intensity of green fluorescence emitted (assigned by the parameter of geometric mean— $GM$ —values). Thus, an increased membrane potential is indicated by a higher fluorescence intensity (i.e., higher  $GM$  value), with the reverse being seen for lower membrane potentials.

### DEP experiments

Drug-resistant and -sensitive cells (including those incubated for 30 min at 37°C with XR9576) were centrifuged at room temperature at  $0.19 \times g$  for 5 min. The pellets were washed and resuspended in isotonic medium consisting of 8.5% (w/v) sucrose plus 0.3% (w/v) dextrose buffer (Gascoyne et al., 1997). The sample conductivity was adjusted to  $2.5 \text{ mS m}^{-1}$  using PBS and the final conductivity was verified with a conductivity meter (RS Components, London, UK). The final cell population was counted using a hemocytometer and adjusted to  $\sim 3 \times 10^5$  cells per ml ( $\pm 15\%$ ) for DEP measurements. To reduce the effect of variation in cell number in each sample, the experiments were repeated many times (generally 4–6) with different populations, which were summed before modeling.

As shown in Fig. 1, the system consisted of a signal generator, a light microscope, and the dielectrophoresis chamber in which the cell suspension was placed. The dielectrophoretic forces were generated by two needle-shaped electrodes, pointing toward each other, with opposing tips spaced  $100 \mu\text{m}$  apart in a glass Petri dish. The needles were formed by cutting a thin stainless steel rod at a shallow angle, such that cell collection occurred along the sharpened edge. These edges were placed facedown on the bottom of the chamber to ensure that cell collection always occurred within the field of focus of the microscope. The cell suspension was added to the electrodes by micropipette. The electrodes were energized by a Thurlby Thandar (Huntingdon, UK) signal generator in the range 10–20 MHz at an applied voltage of 20 V (peak-to-peak). Recordings were taken by exciting the electrodes for 1 min and counting the collected cells using a handheld tally counter, as described by Pohl (1978). The cell density was sufficiently low for the cell population to be disperse enough for cell-to-cell interactions (pearl-chaining) not to affect the results. Similarly, the cells were counted at arrival to the electrode edge to ensure accuracy. The interelectrode gap was chosen to be sufficiently large, and the time over which the experiment was performed ensured that the volume over which cells were attracted to the electrodes was sufficiently large, for variations in the local electric field gradient due to cell collection to have a minimal effect on the behavior of the cell collection process. Measurements were taken at five frequencies per decade, between 10 kHz and 20 MHz. Experiments were observed using an inverted microscope (Carl Zeiss Group, Oberochen, Germany) and charge-coupled device camera with videotaping for subsequent analysis. The dielectric parameters for each cell line in the absence or presence of XR9576 were obtained by fitting the measurement spectra to the single shell model (Irimajiri et al., 1979).

## RESULTS

### MDR confirmation of P-gp

As illustrated in Table 1, the  $IC_{50}$  value (defined as the concentration of the drug that reduces cell viability by 50% relative to untreated controls) for K562 was  $0.58 \mu\text{M}$  ( $\pm 0.28$ ) and  $8.13 \mu\text{M}$  ( $\pm 1.18$ ) respectively, indicating a resistance of 14-fold. The treatment of K562AR cells with doxorubicin in combination with the modulator XR9576 reduced the  $IC_{50}$  value to resemble that of the parent cell line ( $0.48 \mu\text{M} \pm 0.08$  and  $0.58 \mu\text{M} \pm 0.28$ , respectively), indicative of resistance reversal. Fig. 2 shows that the P-gp (relative molar mass,  $Mr = 170,000$ ) is expressed highly in the MDR cell line (K562AR), but is not detected in the parental (K562) cells.

### Flow cytometry

Membrane potentials of drug-sensitive and -resistant cancer cells were assessed after 30 min exposure to the dye. As shown in Fig. 3, *a* and *b*, the green fluorescence intensity emitted by the dye was markedly decreased in K562AR ( $GM = 36$ ) relative to the K562 parental cell line ( $GM = 124$ ). The modulator XR9576 did not have a noticeable effect on the green fluorescence intensity in K562, with  $GM$  values unchanged after treatment with XR9576 (126). However, the use of XR9576 with K562AR had a striking effect on the fluorescence intensity giving  $GM$  values of 492 relative to 124 for the parent K562 cell line.

### DEP experiments

Fig. 4, *a* and *b*, show spectra of cell collection versus frequency for K562 and K562AR respectively. The cells were suspended in isotonic sucrose/dextrose medium with

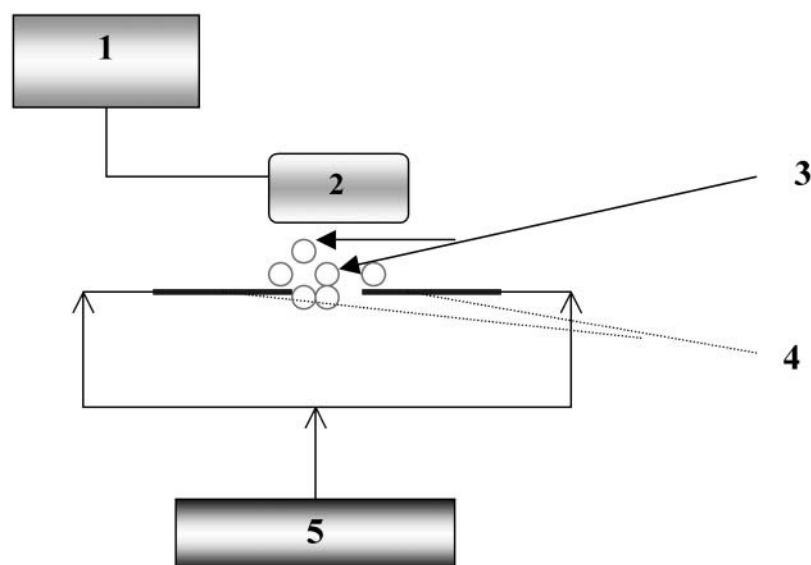


FIGURE 1 A schematic diagram of DEP arrangement. (1) TV and video screen; (2) microscope; (3) cells; (4) electrodes, placed such that the tips are opposing; and (5) signal generator.

**TABLE 1** A summary of the MTT-cytotoxicity results, showing the  $IC_{50}$  values for K562 and K562AR using doxorubicin and XR9576

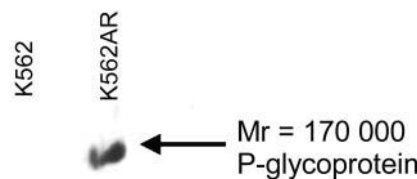
Cell line	Average $IC_{50}$ with doxorubicin $\mu\text{M}$ ( $\pm$ SD)	Average $IC_{50}$ with doxorubicin and XR 9576 $\mu\text{M}$ ( $\pm$ SD)
K562	0.58 (0.28)	0.35 (0.07)
K562AR	8.13 (1.18)	0.48 (0.08)

A summary of the MTT results (of at least three experiments), using doxorubicin and XR9576 (100 nM) showing the  $IC_{50}$  values for each cell line after 72 h incubation and of at least three experiments. The MDR phenotype is further confirmed in K562AR relative to K562, as the  $IC_{50}$  value increased from 0.58  $\mu\text{M}$  to 8.13  $\mu\text{M}$ , respectively. Key: AR, Adriamycin(now known as doxorubicin)-resistant; SD, standard deviation.

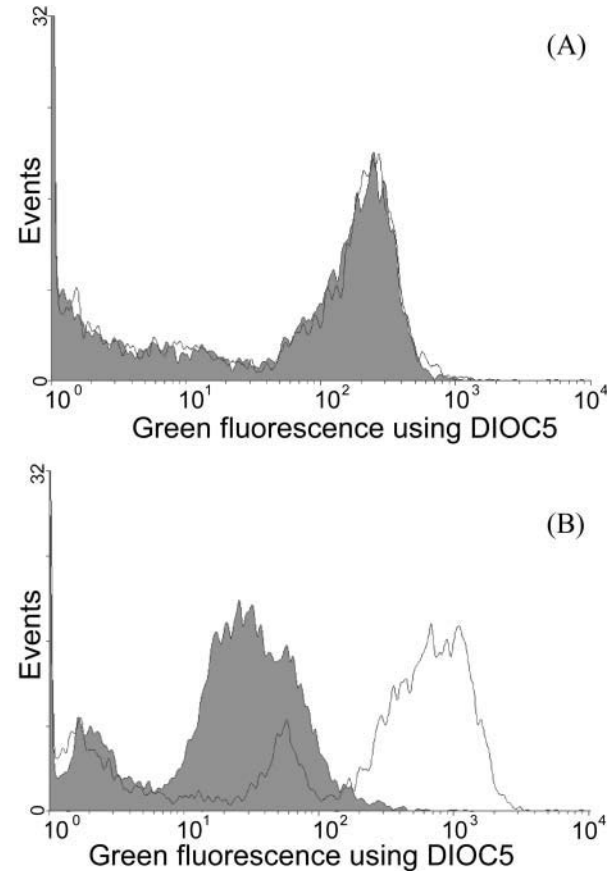
a conductivity of 2.5  $\text{mS m}^{-1}$ . Both figures indicate that the cells begin collecting after 10kHz in frequency. However, the collection rate shows a decline above 2–3 MHz for K562, unlike K562AR where the decline begins at 8–10 MHz. Similar measurements were recorded for K562 and K562AR after modulator treatment (not shown).

# DISCUSSION

The dielectric parameters for both cell types were estimated using a single-shell dielectric model (Huang et al., 1992) to model the DEP response. The best fit to experimental data was found by scaling the polarizability by an arbitrary factor to match the curve to the measured data, and then altering the permittivity and conductivity of the membrane and cytoplasm until a best match was found. Similar methods have been successfully used in the literature for the study of cells (Burt et al., 1990), viruses (Hughes et al., 1998), and latex beads (Bakewell and Morgan, 2001). The parameters determined by this method are shown in Table 2. The relative permittivity and conductivity of the medium were 78 and 2.5  $\text{mS m}^{-1}$ , respectively. The cells were modeled as being of diameters 8  $\mu\text{m}$  and 8.6  $\mu\text{m}$  for K562 and K562AR, respectively. As mentioned in a previous study (Yang et al., 1999), the use of a single-shell model represents an approximation in the values obtained of the structurally complicated cells; although a larger number of shells can be used (e.g., Griffith and Cooper, 1998), it is increasingly difficult to reliably determine a unique set of parameters. The single-shell model can provide a useful method of



**FIGURE 2** A Western blot to demonstrate the expression of the MDR protein (P-glycoprotein) in K562AR (right lane) and its absence in K562 (left lane), thus confirming the MDR phenotype in K562AR.



**FIGURE 3** A representative of at least four experiments showing flow cytometry results, using 10  $\mu\text{M}$  DIOC5 in human leukemic cell lines. The figure shows the differences in the green fluorescence emitted by the membrane-potential-sensitive dye DIOC5 for K562 (A) and K562AR (B) before and after treatment with 100 nM XR9576. The filled graphs show data recorded before treatment with XR9576, and the unfilled graphs show data taken subsequent to treatment.

comparison of the differences in the dielectric properties of the cell interior. Although this does not take into account any differences in the fine structure of the cell and any implications this has for the derived dielectric data, it has proven to be a useful tool for comparison of cells in a range of contexts (Yang et al., 1999).

It can be seen from Table 2 that the drug-sensitive K562 appears to exhibit a lower cytoplasmic conductivity (0.23  $\text{S m}^{-1}$ ) than its MDR counterpart K562AR (0.50  $\text{S m}^{-1}$ ), before and after treatment with XR9576. This indicates different ionic strengths in the cytoplasm of the parent cells compared to MDR cells.

Despite the presence of P-gp, the DEP results indicate that the electrical properties remained the same after treatment with XR9576 in both K562 and K562AR cells. Also, there do not appear to be any significant differences in the membrane conductivity or capacitance observed between cell lines, implying no significant morphological differences. However, K562 exhibited a significantly lower cytoplasmic

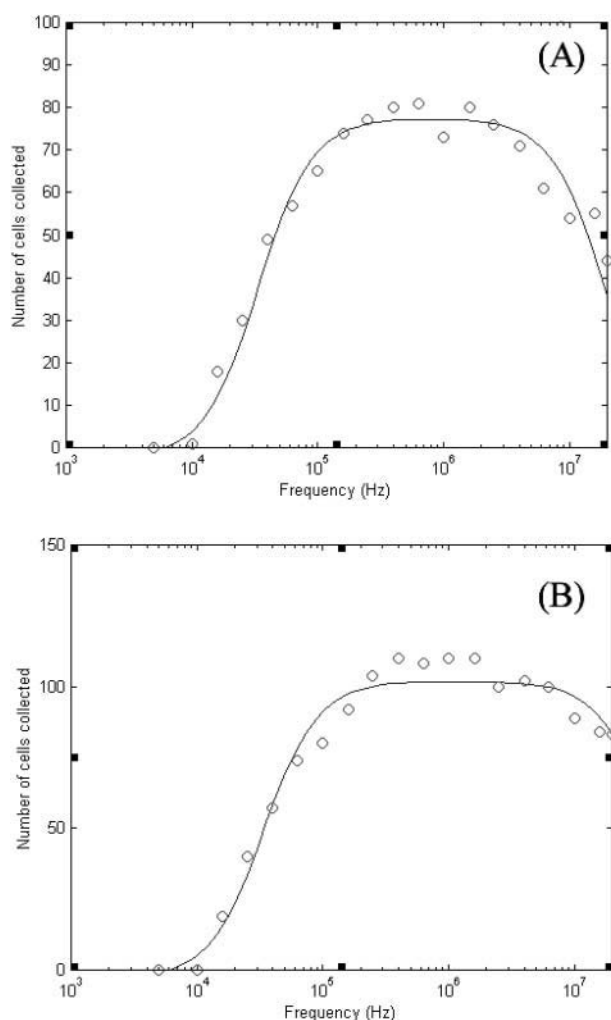


FIGURE 4 The average DEP collection spectrum for K562 and K562AR of at least five experiments with the polarization model scaled to fit. (A) DEP collection spectra for K562, with collection starting from 10 kHz that starts to decrease at  $\sim 3$  MHz. (B) DEP collection spectra for K562AR showing that the collection also starts at  $\sim 10$  kHz but starts decreasing at  $\sim 8$  MHz.

conductivity than K562AR ( $0.23 \text{ S m}^{-1}$  vs.  $0.50 \text{ S m}^{-1}$ , respectively) indicating a lower cell ionic content. Thus, these data have discriminated the cell lines according to their different dielectric properties. DEP has, therefore, brought attention to differences in the cytoplasm, a part of the cell that is not often considered in MDR.

By comparing the results obtained from DEP and flow cytometry, it can be seen that DEP results clearly do not directly agree with those obtained by flow cytometry. However, examination of these differences can yield useful information. The DEP results show that neither the cytoplasmic or the membrane permittivities, or the conductivities, were altered in the presence of XR9576 for K562AR (Table 2), despite P-gp expression. However, flow cytometry (Fig. 3, *a* and *b*), suggested an increase in membrane potential after modulator treatment for K562AR. This con-

**TABLE 2** A summary of the DEP results showing the electrical parameters of both the cytoplasm and the membrane of K562 and K562AR before and after modulation with XR9576

Cell line	Cytoplasm		Membrane	
	$\sigma$ (S/m)	$\epsilon$	$K_s$ (nS)	$C_{\text{spec}}$ (mF m $^{-2}$ )
K562	0.23 (0.21–0.24)	40–80	0.008	8.2 (7.6–7.0)
K562AR	0.50 (0.48–0.51)	40–80	0.008	7.6 (6.3–8.8)
K562 + XR9576	0.20 (0.19–0.21)	40–80	0.008	8.2 (7.6–8.8)
K562AR + XR9576	0.55 (0.51–0.58)	40–80	0.008	7.6 (6.3–8.8)

A summary of the DEP results, including the conductivity ( $\sigma$ ) and relative permittivity ( $\epsilon$ ) of both the cytoplasm and membrane for K562 and K562AR before and after treatment with XR9576. The table shows the increase in the cytoplasmic conductivity in K562AR relative to K562 ( $0.50 \text{ S/m}$  and  $0.23 \text{ S/m}$ , respectively). Treatment with XR9576 caused no changes in any of the biophysical parameters. It can be seen that there appeared to be no changes taking place in the cytoplasmic permittivity or the surface conductance ( $K_s$ ). Key: AR, adriamycin (now known as *doxorubicin*)-resistant;  $\sigma$ , conductivity;  $\epsilon$ , permittivity;  $K_s$ , surface conductance; and  $C$ , capacitance.

tradictory result may, we suggest, indicate that without the modulator, the P-gp could be pumping DIOC5 dye out of the cell, giving an artifactual result.

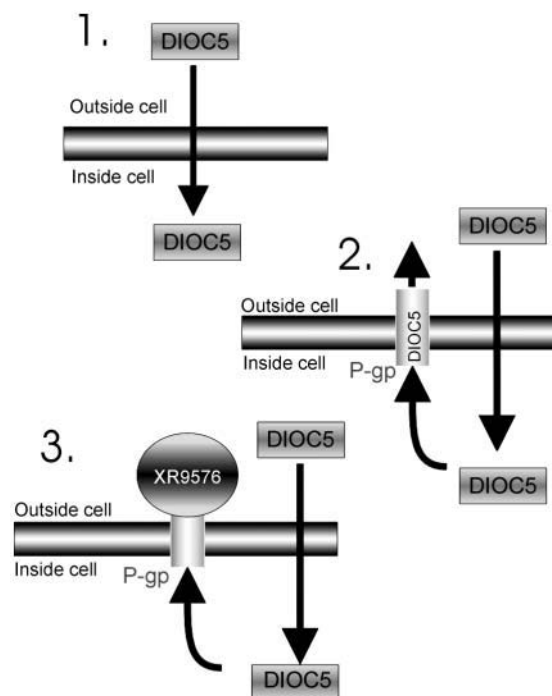


FIGURE 5 An outline of the proposed mechanism of action of XR9576 using DEP and flow cytometry. 1, A drug-sensitive cell, where DIOC5 dye permeates the membrane, thus giving a high level of fluorescence (indicative of membrane potential); 2, a drug-resistant cell before XR9576 treatment—DIOC5 permeates the membrane, but is then expelled from the cell via P-glycoprotein, thus giving an artifactually low level of fluorescence (indicating low membrane potential); and 3, a drug-resistant cell subsequent to XR9576 treatment, wherein XR9576 blocks P-glycoprotein and therefore prevents DIOC5 from being expelled, thus giving rise to increased cellular fluorescence relative to 2 (i.e., increased, true membrane potential).

We suggest that the contradictory results can be resolved if XR9576 is considered to be blocking the P-gp pump and thus preventing the efflux of the dye. In this regime, the fluorescent dye entering the membrane of the drug-resistant cells is pumped out, resulting in an artificially low fluorescence and implied low membrane potential. When P-gp is blocked, this pumping action does not occur and the high fluorescence level truly reflects the high cytoplasmic ionic strength. This resolves the differences between the two results, and also highlights the P-gp blocking effect of the XR9576 compound. This mechanism of action is illustrated in Fig. 5. This finding is in agreement with a previous study that suggested DIOC5 as being a substrate for ABC transporters (Gollapudi and Gupta, 1992). It is also in agreement with the observation that the DIOC5 result obtained for K562AR in the presence of XR9576 was somewhat higher than that seen for the K562 parental line, indicative of a higher cytoplasmic conductivity. There may be further effects on the cellular uptake of DIOC5 in the presence of XR9576 in K562AR cells that give rise to this effect. In contrast, DEP did not reveal any significant differences in the electrical nature of the membranes of K562 and K562AR cells.

## CONCLUSIONS

We have shown that DEP can be used as a technique to probe the biophysical differences between cell lines, with MDR cells exhibiting a higher cytoplasmic conductivity than the parental cells. These data point to a difference in the relative ionic content of the cells. XR9576, a P-gp specific modulator did not exert any effects on the cytoplasm or membrane parameters. Our data, therefore, show no significant changes in the biophysical properties of drug-sensitive or drug-resistant cancer cells after modulation therapy. There has been a disparity between DEP and flow cytometry results. DEP and flow cytometry in combination indicated that DIOC5 is a substrate for P-gp. Furthermore, DEP has provided a more informative and a rigorous approach in revealing that it is the cytoplasm that varies between MDR- and drug-sensitive cells. The study has demonstrated the application of DEP as a novel approach to aid a better understanding of the MDR phenotype in cancer cells.

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