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Inhibition of P-glycoprotein function by XR9576 in a solid tumour model can restore anticancer drug efficacy

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Abstract

Resistance to cancer chemotherapy involves both altered drug activity at the designated target and modified intra-tumour pharmacokinetic properties (e.g. uptake, metabolism). The membrane transporter P-glycoprotein (P-gp) plays a major role in pharmacokinetic resistance by preventing sufficient intracellular accumulation of several anticancer agents. Whilst inhibiting P-gp has great potential to restore chemotherapeutic effectiveness in blood-borne cancers, the situation in solid tumours is less clear. Therefore, the degree of resistance tumours pose to the cytotoxicity of vinblastine and doxorubicin was characterised using the multicellular tumour spheroid model. Tumour spheroids were generated from either drug-sensitive $MCF7^{WT}$ breast cancer cells or a resistant P-gp-expressing variant (NCI/ADR^{Res}). Drug-induced cytotoxicity in tumour spheroids was measured using an outgrowth assay and compared with that observed in monolayer cultures. As anticipated, the 3-D organisation of $MCF7^{WT}$ in tumour spheroids was associated with a reduction in the potency of doxorubicin and vinblastine—i.e. the inherent multicellular resistance phenomenon. In contrast, tumour spheroids from NCI/ADR^{Res} cells did not display multicellular resistance. However their constitutive expression of P-gp reduced the potency of both anticancer drugs. Moreover, the highly potent P-gp inhibitor, the anthranilic acid derivative, XR9576, was able to restore the cytotoxic efficacy of both drugs in tumour spheroids comprising NCI/ADR^{Res} cells. The results suggest that inhibition of P-gp in solid tumours is achievable and that generation of potent inhibitors will provide a significant benefit towards restoration of chemotherapy in solid tissues.

Keywords: XR9576; P-glycoprotein; Multidrug resistance; Solid tumour model

1. Introduction

The previous two decades have been witness to a significant improvement in the quality of treatment afforded by chemotherapy in the treatment of cancer. In particular, haematological and testicular cancers that rely on chemotherapy as first-line treatment are now associated with high curative rates in many instances [1–4]. Yet despite recent advances in chemotherapy, the true potential of new agents has not been realised due to the multitude of resistance pathways that impair chemotherapeutic efficacy (for review, see Refs. [5–11]). These resistance pathways can be viewed more simply as comprising both pharmacodynamic and pharmacokinetic factors acting synergistically to provide a significant barrier to chemotherapy. Pharmacodynamic influences comprising drug efficacy in tumours include heterologous cell populations (e.g. proliferating and quiescent), DNA repair systems and altered responses to apoptotic stimuli. Resistance also arises from many pharmacokinetic factors that result in reduced drug distribution within tumour tissue, sequestration of drug within specific cellular pools, increased metabolic inactivation and reduced cellular uptake. The reduced cellular uptake mechanism may be considered a 'front-line' defence produced by cancer cells to combat anticancer agents and is conferred by a number of drug transporters. The most widely investigated and characterised transporter is the adenosine triphosphate (ATP)-Binding Cassette (ABC) family member P-glycoprotein (P-gp) [6–12]. P-gp expression has been demonstrated in a large number of different cancer types [13–19] and in many cases the presence of P-gp has been correlated with lower rates of remission and shorter survival times.

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Due to its widespread appearance in cancerous tissue, P-gp has been the target of intense academic, clinical and pharmaceutical research aimed at pharmacologically inhibiting its actions. However, many of the early 'first and second generation' P-gp inhibitors resulted in poor efficacy due to low potency or selectivity and often caused many unwanted toxic side-effects *in vivo*. Despite this, a number of these early generation P-gp inhibitors were trialled clinically with predictably disappointing results [20–23]. In recent years, pharmaceutical chemical screening programmes have yielded a third generation of P-gp inhibitors with high potency and selectivity [24–27]. Several including PSC833 [24], LY79553 [22] and XR9576 [28] have proceeded to clinical trials. Results arising from clinical trials and many scientific investigations indicate that the inhibition of P-gp function in haematological malignancies does produce clinical benefit; however, the situation in solid tumours is less clear.

As mentioned above, drug resistance to chemotherapy is a multifactorial process and it also depends on the chemical nature of drugs involved in treatment and also the cellular profile within a tumour. In solid tumours, cells are organised into a 3-D architecture that ultimately produces a hostile local microenvironment (↓pH, Δredox state, ↓pO_2, ↑interstitial pressure, extracellular matrix (ECM), etc.) and leads to the presence of a large proportion of unresponsive or non-proliferating cells. These factors have been collectively termed 'multicellular resistance' (MCR) and are inherent to solid tumour tissue [29–31]. It has been argued that in the face of MCR, the influence of P-gp is negligible [32–34]. If P-gp does prove to be a negligible influence on resistance, then pharmacological modulation of its activity would not affect the ability of anticancer drugs to produce cytotoxicity in a solid tumour. In order to address this issue, it was necessary to determine first the relative cytotoxicities of the clinically important compounds, vinblastine and doxorubicin, in monolayers versus an *in vitro* tumour model; the multicellular tumour spheroid (MCTS) system. Tumour spheroids provide a faithful representation of solid tumour architecture, cellular heterogeneity and hostile microenvironment [30]. Secondly, by generating tumour spheroids from drug-sensitive and P-gp-expressing cells, it was possible to model what role P-gp played within solid tumours and whether its activity could be modulated.

2. Materials and methods

2.1. Materials

Dulbecco's minimum essential medium (DMEM), penicillin/streptomycin and fetal calf serum were obtained from Invitrogen (Paisley, UK). Methylthiazoletetrazolium (MTT), methylene blue trihydrate, vinblastine sulphate and doxorubicin hydrochloride were purchased from Sigma Aldrich (Poole, UK). Gill's Haematoxylin, DPX mountant and Aquamount were obtained from BDH. The 1% Eosin solution was obtained from RA Lamb (Eastbourne, UK). The monoclonal antibromodeoxyuridine (Bu20a) was provided by the Leukaemia Research Fund Immunodiagnostics Unit, Nuffield Department of Clinical Laboratory Sciences, University of Oxford. XR9576 was generously provided by Xenova Ltd (Slough, UK).

2.2. Cell lines

Drug-sensitive ($MCF7^{WT}$) human breast cancer cells and the P-gp-expressing resistant cell line (NCI/ADR^{Res}) were obtained from Dr Phillips (University of Bradford, UK) and Prof. Cowan (NCI, USA), respectively. Both cell lines were grown as monolayer cultures in DMEM with glutamax and supplemented with 10% (v/v) fetal calf serum and penicillin/streptomycin (100U ml^{-1} and 100 mg ml^{-1}, respectively). The resistant NCI/ADR^{Res} cell line was cultured in the presence of 3 μM doxorubicin for a single passage every 10 passages to maintain the selection pressure [35]. This cell line was formerly known as the $MCF7^{Adr}$ line and has been redesignated as described in Ref. [36].

2.3. Growth of tumour spheroids

Tumour spheroids were grown using the liquid overlay technique described in Ref. [37] in 96-well tissue-culture plates. The 96-well plates were given a 100 μl base-coat of 0.75% (w/v) agar that had been prepared in DMEM. Freshly trypsinised cells, taken from exponentially-growing cultures, were overlaid on solid agar base-coats at a density of 4×10^3 cells per 200 μl DMEM (supplemented as described for monolayers). The $MCF7^{WT}$ cell lines were kept still for 24 h (37 °C, 5% CO_2) after which the plates were transferred to a Titramax100 (Heidolph Instruments) and shaken at 300 rotations per minute (r.p.m.) in a tissue culture incubator (37 °C, 5% CO_2). Tumour spheroids from NCI/ADR^{Res} cells were kept stationary for 48 h prior to shaking due to a greater fragility at early stages of spheroid growth. Tumour spheroids from $MCF7^{WT}$ cells were fully formed within 48 h and could be routinely cultured for up to 10 days under these conditions, subject to replacement of medium at 3-day intervals. In contrast, tumour spheroids from NCI/ADR^{Res} cells required 72 h to fully form regular, compact morphology. At such time the tumour spheroids were used in the subsequent assays on drug cytotoxicity. Growth of tumour spheroids was assessed by measurement of the tissue diameter using a calibrated eye-piece graticule (100 points per 10 mm) (Pyser-SGI, UK).

2.4. Drug cytotoxicity assessment in monolayer cultures

Cells were seeded at a density of 3×10^3 per well of a 96-well tissue culture plate in a volume of 100 μl DMEM and left to attach for 24 h. Drugs were prepared in medium at twice the desired concentration and a 100 μl volume added to each well. The cells were incubated in the presence of drug for 1, 24 or 72 h. Cells that had been exposed to drug for 1 or 24 h were washed twice in medium and then incubated for a further 71 or 48 h, respectively. Following the total incubation period of 72 h, the number of viable cells was determined using the MTT assay. Briefly, 20 μl of a 5 mg/ml MTT solution in phosphate-buffered saline (PBS) was added to the wells and incubated for 4 h at 37 °C. Following incubation, the medium was aspirated, the formazan crystals produced by viable cells were dissolved in 150 μl dimethyl sulphoxide (DMSO) and the absorbance measured at 550 nm in a SpectraMAX 250 spectrophotometer (Molecular Devices). Cell viability at each drug concentration was determined as a percentage of the number of viable cells detected in the absence of drug treatment. Doxorubicin and vinblastine were stored as 50 mM stocks in DMSO and added to cells in the range 10^{-12}–10^{-4} M, whilst ensuring that the final solvent concentration was less than 0.1%. To determine its ability to sensitise cells to anticancer agents, XR9576 was added to cells (3×10^{-9}–3×10^{-7} M) 12 h prior to the administration of vinblastine or doxorubicin.

2.5. Measurement of drug cytotoxicity in tumour spheroid cultures

Cytotoxicity of drugs in tumour spheriods was determined by measuring the amount of cellular outgrowth from the tissue following or during drug exposure. Tumour spheroids were grown from a starting density of 4×10^3 cells per well and drugs were then added as described in the previous section once the tissue had assumed a compact structure. The tumour spheroids were incubated with the drugs for either 1, 24 or 72 h. Where drug exposures were selected for 1 or 24 h, following appropriate incubation the drug containing medium was carefully discarded, the tumour spheroid washed twice in medium and then cultured 'drug-free' for a further 71 or 48 h. After a total incubation time of 72 h, the tumour spheroids were transferred to 'uncoated' 96-well tissue culture plates and gently washed two times in 200 μl drug-free medium. The tumour spheroids attached to the plates and were incubated for a period of 72 h to allow cellular outgrowth from the tissue—this procedure is referred to as the radial outgrowth assay. Following outgrowth, the medium was aspirated and replaced with 150 μl 5 g/l methylene blue in 50% (v/v) methanol to fix and stain cells. The wells were washed three times in PBS and the radial outgrowth measured using a graduated microscope eye-piece graticule. The radius of the tumour spheroids was subtracted from the measurements and the degree of outgrowth was expressed as a percentage of that obtained in the absence of drug treatment. The concentrations of all drugs tested were identical to those listed for the monolayer cytotoxicity assays above.

2.6. Routine histological procedures

To enable histological assays, tumour spheroids were harvested, washed in PBS and fixed in neutral-buffered formalin, pH 7.0. The tumour spheroids were transferred to cut-off sections of 1 ml round-bottom cryogenic vials (Nalgene, Rochester, USA), which acted as casting moulds for embedding. The tumour spheroids were allowed to settle, formalin was removed and the casting moulds filled with molten 2% (w/v) agar in 4% (v/v) formaldehyde. The agar was allowed to set, and embedded tumour spheroids were subsequently removed from the casting moulds and placed in a tissue culture cassette (RA Lamb, Eastbourne, UK) for routine processing by the Histopathology Department (John Radcliffe Hospital, Oxford UK). The processed tumour spheroids (in agar) were then embedded in paraffin wax, 5 μm sections were cut and adhered to Superfrost Plus microscope slides (BDH, Leicester, UK). To undertake routine histology or immuno-cytochemistry, the sections were de-waxed using Citroclear (HD Supplies, Ayelsbury, UK) and passed through a graded series of alcohol and finally rehydrated in distilled H_2O. Haematoxylin and eosin staining of sections was done according to standard protocols described in Ref. [38].

2.7. Measurement of cell proliferation and P-gp expression in tumour spheroids

The proliferation status of cells within tumour spheroids was determined by measurement of 5-bromo-2′-deoxy-uridine (BrdU) incorporation using a peroxidase-based immunohistochemical assay. Tumour spheroids were incubated with BrdU (10 μM) for 12 h prior to harvesting and histological preparation. The paraffin-embedded tissue sections were completely de-waxed as described above and then re-hydrated with PBS. Antigen retrieval was achieved by placing slides in 1 M HCl at 60 °C for 10 min and then washing twice in PBS. Endogenous peroxidase activity was inhibited with PEROXIDAZED 1 (BioCarta, Hamburg, Germany) for 10 min and slides were subsequently washed thoroughly with PBS. Sections were covered with the mouse monoclonal anti-BrdU antibody Bu20a [39] for 45 min at room temperature in a humid environment and subsequently washed twice in PBS. The goat anti-mouse,

poly-horse radish peroxidase (HRP)-linked secondary antibody MACH2™ (BioCarta, Hamburg, Germany) was added for 30 min at room temperature in a humid environment and subsequently washed twice in PBS. Detection was achieved using the commercial 3,3-diaminobenzidine (DAB) substrate-chromogen (DAKO, Carpinteria, USA) for 5–10 min at room temperature. Once the colour reaction had proceeded to desired levels, the sections were washed in PBS and coverslips mounted using Aquamount™.

P-gp expression in tumour spheroids and monolayers was also measured using a peroxidase-based immunohistochemical assay with the UIC2 monoclonal anti-Pgp antibody (Immunotech, France). Expression in tumour spheroids was done using paraffin-embedded sections, whilst monolayers were grown on coverslips. The monolayers were fixed by incubation in 4% neutral-buffered formalin in PBS for 30 min at room temperature. Antigen retrieval in tumour spheroids was achieved by heating slides in 0.1 M citrate (pH 6.0) for 7 min at low power in a conventional microwave. Endogenous peroxidase activity was inhibited as described above. The slides or coverslips were 'blocked' with 1% bovine serum albumin (BSA) in PBS for 60 min at room temperature. Tissues were overlaid with UIC2 antibody at 2 μg/ml (in 1% BSA) for 120 min, washed three times in PBS and then incubated with 10 μg/ml antimouse Ig-HRP (DAKO, USA). Detection and mounting procedures were identical to that described for the BrdU assay above.

2.8. Data analysis

Drug toxicity in monolayers and tumour spheroids was quantified using non-linear regression of the general dose-response equation [40]:

$$F = F_{\min} + \frac{(F_{\max} - F_{\min})}{(1 + 10^{(\log_{10}\mathrm{IC}_{50} - D)})}$$

where: F = viable cell number, F_{max} = maximum viable cell number, F_{min} = minimum viable cell number, IC_{50} = drug concentration causing a 50% decrease in cell viability, D = logarithm of drug concentration.

The viable cell number at each drug concentration was expressed as a percentage of the MTT absorbance at 550 nm obtained in the absence of drug. In the case of assays involving cytotoxicity in tumour spheroids, the cell viability is replaced with radius of outgrowth (μm).

Statistical comparisons between mean values of two parameters were done using the Student's *t*-test and comparison of >2 values was achieved using ANOVA with a Tukey's *post hoc* test. In both cases, a P value <0.05 was considered statistically significant.

3. Results

3.1. Characterisation of tumour spheroids morphology and proliferative status

The $MCF7^{WT}$ cells rapidly produced compact tumour spheroids and the starting cell number of 4×10^3 cells produced tissue with a diameter of 473 ± 4 μm prior to drug administration. Tumour spheroids derived from the $MCF7^{WT}$ cell line will be termed TS^{WT} and a similar nomenclature will be adopted for the P-gp-expressing cell line (i.e. TS^{Res}). P-gp-expressing TS^{Res} cells took longer to form compact structures and the diameters were 521 ± 8 μm. The morphological features of tumour spheroids derived from both cell types are shown in Fig. 1(a)–(d). The brown-staining nuclei highlight the proliferating cell population that had incorporated BrdU. The proliferating cells in TS^{WT} highly localised to the outer layers of the tissue, with haematoxylin-stained nuclei predominating at deeper levels. In TS^{Res} cells, the proliferating cells were not as strictly localised with numerous examples at deeper layers in the tissue compared with the TS^{WT}. Fig. 2 demonstrates the relative expression levels of P-gp in the two MCF7 cell lines, TS^{WT} and TS^{Res}. There was no detection of P-gp in $MCF7^{WT}$ cells (Fig. 2a), which is in stark contrast to that observed in the drug-resistant cells (Fig. 2b). The presence of P-gp in the NCI/ADR^{Res} cells corresponds to the brown staining and the expression appears at the cell periphery, consistent with the plasma membrane localisation of P-gp. Nuclei were counterstained blue with haematoxylin for each sample. There was some evidence of low-level P-gp expression in the TS^{WT} at the deeper or quiescent cell layers (Fig. 2c). The low level

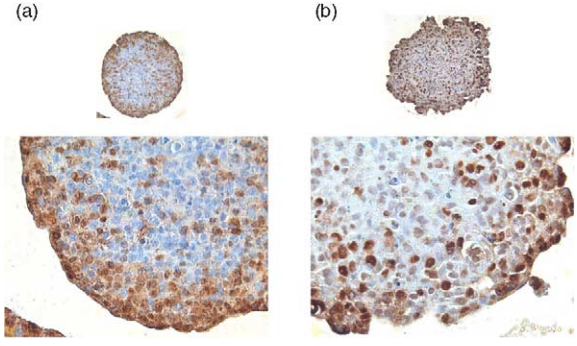

Fig. 1. Cellular proliferative status in TS comprising drug-sensitive or drug-resistant cells. Tumour spheroids were generated from $MCF7^{WT}$ and NCI/ADR^{Res} cells using the liquid overlay technique and incubated in 10 μM 5-bromo-2′-deoxy-uridine (BrdU) for 12 h prior to harvesting. Histological sections (5 μm) were subjected to immunocytochemical detection of incorporated BrdU (brown nuclei). Haematoxylin was used to counterstain (blue nuclei) cells that had not incorporated BrdU: (a) low (upper) and high (lower) magnification images of sections from TS^{WT}; (b) low (upper) and high (lower) magnification images of sections from TS^{Res}.

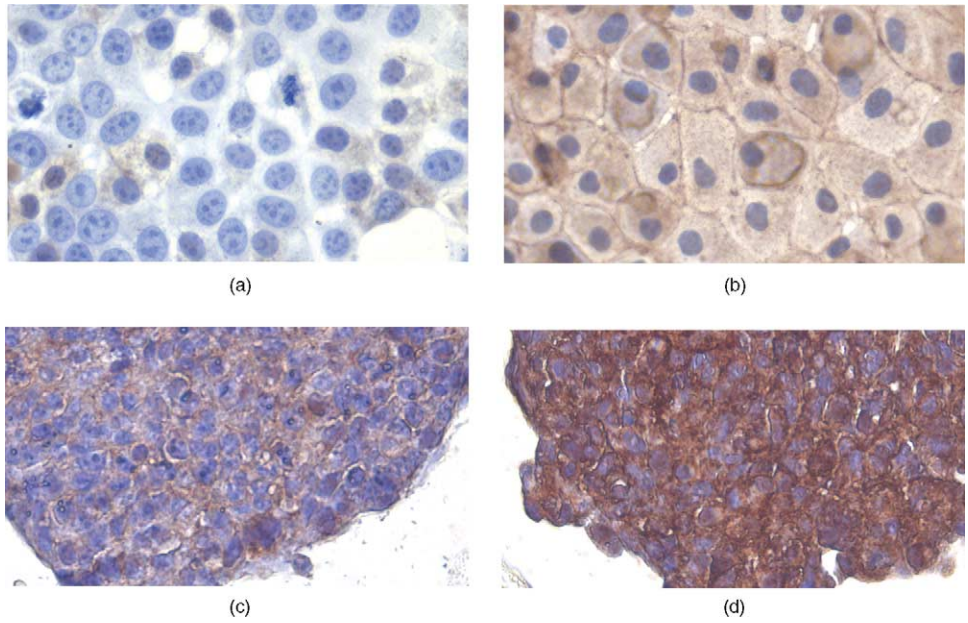

Fig. 2. Expression of P-gp in monolayers and tumour spheroids. The relative expression levels of P-gp were determined using immunocytochemical detection with the monoclonal antibody UIC2. (a) and (b) demonstrate the expression of P-gp (brown staining) in monolayer cultures of $MCF7^{WT}$ or NCI/ADR^{Res} cells. Nuclei were counterstained in all cases with haematoxylin; (c) and (d) represent TS^{WT} and TS^{Res}, respectively.

expression of P-gp has previously been reported to be induced in the central hypoxic environment of tumour spheroids [41–43]. However, the presence of P-gp in TS^{Res} revealed localisation throughout the tissue. Moreover, it appeared to indicate significantly greater levels of expression than those observed in TS^{WT} (Fig. 2d).

3.2. The cytotoxicity of doxorubicin and vinblastine administered to monolayers for varying times

Both drug-sensitive and drug-resistant cell lines grown as monolayers were exposed to doxorubicin and vinblastine for varying times to determine the degree of drug-induced cytotoxicity. A drug exposure as short as 1 h was sufficient for doxorubicin to produce cytotoxicity in $MCF7^{WT}$ cells characterised by a potency of $IC_{50} = 0.062 \pm 0.037$ μM (Table 1). By extending the drug exposure time to 72 h, there was an approximately 3-fold improvement in the potency of doxorubicin to produce cytotoxicity in $MCF7^{WT}$ cells, although this was not statistically significant (ANOVA). By comparison, the potency of a 1-h doxorubicin exposure to elicit cytotoxicity in the P-gp-expressing NCI/ADR^{Res} cell line versus $MCF7^{WT}$ was significantly reduced ($P < 0.05$) as reflected by the 84-fold higher value of $IC_{50} = 5.2 \pm 2.9$ μM. This reduction in potency observed in NCI/ADR^{Res} cells was expressed as the unicellular resistance factor (UCRF) in Table 1, a parameter that reflects the degree of resistance afforded by expression of P-gp. Regardless of the drug exposure time, the potency of doxorubicin to produce cytotoxicity was consistently and significantly lower ($P < 0.05$) in the NCI/ADR^{Res} cells compared with the parental $MCF7^{WT}$ line. Although there was no significant change in the UCRF following an increase in drug exposure from 1 to 24 h, the ratio increased dramatically from approximately 100–660-fold at 72 h.

Vinblastine also produced cytotoxicity in $MCF7^{WT}$ cells (Table 1), and the potency following a 1-h exposure ($IC_{50} = 2.6 \pm 0.7$ nM) was 23-fold higher compared with doxorubicin under equivalent conditions. The NCI/ADR^{Res} cells displayed significant resistance to a 1-h exposure to vinblastine as shown by the 8585-fold higher IC_{50} value compared with the parental cells. There appeared to be a clear relationship between the potency of vinblastine to produce cytotoxicity and exposure time in the $MCF7^{WT}$ cells as demonstrated by the almost 9-fold increase in potency from 1 ($IC_{50} = 2.6 \pm 0.7$ nM) to 72 h ($IC_{50} = 0.30 \pm 0.06$ nM) ($P < 0.05$). An identical increase in drug exposure time increased (59-fold) the potency of vinblastine to produce cytotoxicity in the P-gp-expressing NCI/ADR^{Res} cells. However, whilst the NCI/ADR^{Res} cells remained highly resistant to vinblastine at each time point, by comparison to their drug-sensitive counterparts, the magnitude of the differences in potencies was reduced from >8600-fold at 1 h to approximately 1300-fold after 72 h of drug exposure. At each drug exposure time investigated, the degree of resistance afforded by P-gp to vinblastine in the NCI/ADR^{Res} cells was greater than that towards doxorubicin. However, the gap between the levels of resistance (UCRF) 'narrowed' at longer exposure times.

Table 1
Cytotoxicity of doxorubicin (DOX) and vinblastine (Vbl) in monolayers and tumour spheroid cultures of drug-sensitive and drug-resistant cells

	Dox (μM)	UCRF	MCRF	Vbl (nM)	UCRF	MCRF
1-h exposure						
$MCF7^{WT}$	0.06±0.04			2.6±0.7		
NCI/ADR^{Res}	5.2±2.9	84		22 320±4510	8585	
TS^{WT}	1.2±0.3		19	10.5±2.8		4
TS^{Res}	9.2±2.9	8	2	3740±616	356	0.2
24-h exposure						
$MCF7^{WT}$	0.16±0.07			1.9±0.8		
NCI/ADR^{Res}	15.7±4.8	98		7661±1970	4032	
TS^{WT}	0.75±0.24		5	6.0±0.8		3
TS^{Res}	11.8±3.9	16	0.8	1950±211	325	0.3
72-h exposure						
$MCF7^{WT}$	0.023±0.01			0.3±0.1		
NCI/ADR^{Res}	15.3±2.4	660		379±39	1263	
TS^{WT}	0.63±0.15		27	3.3±1.1		11
TS^{Res}	6.4±1.3	10	0.4	1062±180	322	3

Monolayers and tumour spheroids were exposed to doxorubicin or vinblastine for a period of 1, 24 or 72 h and cytotoxicity was subsequently determined by the MTT or radial outgrowth assays, respectively. The potency to affect cytotoxicity was expressed as an IC_{50} value determined from dose–response curves as described in the Methods. All values were obtained from four to six independent observations and are shown as mean±standard errors of the means. The UCRF refers to the resistance afforded by P-gp and corresponds to the ratio of IC_{50}s produced in NCI/ADR^{Res} versus $MCF7^{WT}$ monolayers or TS^{WT} versus TS^{Res}. The MCRF refers to the ratio of IC_{50}s produced in tumour spheroids versus monolayers for each cell line. MTT, methylthiazoletetrazolium.

3.3. The cytotoxicity of doxorubicin and vinblastine administered to tumour spheroids for varying times

Subsequent investigations were tailored to assess the impact of P-gp expression in solid tissues on the potency of drug cytotoxicity. Drug cytotoxicity in tumour spheroids was determined using an outgrowth assay where cells from the peripheral layers of the attached spheroid (radius = R_1) grow outwards in a radial fashion to a distance of R_2. By 4 days of incubation, the radius of outgrowth (R_{out}) was determined by $R_{out} = R_2 - R_1$. The outgrowth measured using this assay was plotted as a function of drug concentration. Dose–response analysis was undertaken to determine the potency (IC_{50}) of drug to retard radial outgrowth, thereby providing a measure of cytotoxicity in both TS^{WT} and TS^{Res}. The cytotoxicity data has previously been shown to correlate with the production of cellular damage/death as adjudged by microscopy [44].

Doxorubicin was able, in a dose-dependent manner, to completely retard the cellular outgrowth from tumour spheriods. The data shown in Table 1 indicates the potency of doxorubicin to produce this effect following exposure times of 1, 24 and 72 h, respectively. Similar to the observations in the monolayer cultures, the potency of doxorubicin to produce cytotoxicity in either TS^{WT} or TS^{Res} did not show a significant dependence on exposure time (Table 1). The cytotoxic potency of doxorubicin in TS^{WT} following a 1-h exposure was 1.2±0.3 μM, which was significantly different from the value in monolayers, and the 19-fold disparity reflects the contribution of multicellular resistance (MCR) factors. The degree of multicellular resistance in TS^{WT} did not alter significantly by increasing the exposure time to 72 h, where the MCR ratio indicated a 27-fold higher IC_{50} for doxorubicin in solid tissues compared with monolayers. The data obtained for TS^{Res} reflect the impact of P-gp on MCR. It was observed that expression of P-gp did contribute to the overall resistance spectrum in tumour spheroids since the IC_{50} was increased 8-fold ($P < 0.05$) in TS^{Res} ($IC_{50} = 9.2 \pm 2.9$ μM) compared with that in TS^{WT} ($IC_{50} = 1.2 \pm 0.3$ μM) following a 1-h incubation in doxorubicin. Unlike monolayer NCI/ADR^{Res} cultures, increasing the exposure time to 24 (UCRF = 16-fold) or 72 h (UCR = 10-fold) did not produce higher levels of multicellular resistance in TS^{Res}. The degree of resistance produced by P-gp (i.e. TS^{WT} versus TS^{Res}) in this 3-D setting was also significantly lower than in the corresponding monolayer system. Moreover, there was no inherent multicellular resistance (MCRF < 1) in TS^{Res} tissue compared with their corresponding monolayer cultures of NCI/ADR^{Res} cells.

Vinblastine displays different physicochemical properties, a separate cellular target and a higher affinity for P-gp than doxorubicin; therefore the influence of P-gp expression on vinblastine efficacy in the tumour spheroid model was also investigated. As shown in Table 1, vinblastine did produce cytotoxicity in TS^{WT} and the $IC_{50} = 10.5 \pm 2.8$ nM following a 1-h incubation with drug was 4-fold higher than that observed in $MCF7^{WT}$ monolayers. However, the potency of vinblastine cytotoxicity in TS^{WT} was time-dependent as manifest by decreasing values for IC_{50} obtained following 24

($IC_{50}=6.0\pm0.8$ nM) or 72 ($IC_{50}=3.3\pm1.1$ nM) h exposure to the drug. However, whilst a MCR component was clearly evident, the MCRF in TS^{WT} did not vary markedly for 1- (4-fold), 24- (3-fold) or 72- (11-fold) h exposures to vinblastine.

As was the case with TS^{WT}, there is also a time-dependent increase in the potency of vinblastine to produce cytotoxicity in the corresponding TS^{Res} tissue. This was evident from the decrease in IC_{50} from 3740 ± 616 nM at 1 h to 1062 ± 180 nM following 72 h exposure. The potency to elicit cytotoxicity in TS^{Res} was lower than that observed in monolayer cultures of NCI/ADR^{Res} cells, indicating the absence of a MCRF influence at 1- and 24-h drug exposures. A modest 3-fold MCRF was observed following a 72-h exposure. However, there was a significant UCRF component since vinblastine displayed a significantly reduced potency to cause cytotoxicity in TS^{Res} compared with that in TS^{WT} at each time point investigated. The level of this P-gp-dependent resistance was greater than 300-fold and did not alter appreciably if the exposure time was increased. Although the UCRF was significant, the absolute protection afforded was considerably lower than the levels observed in monolayer cultures (1310–8585-fold). Despite differences in the extent of protection P-gp confers in spheroid versus monolayer cultures, the results clearly demonstrate that P-gp was able to influence the cytotoxicity of doxorubicin and vinblastine in solid tumour models. Therefore, subsequent investigations focused on determining whether inhibition of this protein could restore drug potency.

3.4. The relative ability of XR9576 to reverse resistance to vinblastine or doxorubicin in monolayer cultures

The ability of XR9576 to restore drug cytotoxicity in monolayers was determined using a 24-h exposure to anticancer drug subsequent to a 12-h pre-exposure to the P-gp inhibitor XR9576. At the end of this co-exposure to anticancer drug and P-gp inhibitor, the cells were allowed to recover for 48 h prior to measurement of cytotoxicity. The sensitivity of $MCF7^{WT}$ monolayer cultures to doxorubicin ($IC_{50}=0.151\pm0.028$ μM) was not significantly affected by XR9576, even at concentrations as high as 300 nM ($IC_{50}=0.274\pm0.067$ μM) (Table 2). The NCI/ADR^{Res} cells displayed a 104-fold resistance to doxorubicin in the absence of XR9576 ($IC_{50}=15.7\pm4.8$ μM). However, in contrast to the wild-type cells, XR9576 caused a dose-dependent increase in the potency of doxorubicin to produce cytotoxicity. This increased potency was associated with a concomitant reduction in the level of resistance (UCRF) observed in the P-gp-expressing NCI/ADR^{Res} cells. The resistance to doxorubicin could not be completely abolished by XR9576 since a residual level remained (7-fold) in the presence of a 300 nM concentration. To quantify the ability of XR9576 to restore cytotoxicity, secondary plots of the normalised IC_{50} for doxorubicin (assigned a value of 1 in the absence of inhibitor) as a function of XR9576 concentration were constructed. The plot produced for doxorubicin in NCI/ADR^{Res} monolayers was characterised by an EC_{50} value of 17 nM. This value describes the concentration of XR9576 required to reduce the level of resistance to cytotoxic drug to half the value in the absence of P-gp inhibitor. This EC_{50} value is subsequently referred to as the 'reversal potency' for XR9576. The results therefore demonstrate that XR9576 may restore sensitivity of NCI/ADR^{Res} cells to doxorubicin by potent inhibition of P-gp.

Similarly to doxorubicin, XR9576 did not produce any pronounced effects on the cytotoxicity of vinblastine in $MCF7^{WT}$ cells at concentrations up to 100 nM (Table 3). However, the potency of vinblastine to produce a cytotoxic effect was increased marginally at an XR9576 concentration of 300 nM ($IC_{50}=0.31\pm0.17$ nM), perhaps reflecting an adverse drug interaction at this high concentration of P-gp inhibitor. The potency of vinblastine to produce cytotoxicity in NCI/ADR^{Res}

Table 2
The ability of XR9576 to restore the cytotoxicity of doxorubicin in monolayers and tumour spheroid cultures of drug-sensitive and resistant-cells

[XR9576] (nM)	Monolayers			Tumour spheroids		
	$MCF7^{WT}$	NCI/ADR^{Res}	UCRF	$MCF7^{WT}$	NCI/ADR^{Res}	UCRF
0	0.151±0.028	15.7±4.8	104	1.02±0.34	28.6±17.1	28
3	0.206±0.060	13.4±4.9	65	1.46±0.64	19.0±9.0	13
10	0.228±0.071	14.0±7.8	61	0.94±0.29	3.7±2.4	4
30	0.256±0.061	2.2±0.2	9	1.72±0.36	0.55±0.24	0.3
100	0.212±0.049	1.8±0.2	8	2.81±0.30	0.66±0.16	0.2
300	0.274±0.067	2.0±0.4	7	0.65±0.27	0.32±0.18	0.5

Monolayer and tumour spheroid cultures were incubated in the presence of varying XR9576 concentrations for 12 h prior to the addition of doxorubicin (0–100 μM) for a further 24 h. Cytotoxicity was measured by the MTT colorimetric assay in monolayers or the extent of radial outgrowth in tumour spheroids. The potency of doxorubicin to produce cytotoxicity (IC_{50}) in the presence of XR9576 was determined by non-linear regression of the dose–response curve as described in the Methods. All values of IC_{50} are expressed as means±SEM in units of μM, obtained from at least four independent observations. The UCRF approximates the degree of P-gp-mediated resistance and is the ratio of IC_{50} values from NCI/ADR^{Res} versus $MCF7^{WT}$ monolayers or tumour spheroids. MTT, methylthiazoletetrazolium.

Table 3
The ability of XR9576 to restore the cytotoxicity of vinblastine in monolayers and tumour spheroid cultures of drug-sensitive and drug-resistant cells

[XR9576] (nM)	Monolayers			Tumour spheroids		
	$MCF7^{WT}$	NCI/ADR^{Res}	UCRF	$MCF7^{WT}$	NCI/ADR^{Res}	UCRF
0	1.29±0.47	3010±329	2333	12.7±4.0	2271±473	179
3	0.73±0.31	2860±496	3918	13.8±5.2	2504±434	181
10	1.76±1.04	2620±411	1489	15.2±9.6	2026±398	133
30	2.74±1.91	3590±339	1310	14.5±9.5	569±136	39
100	0.68±0.27	8.81±2.51	13	12.4±4.1	2.9±1.3	0.3
300	0.31±0.17	2.38±0.61	8	7.5±2.6	8.2±5.4	1.1

Monolayer and tumour spheroids cultures were incubated in the presence of varying XR9576 concentrations for 12 h prior to the addition of vinblastine (0–100 μM) for a further 24 h. Cytotoxicity was measured by the MTT colorimetric assay in monolayers or the extent of radial outgrowth in tumour spheroids. The potency of vinblastine to produce cytotoxicity (IC_{50}) in the presence of XR9576 was determined by non-linear regression of the dose–response curve as described in the Methods. All values of IC_{50} are expressed as means±SEM in units of nM, obtained from at least four independent observations. UCRF approximates the degree of P-gp-mediated resistance and is the ratio of IC_{50} values from NCI/ADR^{Res} versus $MCF7^{WT}$ monolayers or tumour spheroids. MTT, methylthiazoletetrazolium.

cells (IC_{50} = 3010±329 nM) reflected a 2333-fold resistance, which was sensitive to the effects of XR9576. This high level of resistance to vinblastine was reduced to a residual level of approximately 7-fold; an analogous situation to that observed for the chemically unrelated compound doxorubicin. The 'reversal potency' of XR9576 for vinblastine in the NCI/ADR^{Res} cell line was determined to be 68 nM suggesting that the resistance to vinblastine is less readily overcome compared with that observed for doxorubicin which was characterised by a 4-fold lower value.

3.5. The relative ability of XR9576 to reverse resistance to vinblastine or doxorubicin in tumour spheroid cultures

Can XR9576 also restore the sensitivity to vinblastine and doxorubicin in the TS^{Res} model? To answer this question, TS^{Res} were exposed to a range of vinblastine concentrations in the presence or absence of several XR9576 concentrations and the cytotoxicity determined using an outgrowth assay. The potency of vinblastine to produce cytotoxicity at several concentrations of XR9576 are summarised in Table 3. The data indicate that the 179-fold unicellular resistance to vinblastine in TS^{Res} compared with the TS^{WT} could be completely overcome with the P-gp inhibitor XR9576 and unlike the situation in monolayers, there was no evidence of residual resistance, even at high inhibitor concentrations. The 'reversal potency' of XR9576 to vinblastine in TS^{Res} was described with an EC_{50} value of 17 nM, which was 4-fold higher than that observed in monolayer cultures.

The 28-fold lower potency of doxorubicin to produce cytotoxicity in TS^{Res} was also reduced by XR9576 in a dose-dependent manner (Table 2). The 'reversal potency' was 6 nM, which is approximately 3-fold higher than required for XR9576 to restore the cytotoxic effects of vinblastine. The ability of XR9576 to fully 'sensitise' TS^{Res} to both vinblastine and doxorubicin demonstrates that the UCR component is in fact P-gp-mediated. In addition, the restoration potencies for XR9576 to improve the effectiveness of anticancer drugs appeared to depend on the level of resistance conferred by P-gp in the specific system (monolayer or tumour spheroids).

4. Discussion

Drug resistance in solid tumours represents a complex and interconnected series of resistance pathways that are both inherent and acquired [1]. Many of the inherent pathways have been classified under the banner of 'multicellular resistance' and provide a barrier to chemotherapy by adversely affecting intratumour drug pharmacokinetics [30,45]. The aetiological factors underlying the generation of these pathways are unclear, but they are thought to arise due to the hostile microenvironment produced in solid tumours [45,46] and even factors such as adhesion or cell–cell contact [47]. Given the complexity and enormity of the barrier presented by MCR, do specific mediators of drug resistance such as P-gp play a significant role in the overall drug resistance phenotype *in vivo*? There are a plethora of medical investigations correlating drug resistance with P-gp expression, thereby implicating this protein with overall prognosis to chemotherapy [17,19,48,49]. Controversy surrounds the issue and it has been argued that the correlations are not direct proof of a causal link between P-gp expression and drug resistance [32–34]. The present investigations have utilised the tumour spheroid model to provide quantitative analysis indicating that P-gp expression does attenuate drug efficacy in solid tissues. Moreover, the pharmacological inhibition of P-gp activity can be achieved in solid tumour models, resulting in the restoration of drug sensitivity.

The large number of protocols used for *in vitro* investigations on monolayer systems use a 1-h exposure period [50–52], but is this sufficient, particularly in solid

tumours? A major factor influencing the success of chemotherapy is the attainment of an appropriate exposure time to permit sufficient drug distribution within solid tumours. The rate of drug distribution within tumour spheroids is highly dependent on the physico-chemical properties of individual compounds [53,54]. For example, hydrophilic molecules such as cisplatin equilibrate rapidly, whereas hydrophobic agents such as doxorubicin and vinblastine may be efficiently sequestered, thereby preventing widespread distribution [43,55,56]. A mathematical model predicts that an incubation period of 5–15 h is necessary to achieve both (i) efficient accumulation of drugs and (ii) to ensure that the majority of cells have entered a proliferative status [57]. The time-dependent behaviour in the potency of vinblastine induced cytotoxicity in monolayer cultures, and to a lesser extent in tumour spheroids, is presumably due to the cell-cycle specificity of this agent [51,58]. Drugs were added to monolayer cultures shortly after re-seeding and, as a consequence, the cell population was not in the exponential growth phase. Consequently, the more dramatic effect in monolayer cultures reflects the progression of cells to a proliferative state, whereas in tumour spheroids, most cells in the outer layers were already undergoing proliferation at the time of drug addition. In contrast, doxorubicin did not display clear time dependence in the generation of cytotoxicity in either monolayers or tumour spheroids, which possibly correlates with the multiple routes of anthracycline-mediated cytotoxicity. Although predominantly acting to inhibit topoisomerase II during the cell cycle S-phase, cytotoxic actions have also been attributed to intercalation with DNA and peroxidative damage to cellular macromolecules [58]. Consequently, this ability of doxorubicin to act via multiple mechanisms may uncouple drug activity from the cell cycle.

The high levels of doxorubicin and vinblastine resistance are well established to occur due to their reduced intracellular accumulation as a result of the drug efflux activity of P-gp [59]. The NCI/ADR^{Res} cells used in the present investigation have been generated by drug selection; consequently, they highly overexpress P-gp and this is the major resistance mechanism. However, NCI/ADR^{Res} cells also exhibit resistance mechanisms other than increased drug efflux [60], and this may account for the low-level residual drug resistance observed following inhibition of P-gp by XR9576. The presence of P-gp in tumour spheroids did significantly reduce the potency of doxorubicin (10–20-fold) and vinblastine (200–300 fold) to produce cytotoxicity. However, the extent was approximately an order of magnitude lower than that in monolayer cultures. The resistance of monolayers to chemotherapeutic agents has always been viewed as significantly greater than that encountered in clinical tissues [1]. Furthermore, the level of resistance rarely correlates with the difference in the accumulation of drug and reported levels of multicellular resistance are significantly lower, yet clearly sufficient to interfere with chemotherapy.

The presence of inherent multicellular resistance in TS^{WT} was evident due to the reduced potency of doxorubicin and vinblastine to generate cytotoxicity compared with the MCF7^{WT} monolayers. Both drugs displayed a quantitatively similar 10–20-fold degree of MCR in TS^{WT}. This apparent 'low' degree of resistance requires that the reporting assay be taken into account. The radial outgrowth assay is a specific reporter for the proliferating outer cell layers of tumour spheroids [61]. Cytotoxic damage to the outer layers causes reduced tumour spheroids growth due to shedding of damaged cells, yet, at the same time, it will lead to the recruitment of quiescent populations to the proliferating pool [44]. Given that the distribution and accumulation of doxorubicin or vinblastine in the quiescent layer is low [43,56], then these inner cell layers in tumour spheroids provide a rich source of cells capable of recovering tissue growth following the initial cytotoxic onslaught. Whilst the radial outgrowth assay provides a direct measure of the initial onslaught produced by anticancer drugs in the proliferating peripheral cells, it does not take into account the longer term 'recovery potential' of the inner layers [44].

Consequently, the modest degree of MCR is a likely underestimation of the true resistance capacity of tumour spheroids. A surprising finding of the current investigations was the apparent absence of MCR in the P-gp-expressing TS^{Res} tissue. The reasons for this are unclear at present and may possibly result from either differences in (i) metabolic and biophysical characteristics of the NCI/ADR^{Res} cells compared with the parental line [35,60,62], or (ii) the different morphology of the TS^{Res}. A less compact 3-D organisation would produce lower interstitial pressures and reduced cell–cell contact, thereby profoundly influencing the distribution, equilibration and potency of anticancer drugs within the tumour spheroids [53,55,63]. This may also explain the lower P-gp-mediated effect, particularly since prolonged or increased exposure of cells to drug may partially overcome P-gp-mediated resistance [64].

A major finding of the current investigation is that P-gp does play a role in defence against the early stages of drug-induced cytotoxicity in a model of solid tumour architecture. It was of major interest therefore to determine whether this effect could be modulated pharmacologically? Although it has been suggested that P-gp-mediated resistance may be overcome by continuous drug infusion [64], this may not always be possible in the clinical setting. Consequently, XR9576, like GF120918, PSC833 and LY79553 [22,24,26], has been specifically developed to potently and selectively counter the actions of P-gp in tumour tissue [28]. In NCI/ADR^{Res} cells, the use of XR9576 provided a means to

restore the effectiveness of doxorubicin and vinblastine in retarding cell growth in monolayers, a property that was produced at clinically achievable concentrations. This promising *in vitro* activity of XR9576 has led to the establishment of clinical trial with the aim of restoring sensitivity to chemotherapy; and in the case of P-gp-expressing lymphocytes, this is clearly achievable [28]. The data using tumour spheroids now indicates that XR9576 is also capable of potently restoring the sensitivity to anticancer drugs in a solid tissue environment. That the restoration of drug sensitivity was possible in the outer layers of tumour spheroids, despite the relatively high level of P-gp expression, provides hope that XR9576 may provide demonstrable benefit in clinical tissues where the amount of P-gp will be considerably lower. However, the positive response to XR9576 may only reflect inhibition of P-gp in the surface-located proliferating cell population. For certain drugs (doxorubicin), it has been reported that the inhibition of P-gp function may reduce penetration [42] through solid tissue. However, the results of a more recent report [54] indicate that the inhibition of P-gp will impact on drug distribution/penetration in a manner dependent on the physico-chemical properties of the drug. For example, in comparison to doxorubicin [42], paclitaxel derivatives will distribute throughout tumour spheroids following inhibition of P-gp. It has previously been demonstrated that P-gp expression may develop in the hypoxic or quiescent layers of tumour spheroids and possibly clinical tumours [41–43]. Therefore, quiescent cells that have been recruited to the proliferating population will possess a P-gp-mediated resistant phenotype. To counter this possibility, careful dosing schedules of P-gp inhibitor and cytotoxic agent will be required to maintain chemosensitivity. Alternatively, should XR9576 distribute extensively throughout solid tissue (e.g. to quiescent cells) and effect extensive P-gp inhibition, it may promote accumulation that will only manifest as cytotoxicity once the quiescent cell re-enters the cell cycle.

In summary, the present investigations indicate that P-gp expression in cells within a solid tumour will modulate the effectiveness of drug therapy and depending on the exact morphology of the tissue, augment the inherent MCR. Moreover, the results provide 'proof of principle' for pharmacological inhibition of P-gp in order to restore chemotherapeutic efficacy in solid tumour tissue.

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