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Incidence of P-glycoprotein overexpression and multidrug resistance (MDR) reversal in adult soft tissue sarcoma

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Abstract

Multidrug resistance (MDR) is a widespread problem in the treatment of neoplastic diseases and may limit the effectiveness of treatment of adult soft tissue sarcomas (STS). We examined the levels of expression of the MDR marker P-glycoprotein (Pgp) in fresh, surgical material and matched paraffin-embedded tissue using MRK-16 and JSB-1 monoclonal antibodies. Using fresh tumour material in short-term culture an assessment of doxorubicin sensitivity (MTT assay) and MDR modulation using PSC-833 in daunorubicin (DNR) accumulation experiments (FACS analysis) was carried out. 44 patients were studied at various disease stages with a mean follow-up duration of 487 days (range: 45–1095 days). Immunocytochemistry and immunohistochemistry showed 62% and 58%, respectively, of STS samples were positive for Pgp. Patients showing negative Pgp expression had a median survival of 544 days versus 431 days for Pgp-positive patients ($P=0.311$), with disease-free survival medians of 508 and 355 days, respectively ($P=0.203$). *In vitro* doxorubicin sensitivity was not informative in this respect and there was no apparent relationship between this and Pgp expression. Eleven out of 29 samples evaluated for MDR modulation showed enhanced tumour cell DNR accumulation. However, the effects of PSC-833 on drug accumulation in clinical material were modest compared with those seen for MDR cell lines, with a maximum of only 20% enhancement. Moreover, there was no relationship between the extent of PSC-833 effects on accumulation and the levels of Pgp seen in the STS samples. Nevertheless, we show evidence that a proportion of cases of STS express moderate to high levels of Pgp. There may be a role for MDR modulating agents in association with doxorubicin in the treatment of these tumours, either in the adjuvant setting or at first relapse.

Keywords: Sarcoma; P-glycoprotein; Doxorubicin sensitivity; MDR reversal

1. Introduction

Doxorubicin is the mainstay of chemotherapy in adult soft tissue sarcomas (STS), along with ifosfamide, but the response rate for this disease with combination chemotherapy is only 20–30%. Poor clinical response to resistance to cancer chemotherapy, which is a commonplace occurrence for a number of neoplasms, is also prevalent in STS. Multidrug resistance (MDR) mediated via the *MDR1* gene is perhaps the best known form of anticancer drug resistance which has, to date, been extensively described in drug-resistant cell line models with acquired resistance to natural product cytotoxic agents such as the anthracyclines. The *MDR1* gene codes for a 1280 amino acid transmembrane glycoprotein of 170 kDa, termed *P*-glycoprotein (Pgp).

Pgp positivity may be seen in a number of patients' tumours during the course of their disease. However, there are some indications that a proportion of relapsed tumours may remain Pgp negative during the course of therapy and thereafter. Thus, other mechanisms underlying the evolution of a drug-resistant phenotype must be considered. A number of studies do, however, suggest Pgp overexpression is a feature of STS [1,2]. Specifically, Pgp expression has been examined in 15 cases of matched primitive neuroectodermal tumour (PNET) and Ewing's tumours before and after treatment, 7 of which were positive for Pgp prior to treatment. Subsequently, 4 of the originally negative group became positive following treatment [3]. In contrast, a study by Vergier and colleagues [4] found only 1 out of 22 cases

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of sarcoma were positive for Pgp, but the authors stress the importance of assessing the various techniques available for detection before drawing any conclusions.

A number of studies used immunohistochemistry [2,5,6], whilst others studied *MDR1* mRNA expression by dot blot hybridisation [7,8,9]. The present study made use of both fresh and paraffin-embedded material from cases of adult STS. In addition, by using fresh tumour material in short-term culture we were able to make an assessment of MDR function by the combined use of cyclosporins and anthracyclines *in vitro*. This work, therefore, represents an *in vitro* feasibility study into the potential for the use of MDR reversal strategies in this disease group. Our overall objective was to investigate the possibility of identifying a cohort of STS patients who may respond to a regimen of MDR reversal. Such a strategy may result in an improvement in the management of this typically chemorefractory disease. Preliminary results on a selection of data taken from this study have been published in abstract and lecture form [10–12].

2. Patients and methods

Wherever possible the methodological guidelines laid down by the St Jude's MDR workshop [10] were adhered to.

2.1. Patients

Details are summarised in Table 1 and represent 44 cases of adult STS of various histological types and grade. The patients included in this study were either pretreated, as detailed in Table 2, or chemotherapy-naive. Samples were received from cases at various disease stages, as detailed in Table 3. All patients underwent surgery performed at the Royal Marsden Hospital (RMH), London, UK. Histopathological assessment and diagnosis was made by the Department of Histopathology, RMH. Ethical approval for this study was obtained from the Committee for Clinical Research and the Ethics Committee, RMH with signed consent from all patients involved.

2.2. Culture of fresh tumour material

All tissue culture reagents were obtained from Life Technologies (UK) Paisley, UK, unless otherwise stated. Freshly acquired surgical samples immersed in L-15 (Leibovitz) medium with antibiotics were received in the laboratory within 48 h. Samples were subjected to washing with L-15 tissue culture medium to remove blood and were then subjected to mincing using crossed scalpels. Collagenase type I (Sigma Chemicals, Poole, Dorset, UK), was used at a concentration of 0.25% to further disaggregate the sample with incubation at 37°C in 5% CO_2 for 20 min–2 h depending on the consistency of the sample. Samples were subjected to further mechanical disaggregation by pipetting the resulting cellular suspension to create a quasi single cell suspension. If samples were heavily bloodstained a sucrose gradient (Histopaque 1077, Sigma) was used to separate out blood cells from tumour cells. For short-term cultures, cell suspensions were added in Ham's F-12 with Glutamax, 10% heat inactivated fetal calf serum (FCS) and antibiotics and incubated in an humidifying gassing incubator at 37°C, 5% CO_2. All cultures which were successfully established grew as monolayers. Cells were detached from tissue culture flasks by the use of trypsin-EDTA (Life Technologies), or by the use of non-enzymic cell dissociation fluid (Sigma) for use in drug accumulation experiments to assess MDR reversal with PSC-833.

2.3. Control MDR cell lines used throughout the study

The Fuji human synovial sarcoma line [14] which overexpresses the *MDR1* gene (data not shown) and has a constitutive low-level Pgp expression was used for immunocytochemistry. The CH1 (human ovarian cancer line; [15]) and its doxorubicin-resistant variant CH1DOXR (with 25-fold resistance; [16]) was used alongside the HSN rat fibrosarcoma line (showing

Table 1
Demographic and histological features of soft tissue sarcomas (STS)

Characteristic	No.	%
Sex:		
Male	24	55
Female	20	45
Age (years)		
10–30	1	3
31–50	17	39
51–70	16	36
71–90	10	23
Histological subtype:		
MFH	9	20
Leiomyosarcoma	7	16
Liposarcoma	3	7
Myxoid liposarcoma	5	11
Synovial	5	11
Fibromatosis	4	9
PNET	1	2
MPNT	2	5
Others	8	19
Grade		
Benign	4	9
Low	7	16
Intermediate	9	20
High	22	50
Unclassified	2	5

MFH, malignant fibrohistiocytoma; PNET, primitive neuroectodermal tumour; MPNT, malignant peripheral nerve sheath tumour.

Table 2
Patients treated prior to sampling

Sample/diagnosis	P-glycoprotein (Pgp) status	Pretreatment
MS4/Leiomyosarcoma (1st relapse)	Positive	Radiotherapy
MS36/synovial sarcoma (pulmonary recurrence)	Positive	MAID[a]
MS40/Liposarcoma (new primary)	Negative	Doxorubicin
MS42/Myxoid liposarcoma (1st relapse)	Positive	Radiotherapy
MS66/Leiomyosarcoma (3rd relapse)	Positive	Radiotherapy
MS67/Mesenchymoma (primary surgery)	Positive	Radiotherapy for previous Hodgkin's disease

[a] MAID = mesna, doxorubicin, ifosfamide, DTIC (dacarbazine).

constitutive low-level Pgp expression) with its doxorubicin-resistant variants HSNDOX1 (approximately 6-fold resistant) and HSNDOX* (approximately 30-fold resistant; [17]) as controls for immunocytochemistry and MDR reversal with cyclosporins. The overexpression of Pgp has been previously confirmed by Western blotting of all the control cell lines used in the study using the C-219 antibody (data not shown). Maintenance doses of doxorubicin were used to retain the MDR phenotypes of the drug-resistant lines until at least 48 h before the start of experiments. All cell lines were routinely screened for mycoplasma. Monolayer cultures were subjected to gentle trypsinisation and reduced to a single cell suspension prior to use as controls for cytospins used in immunocytochemistry. Alternatively, cell lines used as controls for drug accumulation experiments to assess resistance reversal were subjected to two gentle washes with non-enzymic dissociation fluid (Sigma) in order to detach and to achieve a single cell suspension. Exponentially growing cells were harvested for use in experiments.

2.4. Chemosensitivity testing

STS cells at a density of $1–5\times10^5$ cells/ml in Ham's F-12 containing 10% FCS, Glutamax and antibiotics (all obtained from Gibco Life Technologies) were dispensed into 96-well plates in volumes of 200 μl and left to equilibrate for 24 h at 37°C and 5% CO_2. Doxorubicin (obtained from Sigma) was dissolved in sterile distilled water and stored as a stock solution in frozen aliquots. Freshly thawed drug solution was diluted in tissue culture medium containing 10% FCS and added in a volume of 50 μl in increasing concentrations. Control wells containing cell suspension were supplemented with a similar volume of tissue culture medium only. Following a 72 h incubation, cultured cells were subjected to the lactate dehydrogenase (LDH) assay for assessment of cell number, according to the previously published method [18]. Results were expressed as the IC_{50}, i.e. the concentration of cytotoxic drug that reduced the cell number by 50%, relative to the control (untreated cells).

Table 3
Setting of sarcoma samples and incidence of P-glycoprotein (Pgp) expression

Sample setting	*n*	Pgp positive *n* (%)
Primary surgery	27	11 (41)
1st relapse	12	10 (83)
2nd relapse	1	1 (100)
3rd relapse	2	1 (50)
Metastatic disease	2	0 (0)

2.5. Estimation of p-glycoprotein expression

2.5.1. Immunocytochemistry

Cytospin preparations were made of tumour cells in suspension using a cytological centrifuge, allowed to air dry and then were stored at −20°C wrapped in foil until required. Cytospin preparations were fixed in acetone: methanol (1:1) for 90 s and normal rabbit serum was used to block non-specific staining. Pgp expression was assessed using the murine monoclonal antibody MRK-16 (TCS Biologicals, Boltolph, Claydon, UK). Incubation with the primary antibody or the appropriate isotype control (i.e. IgG2a using purified murine immunoglobulin obtained from Sigma) diluted and used at the same protein concentration as the primary antibody was followed by the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique (reagents obtained from DAKO Ltd., Ely, UK) using Fast Red as the chromogenic endpoint and 0.1% Mayer's haematoxylin solution as a counterstain (Sigma). Slides were scored blind to other results and recorded as the percentage positive cells with correction made for the isotype staining. In addition, slides which were graded as positive for Pgp expression showed staining in multiple tumour cells in multiple microscopic fields. All slides were read by at least two independent viewers with the consensus results being reported. The grading of staining intensity was also recorded as (−) negative, (−/+) weak, (+) low, (++) intermediate, (+++) high and (++++) ultrahigh. Positive expression was regarded as (+) and above. Thus, percentage scores were the summation of the number of cells with (+) to (++++) staining.

2.5.2. Immunohistochemistry

Five micron sections of formalin-fixed tumour tissue were used to assess Pgp status, alongside the replicate cytospins made from replicate fresh material. Microwave oven treatment was used to unmask the antigen sites in the presence of citrate buffer at pH 6.0. For immunostaining, the avidin–biotin peroxidase complex methodology was used with the JSB-1 antibody (TCS Biologicals), with a secondary biotinylated rabbit anti-mouse antibody (DAKO). The colour reaction was developed using 0.02% 3-,3′-diaminobenzidine tetrachloride with 0.02% hydrogen peroxide in Tris buffer (pH 7.4). After immunostaining sections were counterstained with Harris' haematoxylin, dehydrated in graded alcohols, cleared in xylene and mounted. Scoring was in line with the grading system used for the immunocytochemistry, i.e. (−) negative to (+ + + +) ultrahigh.

2.5.3. Quantitation using flow cytometric analysis

Cells were pelleted by centrifugation, washed twice with 0.1% bovine serum albumin (BSA) in sterile phosphate buffered saline (PBS) and then resuspended in 100 μl of ice-cold 70% methanol in PBS in order to fix and permeabilise the cells. Cells were then vortexed vigorously and stored at −20°C for up to 4 weeks. In order to detect Pgp, fixed cells were incubated for 1 h at room temperature with mouse monoclonal antibody (MAb) MRK-16 at 10 μg/ml (TCS Biologicals) or the appropriate isotype control mouse IgG2a (at the same protein concentration as the primary antibody; Sigma Chemicals). Cells were then washed twice in 0.1% BSA in PBS, resuspended in 1 ml and goat-anti-mouse fluorescein isothiocyanate (FITC; Sigma) diluted 1:100 in distilled water added, followed by a 45 min incubation in the dark. Fluorescence was analysed with an ESP Elite flow cytometer, which was equipped with an argon laser. The fluorescence of 10 000 events was logarithmically measured at a laser excitation wavelength of 488 nm. The fluorescence of daunorubicin was collected through a band filter of 575 nm. The logarithmically amplified signals were converted into values on a linear scale, expressed as relative fluorescence units from which the mean fluorescence was computed. The mean of the fluorescence of the MRK-16 labelled cells was divided by the mean of the fluorescence of the isotype control-labelled cells. The range of MDR cell lines used in these studies enabled a calibration graph to be constructed, indicating the linearity of the method ($R^2 = 0.978$). Cell lines showed approximately 20–75% Pgp positivity with a grading of intensity +−+ + (data not shown).

2.6. Drug accumulation studies for resistance reversal using PSC-833

Cells in single suspension ($0.5–1 \times 10^6$) were incubated for 30–60 min at 37°C in the absence or presence of PSC-833 (a kind gift from Novartis, Camberley, UK) at 1 μM in Dulbecco's phenol red-free medium with 10% FCS in 1 ml aliquots. This dose of modulator was shown to effectively reverse Pgp-mediated drug resistance in chemosensitivity testing using control cell lines in this study (data not shown). It is also a dose that has been used in other studies and is within the clinically achievable dose range for PSC-833. Daunorubicin (Sigma) at a concentration of 5 μM was added with a further 60 min incubation to achieve steady-state levels. Cells were then pelleted by centrifugation at 400*g*, at 4°C and resuspended in fresh medium, again in the presence of PSC-833. Cells were allowed to efflux the daunorubicin for a further 60 min at 37°C and pelleted as before.

Following resuspension in drug-free medium, cells were stored on ice prior to analysis. Throughout the incubation periods, cells were gently agitated at regular intervals to prevent excessive clumping. A control untreated cell suspension was run at the beginning of each analysis, to allow elimination of clumped cells and debris by gating out. The fluorescence of daunorubicin was collected through a band filter of 575 nm. Results were calculated as the ratio of the mean daunorubicin-associated fluorescence peak in the presence of PSC-833 to that obtained in the absence of PSC-833. Thus, any values obtained giving a ratio of > 1.00 were deemed to be positive.

2.7. Statistics

Non-parametric methods were used for statistical analysis. Spearman's rank correlation coefficient was used to compare nominal data, Wilcoxon signed rank test to compare paired matched samples and the Mann–Whitney U test to assess differences between groups of patients. Survival was calculated using Kaplan–Meier analysis and was measured from time of diagnosis to time of death or the last recorded contact with the patient (up to August 1998). The log rank test (with Yates correction) was used to compare survival curves.

3. Results

The mean duration of follow-up on the subjects in the study was 487 days (range: 45–1095 days).

3.1. Chemosensitivity

Twenty-nine samples were deemed suitable for chemosensitivity testing. The range of doxorubicin IC_{50} values obtained are shown in Fig. 1 (which shows a selection of data) ranging from 0.14 to > 2.00 μM. The overall mean for the group was 1.09 μM, median 0.71 μM. There was no statistically significant difference

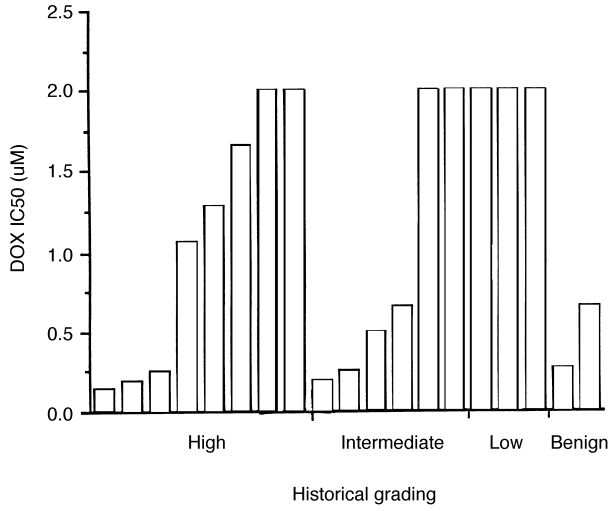

Fig. 1. Relationship between doxorubicin sensitivity and histological grading of adult soft tissue sarcoma (STS).

between the survivals of those patients with *in vitro* doxorubicin sensitivities below or above the median value ($P = 0.928$). As seen in Fig. 1, histological grading had no influence on *in vitro* doxorubicin sensitivity. Benign tumours (fibromatoses) appeared the most chemosensitive of the entire series tested [19].

3.2. Pgp estimation

3.2.1. Immunocytochemistry

For cytospins of fresh tumour material, the level of Pgp assessed on 42 samples ranged from 0 to 87%, with positivity in 26 (62%) of samples (Fig. 2). In most cases Pgp was localised to the plasma membrane, although occasional cytoplasmic staining was seen, but in the presence of membrane staining. The scoring of the staining intensity tended to be (+)–(++). Heterogeneous Pgp expression was evident in some samples. Fig. 2 indicates an even distribution of Pgp expression for high, intermediate and low histological grades.

3.2.2. Immunohistochemistry

Of the 33 samples evaluated, 19 (58%) showed varying degrees of Pgp positivity, from (+) to (++++) (data not shown). As the JSB-1 antibody recognises an internal epitope of Pgp some variation in the results could be expected when comparing them with those obtained using MRK-16 antibody in the same samples. However, the two sets of data were in good agreement. The subcellular Pgp localisation was not so clear as that seen for the disaggregated samples used for the immunocytochemistry. However, tissue structures were clearly located, in particular blood vessels, which stained positively in a number of sample sections.

3.3. FACS analysis

Control cell line values for quantitation of Pgp were as follows (stated as standard error of the mean, SEM; and standard deviation, S.D.; in parentheses: FACS ratio of MRK-16 reactive cell population relative to the matched isotype control): Fuji 1.21 (0.05); HSN 1.13 (0.03); HSNDOX1 1.39 (0.075); HSNDOX* 3.2. (0.094). The relationship between these measurements and immunocytochemistry per cent (ICC%) positivity are depicted in Fig. 3. The correlation between FACS ratio and per cent immunocytochemistry gave an r value of 0.48 using Spearman's rank correlation analysis for the tumour samples. A number of factors may contribute to the lack of linearity, such as heavy isotype

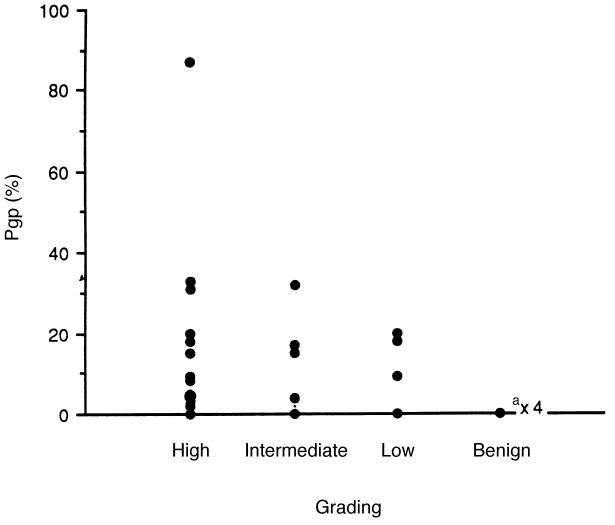

Fig. 2. Relationship between extent of Pgp positivity (immunocytochemistry) and histological grading of adult soft tissue sarcoma (STS). [a]Representative of four samples.

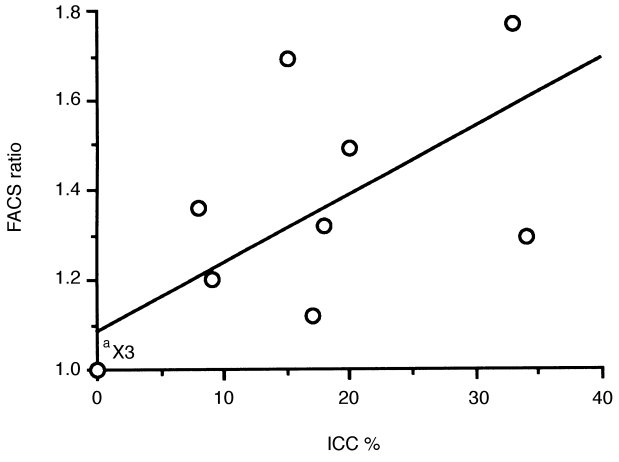

Fig. 3. Graph showing relationship between FACS ratio and % positivity with immunocytochemistry (ICC) for determination of Pgp expression. [a]Three samples were zero.

control reactivity and variations in antigen density. For some samples, two populations of cells expressing varying levels of Pgp were seen which were also evident from the data obtained for the MDR reversal effects of PSC-833, i.e. samples MS80, MS90.

3.4. Drug accumulation studies for resistance reversal using PSC-833

Control MDR cell lines were run in each batch of clinical samples evaluated. Results obtained as ratios of cellular daunorubicin fluorescence in the presence and absence of PSC-833 (as the mean and standard deviation in parentheses) were for HSN 1.36 (0.170); HSNDOX* 5.89 (1.11); CH1DOXR 6.8 (1.08). Results obtained for clinical material are shown in Table 4. Of the 29 samples evaluated 11 (38%) showed some modification, with ratios ranging from 1.03 to 1.20. We were able to see a relationship between the mean level of Pgp expression of a sample and its modification by PSC-833. Samples showing positive function had a mean Pgp level of 25%, compared with 8% for samples with no function, $P=0.010$ (Mann–Whitney U). However, there was no apparent overall relationship between the magnitude of the PSC-833 effect on daunorubicin accumulation and the extent of Pgp positivity (or FACS ratio), (Fig. 4).

3.5. Relationship between clinical parameters and Pgp expression

The median survival for patients with Pgp-positive tumours was 431 days, versus 544 days for patients with Pgp-negative tumours, $P=0.311$. The median disease-free survival for patients with Pgp-positive tumours was 355 days versus 508 days for Pgp-negative patients, $P=0.203$ (data not shown). Table 3 details the grouping of patients according to clinical setting of their sample and Pgp positivity. It can be seen that the majority of cases were taken at either primary surgery or at first relapse (39/44; 89% of the total sample). Pgp positivity was less frequent in the former (11/27; 41%) than in the latter group of patients (10/12; 83%). Low sample numbers from the other specimen groupings made this assessment inconclusive with respect to disease progression and Pgp positivity. Table 2 details Pgp status on patients who had undergone treatment prior to sampling. 5/6 subjects were positive for Pgp following various treatment modalities. Sample MS40 (new primary tumour) which was from the only patient to be pretreated with single agent doxorubicin was negative for Pgp.

A total of 13 patients received chemotherapy and/or radiotherapy following surgical biopsy for this study, as detailed in Table 5. The three samples showing high Pgp expression (MS33,49,55) and 2/3 with intermediate expression (MS39,54,69) were all from patients who died within the time-frame of the study. Of the remaining negative and low Pgp-expressing samples, 6/7 were alive at the close of the study.

4. Discussion

Several mechanisms underlying anticancer drug resistance have been described but MDR due to Pgp is perhaps the best studied to date. Hence, the present study has investigated the association between functional Pgp expression and chemosensitivity with the clinical behaviour of adult STS, a chemorefractory group of tumours. Data have been derived from the examination of fresh, surgical material in short-term culture and formalin-fixed tissue. In addition to assessing the role of

Table 4
Resistance reversal data using PSC-833 obtained from adult STS samples

Sample	Resistance reversal ratio	Ppg level (%)	FACS ratio
MS33/Synovial sarcoma	1.16	87	ND
MS43/Extraskeletal	1.07	34	1.29
MS49/MFH	1.03	32	ND
MS51/MFH	1.03	33	1.77
MS59/Leiomyosarcoma	1.14	8	ND
MS62/Myxoid chondrosarcoma	1.17	9	1.20
MS66/Leiomyosarcoma	1.14	29	3.40
MS67/Mesenchymoma	1.20	15	1.67
MS80/Myxoid liposarcoma	1.04; 1.11 (2 peaks seen)	18	1.00; 1.32
MS88/Leiomyosarcoma	1.18	4	ND
MS90/MFH	1.00; 1.10 (2 peaks seen)	9	1.00; 1.36

ND, not done due to insufficient sample; MFH, malignant fibrohistiocytoma. Only samples showing a positive result are shown.

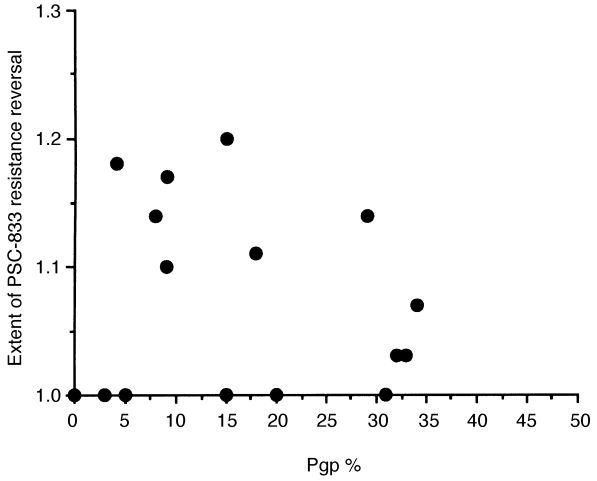

Fig. 4. Scatter plot indicating lack of correlation between level of Pgp expression and extent of resistance reversal by PSC-833.

Table 5
Postsampling chemotherapy for sarcoma patients

Sample/diagnosis	Post-operative treatment	Pgp status	Outcome
MS10/Synovial (Primary surgery)	Doxorubicin, ifosfamide, radiotherapy	Positive	Disease free
MS33/Synovial (2nd local recurrence)	Doxorubicin	Positive	No response, patient died
MS39/MPNT (1st local recurrence)	Doxorubicin	Positive	Rapid relapse with lung metastases, patient died
MS48/PNET (primary surgery)	Doxorubicin, vincristine, ifosfamide	Negative	Disease free
MS49/MFH (primary surgery)	Radiotherapy	Positive	Rapid relapse, patient died
MS54/Synovial (primary surgery)	Ifosfamide	Positive	Complete response, disease free
MS55/Spindle cell (1st relapse)	Doxorubicin, DTIC	Positive	Persistent disease, brain and lung metastases, patient died
MS57/MFH (primary surgery)	Doxorubicin	Positive	Partial response, late relapse, patient alive
MS67/Mesenchymoma (primary surgery)	Ifosfamide	Positive	Stable disease, rapid relapse, patient died
MS69/Pleomorphic (primary surgery)	Radiotherapy	Positive	Persistent disease, patient died
MS73/Myxoid liposarcoma (primary surgery)	Ifosfamide	Negative	Lung metastases, further ifosfamide given, stable disease, patient alive
MS79/Mesothelioma (primary surgery)	MVP	Positive	Partial response, patient alive
MS90/MFH (1st recurrence)	Doxorubicin	Positive	Partial response, patient alive

DTIC, dacarbazine; MPV, methotrexate/vinblastine/procarbazine; MFH, malignant fibrohistiocytoma; MPNT, malignant peripheral nerve sheath tumour.

Pgp in adult STS, we have looked at the potential of the MDR reversing agent PSC-833 in this context.

None of the data obtained from this study achieved a level of statistical significance (at the $P < 0.05$ level). However, whilst the number of patients in the present study is relatively small, our data compare favourably with similar sized studies [2,20]. Our findings are in agreement with these reports describing Pgp expression as a prognostic indicator for adult STS. In addition to measuring Pgp, an assessment of doxorubicin sensitivity was made (using chemosensitivity testing in short-term culture) and we conclude that Pgp-positive and -negative tumours could not be distinguished in terms of this *in vitro* parameter ($P = 0.858$). 11 of the 13 patients assessable for response to postoperative chemotherapy and/or radiotherapy were Pgp positive, Table 5. However, as only 8 of these were given MDR-related cytotoxic agents (such as doxorubicin, vinblastine) no conclusions can be drawn with respect to prediction of chemosensitivity *in vivo*.

In this study we used two MAbs, MRK-16 and JSB-1, directed against external and internal Pgp epitopes, respectively. The different approaches of using fresh, disaggregated and fixed paraffin-embedded tumour tissue are each informative in their own right. Immunohistochemistry, although not as sensitive as PCR-based techniques, has the advantage of enabling identification of areas and/or particular structures therein that may be Pgp-rich. Immunohistochemistry and immunocytochemistry can also facilitate histological confirmation of each cell with respect to its malignant character. Clearly, accurate quantitation of Pgp expression in a tumour, although highly desirable, is difficult to achieve, as the present study suggests. The FACS analysis of Pgp content was able to pick out subpopulations of cells with varying levels of Pgp expression in some samples. However, as acknowledged by others [21] there is evidence of highly overlapping subpopulations of Pgp expressing tumour cells which may mask the extent of heterogeneity of some samples. Other technical difficulties were evident when using FACS analysis for Pgp quantitation, i.e. non-specific, high isotype control binding and an overall lack of sensitivity relating to choice of fluorochrome (FITC) and the need for higher affinity anti-Pgp antibodies.

Clinical trials involving the use of MDR reversal strategies in solid tumours have hitherto been disappointing. We show evidence that a proportion of cases of adult STS may express moderate to high levels of Pgp. Furthermore, a cohort of those patients may benefit from use of modulation strategies, such as PSC-833. However, our data show that PSC-833 enhancement of anthracycline accumulation in clinical material is considerably more modest than that seen for MDR cell lines even those with low levels of acquired doxorubicin resistance. Indeed, the Fuji human synovial sarcoma cell line, showing constitutive *MDR1* and Pgp expression (data not shown) shows a complete lack of chemosensitisation and no enhancement of anthracycline accumulation with combined PSC-833 treatment (data not shown). Whether disaggregation of the tumour sample causes disruption of MDR transporter integrity is not known. It may be that the most appropriate place to use Pgp-modulating agents is at the time of initial chemotherapy in order to prevent the emergence of resistance. In a prospective randomised trial designed to test such a hypothesis, time to progression would perhaps be a better endpoint than response rate

and this would be the best setting in which to test the validity of pretreatment *in vitro* assessment of reversible drug resistance.

Data emerging from clinical trials on MDR modulating agents tend to support the findings of the present study in that only modest effects, if any, have been seen for adult solid tumours. Our findings support a renewed search for potent, more specific MDR reversing agents.

References

1. Serra M, Scotlandi K, Manara MC, *et al. Cytotechnology* 1996, **19**, 253–256.
2. Nakanishi H, Myoui A, Ochi T, Aozasa K. P-glycoprotein expression in soft tissue sarcomas. *J Cancer Res Clin Oncol* 1997, **123**, 352–356.
3. Hijazi YM, Constantine AA, Navarro S, Steinberg SM, Horowitz ME, Tsokos M. Immunohistochemical detection of p-glycoprotein in Ewing's sarcoma and peripheralprimitive neuroectodermal tumors before and after chemotherapy. *Anatomic Path* 1994, **102**, 61–67.
4. Vergier B, Cany L, Bonnet F, Robert J, de Mascarel A, Coindre JM. Expression of MDR1/Pglycoprotein in human sarcomas. *Br J Cancer* 1993, **68**, 1221–1226.
5. Gerlach JH, Bell DR, Karakousis C, *et al.* P-glycoprotein in human sarcoma: evidence formultidrug resistance. *J Clin Oncol* 1987, **5**, 1452–1460.
6. Chan HSL, Thorner PS, Haddad G, Ling V. Immunohistochemical detection of p-glycoprotein: prognostic correlation in soft tissue sarcoma of childhood. *J Clin Oncol* 1990, **8**, 689–704.
7. Goldstein LJ, Galski H, Fojo A, *et al.* Expression of a multidrug resistance gene in humancancers. *J Natl Cancer Inst* 1989, **81**, 116–124.
8. Toffoli G, Frustaci S, Tumiotto L, *et al.* Expression of MDR1 and GST pi in human soft tissue sarcomas: relation to drug resistance and biological aggressiveness. *Ann Oncol* 1992, **3**, 63–69.
9. Oda Y, Schneider-Stock R, Rys J, Gruchala A, Niezabitowski A, Roessner A. Expression of multidrug resistance associated protein gene in human soft tissue sarcomas. *J Cancer Res Clin Oncol* 1996, **122**, 161–165.
10. Coley HM, Verrill MV, Gregson SE, Judson IR. Drug resistance mechanisms in adult soft tissue sarcomas (STS) using primary and established cultures. *Proc AACR* 1996, **37**, 2119.
11. Coley HM, Verrill MW, Judson IR, Fisher C. P53 abnormalities influence modulation ofMDR in primary cultures of adult soft tissue sarcoma. *Proc AACR* 1997, **38**, 2612.
12. Coley HM. Drug resistance studies using fresh human ovarian carcinoma and soft tissue sarcoma samples. *Keio J Med* 1997, **46**, 142–147.
13. Beck WT, Grogan TM, Willman CL, *et al.* Methods to detect P-glycoprotein-associated multidrug resistance in patients' tumors: consensus recommendations. *Cancer Res* 1996, **56**, 3010–3020.
14. Nojima T, Yi-Song W, Abe S, Matsuno T, Yamawaki S, Nagashima K. Morphological and cytogenetic studies of a human synovial sarcoma xenotransplanted into nude mice. *Acta Pathologica Japonica* 1990, **40**, 486–493.
15. Hills CA, Kelland LR, Abel G, Siracky J, Wilson AP, Harrap KR. Biological properties often human ovarian carcinoma cell lines: calibration *in vitro* against four platinum complexes. *Br J Cancer* 1989, **59**, 527–534.
16. Yanagisawa T, Newman A, Coley HM, Renshaw J, Pinkerton CR, Pritchard-Jones K. BIROCODAR (VX-710; Incel™) — an effective chemosensitiser in neuroblastoma. *Br J Cancer* 1999, **80**, 1190–1196.
17. Sosabowski J, Carnochan P, Coley HM, Judson IR. Development and evaluation of novel PET radiotracer probes for multidrug resistance (MDR) using a transplantable rat sarcoma. *Proc AACR* 1997, **38**, 3979.
18. Coley HM, Lewandowicz G, Sargent JM, Verrill MW. Chemosensitivity testing of fresh and continuous tumour cell cultures using lactate dehydrogenase. *Anticancer Res* 1997, **17**, 231–236.
19. Verrill MW, Coley HM, Judson IR, Fisher C. Susceptibility of fibromatosis cells in short term culture to ifosfamide: a possible new approach to the treatment of clinically aggressive cases. *Sarcoma* 1999, **3**, 79–84.
20. Levine A, Holzmayer T, Bacus S, *et al.* Evaluation of newer prognostic markers for adult soft tissue sarcomas. *J Clin Oncol* 1997, **15**, 3249–3257.
21. Broxterman HJ, Sonneveld P, Feller N, *et al.* Quality control of multidrug resistance assays in adult acute leukaemia: correlation between assays for p-glycoprotein expression and activity. *Blood* 1996, **87**, 4809–4816.