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BIRICODAR (VX-710; Incel™): an effective chemosensitizer in neuroblastoma

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Summary Clinical studies have suggested that both MDR1 and MRP may play a significant role in the chemosensitivity and outcome of neuroblastoma. To clarify the nature of multidrug resistance (MDR) in this tumour a series of six neuroblastoma cell lines have been characterized with regard to P-glycoprotein, MRP and LRP expression using immunocytochemistry and expression of MDR1, MRP, LRP and topoisomerase II genes using reverse transcription polymerase chain reaction (RT-PCR). By RT-PCR, all lines expressed MRP, five expressed LRP and four expressed MDR1, but protein levels of each of these were variable. Chemosensitization to a range of MDR-associated drugs (vincristine, doxorubicin, etoposide, taxotere, topotecan) and non-MDR-associated drugs (cisplatin, melphalan) by three modulating agents, cyclosporin A, PSC 833 and the novel Biricodar (VX-710; Incel™), was evaluated using a colourimetric cytotoxicity assay (MTS). Alteration of daunorubicin efflux by these agents was evaluated using FACS analysis. Clonogenic assay was used to study the influence of these chemosensitizers on vincristine cytotoxicity. Marked sensitization to vincristine was observed in MDR1-positive lines, and a similar but less consistent effect was seen with taxotere, doxorubicin and etoposide. With MRP-positive, MDR-negative lines, only VX-710 caused consistent sensitization. These data confirm MDR1 and MRP expression as contributory factors in chemoresistance in neuroblastoma and indicate that VX-710 may be a useful modulator of both mechanisms and worthy of clinical evaluation in this tumour.

Although half of all children with neuroblastoma will be cured of their disease, there are still subgroups in whom the outlook remains poor. These include children with metastatic disease, particularly affecting bone, or those with regional disease in whom the tumour shows N-MYC amplification or chromosome 1p deletion. In these patients, despite intensive multiagent chemotherapy often followed by high dose chemotherapy with stem cell rescue, less than one-quarter will be long-term survivors. Since the introduction of cisplatin–etoposide combinations there have been few new chemotherapy agents which have proven to be active in neuroblastoma. Alternative strategies, such as dose escalation or high dose intensity regimens, may have improved remission rates but have had only modest impact on cure rates. There is therefore an urgent need for new approaches in this disease.

As in other childhood tumours, drug resistance in neuroblastoma is multifactorial, but there is evidence that multidrug resistance (MDR) involving P-glycoprotein (P-gp), the product of the MDR1 gene or MDR-related protein, the product of the MRP gene may influence both the biological behaviour of these tumours and response to chemotherapy (Bourhis et al, 1989; Chan et al, 1991; Bordow et al, 1994; Norris et al, 1996). Resistance to several of the most important drugs used in children's cancer, for example anthracyclines, vincristine, etoposide and actinomycin, has been shown to involve these mechanisms.

Modulation of P-gp function has been demonstrated in a range of tumour cell lines using a variety of compounds. The most extensively evaluated in clinical practice are verapamil and cyclosporin A (Cairo et al, 1989; Lum et al, 1993; Cowie et al, 1994, 1995; Chan et al, 1995). The cyclosporin analogue PSC 833 has been introduced more recently (Helson et al, 1994; Boote et al, 1996) and is currently undergoing a dose-finding study in children. The major drawback of MDR modulation strategies thus far has been the unacceptable level of toxicity of the modulating agent. In the case of verapamil, it has proven difficult to achieve blood drug levels in patients comparable to those shown to be effective in vitro due to cardiac toxicity. Similarly, with high dose cyclosporin, dose-limiting nephrotoxicity and hepatic toxicity have compounded the extramedullary toxicity of the cytotoxic chemotherapy. There is, therefore, a need for alternative reversal agents with a lesser toxicity to normal tissue.

VX-710 is a non-macrocyclic pipecolinate derivative which binds the FK506 receptor protein. VX-710 has been shown to restore sensitivity in a range of multidrug-resistant cells, including myeloma, melanoma, carcinoma and leukaemia (Germann et al, 1997*a*). The drug inhibits the efflux activity of P-gp and stimulates vanadate-sensitive P-gp ATPase activity, suggesting a direct high affinity interaction between VX-710 and P-gp (Germann et al, 1997*a*). Of particular importance are results from Phase I clinical studies in adults which have shown this agent to have an encouraging toxicity profile with doses up to 120 mg m^{-2} per hour as a 24 to 96-h infusion. Minimal adverse reactions. These included headaches, mild to moderate nausea and mild reversible hypotension. At the highest dose there was mild hyperbilirubinaemia (Rowinsky et al, 1998). Steady-state concentrations of VX-710 (≥8 μM) were 2–3 fold higher than level effective in vitro as an MDR modulator. Moreover, hepatic retention of sestamibi increased up to eightfold during VX-710 administration, suggesting inhibition of P-gp in the liver (Peck et al, 1995).

In the present study, a range of neuroblastoma cell lines have been characterized with regard to drug resistance phenotype and the chemosensitization to several cytotoxic agents using PSC 833, cyclosporin and VX-710. Three methods were used: microtitre

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Table 1 Clinical data on origins of the neuroblastoma cell lines

Cell line	Patient age (months)	Tissue source	Prior therapy	Response to therapy
SKNBE	24	Bone marrow	RT, VCR, CTX, ADR, ACT	None
SKNSH	48	Bone marrow	RT, VCR, CTX, DNR, FMdU, ADR	None
HX-142	19	Primary tumour	VCR, CDDP, VM-26, CTX	PR
HX-138	72	Primary tumour	RT, VCR, CDDP, ETOP, CTX	PR
NB-1	33	Cervical lymph node	RT, ACT, CTX, VCR	PR
IMR32	13	Primary tumour	None	

RT = radiotherapy (specimens were not obtained from within the radiation port); VCR = vincristine; CTX = cyclophosphamide; ADR = doxorubicin; ACT = dactinomycin; DNR = daunomycin; FMdU = trifluoromethyl-2′-deoxyuridine; CDDP = cisplatin; VM-26 = teniposide; ETOP = etoposide; DTIC = dacarbazine; PR = partial response.

tetrazolium–formazan assay (MTS), clonogenic assay and daunorubicin efflux with FACS.

METHODS

Cell lines and culture conditions

The derivation and characterization of the six established human neuroblastoma cell lines used in this study are outlined in Table 1. SKNBE, IMR-32, SKNSH and SKNBE cells were obtained from the American Tissue Culture Collection (Rockville, MD, USA). NB-1 cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). Hx-138 and Hx-142 were provided by Professor G Steele (Radiotherapy Unit, Institute of Cancer Research, Sutton, UK) (Tumilowicz et al, 1970; Biedler et al, 1973; Inui, 1974; Biedler and Spengler, 1976; Deacon, 1986; Hills et al, 1989; Feller et al, 1995). The doxorubicin-resistant, non-small-cell lung cancer cell line, 2R120, was used as a positive control for MRP expression and provided a negative control for P-gp. The sensitive human ovarian carcinoma line CH-1 and the doxorubicin-resistant derivative line CH-1R were used as negative and positive controls for MDR-1 expression. Cell lines were maintained as monolayers and incubated at 37°C in a humidified atmosphere with 5% carbon dioxide in culture media supplemented with 10% heat-inactivated fetal bovine serum (FBS; GibcoBRL, UK), 1 ml 100 ml^{-1} Glutamax (Gibco), 100 U ml^{-1} of penicillin and 100 μg ml^{-1} streptomycin. RPMI-1640 medium (Gibco) was used for SKNBE, SKNSH, IMR-32 and NB-1, Ham's F12 (Gibco) for HX-138 and HX-142, and DMEM (Gibco) for all control cell lines. Maintenance doses of doxorubicin were used to retain the MDR phenotype of the drug-resistant cell lines until at least 48 h before the start of experiments. All cell lines were routinely screened for mycoplasma by PCR assay (Mycoplasma Primer Set, Stratagene, Cambridge, UK).

MDR characterization by immunocytochemistry

The consensus recommendations of the multicentre workshop were applied, where possible, in this study (Beck et al, 1996). Two antibodies raised against spatially distinct segments of each protein were used for the immunocytochemical detection and semi-quantitative measurement of MRP and P-gp. Control reactions used appropriately matched isotypes or no primary antibody. Cytospin preparations of 50 000 cells were fixed in acetone at –20°C for 5 min, air-dried, preincubated in 10% human AB serum in phosphate-buttered saline (PBS) A, followed by incubation with the primary antibody for 1 h. For detection of P-gp, the mouse monoclonal antibodies, MRK 16 (TCS Biologicals Ltd, Boltolph Claydon, UK), 1:25 dilution, JSB –1, 1:10 and for MRP the antibodies MRPr1, raised in rat (Sigma Chemicals, Poole, UK), dilution 1:50 and MRPm6 (kindly provided by Dr Rik Scheper, Free University, Amsterdam, The Netherlands) raised in mouse at 1:25 were used. The second antibody, rabbit anti-mouse or anti-rat (Dako, Glastrup, Denmark) was used at 1:50 dilution for 30 min. A standard APAAP technique (DAKO) was used for colourimetric development with levamisole (Sigma) to block activity of endogenous alkaline phosphatase. All incubations were at room temperature. Slides were counterstained with Mayer's haematoxylin (Sigma, 2 min). Cells were graded as negative when no staining was apparent and from (+) to (++++), according to the intensity of staining (Beck et al, 1996). Positive control cell lines scored (+++) or (++++) for MRP and P-gp. Reproducibility of the results was confirmed in each case by two repeat experiments.

Reverse transcriptase polymerase chain reaction

Oligonucleotides All sequences of polymerase chain reaction (PCR) primers were as previously reported. Reverse transcription (RT)-PCR analysis of MDR1 and MRP gene expression was performed using two non-overlapping primer pairs, which gave concordant results for each gene in all cell lines (MDR-1: 401-bp product (Futscher et al, 1993); 157-bp product (Bordow et al, 1994); MRP: 565-bp product (Beketic-Oreskovic et al, 1995); 140-bp product (Bordow et al, 1994)). Other primer sets were: topoisomerase II α (topo II α), 322-bp product (Beck et al, 1995), topoisomerase II β (topo II β), 304-bp (Beck et al, 1995), lung-related resistance protein (LRP), 405-bp (Stein et al, 1997), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 358-bp and β_2 microglobulin (β_2M), 120-bp (Bordow et al, 1994). All primer sets spanned an intron to control against amplification of genomic DNA sequences. Two internal controls (GAPDH and β_2M) were used to assess the quality of template in each reaction. All primers were purchased from Oswel DNA Service Lab (Southampton, UK).

Total RNA (1 μg) was reverse transcribed in a 20 μl volume using Superscript II (GibcoBRL) and random hexanucleotides (Pharmacia), according to the manufacturers' instructions. PCR was performed on 50 ng of cDNA in a final volume of 50 μl containing 25 pmol of each primer, 0.4 mM dNTPs (Pharmacia), 20 mM Tris–HCl, 50 mM potassium chloride, 1.8 mM magnesium chloride and 2.5 U μl^{-1} of *Taq* DNA polymerase (Gibco BRL). An initial denaturation for 4 min at 94°C was followed by 27–35 cycles of 45 s at 94°C, 45 s at 55°C, 90 s at 72°C in an Omnigene

Table 2 Expression of MDR-related genes in neuroblastoma and control cell lines

Cell line	Immunocytochemistry staining					RT-PCR		
	MDR-1		MRP		LRP	MDR-1	MRP	LRP
	MRK16	JSB-1	MRP-6 m	MRPr-1	LRP-56			
SKNBE	(+++)	(+++)	(+)	(+++)	(–)	+	+	+
SKNSH	(+++)	(+++)	(+)	(+++)	(–)	+	+	+
HX142	(+)	(+)	(++)	(+++)	(–)	+	+	–
HX138	(+)	(+)	(+)	(++++)	(+++)	+	+	+
NB1	(++)	(++)	(+)	(+)	(–)	–	+	+
IMR 32	(–)	(–)	(–)	(–)	(–)	–	+	+
CH-1P	(–)	(–)	(–)	(–)	(–)	–	+	+
CH-1R	(+++)	(++++)	(–)	(–)	(–)	+	+	+
2R120	(–)	(–)	(+++)	(+++)	(+++)	–	+	+

Immunocytochemistry – intensity of staining recorded as: negative (–), low (+), intermediate (++), high (+++), ultra high (++++). RT-PCR – for MDR-1 and MRP two different primer pairs were used. See Methods.

thermal cycler (Hybaid, UK). PCR products (20 µl) were visualized by gel electrophoresis (2% agarose or 12% polyacrylamide) and ethidium bromide staining. Each sample was assayed at least twice and scored as positive if the correct sized PCR product was detected in two or more experiments with appropriate negative controls (no RNA, no DNA).

Chemicals

Drug sources were as follows: vincristine (Farmitalia Carlo Erba Ltd, Herts, UK), doxorubicin (Eli Lilly, Basingstoke, UK), melphalan and etoposide (Sigma, UK), cisplatin (Johnson Matthey, Reading, UK), taxotere (Rhone-Poulenc, France), topotecan (SmithKline Beecham Pharmaceuticals, Herts, UK), cyclosporin A and PSC 833 (Novartis Pharmaceuticals, Camberley, UK), VX-710 (Vertex Pharmaceuticals Inc., Cambridge, MA, USA). Stock solutions were prepared in saline or water with the exception of: (1) melphalan, which was solubilized in a minimum quantity of 4.5% ethanolic hydrochloric acid and then diluted at least 50-fold in medium before addition to cultures; (2) etoposide and PSC 833, which were solubilized in absolute alcohol and similarly diluted; (3) VX-710 was diluted in the diluent DMEM. Stock solutions were diluted with tissue culture media just before use. The levels of the diluted solvents alone showed no toxic effect on cells (data not shown).

Functional analysis of P-gp and MRP

Cells in single cell suspension ($0.5–1.0 \times 10^6$ in 1 ml aliquots) were equilibrated for 30–60 min at 37°C in the absence/presence of modulating agents (PSC 833 500 nM, VX-710 5 µM) in Dulbecco's phenol-red free medium with 10% FCS. Daunorubicin (Sigma Chemicals), at a concentration of 5 µM was then added, with a further 60 min incubation. Following the fluorescent probe accumulation phase, cells were pelleted by centrifugation at 2000 ***g***, 4°C, and resuspended in fresh medium, again in the absence or presence of the appropriate modulating agent. Cells were allowed to efflux for a further 60 min at 37°C and were then 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. Fluorescence was analysed for 10 000 events using a Coulter Epics ESP Elite flow cytometer.

Cytotoxicity assays

The drug sensitivity and the potencies of the modulators were determined by the MTS cytotoxicity assay (Promega) for all cell lines and clonogenic assay in SKNBE (Goodwin et al, 1995). Cells were seeded at 2500–5000 cells per well to allow exponential growth over not more than four doublings during the incubation period. Plates were preincubated for 24 h, then cytotoxic drugs with or without modulator were added to a final volume of 200 µl. Modulators were used at a concentration that produced less than 10% cell death (PSC833 and cyclosporin A were used at 0.5 µM for all lines; VX710 was used at 5 µM for all lines except HX138 (2.0 µM) and NB-1 (1.0 µM)). Control wells contained medium only or cells alone. All experimental points were set up in quadruplicate wells and all experiments were repeated at least three times. The MTS assay was performed after 72–156 h, depending on the doubling time, to obtain an IC_{50} for each drug. The resistance modifying factor or sensitization ratio was calculated as (IC_{50} with drug alone)/(IC_{50} drug + modulator).

Clonogenic assays were performed using a modification of Pike and Robinson's double layer agar technique (Pike and Robinson, 1970). Five hundred neuroblastoma cells were plated in the presence of 0.0001, 0.001, 0.01 and 1.0 µM vincristine alone or with modulator (0.5 µM cyclosporin A or PSC 833, 5.0 µM VX-710). Cell suspensions were prepared by trypsinization of monolayers and gently passing through a 21-gauge needle. The effectiveness of this procedure to achieve a suspension of single cells was checked microscopically. Each drug concentration and modulator combination was set up in triplicate. Plates were incubated for 14 days. Aggregates of more than 50 cells were scored as colonies. Survival was expressed as a percentage of control colony counts.

The nonparametric, two-sided Mann–Whitney rank sum test was used to analyse the MTS assay data, comparing median sensitization ratios from at least three independent experiments for each data point. Statistical significance was established on the basis of 95% confidence intervals.

RESULTS

MDR expression

Expression of three genes implicated in multidrug resistance (MDR-1, MRP, LRP) was assayed at both the mRNA and protein level in six neuroblastoma and three control cell lines (Table 2). Topoisomerase II α or β were assayed solely by RT-PCR.

Table 3 Sensitization of neuroblastoma cell lines to cytotoxic agents by cyclosporin A, PSC 833 and VX710

Cell line	Vincristine			Taxotere			Doxorubicin			Etoposide		
	CsA	PSC833	VX710	CsA	PSC833	VX710	CsA	PSC833	VX710	CsA	PSC833	VX710
SKNBE	26	33	61	8.3	8.3	14	2.5	3.1	2.8	2.4	2.2	2.8
SKNSH	6.8	7.4	32	4.7	5.8	7.6	2.4	2.4	3.1	1.3	1.5	1.4
Hx142	6.8	5.3	12	3.3	3.4	3.3	1.8	1.5	2.4	3.3	2.8	3.5
Hx138	5.2	4.6	6.7	5.5	4.1	2.4	2.4	2.0	3.5	2.0	3.5	1.1
NB 1	1.2	1.0	1.6	1.5	0.9	1.2	1.1	0.8	1.8	1.0	1.4	1.0
IMR-32	1.0	0.7	1.5	1.2	1.0	1.0	1.0	0.9	0.9	1.0	1.3	1.4

Data shown are median sensitization ratios (SR) from at least three independent experiments. SR = IC_{50} with drug/IC_{50} drug + modulator. IC_{50} values were calculated from dose–response curves of MTS cytotoxicity assays. Modulators (cyclosporin A (CsA), PSC 833 (PSC) and VX710 (VX)) were used at concentrations <IC_{10} for each cell line.

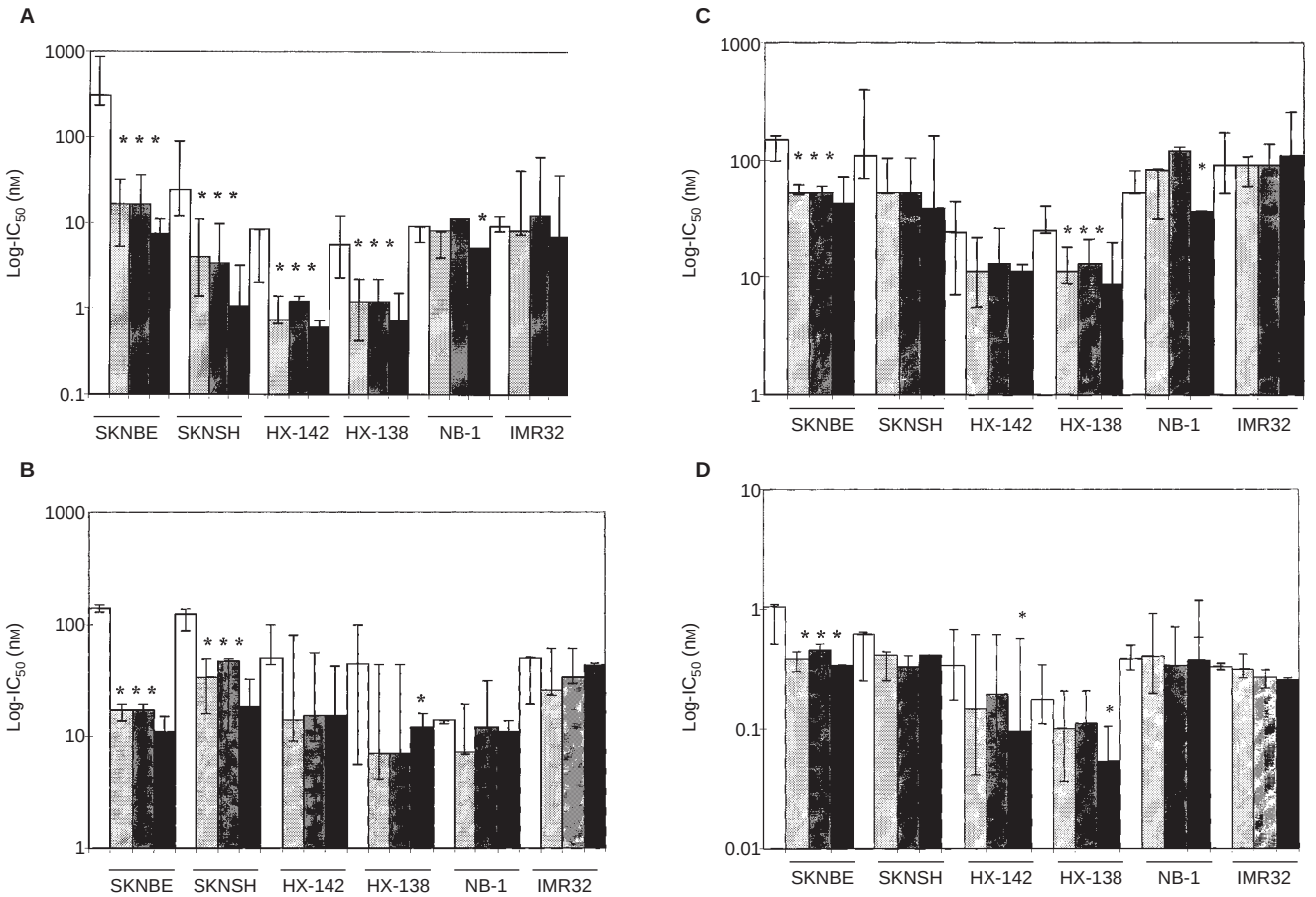

Figure 1 Effects of modulators on cytotoxicity of (**A**) vincristine, (**B**) taxotere, (**C**) doxorubicin and (**D**) etoposide in neuroblastoma cell lines. The results are presented as the median with the range of IC_{50} values obtained from at least three independent experiments. Median IC_{50} values in the presence of modulator which are statistically significant (non-parametric Mann–Whitney rank sum test, $P < 0.05$) from those for the drug alone are asterisked. □, Drug; alone, ▨, +CsA; ■, +PSC833; ■, +Vx710

P-gp expression was detected in 5/6 neuroblastoma cell lines and MDR-1 mRNA by RT-PCR in 4/6. NB1 cells gave a discordant result that was probably due to artefactual binding or cross-reactivity in immunocytochemistry. RT-PCR with two non-overlapping primer sets was consistently negative and P-gp could not be detected by either Western or flow cytometric analysis in this cell line (data not shown). In functional assays, NB1 behaved similarly to IMR32, which was MDR-1/P-gp negative by all methods.

Five lines (all except IMR32) expressed MRP detectable by immunohistochemistry. However, all expressed MRP mRNA detectable by RT-PCR, presumably reflecting the greater sensitivity of the latter technique.

All neuroblastoma lines except one (HX-142) showed expression of LRP by RT-PCR but mainly at low levels, as only one (HX-138) had detectable protein by immunohistochemistry. No cell line showed loss of topoisomerase II α or β expression by RT-PCR.

Table 4 Functional assay of effect of modulators on daunorubicin efflux

Cell line	Efflux ratio	
	PSC 833	VX710
SKNBE	1.5	1.1
	(0.21)	(0.14)
SKNSH	1.2	1.3
	(0.19)	(0.19)
Hx142	2.4	2.2
Hx138	1.3	1.2
NB1	1.4	1.2
	(0.16)	(0.06)
IMR32	1.2	1.3
	(0.10)	(0.16)
CH-1 Pa	1.0	1.0
CH-1 Re	6.2	6.0
	(0.58)	(0.79)
2R120	1.6	1.7
	(0.26)	(0.11)

Efflux ratio = daunorubicin fluorescence in the presence of modulator/daunorubicin fluorescence alone. Data points are either the mean of three or more experiments with standard deviation in parentheses or the mean of two experiments.

Reversibility of drug resistance by MDR modulators

The ability of the three modulators, cyclosporin A, PSC 833 and VX-710, to overcome resistance to a panel of drugs, was examined in all cell lines by an MTS assay. The drug panel included five drugs derived from natural products which are expected to be transported by P-gp (vincristine, doxorubicin, etoposide, taxotere and topotecan) and two drugs (melphalan and cisplatin) which are not. All of these drugs, with the exception of taxotere and topotecan, are commonly used in the treatment of neuroblastoma. In the MTS assay, modulation values were expressed as a sensitization ratio (SR): (IC_{50} drug alone)/(IC_{50} drug + modulator) (Table 3). Examples of histograms of median IC_{50} are illustrated for the four drugs for which statistically significant modulation was detected in the neuroblastoma cell lines (Figure 1 A–D).

The degree of modulation was most marked with vincristine (up to 61-fold) and for VX710 often exceeded the level seen in the MDR-1 positive control cell line, CH-1R. No modulation of IC_{50} was seen in the sensitive control cell line, CH1-P. Modulation of sensitivity to etoposide, doxorubicin and taxotere was variable and generally much less marked (up to 14-fold) than with vincristine. Of interest, no modulation of topotecan sensitivity was seen, although previous studies have demonstrated that topotecan can be transported by P-gp (Hendriks et al, 1992). No modulation was seen in any line with melphalan or cisplatin (Table 3 and data not shown).

The relative efficacy of the three modulators in reversing drug resistance was variable. VX-710 was generally the most effective modulator in the neuroblastoma cell lines, whereas PSC833 gave superior modulation in the two positive control cell lines. Both were generally superior to cyclosporin A.

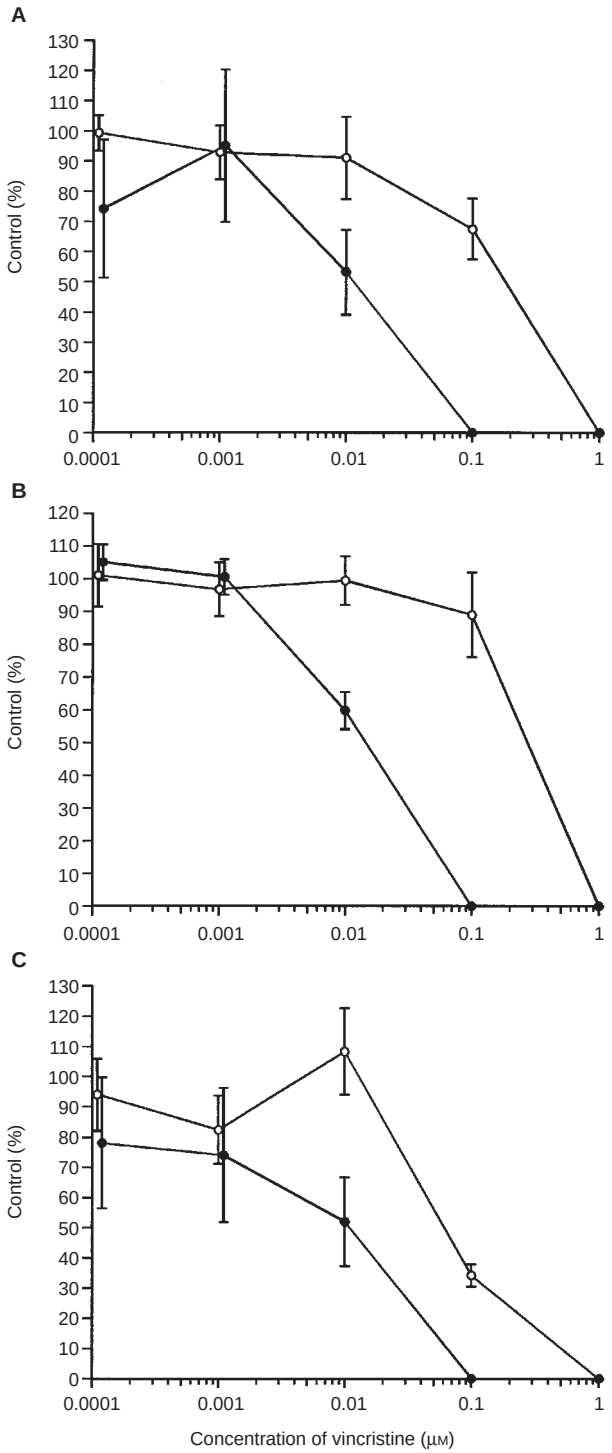

Figure 2 Survival of clonogenic SKNBE neuroblasts exposed to vincristine alone or in combination with modulators. –○– without modulator, –●– with modulator (A = 0.5 μm CSA, B = 0.5 μm, PSC 833, C = 5 μm VX710)

Correlation of modulation of drug resistance with P-gp and MRP expression

No cell line was completely 'clean' in having only one possible mechanism of drug resistance. Such multiplicity of mechanisms is probably common in vivo as well as in vitro and makes simple correlations impossible. All three modulators sensitized both the P-gp- and MRP-positive control cell lines to doxorubicin. The extremely high level of vincristine resistance in the MRP-positive control line 2R120 was not influenced by the modulators, implying additional resistance mechanisms for this drug in this cell line. VX710 gave the greatest number of significant modulations

across the drug panel. In the four neuroblastoma cell lines with both P-gp and MRP expression, VX-710 was the only modulator to significantly sensitize two lines to etoposide and one line to taxotere. VX710 was the only modulator to show an effect in line NB-1, whose drug resistance was ascribed to MRP rather than P-gp (SR = 1.6 for vincristine and 1.8 for doxorubicin).

Clonogenic and functional assays

A subset of modulators and cell lines was further examined by two complementary assays: a clonogenic assay to assess modulation of cytotoxicity on clonogenic cells and a functional assay of drug efflux. The clonogenic assay was only performed for SKNBE exposed to vincristine in combination with each of the three modulators (Figure 2). These results show that all three modulators enhance the killing of clonogenic cells in addition to the overall enhancement of cytotoxicity demonstrated by the MTS assays. Using the functional assay, retention of daunorubicin was clearly enhanced in the CH-1R Pgp-positive control line by both PSC833 and VX-710 (Table 4). This assay showed a more modest degree of modulation in the neuroblastoma cell lines and the MRP-positive control for both VX-710 and PSC 833.

DISCUSSION

This study characterized the MDR phenotype of a range of neuroblastoma cell lines and documented the activity of VX-710 as a drug resistance modulator compared to cyclosporin A and PSC 833. The secondary objective was to determine to what extent any sensitization was linked with known mechanisms of MDR.

As expected from the discussions of the multicentre workshop (Beck et al, 1996) there was some discordance between the characterization of cell lines using different methods. In general, there was a high level of concordance between RT-PCR and immunocytochemistry for both MDR1 and MRP. The staining intensity for both MDR1 and MRP varied depending on the antibody used (Table 2). Discordance occurred only in the case of NB-1 where gene expression was not apparent on RT-PCR despite apparent immunoreactivity. The lack of modulation with cyclosporin A and PSC 833 together with the observation that no significant P-gp levels were detected by either Western of FACS analysis implies that the immunocytochemical staining was a false positive. Functionally significant MRP expression appeared to correlate with positivity in immunocytochemistry rather than RT-PCR. IMR32 and CH-1P were only MRP-positive by RT-PCR and showed no evidence of modulation. All neuroblastoma cell lines expressed the LRP gene but most did not have detectable protein. In no cell line was there evidence of complete absence of topoisomerase II expression by RT-PCR, although a reduction in level of expression would not be detected by this method.

Cytotoxicity and modulation was evaluated using three independent methods, MTS, clonogenic assay and drug efflux modulation. The commonly used MTS or MTT assays have the limitation of assessing overall cell kill, not necessarily cells capable of repopulation. In this study there was close correlation between the modulation observed with all three modulators as evaluated by either MTS or clonogenic assay. Efflux assay using either daunorubicin or rhodamine may shed light on the mechanism by which VX-710 influences cytotoxicity. It is clear, however, that changes in drug efflux are not necessarily translated into cell kill. The degree of sensitization to anthracyclines using VX-710 or PSC 833 were more marked with MTS assay than might have been predicted with functional assay, particularly in SKNBE and HX138. A close correlation between cytotoxicity as measured by MTS assay and drug efflux was apparent for the P-gp control cell lines. The sensitive parental line, CH1-P, showed no evidence of modulation whereas in the resistant line, CH-1R, marked enhancement of drug retention was reflected in high levels of cytotoxicity to both vincristine and doxorubicin. In clinical practice, the functional assay is generally the method of choice for evaluating leukaemia, where fresh tumour cells can be readily evaluated. For solid tumours this is difficult and the MTS assay may be more practical.

In the present study the highest dose used for the VX-710 studies was 5 μM. This is a concentration which is readily achieved in clinical studies (Rowinsky et al, 1998). Even lower concentrations may be effective as dose–response studies (data not shown) failed to show any significant difference in the modulation of SKNBE with vincristine over a VX-710 concentration range of 0.5–5 μM.

VX-710 functions in a similar way to the modulators cyclosporin A and PSC 833, influencing the drug accumulation defect by direct interaction with P-g as indicated by photoaffinity labelling experiments. Further evidence of direct high affinity interaction between VX-710 and P-gp is found in the concentration-dependent stimulation of P-gp ATPase activity. The concentrations of VX-710 required to reverse MDR in vitro in a range of cell lines assessed using rhodamine, sestamibi and labelled doxorubicin are similar to cyclosporin A (Germann et al, 1997*a*, 1997*b*). It is unclear to what extent the compound acts as a competitive ligand for binding sites of cytotoxics.

The main attraction of VX-710 in clinical practice is its striking lack of organ toxicity. Other agents active in vitro such as verapamil and high-dose cyclosporin are significantly limited in clinical use, particularly with combination chemotherapy. Both have inherent toxicity and alter chemotherapy pharmacokinetics. Dose-finding studies in adults have demonstrated that the maximum tolerated dose of VX-710 is well in excess of that required to achieve drug levels found to effectively influence drug efflux in vitro. Moreover, preliminary studies in vivo using sestamibi have shown that VX-710 will enhance both hepatic and tumour retention of this surrogate marker for P-gp-related drugs (Peck et al, 1995).

In conclusion, it is apparent that in a range of neuroblastoma cell lines, both VX-710 and PSC 833 are effective sensitizers of MDR-modulated chemotherapy. Although this sensitization is predominantly P-gp-dependent, VX710 did appear to have a broader spectrum of modulator activity. In the two MDR-1-negative lines, which were both MRP-positive, there was some evidence of sensitization using VX-710, which was not apparent with PSC 833 or cyclosporin A. In the MRP control line 2R120, VX-710 was as effective as the other agents in sensitizing to doxorubicin. This is an indication for extension of dose-finding studies in adults to children with refractory or recurrent neuroblastoma or other solid tumours known to be P-gp-positive.

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