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Original Paper

The Influence of P170-glycoprotein Modulators on the Efficacy and the Distribution of Vincristine as well as on *MDR*1 Expression in BRO/mdr1.1 Human Melanoma Xenografts

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Multidrug resistance modulators may increase the antitumour efficacy of drugs affected by P170-glycoprotein (Pgp) in Pgp-positive tumours *in vivo*. Inhibition of Pgp function in normal tissues, however, may enhance side-effects. Dexniguldipine-HCl, its analogues B9203-009 and B9303-036, and the dipyridamole derivative BIBW22BS could reverse vincristine (VCR) resistance in BRO/mdr1.1 cells (transfected with full-length *MDR*1 cDNA) and 2780^{AD} cells (selected for doxorubicin resistance) *in vitro*. VCR resistance in BRO/mdr1.1 xenografts grown subcutaneously (s.c.) in the nude mouse was not or only slightly affected by the Pgp modulators. VCR concentrations in normal mouse tissues increased with the dose of the Pgp modulator administered and this was most pronounced in liver, kidney, small gut and colon. Dexniguldipine 40 mg/kg intraperitoneally (i.p.) given once 4 h before VCR 1 mg/kg intravenously (i.v.) resulted in increased VCR concentrations in BRO/mdr1.1 xenograft tissue. Surprisingly, when dexniguldipine 40 mg/kg i.p. was administered daily ×3 before VCR, tumour VCR concentrations were not affected. This phenomenon was not observed in normal mouse tissues. Upregulation of *MDR*1 mRNA to 2.7- to 3.8-fold higher levels than control mRNA in BRO/mdr1.1 xenograft tissue occurred after VCR or dexniguldipine at 4–8 h and up to 1.7-fold at 24–28 h after injection. The combination showed 3.6- to 3.7-fold increased levels at 4 h after VCR injection. The lower VCR concentrations measured in BRO/mdr1.1 xenograft tissue after pretreatment with dexniguldipine for 3 days relative to animals treated with dexniguldipine only once will likely be caused by a gradual increase of Pgp expression as a response to the upregulation of *MDR*1. © **1999 Elsevier Science Ltd. All rights reserved.**

Key words: P170-glycoprotein, vincristine, dexniguldipine-HCl, BRO/mdr1.1 human melanoma xenografts, *MDR*1 upregulation
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INTRODUCTION

IN RECENT years it has become evident that several cellular mechanisms may account for drug resistance in malignancies. The 'classical' multidrug-resistance phenotype has been studied most extensively. The usual pattern of cross-resistance in cells that exhibit this phenomenon includes a large variety of hydrophobic natural products, semisynthetic analogues of such products, and synthetic organic compounds [1]. It is well recognised that such cells show increased expression of an ATP-dependent efflux pump in the cell membrane, designated as P170-glycoprotein (Pgp) [2]. Pgp is encoded by the *MDR*1 gene [3]. Pgp-positive cells may occur in tumour types derived from tissues that normally express Pgp, such as colon cancer and renal cell cancer. Pgp expression may also be present *de novo* upon activation of the expression of the *MDR*1 gene during the process of malignant transformation or may be acquired in tumours that relapse during or after chemotherapy [1].

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Pgp-positive cells do not only show an increased efflux of a variety of cytotoxic agents, but drugs can also bind to the protein competitively. This observation has resulted in attempts to combine relatively non-toxic compounds that inhibit the multidrug transporter with cytostatic agents affected by Pgp [1]. Reversal of multidrug resistance (mdr) *in vitro* is relatively easy in cell lines expressing Pgp, that is either acquired upon selection against increasing drug concentrations or obtained after transfection with the *MDR*1 gene. Few reports have appeared on the successful reversal of resistance in tumour models grown in mice. Examples are the addition of verapamil, the nifedipine analogue AHC-52, the triazinoaminopiperidine derivative S 9788, or the cyclosporine analogue PSC 833 to vincristine in P388/VCR leukaemia [4–7], PSC 833 added to doxorubicin in L1210 leukaemia transfected with *MDR*1 cDNA [8], and the acridonecarboxamide derivative GF120918 combined with doxorubicin in P388/DOX leukaemia [9]. In contrast to positive results in intraperitoneal (i.p.) mouse leukaemias, it is far more difficult to reverse resistance in subcutaneously (s.c.) grown Pgp-positive solid tumours. Verapamil combined with VCR did not influence the growth of Rh18/VCR human rhabdomyosarcoma xenografts or KB8-5 human epidermoid xenografts [10, 11]. In BRO human melanoma xenografts transfected with *MDR*1 cDNA we could only slightly increase the growth inhibition by VCR upon addition of the dipyridamole derivative BIBW22BS, but this reversal was not complete when compared with the efficacy of VCR in non-*MDR*1 BRO xenografts [12].

A large number of substrates of Pgp have been studied in the clinic, such as the calcium-channel blockers and the cyclosporines. These trials have not always been conclusive which can be explained by a number of factors, such as: (a) the inability to achieve adequate concentrations of the potential modulating compound because of side-effects; (b) drug levels of both modulator and cytostatic agent were not always measured, making it difficult to assess the pharmacological consequences of drug interactions; (c) the free fraction of the modulating agent available for Pgp binding might be affected by plasma proteins *in vivo*; and (d) measurement of *MDR*1 expression in tumour cells was not always performed [13]. Few promising results in modulating mdr have been obtained in haematological malignancies: acute myeloid leukaemia, lymphoma and multiple myeloma [14]. The most cumbersome problem in the modulation of mdr can be attributed to a pharmacological drug interaction at the site of metabolism or the inhibition of Pgp in normal excretory tissues, specifically the biliary tract and proximal renal tubules, resulting in enhanced side-effects caused by increased plasma areas under the curve of cytotoxic agents and their metabolites [14]. Enhanced antitumour efficacy may also be the result from increased drug exposure rather than from inhibition of Pgp.

Research has focused on the development of better effective and less toxic Pgp modulators for use in the clinic, such as the calcium-channel blocker dexniguldipine and the dipyridamole derivative BIBW22BS. In the present experiments we determined the reversing capacity of dexniguldipine, its more potent analogues B9203-009 and B9303-036, as well as BIBW22BS in Pgp-positive cells grown *in vitro* and *in vivo*. In addition, possible pharmacological drug interactions were investigated by measuring VCR concentrations in plasma, in tumours and normal organs of mice bearing BRO or BRO/mdr1.1 xenografts. VCR levels were compared with those from BRO/mdr1.1-bearing mice pretreated with Pgp modulators. Lastly, the influence of VCR and/or dexniguldipine was assessed on *MDR*1 expression in BRO/mdr1.1 xenografts in order to explain the negative outcome of the modulation experiments in BRO/mdr1.1 cells grown *in vivo*.

MATERIALS AND METHODS

Drugs

VCR (Eli Lilly, Nieuwegein, The Netherlands) was purchased as a solution of 1 mg/ml. Dexniguldipine-HCl (B8509-035 or (-)-3-[3-(4,4-diphenyl-1-piperidinyl)propyl]-5-methyl-1,4-dihydro-2,6-methyl-4(*R*)-(3-nitrophenyl)pyridine-3,5-dicarboxylate-hydrochloride), B9203-009 (5-acetyl-2,6-dimethyl-3-[8-(4,4-diphenyl)-1-piperidinyl)octanoyl]-4-(3-nitrophenyl)-pyridine-hydrochloride) and B9303-036 (3-[8-(4,4-diphenyl-1-piperidinyl)octanoyl]-4-(3-nitrophenyl)-2-methyl-5-oxo-5,6,7,8-tetrahydro-chinolin) were provided by Byk Gulden GmbH (Konstanz, Germany). Dexniguldipine was dissolved in polyethylene glycol 400 plus 0.01 N HCl (1:1) to 10^{-2} M and H_2O to 10^{-3} M. B9203-009 and B9303-036 were dissolved in dimethyl sulphoxide to a concentration of 3×10^{-3} M. BIBW22BS (4-[N-(2-hydroxy-2-methyl-propyl)-ethanolamino]-2,7-bis(cis-2,6-dimethyl-morpholino)-6-phenylpteridine) (Dr. Karl Thomae GmbH, Biberach an der Riss, Germany) was dissolved in HCl 0.1 N to 20 mg/ml and further diluted in NaCl 0.9% to 2 mM at pH 2.7. Stock solutions were stored at room temperature in the dark. For *in vitro* use, drugs were further diluted in tissue culture medium.

For *in vivo* use, VCR 1 mg/ml was further diluted in NaCl 0.9% to 0.2 mg/ml. Dexniguldipine, B9203-009 and B9203-36 were provided as a micro-emulsion 20 mg/ml and were further diluted in glucose 5% to 1.25, 5 and 5 mg/ml, respectively. BIBW22BS 20 mg/ml was further diluted in NaCl 0.9% to 2.5 mg/ml.

Cell lines and drug sensitivity

The Pgp-positive subline BRO/mdr1.1 (BRO/pFRmdr1.6 clone 1.1) was obtained by transfection of the parent human melanoma cell line BRO with a full-length *MDR*1 cDNA [15]. The Pgp-positive subline 2780^{AD} [16] of ovarian cancer cell line A2780 was obtained by stepwise selection against increasing concentrations of doxorubicin in tissue culture. The cell lines were grown in Dulbecco's modified Eagle's medium (Flow, Irvine, Scotland) supplemented with 10% heat-inactivated fetal calf serum (Sanbio, Uden, The Netherlands), 2 mM L-glutamine, 50 IU/ml penicillin and 50 µg/ml streptomycin (Flow) in a humidified atmosphere containing 5% CO_2 at 37°C. The resistant sublines were cultured in the presence of a selective drug (VCR 10 nM for BRO/mdr1.1 and doxorubicin 2 µM for 2780^{AD}) until 3 days before the experiments.

Antiproliferative effects of VCR alone or in the presence of modulators were determined by means of the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay [17]. Single-cell suspensions were prepared and plated in quadruplicate into 96-well plates. After 24 h drugs were added and the assay was terminated after an incubation period of 4 days. The absorbance of each well was measured at a wavelength of 540 nm using a microtitre plate reader (Reader Microelisa System, Organon Teknika, Turnhout, Belgium). Readings in experimental wells were linear with cell concentrations between 2500 and 100 000 cells/well.

Wells with cells grown for 24 h before adding the drugs were used to check for adequate cell growth after the 4-day culture period in control wells. Experiments were repeated at least four times to obtain reproducible results. Drug effects were expressed as the IC_{50}, which is the concentration of the drug inducing 50% inhibition of cell growth of treated cells when compared with control cell growth.

Xenografts and drug sensitivity

Female nude mice (Hsd: athymic nude-*nu*) were purchased at the age of 6 weeks (Harlan, Horst, The Netherlands) and kept in a clean room under specified atmospheric conditions. For animal handling, transplantation procedures, treatment and biodistribution experiments, permission was obtained from the university ethical committee on experimental animals. BRO and BRO/mdr1.1 xenografts were established from cell lines grown in tissue culture. Mice were inoculated s.c. with 10^7 cells in both flanks. The solid tumours arising at the inoculation site (passage 1) were transferred as tissue fragments with a diameter 2–3 mm through a small skin incision into both flanks of 8- to 10-week-old mice. Treatment and biodistribution experiments were carried out in passage 2 or higher passages.

Upon tumour growth, mice were weighed twice-a-week and BRO and BRO/mdr1.1 xenografts were measured in three dimensions with a vernier caliper. The tumour volume was calculated by the equation length×width×thickness×0.5, and expressed in mm^3. One volume doubling time of BRO xenografts is 2.5 (S.D. ± 0.7) days and that for BRO/mdr1.1 xenografts is 3.4 (S.D. ± 0.5) days. BRO xenografts do not express the *MDR*1 gene and are sensitive to VCR. BRO/mdr1.1 xenografts have been demonstrated to retain the *MDR*1 gene and the drug-resistant phenotype up to at least 15 passages [12].

For treatment experiments, groups of six tumour-bearing mice (10–12 tumours) were formed to provide a mean tumour volume between 75 and 150 mm^3 in each group (designated as day 0). Pilot experiments contained 3–4 animals per group. VCR was injected weekly ×2 at the maximum tolerated dose of 1 mg/kg for this schedule. At the maximum tolerated dose nude mice should demonstrate a reversible weight loss of approximately 10% of the weight at the start of treatment [18]. The modulators were injected at various doses and intervals preceding VCR based on information given by the suppliers.

For evaluation of drug efficacy, tumour volumes were converted to values related to the initial volume [18]. This relative volume was expressed by the formula V_T/V_0, where V_T is the volume on any given day and V_0 the volume on day 0. The ratio of the mean relative volume of treated tumours versus that of control tumours (T/C) was assessed on each day of measurement. The percentage of growth inhibition (GI%) was calculated as 100%–T/C%. In addition, for individual tumours two volume doubling times were calculated from day 0 (expressed as $T_{D1\rightarrow4}$). For evaluation of drug toxicity, mean relative body weight loss was calculated as a percentage of the mean body weight on day 0.

Drug concentrations in tissues

Nude mice were divided in groups of three animals to provide equal tumour volumes (mean volume between 200 and 500 mm^3) in each group. Mice were injected with VCR 1 mg/kg intravenously (i.v.) preceded or not by dexniguldipine or BIBW22BS. At 4 and 24 h the animals were bled under ether anaesthesia. Normal tissues and tumours were dissected and rinsed in NaCl 0.9% to minimise blood residues. Plasma was stored at − 20°C and tissues were snap-frozen in liquid N*2* and stored at − 70°C until analysis.

VCR concentrations were quantitated using high-performance liquid chromatography (HPLC) analogous to a method previously described for vinblastine [19]. Tissues were accurately weighed and homogenised (approximately 0.2 g/ml) in blank (drug free) human plasma. Aliquots (200–500 µl) of plasma or tissue homogenates were pipetted into glass tubes provided with a teflon lined screw cap. A volume of 10 µl of 25 µg/ml of desacetylvinblastine (internal standard) in acetonitrile and 4 ml of diethyl ether was added to each tube. The tubes were mixed vigorously for 10 min and then centrifuged (10 min, 3000 ***g***, 4°C). The bottom (aqueous) layer was frozen in an ethanol-dry ice bath and the supernatant organic fraction decanted into a clean glass tube. The organic fluid was evaporated at 37°C under a gentle stream of nitrogen and the residue reconstituted in 150 µl of acetonitrile. A volume of 100 µl was subjected to HPLC using a stainless steel (250×2 mm) column packed with 5 µm Spherisorb Si material. The mobile phase was composed of acetonitrile: 10 mM citrate buffer pH 3.0 (85:15; v/v) containing 10 mM tetrabutylammonium bromide and was delivered at a flow rate of 0.2 ml/min. Detection was performed with a UV SF757 detector (Kratos, Ramsey, New Jersey, U.S.A.) operating at 300 nm.

Dexniguldipine concentrations were measured in the homogenised tissue samples with the use of HPLC with UV-detection at 230 nm [20]. Blank homogenised mouse tissues spiked with a dexniguldipine standard solution were used as a reference.

MDR*1 expression*

Determination of the *MDR*1 gene expression in tumour tissue *in vivo* was carried out with the RNase protection assay. In short, after pulverisation of frozen xenograft tissue in a microdismembrator total cellular RNA was extracted by homogenisation in guanidine isothiocyanate followed by an ultracentrifugation step on a caesium chloride cushion. An NP40 procedure was used to isolate cytoplasmic RNA from control cell lines. RNAse protection was performed as described earlier [21, 22]. Briefly, 10 µg of total RNA was hybridised with a [^{32}P]CTP-labelled anti-sense RNA probe, specific for human *MDR*1-mRNA, which was obtained by transcription of a 301 nucleotide cDNA fragment (positions 3500–3801) with SP6 RNA polymerase. A γ-actin probe was included as an internal control for determination of RNA loading. The hybridised probe was visualised after electrophoresis through a denaturing 6% acrylamide gel. For autoradiography, the gel was exposed at − 70°C to a Kodak XS film overnight.

Statistics

Differences in treatment results expressed as $T_{D1\rightarrow4}$ and in VCR concentrations in tumour tissues were evaluated with Student's *t*-test for unpaired data.

RESULTS

Modulation of mdr in vitro

BRO/mdr1.1 and 2780^{AD} cells were clearly resistant against VCR when compared with the parent cell lines; resistance factors were 112 and 2867, respectively (Table 1).

Table 1. *Reversal of VCR resistance by modulators*

Modulator		BRO/mdr1.1* IC_{50}†	RF‡	2780^{AD}* IC_{50}†	RF‡
None§		3.7×10^{-8}	112	8.6×10^{-7}	2867
Dexniguldipine	0.1	7.2×10^{-8}	218	5.6×10^{-7}	1867
Dexniguldipine	0.5	4.0×10^{-10}	1.2	9.2×10^{-8}	307
Dexniguldipine	1.0	2.6×10^{-10}	0.8	9.5×10^{-9}	32
B9203-009	0.1	7.5×10^{-8}	227	9.2×10^{-7}	3067
B9203-009	0.5	6.0×10^{-11}	0.2	2.7×10^{-7}	900
B9203-009	1.0	toxic	toxic	8.5×10^{-10}	2.8
B9303-036	0.1	4.0×10^{-8}	121	8.2×10^{-7}	2733
B9303-036	0.5	1.3×10^{-11}	0.04	5.0×10^{-9}	17
B9303-036	1.0	toxic	toxic	2.3×10^{-10}	0.8
BIBW22BS	0.1	5.4×10^{-9}	16	4.4×10^{-7}	1467
BIBW22BS	0.5	2.2×10^{-10}	0.7	2.6×10^{-9}	8.7
BIBW22BS	1.0	1.1×10^{-10}	0.3	4.0×10^{-10}	1.3

*Mean IC_{50} value of VCR in BRO cells was 3.3×10^{-10} M and in A2780 cells 3.0×10^{-10} M; †Mean value in M of three independent experiments; ‡Resistance factor, ratio of the IC_{50} in experimental cells versus the IC_{50} of VCR alone in parent cells; §Pgp-positive cells treated with VCR alone.

Upon addition of dexniguldipine or BIBW22BS in non-toxic concentrations of 0.5–1.0 μM reversal of VCR resistance was complete in BRO/mdr1.1 cells, but only for BIBW22BS at 1.0 μM in 2780^{AD} cells. B9203-009 and B9303-036 appeared to be slightly more potent than dexniguldipine in the modulation of Pgp in 2780^{AD} cells, but at concentrations of 0.5–1.0 μM the compounds were toxic in BRO/mdr1.1 cells.

Modulation of mdr in vivo

Previously, we have shown that VCR 1 mg/kg i.v. weekly×2 is not effective in well-established s.c. BRO/mdr1.1 xenografts in the nude mouse, while BRO tumours are sensitive to VCR [12]. In the present experiments these findings were confirmed (Tables 2 and 3) and a typical experiment is shown in Figure 1. Upon VCR treatment the time to double twice in volume increased at least 2-fold in BRO tumours, whilst this was not significantly affected in BRO/mdr1.1 tumours.

Table 2 shows the influence of the Pgp modulator dexniguldipine on the efficacy of VCR *in vivo*. Dexniguldipine was given at a dose of 25 mg/kg i.p. on days 0–4 and 7–11, while VCR 1 mg/kg i.v. was administered on days 2 and 9 with an interval of 4 h after dexniguldipine. The schedule was selected because: (a) a dose of 25 mg/kg i.p. was well tolerated by tumour-bearing nude mice; (b) tissue distribution of ^{14}C-labelled dexniguldipine revealed maximum concentrations between 8 and 24 h after administration; (c) tumour concentrations were considered to be sufficient for reversal of VCR (Byk Gulden GmbH, unpublished data). Dexniguldipine alone was not toxic and did not affect tumour growth. Upon addition of dexniguldipine to VCR the mean weight loss increased, as well as the time for BRO and BRO/mdr1.1 tumours to double their volume. The enhanced inhibition of growth was only significant in BRO/mdr1.1 tumours ($P < 0.05$) compared with VCR alone. While tumour-bearing mice tolerated dexniguldipine 25 mg/kg i.p. well, the dose was increased to 40 mg/kg i.p. In the same treatment schedule in mice bearing BRO/mdr1.1 tumours no additional toxicity was observed, but further reversal of VCR resistance was not evident. In a pilot study dexniguldipine was increased to 50 mg/kg i.p., but this resulted in two deaths within 5 min after administration. VCR was not effective in mice treated with the combination of dexniguldipine 50 mg/kg i.p. followed by VCR 1 mg/kg i.v. weekly ×2.

From Table 1 it appeared that B9303-036 and B9203-009 were more potent than dexniguldipine in the reversal of VCR resistance *in vitro*. In a pilot study, BRO/mdr1.1-bearing mice were treated with B9303-036 20 mg/kg i.v. 1 h preceding

Table 2. *Influence of dexniguldipine on the toxicity and the efficacy of VCR in tumour-bearing nude mice*

Drug*	Days	Modulator†	Dose mg/kg i.p.	Days	N‡	Weight loss mean ± S.D.	Relative body weight†† day 14 mean ± S.D.	GI%§	$T_{D1\to4}$‖ days ± S.E.M.
BRO									
Control		none			6	n.a.	1.21 ± 0.06	n.a.	6.0 ± 0.5
None		dexnigul.	25	0-4, 7–11	6	2 ± 2%	1.19 ± 0.15	0%	6.6 ± 0.8
VCR	2, 9	none			6	0 ± 4%	1.01 ± 0.03	74%	14.9 ± 2.7¶
VCR	2, 9	dexnigul.	25	0–4, 7–11	6	10 ± 7%	0.96 ± 0.06	85%	19.2 ± 1.5¶
BRO/mdr1.1									
Control		none			6	n.a.	1.14 ± 0.10	n.a.	5.7 ± 0.7
None		dexnigul.	25	0–4, 7–11	6	1 ± 2%	1.11 ± 0.05	36%	8.4 ± 1.1
VCR	2, 9	none			6	1 ± 9%	1.10 ± 0.05	8%	6.1 ± 0.5
VCR	2, 9	dexnigul.	25	0–4, 7–11	6	10 ± 7%	0.99 ± 0.10	55%	9.5 ± 1.3¶, **
BRO/mdr1.1									
Control		none			6	n.a.	1.12 ± 0.04	n.a.	8.2 ± 1.0
None		dexnigul.	40	0–4, 7–11	5	3 ± 6%	1.08 ± 0.11	15%	8.6 ± 1.3
VCR	2, 9	none			5	1 ± 9%	1.05 ± 0.11	34%	9.4 ± 0.8
VCR	2, 9	dexnigul.	40	0–4, 7–11	5	9 ± 6%	1.02 ± 0.06	56%	11.6 ± 1.2¶
BRO/mdr1.1									
Control		none			3	n.a.	1.06 ± 0.01	n.a.	9.1 ± 1.3
None		dexnigul.	50	0, 7	3	6 ± 8%	0.96 ± 0.11	0%	8.9 ± 0.1
VCR	0, 7	dexnigul.	50	0, 7	4	8 ± 1%	1.02 ± 0.03	0%	9.3 ± 0.8

*VCR 1 mg/kg i.v.; †Dexniguldipine preceded VCR by an interval of 4 h; ‡Number of mice; §Growth inhibition %; ‖Days for tumours to double twice in volume; ¶Better effective than control ($P < 0.05$); **Better effective than VCR alone ($P < 0.05$); n.a., not applicable. ††Ratio of the body weight on day 14 compared with day 0.

Table 3. Influence of B9303-036 and B9203-009 on the toxicity and the efficacy of VCR in tumour-bearing nude mice

Drug*	Days	Modulator†	Dose mg/kg i.p.	Days	N‡	Weight loss mean ± S.D.	Relative body weight day 14** mean ± S.D.	GI%§	$T_{D1\rightarrow4}$‖ days ± S.E.M.
BRO									
Control		none			5	n.a.	1.16 ± 0.06	n.a.	8.3 ± 1.2
None		B9303-036	20 (1×)	0, 7	3	0%	1.27 ± 0.13	0%	7.3 ± 0.9
VCR	0, 7	none			5	6 ± 3%	1.10 ± 0.06	19%	8.0 ± 0.5
VCR	0, 7	B9303-036	20 (1×)	0, 7	3	11 ± 7%	1.06 ± 0.05	36%	8.8 ± 0.7
BRO/mdr1.1									
Control		none			6	n.a.	1.17 ± 0.06	n.a.	8.7 ± 1.2
None		B9303-036	20 (3×)	0, 7	6	0 ± 4%	1.11 ± 0.10	30%	9.1 ± 1.2
VCR	0, 7	none			6	7 ± 3%	1.08 ± 0.12	57%	11.3 ± 2.2
VCR	0, 7	B9303-036	20 (3×)	0, 7	6	15 ± 4%	0.99 ± 0.05	67%	13.1 ± 1.0¶
BRO/mdr1.1									
None		none			3	n.a.	1.03 ± 0.04	n.a.	9.2 ± 2.3
VCR	0, 7	B9203-009	20 (1×)	0, 7	3	18 ± 2%	0.94 ± 0.03	34%	10.3 ± 1.3

*VCR 1 mg/kg i.v.; †B9303-036 (1×) and B9203-009 preceded VCR by an interval of 1 h, while B9303-036 (3×) was given − 1 h, + 7 h, + 24 h; ‡Number of mice; §Growth inhibition %; ‖Days for tumours to double twice in volume; ¶Better effective than control ($P < 0.02$); **Ratio of the body weight on day 14 compared with day 0. n.a., not applicable.

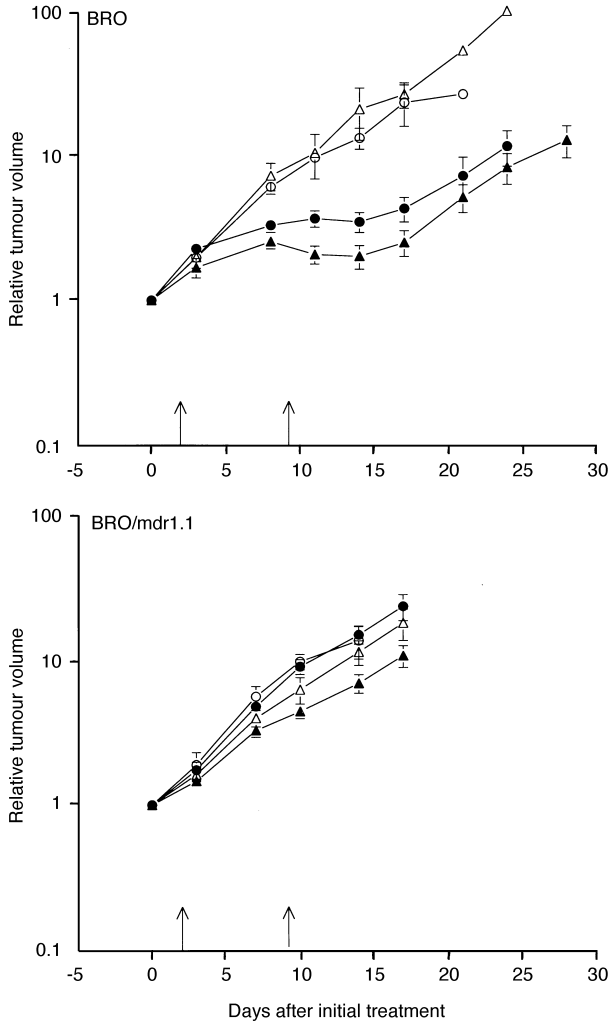

Figure 1. Growth curves of BRO or BRO/mdr1.1 xenografts of mice not treated (○), treated with VCR 1 mg/kg i.v. on days 2 and 9 (●), treated with dexniguldipine 25 mg/kg i.p. on days 0–4 and 7–11 (△) or the combination of VCR and dexniguldipine (▲). VCR injection days are indicated with arrows. Results are expressed as mean ± S.E.M.

VCR 1 mg/kg i.v. weekly ×2 (Table 3). There was no additional toxicity, but reversal of VCR was not evident. In the next experiment, mice were treated with B9303-036 20 mg/kg i.v. 3 times (− 1 h, + 7 h, + 24 h) combined with VCR 1 mg/kg i.v. (0 h) weekly ×2. The combination was significantly more effective when compared with control tumours ($P < 0.02$), but not when compared with VCR-treated tumours. Further experiments with B9303-036 were not considered as the combination had reached a maximum weight loss of 15 (S.D. ± 4)%. A pilot experiment with B9203-009 20 mg/kg i.v. preceding VCR 1 mg/kg i.v. with an interval of 1 h weekly ×2 showed a maximum weight loss of 18 (S.D. ± 2)% and no increase of the efficacy of VCR.

BIBW22BS 50 mg/kg i.v. was given 1 h preceding VCR 1 mg/kg i.p. weekly ×2. Earlier it had been established that BIBW22BS in a dose of 12.5 mg/kg i.v. in mice resulted in rapid plasma pharmacokinetics of 18.7 μM (5 min), 9.0 μM (60 min) and 4.9 μM (4 h) as measured by HPLC (data from Dr. Karl Thomae GmbH). BIBW22BS 50 mg/kg i.v. was the highest possible dose as the compound induced muscular relaxing and sedating effects with recovery within 2 h after administration [12]. BIBW22BS alone neither induced weight loss nor affected tumour growth. Upon addition of BIBW22BS to VCR the mean weight loss increased from 4 (S.D. ± 4)% to 14 (S.D. ± 8)% in mice bearing BRO/mdr1.1 xenografts. The time for BRO/mdr1.1 tumours to double their volume was significantly increased ($P < 0.01$) from 6.4 (S.D. ± 0.5) days to 12.9 (S.D. ± 1.1) days [12].

Distribution of VCR

In an attempt to obtain insight into the possible increase in retention of VCR in BRO/mdr1.1 xenografts induced by Pgp modulators we carried out three separate distribution experiments.

In the first experiment BRO-bearing mice were treated with VCR 1 mg/kg i.v. alone. Mice implanted with BRO/mdr1.1 tumours were treated with VCR 1 mg/kg i.v. alone or dexniguldipine either 25 mg/kg i.p. or 40 mg/kg i.p. days 0, 1, 2, 3 combined with VCR on day 2 (4 h after dexniguldipine). Mice were autopsied at 4 and 24 h after VCR administration. Plasma, tumours, liver, kidney, heart, lung, spleen, stomach,

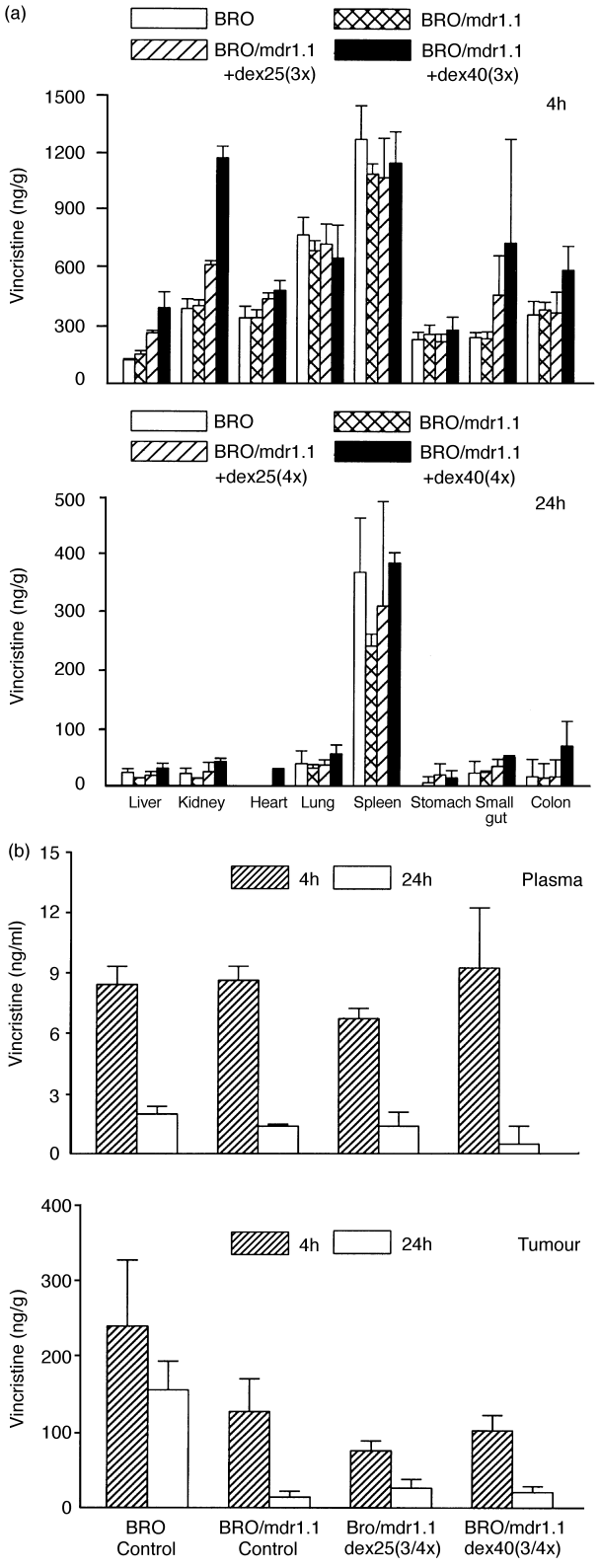

Figure 2. Distribution of VCR in (a) normal organs; (at 4 and 24 h) or (b) plasma and tumour tissue of mice bearing BRO or BRO/mdr1.1 xenografts. Mice were treated with VCR 1 mg/kg i.v. on day 2 while additional groups of BRO/mdr1.1-bearing mice were pretreated with dexniguldipine 25 or 40 mg/kg i.p. days 0, 1, 2, 3 (4 h before VCR on day 2). Mice were autopsied at 4 or 24 h after VCR injection. Each column represents organs of three mice. Results are expressed as mean ± S.D.

small gut and colon were analysed and the results are shown in Figure 2. For normal tissues in BRO- and BRO/mdr1.1-bearing mice treated with VCR alone there were no differences between VCR concentrations. Spleen VCR levels were highest (1180 ng/g at 4 h and 320 ng/g at 24 h) followed by lung VCR levels (730 ng/g and 35 ng/g, respectively). Levels in other tissues varied slightly; 200–400 ng/g at 4 h and 0–40 ng/g at 24 h. In BRO tumours, however, VCR levels at 4 h were 239.3 (S.D. ± 87.5) ng/g, whilst in BRO/mdr1.1 tumours levels were 1.9-fold lower being 127.4 (S.D. ± 42.7) ng/g ($P < 0.05$); at 24 h the respective levels were 155.6 (S.D. ± 37.7) ng/g and 13.8 (S.D. ± 7.9) ng/g being 11.3-fold lower ($P < 0.001$). VCR levels in liver, kidney, small gut and colon increased with the pretreatment dose of dexniguldipine. This increase was less obvious in the other organs and in plasma. In contrast with our expectations, BRO/mdr1.1 tumour tissue did not contain higher VCR levels when pretreated with dexniguldipine.

Because BIBW22BS appeared to be slightly more effective than dexniguldipine in the reversal of VCR resistance in BRO/mdr1.1 xenografts *in vivo*, a second distribution experiment was carried out including BIBW22BS. Mice bearing BRO or BRO/mdr1.1 were treated with VCR 1 mg/kg i.v. alone. Other BRO/mdr1.1-bearing mice were given VCR 1 mg/kg i.v. preceded by dexniguldipine 40 mg/kg i.p. (interval 4 h) or BIBW22BS 50 mg/kg i.v. (interval 1 h). Mice were autopsied at 1 min, 30 min, 4 h and 24 h after VCR administration. Plasma, tumours, liver, kidney, heart and small gut were analysed and the mean values were used to calculate the areas under the curve over the first 24 h (Table 4). Again, there was no major difference between the VCR areas under the curve for normal tissues of BRO- and BRO/mdr1.1-bearing mice. The VCR area in BRO/mdr1.1 tumours was 2.4-fold lower than that in BRO tumours. There was a remarkable increase in the VCR area under the curve of liver, kidney, heart and small gut upon pretreatment with dexniguldipine, whilst the increase was even more pronounced in the presence of BIBW22BS. The VCR area under the curve of BRO/mdr1.1 tumours pretreated with dexniguldipine or BIBW22BS was similar to that of BRO tumours.

The third experiment was designed to compare the influence of dexniguldipine 40 mg/kg i.p. days 0, 1, 2, 3 combined with VCR 1 mg/kg i.v. day 2 (4 h after dexniguldipine) and dexniguldipine 40 mg/kg i.p. preceding VCR with an interval of 4 h. Control groups were BRO- and BRO/mdr1.1-bearing

Table 4. VCR areas under the curve in mouse xenograft tissues*

Tissue	BRO VCR	BRO/ mdr1.1 VCR	BRO/ mdr1.1 VCR + dex 40 (1×)†	BRO/ mdr1.1 VCR + BIBW22BS‡
Liver	188.1	177.5	518.9	672.1
Kidney	448.4	417.9	904.6	1504.1
Heart	263.4	352.4	443.3	832.4
Small gut	318.5	356.1	791.6	935.1
Tumour	193.3	78.9	177.9	204.3
Plasma	27.8	15.3	35.5	33.3

*Mice were autopsied 1 min, 3 min, 4 h and 24 h after VCR 1 mg/kg i.v. The area under the curve over the first 24 h was calculated from the mean values of groups of three mice each and expressed as μg/g.min or μg/ml.min; †Dexniguldipine 40 mg/kg i.p. preceding VCR with an interval of 4 h; ‡BIBW22BS 50 mg/kg i.v. preceding VCR with an interval of 1 h.

mice treated with VCR alone. Mice were autopsied at 4 and 24 h after VCR administration. As the results were similar to the corresponding groups in the first and the second experiment, the data were pooled and expressed as mean values (± S.E.M.) (Figure 3). VCR levels in normal tissues in BRO- and BRO/mdr1.1-bearing mice were similar. Regardless of the schedule of dexniguldipine, these levels increased to the same extent. Plasma VCR levels were not clearly affected. Similar to the second experiment: when one injection of dexniguldipine preceded VCR in BRO/mdr1.1 tumours, the VCR levels at 4 h reflected levels in BRO tumours, but the 24-h level was 40% of that in BRO. Similar to the first experiment: when dexniguldipine treatment for 3 days preceded VCR, the VCR levels were 57% at 4 h and only 11% at 24 h in BRO/mdr1.1 tumours when compared with the respective time-points in BRO tumours. VCR concentrations in BRO/mdr1.1 tumours treated with VCR alone or VCR preceded by dexniguldipine for 3 days were not significantly different.

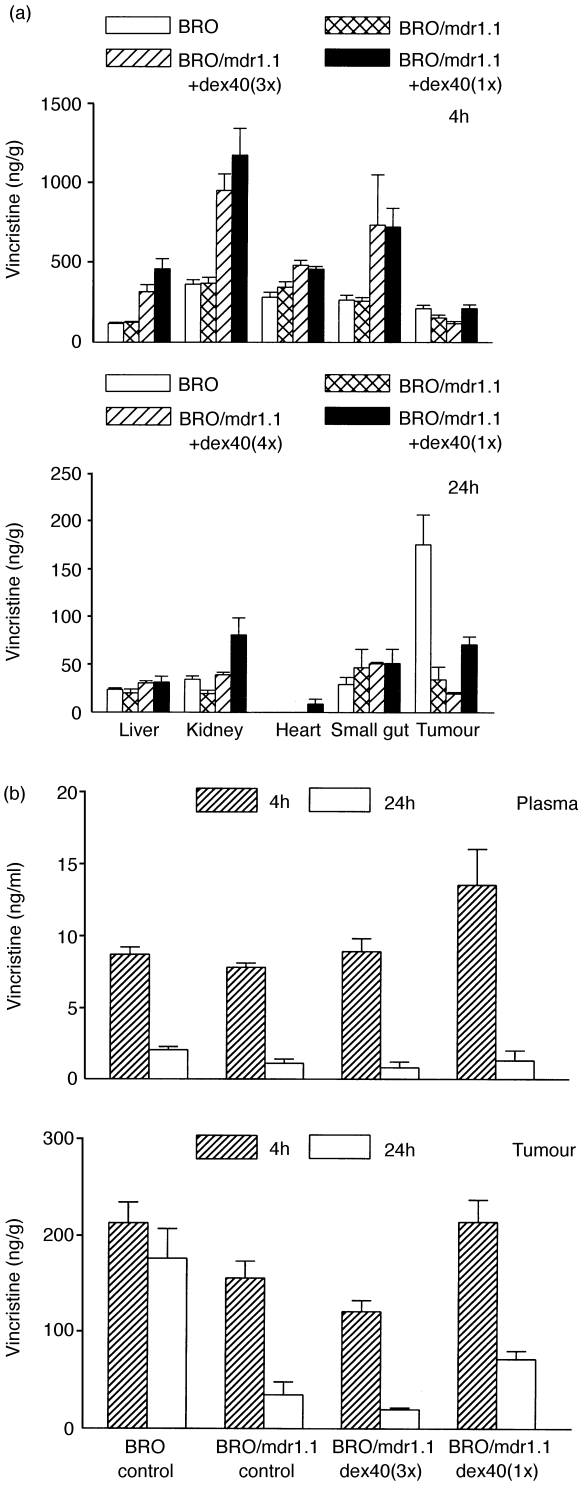

Figure 3. Distribution of VCR in (a) normal organs; (at 4 and 24 h) or (b) plasma and tumour tissue of mice bearing BRO or BRO/mdr1.1 xenografts. Mice were treated with VCR 1 mg/kg i.v. on day 2 while additional groups of BRO/mdr1.1-bearing mice were pretreated with dexniguldipine 40 mg/kg i.p. days 0, 1, 2, 3 (4 h before VCR on day 2) or once 4 h before VCR. Mice were autopsied at 4 or 24 h after VCR injection. Each column represents organs of 6–9 mice. Results are expressed as mean ± S.E.M.

Distribution of dexniguldipine

Mice bearing BRO/mdr1.1 xenografts were treated with dexniguldipine 40 mg/kg i.p. days 0, 1, 2, 3 combined with VCR 1 mg/kg i.v. on day 2 (4 h after dexniguldipine). The uptake of the Pgp modulator in normal as well as in tumour tissues was measured on day 2 at 8 h and on day 3 at 4 h after dexniguldipine injection. Table 5 shows that kidney, lung, spleen and stomach contained relatively high dexniguldipine levels varying between 53 and 63 μg/g at 4 h. The levels were reasonably well sustained at 8 h levels (Table 5). In contrast, levels in BRO/mdr1.1 tissue were low (3.3–4.1 μg/g) and accounted for approximately 5.4–6.7 nmol/g tissue.

MDR*1 expression* in vivo

In an attempt to understand the low VCR levels in BRO/mdr1.1 tissue upon dexniguldipine pretreatment for 3 days when compared with the increased VCR levels if dexniguldipine was given only once, we measured *MDR*1 mRNA expression. Apart from control tumours, groups of BRO/mdr1.1-bearing mice were treated with VCR 1 mg/kg i.v., dexniguldipine 40 mg/kg i.p. days 0, 1, 2, 3 or dexniguldipine only once and the two combinations of dexniguldipine preceding VCR with an interval of 4 h (on day 2). Mice were autopsied 4 and 24 h after VCR and BRO/mdr1.1 tumours were collected. The RNase protection assay was carried out 3–5 times in two tumours for each time-point. Figure 4 clearly shows that compared with control tumours, *MDR*1

Table 5. *Distribution of dexniguldipine* in mouse and xenograft tissues*

Tissue	Dexniguldipine at 4 h μg/g ± S.D.	Dexniguldipine at 8 h μg/g ± S.D.
Liver	41.9 ± 2.8	30.6 ± 4.7
Kidney	60.2 ± 11.6	61.1 ± 3.0†
Heart	17.7 ± 1.2	11.8 ± 6.6†
Lung	56.2 ± 9.1	49.4 ± 21.9
Spleen	63.3 ± 15.4	37.6 ± 2.6
Stomach	53.9 ± 11.4	31.3 ± 9.9
Small gut	32.2 ± 8.2	15.7 ± 5.5
Colon	37.3 ± 7.3	15.2‡
BRO/mdr1.1	3.3 ± 0.7	4.1 ± 1.1

*Dexniguldipine 40 mg/kg i.p. days 0, 1, 2, 3 in combination with VCR 1 mg/kg i.v. on day 2 (4 h interval); three mice were autopsied on day 2 at 8 h after dexniguldipine and on day 3 at 4 h after dexniguldipine; †Results of two organs; ‡Result of one organ.

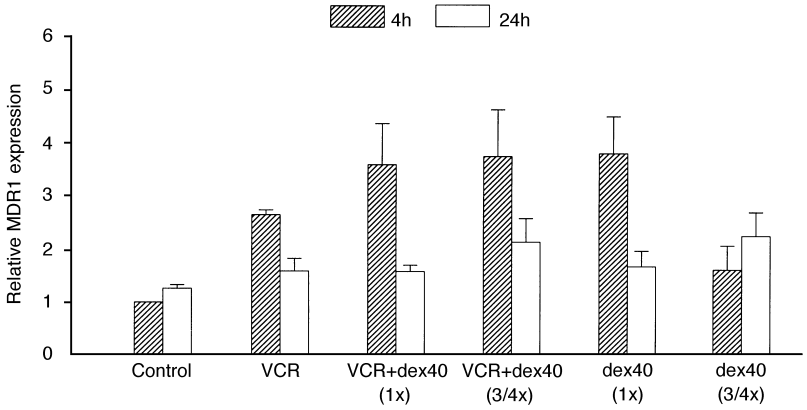

Figure 4. Relative *MDR*1 mRNA expression in BRO/mdr1.1 xenograft tissue in mice treated with VCR and/or dexniguldipine. The value of BRO/mdr1.1 control (untreated) tumours at 4 h was set at 1. Mice were treated with VCR 1 mg/kg i.v., dexniguldipine 40 mg/kg i.p., dexniguldipine 40 mg/kg i.p. days 0, 1, 2, 3, VCR preceded by dexniguldipine with an interval of 4 h, VCR on day 2 combined with dexniguldipine days 0, 1, 2, 3 (interval of 4 h before VCR on day 2). Mice were autopsied at 4 or 24 h after VCR injection. In mice treated with dexniguldipine alone (1 ×) autopsy was carried out at 8 or 28 h and in case of dexniguldipine (3/4 ×) this was at 8 h after the third and at 4 h after the fourth injection. Results are of duplicate samples of two separate tumours and are expressed as mean ±S.E.M.

was upregulated 2.7-fold 4 h after VCR. This upregulation was 3.6–3.7-fold when VCR was preceded by dexniguldipine regardless of the schedule. When mice were treated with dexniguldipine alone, *MDR*1 upregulation 8 h after one injection of the modulator was 3.8-fold, but the upregulation was less evident (1.6-fold) in the case of dexniguldipine pretreatment on days 0, 1, 2. Values in mice that received a fourth dexniguldipine injection (day 3) 4 h before tissue analysis had a 2.1- to 2.3-fold higher expression level, whilst in mice that received no treatment around the 24-h time-point *MDR*1 expression was 1.6- to 1.7-fold higher than control levels.

DISCUSSION

Pgp modulators were studied for their efficacy to reverse VCR resistance in BRO/mdr1.1 cells cultured *in vitro* and established as s.c. xenografts. BRO/mdr1.1 cells were transfected with a full-length human *MDR*1 cDNA [15]. Previously, we have shown that the *MDR*1 gene is present and that VCR resistance is retained upon serial transfer of BRO/mdr1.1 xenografts for at least 15 passages [12]. We consider the model more useful than cells selected for 'classical' mdr, because *MDR*1-transfected cells are less likely to co-express other mechanisms of resistance.

VCR measurements in complete tissues using HPLC have not been reported before. Distribution of the drug in normal mouse organs showed the highest concentration and the longest retention in the spleen. Van Tellingen and colleagues [19, 23] have described earlier that vinblastine and vinorelbine showed selective retention in mouse testicular and lymphatic tissues, whereas in most other tissues the drug levels decreased in parallel with the plasma level. With our method we did not detect VCR metabolites in the tissue homogenates. Horton and colleagues [10] have used radioactivity counting for drug level determinations in mouse tissues after administration of [G-^{3}H]VCR. We found a substantial difference in retention of VCR at 24 h. In tissues we measured 5–19% of the concentrations detected at 4 h, while in the study by Horton and colleagues [10] these levels had only reduced to 45–80%. The absolute quantities detected by HPLC were substantially lower, which supports the selectivity of our detection method.

Pgp modulators dexniguldipine, B9203-009, B9303-036 and BIBW22BS were able to reverse resistance against VCR in BRO/mdr1.1 and 2780^{AD} cells grown *in vitro*, but were slightly or not effective in the reversal of VCR resistance in BRO/mdr1.1 xenografts *in vivo*. Perhaps tissue concentrations were not sufficient for complete Pgp inhibition. For dexniguldipine, BRO/mdr1.1 tissue levels varied from 5.4 to 6.7 nmol/g at 4–8 h after injection and were likely too low to expect Pgp modulation of importance. As demonstrated *in vitro*, 0.5 μmol of dexniguldipine for 4 days was required to reverse VCR resistance in BRO/mdr1.1 cells, while 0.1 μmol dexniguldipine was hardly effective. Another factor which has to be taken into account is that most Pgp modulators have high plasma protein binding leaving a small amount of free drug available for inhibition of Pgp [24]. For dipyridamole, the lead compound for BIBW22BS, it is known that > 95% is bound to plasma proteins, particularly to albumin and to α_1-acid glycoprotein [25]. When the mean plasma level of total BIBW22BS was 9.0 μM at 60 min after a dose of 12.5 mg/kg i.v. [12], one would expect this concentration to be sufficient as 0.5 μM of BIBW22BS for 4 days could completely reverse VCR resistance in BRO/mdr1.1 cells *in vitro*. However, tissue levels of free compound *in vivo* may have been too low to inhibit Pgp. Moreover, BIBW22BS plasma levels at 4 h were 25% of peak plasma levels, which may indicate that one injection is not sufficient for prolonged Pgp inhibition.

While dexniguldipine 40 mg/kg i.p. given once 4 h before VCR and BIBW22BS 50 mg/kg i.v. given once 1 h before VCR were not or slightly effective in the increase of the efficacy of VCR in BRO/mdr1.1 xenografts, both compounds could increase the area under the curve approaching the value found in BRO xenografts (Table 4). Other groups have also reported an increase in cytotoxic drug levels in Pgp-positive tumours *in vivo* by Pgp modulators without a clear increase in antitumour effects. This was for instance the case in KB8-5 xenografts where 6-fold higher [G-^{3}H]VCR levels (at 72 h) were measured upon a continuous infusion of verapamil [11] and in P388/DOX leukaemia where doxorubicin levels in the presence of PSC 833 were similar to those measured in the parent P388 cells *in vivo* [26]. The increased VCR area under

the curve in BRO/mdr1.1 xenografts by Pgp modulators did not reflect the shorter retention time of VCR when compared with that in BRO tumours (Figure 3b). It is likely that the rapid clearance rate of VCR from BRO/mdr1.1 tumours will account for our negative findings. The absence of an influence of Pgp modulators on the efficacy of VCR in BRO/mdr1.1 xenografts may also be explained by other factors, such as VCR is measured in whole tissue and not exclusively within tumour cells.

Pgp modulators increased the VCR concentrations in normal mouse tissues without grossly affecting plasma levels. Mice tissues are known to express two different drug-transporting Pgps encoded by *mdr*1a and *mdr*1b that seem to cover the same tasks carried out by the *MDR*1 Pgp in man [27]. Increased cytotoxic drug levels in normal mouse tissues induced by Pgp modulators have been reported before. Horton and associates [10] have demonstrated that increased [G-^{3}H]VCR levels in the presence of verapamil were related with the occurrence of acute neurological symptoms. Neurological toxicity was not observed in our animals.

Since it was not clear why VCR concentrations were reduced in BRO/mdr1.1 tumour tissue when mice had been pretreated with dexniguldipine for 2 additional days, expression levels of human *MDR*1 mRNA were measured. Apart from dexniguldipine it was found that also VCR could enhance *MDR*1 expression which was more pronounced after 4 h than after 24 h. When mice were treated with dexniguldipine for 3 or 4 days this enhancement was less, which was suggestive for quenching of the signal. When VCR was administered to mice that had been pretreated with dexniguldipine for 3 days *MDR*1 upregulation was evident again. Reduced VCR concentrations in BRO/mdr1.1 tumour tissue upon prolonged dexniguldipine exposure were likely the result of a gradual increase of Pgp expression. Even the shorter VCR retention time in BRO/mdr1.1 xenografts upon one injection of a Pgp modulator may have been caused by increased Pgp.

A rapid increase of *MDR*1/Pgp expression by Pgp modulators as well as by cytotoxic agents has been reported before *in vitro* in cell lines selected for 'classical' mdr as well as in cells that express Pgp of origin [28–30]. Drug-induced *MDR*1 upregulation could be maintained for a prolonged period of time after the drugs had been removed [29, 30]. Hu and associates [30] have reported that a 3–4-fold increase in Pgp expression was evident within 24 h after drug exposure. In the study of Herzog and associates [28] there was no increase in the *MDR*1 transcription rate or in nuclear *MDR*1 mRNA, so it was suggested that the upregulation was mediated post-transcriptionally within the nucleus. Down-regulation of *MDR*1 mRNA levels has also been demonstrated as a response to Pgp modulators [31, 32]. Muller and associates [31] have found a decrease in the *MDR*1 proximal promoter activity resulting in reduced *MDR*1 gene transcription. The reason for upregulation of *MDR*1 mRNA in BRO/mdr1.1 xenografts by dexniguldipine or VCR is not clear. For transfection of BRO cells *MDR*1 cDNA was inserted into a eukaryotic expression vector providing the immediate early gene promoter-enhancer of human cytomegalovirus upstream of the cDNA. Future experiments in our laboratory will include the influence of Pgp substrates on *MDR*1 expression *in vivo* in human tumour xenografts that express Pgp of origin.

While VCR concentrations in BRO/mdr1.1 tumour tissue were reduced when dexniguldipine was given for 2 additional days, VCR levels in normal tissues were not clearly affected. Little is known about the regulation of *MDR*1 in normal tissues in man or *mdr*1a and -1b in mice or rats. Upregulation of *mdr*1a and -1b in rat liver, as well as of *MDR*1 in liver of Cynomolgus monkeys could be induced by α-naphthylisothiocyanate-induced cholestasis [33]. In rats treated with 2-acetylaminofluorene a significant increase of biliary excretion of vinblastine was measured as a result of increased Pgp levels [33].

The clinical relevance of a possible upregulation of *MDR*1/Pgp in Pgp-positive human tumour tissue upon treatment with Pgp substrates is not yet clear. In a preliminary report, Abolhoda and associates [34] have described a 3.0-fold increase in *MDR*1 mRNA in lung metastases within 50 min following doxorubicin lung perfusion in sarcoma patients. Whether this phenomenon will occur in other tumour types in patients and will reduce cytotoxic drug levels upon pretreatment with Pgp modulators remains to be determined.

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