

Original article

Biochemical modulation of 'classical' multidrug resistance by BIBW22BS, a potent derivative of dipyridamole

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Summary

Background: Modulators of the 'classical' multidrug resistance (mdr) phenotype have low efficacy in patients with solid tumors. We analyzed BIBW22BS, 4-[N-(2-hydroxy-2-methyl-propyl)-ethanolamino]-2,7-bis(cis-2,6-dimethyl-morpholino)-6-phenylpteridine, a derivative of dipyridamole, for its higher potential to modulate mdr.

Materials and methods: Four human malignant cell lines: BRO, A2780, GLC₄, SW1573, the Pgp-positive sublines: BRO/mdr1.1, 2780^{AD} and the non-Pgp sublines: GLC₄/ADR, SW1573/2R120 were used in vitro to investigate BIBW22BS as a modulator of the antiproliferative effects of vincristine and doxorubicin and to compare the potency of BIBW22BS with that of dipyridamole, verapamil, bepridil and flunarizine. BRO/mdr1.1 s.c. well-established xenografts in nude mice were used to study the modulating properties of BIBW22BS 50 mg/kg i.v. followed after one h by vincristine 1 mg/kg i.p. or doxorubicin 8 mg/kg i.p. weekly × 2.

Results: BIBW22BS was 20- to 100-fold more potent than dipyridamole in the reversal of resistance in the Pgp-

positive sublines. Reversal of resistance was obtained in a dose-dependent manner and was complete at concentrations of 0.5–2.5 μM. At non-toxic, equimolar concentrations of 1.0 μM BIBW22BS showed higher modulating potency than the calcium-channel blockers. BIBW22BS did not affect resistance in the non-Pgp sublines. BRO/mdr1.1 s.c. xenografts have stable multidrug-resistance characteristics upon serial transplantation. BIBW22BS, vincristine, or doxorubicin as single agents were not effective in vivo, while the addition of BIBW22BS could significantly reduce the tumor growth expressed as the T/C% of vincristine from 109% to 48% and that of doxorubicin from 55% to 32%. However, reversal of vincristine resistance in BRO/mdr1.1 xenografts was not complete when compared to the efficacy of vincristine in BRO xenografts.

Conclusion: The results encourage the further preclinical development of BIBW22BS as a modulator of 'classical' multidrug resistance in cancer patients.

Key words: multidrug resistance, BIBW22BS, dipyridamole, verapamil, human tumor xenografts

Introduction

'Classical' multidrug resistance (mdr) is associated with the expression of a 170 kDa P-glycoprotein (Pgp) in the plasma membrane of malignant cells [1, 2]. Pgp acts as an ATP-dependent efflux pump causing a decreased intracellular accumulation of structurally unrelated natural anticancer agents, such as anthracyclines, *Vinca*-alkaloids, epipodophyllotoxins, actinomycin-D and taxol. Cloning of the cDNA for the human *mdr1* gene which encodes Pgp, and the generation of a series of monoclonal antibodies reactive with Pgp, have enabled the detection of the mdr phenotype in patients' tumors [3, 4]. Increased expression of the *mdr1* gene and/or Pgp can be found in tumor types with a low response rate to chemotherapy, such as colon cancer and renal cell cancer, but also in chemoresponsive malignancies although mainly at the time of relapse [1, 2].

Diverse lipophilic compounds are known to reverse

the 'classical' mdr phenotype to varying degrees in vitro [5]. The calcium-channel blockers and cyclosporin derivatives have been mostly studied and appear to interact with Pgp causing a direct inhibition of its function. In patients with hematological malignancies, verapamil and cyclosporin A have demonstrated efficacy as modulators of resistance and specifically in Pgp-positive tumors [6–8]. Low efficacy in solid tumors was reason to search for more potent modulators, such as the D-isomer of verapamil and the cyclosporin A analog SDZ PSC-833 [9, 10]. In parallel, analogs of other classes of lipophilic compounds with modulating properties were developed and BIBW22BS, a derivative of dipyridamole, is one of them.

In vitro, BIBW22BS (Fig. 1) was identified as a more potent modulator of mdr than dipyridamole. Chen et al. [11] have shown that photoaffinity labeling of Pgp could be strongly inhibited by BIBW22BS, indicating that the compound reverses the mdr phenotype by

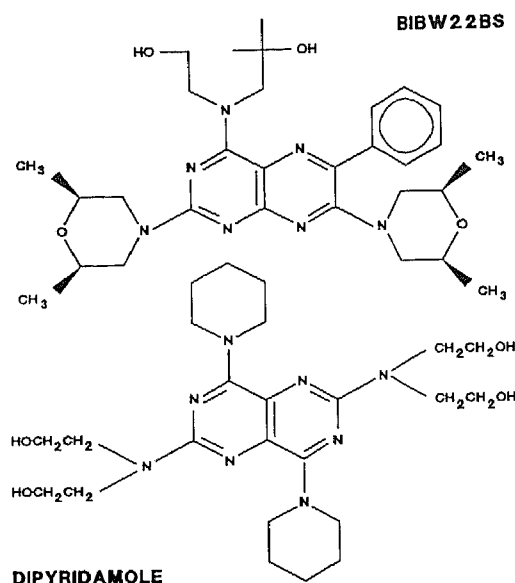


Fig. 1. Structural formulas of 4-[N-(2-hydroxy-2-methyl-propyl)-ethanolamino]-2,7-bis(cis-2,6-dimethyl-morpholino)-6-phenylpyridine (BIBW22BS) and dipyridamole.

interfering with *mdr*-associated Pgp. In the present experiments we compared BIBW22BS with various calcium-channel blockers for its activity to modulate *mdr* in Pgp-positive and non-Pgp cell lines in vitro. In addition, we studied the influence of BIBW22BS on the antitumor activity of vincristine and doxorubicin in well-established Pgp-positive xenografts in an attempt to assess the potential value for clinical use.

Materials and methods

Drugs and resistance modulators

Vincristine (VCR; Eli Lilly, Amsterdam, The Netherlands) was purchased as a solution of 1 mg/ml. Doxorubicin (DOX; Farmitalia Carlo Erba, Nivelles, Belgium) was dissolved in aqua at a concentration of 2 mg/ml. BIBW22BS and dipyridamole (both from Dr. Karl Thomae GmbH) were first dissolved in 0.1 N HCl and then diluted in 0.9% NaCl to a final concentration of 2 mM at pH 2.7. Verapamil (Knoll, Amsterdam, The Netherlands) and bepridil (Organon, Oss, The Netherlands) were provided as a solution of 2.5 mg/ml and 4 mg/ml, respectively. Flunarizine (Janssen, Beerse, Belgium) was dissolved in DMSO at a concentration of 10 mg/ml. Drugs and resistance modulators were further diluted in tissue culture medium when investigated in the cytotoxicity experiments in vitro.

Cell lines and drug sensitivity

Four pairs of human malignant cell lines were used, which were kindly provided by the groups that selected the drug-resistant sublines. The Pgp-positive subline BRO/*mdr*1.1 (BRO/pFR*mdr*1.6 clone 1.1) was obtained by transfection of the parent melanoma cell line BRO with a full length *mdr*1 gene [12]. The Pgp-positive subline 2780^{AD} [13] of ovarian cancer cell line A2780, and the non-Pgp *mdr* sublines GLC₄/ADR [14] of small cell lung cancer cell line GLC₄ and SW1573/2R120 [15] of non-small cell lung cancer cell line SW1573 were selected by a step-wise increase of the doxorubicin concentration in tissue culture medium. All 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% CO₂ at 37 °C. The resistant sublines were cultured in the presence of a selective drug (vincristine 10 nM for BRO/*mdr*1.1 and doxorubicin 2 µM for 2780^{AD}, 1.2 µM for GLC₄/ADR and 0.12 µM for SW1573/2R120) until 3 days before the experiments.

Cellular drug sensitivities were measured using the MTT assay [16]. In this assay the tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] is reduced by the mitochondrial dehydrogenase of metabolically active cells to a blue formazan product. 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 microtiter plate reader (Reader Microelisa System, Organon Teknika, Turnhout, Belgium). Readings in experimental wells were linear with cell concentrations between 2,500 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. Drug effects were expressed as the IC₅₀, which is the concentration of the drug inducing a 50% inhibition of cell growth of treated cells when compared to the growth of control cells.

Xenografts and *mdr*1 expression

Female nude mice (Hsd: athymic nude-nu) were purchased at the age of 6 weeks (Harlan Cpb, Zeist, The Netherlands). The animals were maintained in isolation and animal handling was carried out under sterile conditions. BRO and BRO/*mdr*1.1 xenografts were established from cell lines grown in tissue culture. Mice were inoculated s.c. with 1×10^7 cells in both flanks. The solid tumors 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 experiments were carried out in passage 2 or higher passages.

To determine the retention of the *mdr*1 gene in vivo, the expression was measured in various passages of the BRO/*mdr*1.1 xenografts. In short, after pulverization of frozen xenograft tissue in a microdismembrator total cellular RNA was extracted by homogenization in guanidine isothiocyanate followed by an ultracentrifugation step on a cesium chloride cushion. An NP40 procedure was used to isolate cytoplasmic RNA from control cell lines. RNase protection was performed as described earlier [15,17]. Briefly, 10 µg of total RNA was hybridized with a [³²P]CTP-labeled anti-sense RNA probe, specific for *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 hybridized probe was visualized after electrophoresis through a denaturing 6% acrylamide gel. For autoradiography, the gel was exposed at –70 °C to a Kodak XS film overnight.

Drug sensitivity in vivo

BRO and BRO/*mdr*1.1 xenografts were measured twice a week in 3 dimensions with a vernier caliper. The volume was calculated by the equation length \times width \times thickness \times 0.5, and expressed in mm³. At the start of treatment (day 0), groups of 5–6 tumor-bearing mice (9–12 tumors) were formed to provide a mean tumor volume between 75–150 mm³ in each group. Vincristine and doxorubicin were injected i.v. weekly \times 2 at the maximum tolerated dose (MTD). This dose resulted in a mean weight loss between 5%–15% of the initial weight within 2 weeks after the first injection and was for vincristine 1 mg/kg and for doxorubicin 8 mg/kg. For in vivo use, BIBW22BS was dissolved in HCl 0.1 N at a concentration of 20 mg/ml and further diluted in NaCl 0.9% to 2.5 mg/ml at pH 2.7. The modulating capacity of BIBW22BS was determined by administration of the

MTD i.v. followed after one h by vincristine 1 mg/kg i.p. or doxorubicin 8 mg/kg i.p. weekly \times 2.

For the evaluation of drug efficacy, the tumor 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 at any given day and V_0 the volume at day 0. The ratio of the mean relative volume of treated tumors over that of control tumors multiplied by 100% (T/C%) was assessed at each day of measurement. For the evaluation of drug toxicity the animals were weighed twice-a-week. The mean relative body weight was calculated as the mean of the ratio of the body weight at any given day and that at day 0. Differences in antitumor effects and body weight changes were evaluated with Student's *t*-test.

Results

Modulation of *mdr* in vitro

First, we characterized the 4 malignant cell lines and the drug-resistant sublines for their sensitivity to vincristine and doxorubicin (Table 1). Except for vincristine in GLC₄/ADR, all sublines were resistant to vincristine and doxorubicin. Within the same experiment of quadruplicate samples the SD of the mean was neglectable. However, similar experiments carried out on separate days gave a variation in the IC₅₀ because of which the SD of the mean of resistance factors (RFs) was rather high. Mean RFs were highest in 2780^{AD} cells and amounted to 2878 for vincristine and 900 for doxorubicin, while modest resistance with RFs of respectively 48 and 45 were calculated for SW1573/2R120 cells.

BIBW22BS was compared with dipyridamole for its potency to modulate resistance in the Pgp-positive sublines BRO/*mdr*1.1 and 2780^{AD}. For BIBW22BS the IC₅₀ (range) was in BRO cells 3.3–>10 μ M, in BRO/*mdr*1.1 cells >10 μ M, in A2780 cells 3.4–>10 μ M and in 2780^{AD} cells 1.8–8.0 μ M, while for dipyridamole the

Table 1. Drug concentrations inducing 50% inhibition of cell growth of treated vs. control cells.

Cell line	Vincristine		Doxorubicin	
	nM (\pm SD) ^a	RF ^b (\pm SD)	nM (\pm SD)	RF ^b (\pm SD)
BRO	3.3 (\pm 1.1) \times 10 ⁻¹		7.4 (\pm 3.5)	
BRO/ <i>mdr</i> 1.1	3.7 (\pm 2.2) \times 10	112 (\pm 66)	6.6 (\pm 2.7) \times 10 ²	89 (\pm 37)
A2780	3.0 (\pm 0.2) \times 10 ⁻¹		3.4 (\pm 0.4)	
2780 ^{AD}	8.6 (\pm 4.5) \times 10 ²	2878 (\pm 1498)	3.3 (\pm 1.0) \times 10 ³	900 (\pm 265)
GLC ₄	5.0 (\pm 2.6) \times 10 ⁻¹		5.5 (\pm 2.7)	
GLC ₄ /ADR	4.3 (\pm 2.4) \times 10 ⁻¹	0.9 (\pm 0.5)	3.7 (\pm 0.9) \times 10 ³	700 (\pm 155)
SW1573	2.5 (\pm 0.7) \times 10 ⁻¹		4.4 (\pm 2.3)	
SW1573/2R120	1.2 (\pm 0.8) \times 10	48 (\pm 33)	1.7 (\pm 0.7) \times 10 ²	45 (\pm 18)

^a Mean in M (\pm SD) of \geq 3 separate experiments.

^b Mean resistance factor (\pm SD) expressed as the ratio of IC₅₀ subline/IC₅₀ parent cell line.

IC₅₀ was >10 μ M in all cells. A concentration range of both modulators of 0.1–10 μ M was tested in the absence or the presence of vincristine or doxorubicin. Reversal of resistance was obtained in a dose-dependent manner (Fig. 2). For BIBW22BS complete reversal was reached at 0.5 μ M in BRO/*mdr*1.1 cells and between 1.0–2.5 μ M in 2780^{AD} cells. In contrast, dipyridamole at the highest concentration of 10 μ M tested could achieve reversal of resistance to a degree which was for vincristine similar to BIBW22BS at 0.1–0.5 μ M and for doxorubicin similar to BIBW22BS at 0.1 μ M. This indicates that BIBW22BS is 20- to 100-fold more potent in the modulation of *mdr* when compared to the parent compound. BIBW22BS at non-toxic concentrations of 0.1, 0.5 and 1.0 μ M in combination with vincristine or doxorubicin could not affect resistance in the non-Pgp sublines (data not shown).

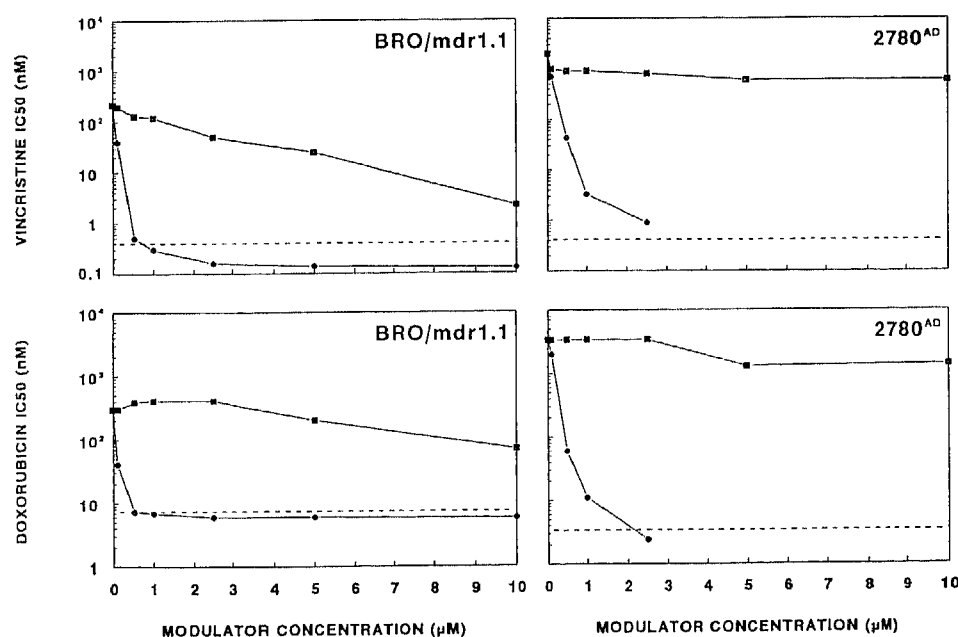


Fig. 2. The modulating capacity of varying concentrations of BIBW22BS (●) and dipyridamole (■) on the antiproliferative effects of vincristine or doxorubicin in the Pgp-positive sublines BRO/*mdr*1.1 or 2780^{AD}. Concentrations >2.5 μ M of BIBW22BS were toxic in 2780^{AD} cells. The dashed line represents the IC₅₀ of the drug in the parent cell lines BRO or A2780.

Several calcium-channel blockers, verapamil, bepridil and flunarizine, were selected to compare their efficacy at equimolar concentrations with that of BIBW22BS. The IC₅₀ of the modulators in the 4 pairs of cell lines was >5.0 μ M except for bepridil with an IC₅₀ of 4.3 μ M in GLC₄/ADR cells. The non-toxic 1.0 μ M concentration was chosen for the comparative study. BIBW22BS appeared to have the best modulating capacity in BRO/mdr1.1 and 2780^{AD} cells, followed by flunarizine, verapamil and bepridil (Table 2). In the parent cell lines and in the non-Pgp sublines GLC₄/ADR and SW1573 no influence on drug sensitivity or resistance was observed by any of the modulators. Only a slight reversal of vincristine resistance was apparent in SW1573/2R120 cells, where the RF was reduced from 70 to approximately 20.

The BRO and BRO/mdr1.1 xenograft model

In order to investigate the potential clinical value of mdr modulators in well-established Pgp-positive solid tumors *in vivo*, we developed the BRO and the BRO/mdr1.1 xenograft model. The model consists of s.c. grown tumors in both flanks of the nude mouse, while treatment is only started when tumors are well measurable after 7–10 days of implantation. Tumor volume doubling times of BRO and BRO/mdr1.1 xenografts are 2.5 (SD \pm 0.7) days and 3.4 (SD \pm 0.5) days, respectively. We characterized the xenografts in passage 2 for their sensitivity to vincristine and doxorubicin. While in BRO/mdr1.1 cells *in vitro* RFs were 112 for vincristine and 89 for doxorubicin, *in vivo* the difference in drug resistance was more pronounced. Vincristine could induce a T/C% of 1.7% in BRO xenografts, while the T/C% was only 78% in BRO/mdr1.1 xeno-

Table 2. Reversal of drug resistance by modulators at 1 μ M.

Cell line	VCR/ DOX RF ^a	BIBW- 22BS RF	Vera- pamil RF	Bepri- dil RF	Fluna- rizine RF
	<i>VCR</i>				
BRO	1	0.9	0.9	0.9	1.3
BRO/mdr1.1	53	0.4	13	25	5.8
A2780	1	1.0	0.8	1.0	0.7
2780 ^{AD}	3103	4.8	690	1103	114
GLC ₄	1	0.6	0.4	1.0	0.5
GLC ₄ /ADR	0.9	0.4	0.5	0.4	0.6
SW1573	1	0.7	0.7	0.8	1.2
2R120	70	21	13	20	20
	<i>DOX</i>				
BRO	1	1.0	1.0	1.0	1.0
BRO/mdr1.1	77	1.0	6.2	23	1.4
A2780	1	1.0	1.3	1.3	0.6
2780 ^{AD}	1200	1.7	185	450	22
GLC ₄	1	0.7	2.2	1.7	2.8
GLC ₄ /ADR	652	652	870	826	1087
SW1573	1	0.5	0.4	1.4	5
2R120	93	71	64	93	93

^a Resistance factor expressed as the ratio of IC₅₀ experimental cells/IC₅₀ parent line treated with drug alone.

grafts. For doxorubicin, however, the respective T/C% were 13% and 45% (Table 3).

In a next study we determined the retention of mdr characteristics in BRO/mdr1.1 xenografts in subsequent passages. The *in vivo* resistance to vincristine and doxorubicin was retained (Table 3 and Fig. 3). Cells were isolated from xenograft tissue in passages 2, 6, 8, and 10 and grown in tissue culture. The RFs for vincristine measured in these cells was similar to the

Table 3. Chemosensitivity of BRO and BRO/mdr1.1 xenografts.

Tumor line	Pas- sage	Vincristine ^a	Doxorubicin ^a
BRO	2	1.7% ^b (14)	13% ^b (12)
BRO/mdr1.1	2	78% (11)	45% ^b (14)
BRO/mdr1.1	5	63% ^b (11)	42% ^b (14)
BRO/mdr1.1	7	55% (17)	nd
BRO/mdr1.1	10	79% (13)	nd
BRO/mdr1.1	15	44% (11)	nd

^a Mean relative volume of treated vs. untreated tumors of that tumor line \times 100% and (day) of measurement.

^b Treatment is significantly different from control ($p < 0.05$).

^c nd, not determined.

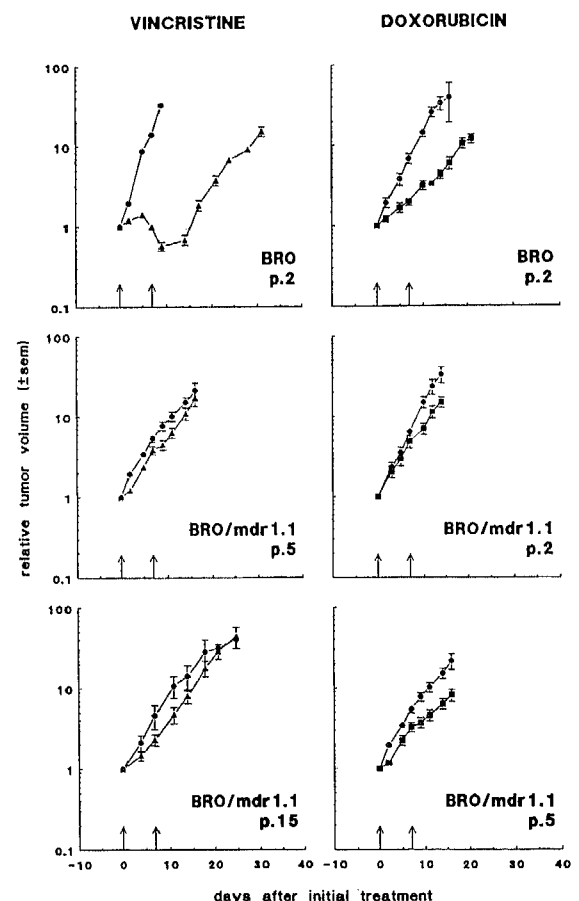


Fig. 3. Growth curves of BRO and BRO/mdr1.1 xenografts of various passages (p) in the nude mouse. The mean relative volume of untreated tumors (\bullet) and the mean relative volumes of tumors treated with vincristine 1 mg/kg *i.v.* weekly \times 2 (\blacktriangle) or doxorubicin 8 mg/kg *i.v.* weekly \times 2 (\blacksquare) are drawn. Arrows indicate the days of treatment and bars represent SE.

RF in the original BRO/*mdr1.1* subline (data not shown). To demonstrate the retention of the *mdr1* gene, mRNA levels were analyzed in BRO/*mdr1.1* xenograft tissue in various passages. In all tissues *mdr1* expression could be detected, indicating the stability of the *mdr1* gene transfection when cells are grown in vivo without a selective agent (Fig. 4).

Modulation of *mdr* in vivo

The influence of BIBW22BS on the antitumor activity of vincristine and doxorubicin was determined in the BRO and the BRO/*mdr1.1* xenograft model. The i.v. route was used for the administration of BIBW22BS, because the low pH of the solution precluded peritoneal injections. An interval of one h was chosen between BIBW22BS and the cytotoxic agents, which were given i.p. This interval was based on rapid pharmacokinetics of BIBW22BS in mice, which was measured by high performance liquid chromatography in a limited number of plasma samples after a dose of 12.5 mg/kg i.v. At 5 min the mean plasma level was 18.7 μ M, at 60 min 9.0 μ M and at 4 h this level was 4.9 μ M (data from Dr. Karl Thomae GmbH).

A pilot study was carried out in groups of 3 non-tumor-bearing mice each to determine the MTD of BIBW22BS given i.v. followed by vincristine 1 mg/kg i.p. or doxorubicin 8 mg/kg i.p. with an interval of one h weekly \times 2. Doses of 12.5 mg/kg, 25 mg/kg and 50 mg/kg were tested. At the higher doses BIBW22BS caused muscular relaxing and sedating effects, but mice recovered within 2 h after administration. The 50 mg/kg dose induced a mean maximum body weight change of 0.79 (SD \pm 0.01) for vincristine and of 0.87 (SD \pm 0.05) for doxorubicin. Two weeks after the first injection the mean body weight change values were 0.93 (SD \pm 0.07) and 0.91 (SD \pm 0.09), respectively.

In the treatment experiments in tumor-bearing mice the 50 mg/kg dose of BIBW22BS was used in order to achieve the highest tumor tissue modulating levels for a prolonged period of time. The treatment regimen was first studied in the BRO/*mdr1.1* xenograft model. As

compared to untreated xenografts, growth in BRO/*mdr1.1* tumors treated with BIBW22BS, vincristine, or doxorubicin as single agents was not significantly affected (Table 4). However, the addition of BIBW22BS to the vincristine or the doxorubicin schedule could significantly reduce the T/C% obtained with vincristine alone from 109% to 48% and that of doxorubicin alone from 55% to 32%. Thereafter, the same treatment regimen was studied in the BRO xenograft model. BIBW22BS alone was not effective, while vincristine and doxorubicin induced a T/C% of 7% and 35%, respectively. The addition of BIBW22BS to the cytotoxic agents did not further affect the growth of BRO tumors.

The influence of body weight loss on the antitumor

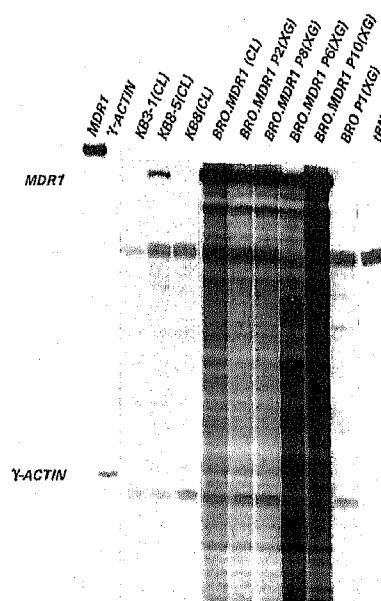


Fig. 4. *Mdr1* gene expression detected by the RNase protection assay in the BRO/*mdr1.1* subline (CL), in BRO xenograft (XG) tissue (passage 1), and in BRO/*mdr1.1* xenograft tissue (passages 2, 6, 8 and 10). Cell lines KB3-1 (Pgp-negative), KB8 (low Pgp expression) and KB8-5 (Pgp-positive) are controls. A γ -actin probe is included as a control for RNA recovery. A tRNA control is hybridized with both the *mdr1* and the γ -actin probe.

Table 4. The effect of BIBW22BS on the antitumor efficacy of vincristine or doxorubicin in BRO and BRO/*mdr1.1* xenografts.

Drug	Dose mg/kg i.p.	BIBW22BS mg/kg i.v.	Days	BRO		BRO/ <i>mdr1.1</i>	
				T/C% ^b	Weight ^c	T/C% ^b	Weight ^c
None		50	0,7	99%	1.23 \pm 0.06	61%	1.11 \pm 0.02
VCR	1		0,7	7% ^d	1.01 \pm 0.02 ^d	109%	1.10 \pm 0.02
VCR ^a	1	50	0,7	8% ^d	0.84 \pm 0.04 ^{d,e}	48% ^{d,e}	1.01 \pm 0.03 ^{d,e}
None		50	0,7	99%	1.23 \pm 0.06	61%	1.11 \pm 0.02
DOX	8		0,7	35% ^d	1.03 \pm 0.03 ^d	55% ^d	0.87 \pm 0.01 ^d
DOX ^a	8	50	0,7	22% ^d	0.92 \pm 0.01 ^{d,e}	32% ^{d,e}	0.80 \pm 0.02 ^{d,e}

^a Vincristine or doxorubicin preceded by BIBW22BS with 1 h interval.

^b Mean relative volume of treated vs. untreated tumors of that tumor line \times 100% at day 14.

^c Mean relative body weight at day 14 as compared to day 0 \pm SE.

^d Treatment is significantly different from control ($p < 0.05$).

^e BIBW22BS plus drug is significantly different from drug alone ($p < 0.05$).

effects calculated at day 14 was determined. Weight loss was significantly increased in all groups treated with the combination of BIBW22BS plus vincristine or doxorubicin (Table 4), but there were no toxic deaths. Of importance, the increase in weight loss did not result in additional growth inhibition in BRO tumors.

Discussion

Dipyridamole is in clinical use as an agent preventing thrombosis by inhibition of platelet aggregation [19]. Recently, the compound was shown to modulate drug resistance in Pgp-positive tumor cells, but relatively high concentrations ($>10 \mu\text{M}$) were necessary to achieve optimal efficacy [20–22]. In our experiments we could confirm the *mdr*-modulating property of dipyridamole, but at $10 \mu\text{M}$ concentrations only minimal reversal could be obtained. In a structure-activity relationship study, Ramu and Ramu [23] have indicated that some 2,7-dimorpholino-6-phenylpteridine derivatives of dipyridamole have good modulating properties. We could indeed demonstrate the higher potency of BIBW22BS, where concentrations of $0.5\text{--}2.5 \mu\text{M}$ were sufficient to reverse resistance in the Pgp-positive sublines BRO/*mdr1.1* and 2780^{AD}. Dipyridamole as well as BIBW22BS have also been recognized to inhibit nucleoside transport, because of which the cytotoxicity of 5-fluorouracil can be increased [11, 24]. Our recent studies indicate a 2- to 6-fold reduction in the IC₅₀ for 5-fluorouracil when combined with BIBW22BS at $1.0 \mu\text{M}$ in 7/8 human colon cancer cell lines tested [25].

BIBW22BS showed higher potency than verapamil, bepridil and flunarizine in the reversal of resistance in the Pgp-positive sublines. At the non-toxic concentration of $1.0 \mu\text{M}$ none of the modulators could affect resistance in the non-Pgp sublines. Only in the SW1573/2R120 subline a slight reversal of resistance was apparent, which confirmed earlier findings by our group [17]. For dipyridamole in drug-resistant HT1080/DR4 cells which also lack *mdr1* gene expression, a similar finding was reported [26]. It appears that in some *mdr* cells a resistance mechanism other than Pgp can partially be influenced by the present modulators tested.

The BRO and BRO/*mdr1.1* xenograft model was developed in order to analyze the potential value of resistance modulators in the clinical situation of well-established solid tumors. It is of utmost importance to demonstrate the retention of the *mdr* phenotype and the *mdr1* gene expression when cells selected for resistance in vitro are grown without selective agent in vivo. For 2780^{AD} cells injected s.c. in the nude mouse we found partial loss of the *mdr* phenotype as early as the first passage (unpublished data). For BRO/*mdr1.1* cells transfected with the *mdr1* gene, we could demonstrate stability of *mdr* in s.c. xenografts up to 15 serial passages examined.

BIBW22BS in combination with vincristine or doxorubicin could significantly increase the antitumor effects of each of the cytotoxic agents alone in BRO/*mdr1.1* xenografts, but reversal of vincristine was not complete when compared to the efficacy of vincristine in BRO xenografts. The inhibition in tumor growth could not be explained by the increase in body weight change observed with the combination. However, increased body weight loss in these experimental animals following the addition of BIBW22BS to vincristine or doxorubicin may suggest the presence of a drug interaction. Much interest is now focussed on the possible interaction between resistance modulators and cytotoxic agents and more specifically on the function of Pgp in normal cells, such as the biliary tract and the renal tubules. Evidence is given that verapamil can increase the area under the concentration-time curve as shown for anthracyclines in cancer patients [27, 28] and for vincristine in human tumor-bearing nude mice [29]. A recent study carried out in bile canalicular membrane vesicles by Watanabe et al. [30] has pointed to the role of verapamil as a selective inhibitor of vincristine biliary excretion, while the uptake rate of vincristine in isolated hepatocytes was only minimally affected. A drug interaction between the cyclosporin analog SDZ PSC-833 and etoposide has also been reported [31]. The increased area under the concentration-time curve of a cytotoxic agent may result in enhanced antitumor efficacy as well as additional side effects [29, 31, 32]. Therefore, in the preclinical development of resistance modulators, such as BIBW22BS, analysis of drug pharmacokinetics and (tumor) tissue distribution is mandatory to obtain insight in pharmacological interactions to be anticipated in the clinic.

The increase in the antitumor effects of both vincristine and doxorubicin in the BRO/*mdr1.1* xenograft model may indicate potential usefulness of BIBW22BS in the treatment of Pgp-positive malignancies in the clinic. Thus far, most modulators have been tested in vivo in murine hematological malignancies and efficacy appeared most prominent in patients with multiple myeloma or lymphoma [6–8]. In patients with solid tumors the modulating capacity will not only depend on the tumor tissue levels of the compounds for a particular time period, but also on the extent of plasma protein binding. For dipyridamole it is known that $>95\%$ is bound to plasma protein, particularly to albumin and to α_1 -acid glycoprotein [24]. However, after i.p. administration of 24 mg/m^2 per day concurrently with escalating i.p. doses of etoposide to patients with malignant peritonitis, mean ultrafiltrable dipyridamole concentrations varied between $8.3\text{--}25.3 \mu\text{M}$ which were 25%–35% of the total steady-state peritoneal concentrations [33]. When clinical evaluation of BIBW22BS is considered trials may also include attempts to modulate *mdr* in patients with malignancies restricted to the peritoneal cavity.

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