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The Influence of BIBW22BS, a Dipyridamole Derivative, on the Antiproliferative Effects of 5-Fluorouracil, Methotrexate and Gemcitabine *In Vitro* and in Human Tumour Xenografts

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Dipyridamole is known as a potent inhibitor of facilitated diffusion-mediated nucleoside transport as well as a modulator of 'classical' multidrug resistance. BIBW22BS, a derivative of dipyridamole, has been found to be 20- to 100-fold more potent in the reversal of multidrug resistance when compared to the parent compound. In parallel, we studied the efficacy of BIBW22BS in the modulation of the antiproliferative effects of 5-fluorouracil, methotrexate and gemcitabine in human cancer cell lines. BIBW22BS, at non-toxic concentrations up to 1.0 μM, increased the antiproliferative effects of 5-fluorouracil 2- to 6-fold in seven of the eight colon cancer cell lines tested in a dose-dependent manner. The addition of 1.0 μM BIBW22BS to methotrexate resulted in a slight increase in the antiproliferative effects, but inhibited the activity of gemcitabine 30- to 100-fold in various cancer cell lines. *In vitro*, no notable difference was found between BIBW22BS and dipyridamole in their capacity to modulate the activity of the antimetabolites studied. BIBW22BS did not affect the growth inhibition induced by 5-fluorouracil or gemcitabine in human tumour xenografts grown subcutaneously in nude mice. We confirmed the higher potency of BIBW22BS when compared to dipyridamole in the reversal of drug resistance in the Pgp-positive COLO 320 cell line.

Key words: BIBW22BS, dipyridamole, 5-fluorouracil, methotrexate, gemcitabine, modulation, nucleoside transport

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INTRODUCTION

BIOCHEMICAL MODULATION of the activity of antimetabolites has been the subject of clinical trials to improve the outcome of cancer chemotherapy. In the laboratory, dipyridamole has been shown to inhibit facilitated diffusion-mediated nucleoside transport and, by blockage of nucleoside salvage, to enhance the antiproliferative effects of methotrexate, *N*-phosphonacetyl-L-aspartate (PALA) and acivicin. In addition, dipyridamole can also increase the toxicity of 5-fluorouracil, although interference with thymidine salvage did not appear to be a critical factor [1]. Grem has described an alteration of 5-fluorouracil metabolism by dipyridamole, producing a selective increase in fluorodeoxyuridine monophosphate (FdUMP) levels by a decrease of the efflux of fluorodeoxyuridine.

The modulating capacity of dipyridamole when combined with 5-fluorouracil or methotrexate has been further studied in phase I clinical trials [2, 3]. Dipyridamole did not affect the clinical toxicity, but promoted the total body clearance of 5-fluorouracil [3]. Dipyridamole did not alter the pharmacokinetics of methotrexate, but the toxicity was potentiated, possibly as a result of interference with thymidine salvage which is necessary for normal physiological processes [2]. Due to high protein binding, the steady state concentration of free dipyridamole given by continuous infusion at the maximum tolerated concentration was 20–25 nM and this would be expected to have minimal effect considering *in vitro* data on nucleoside transport inhibition [1]. More potent derivatives of dipyridamole may be more effective at increasing the efficacy of 5-fluorouracil and methotrexate.

BIBW22BS, 4-[*N*-(2-hydroxy-2-methyl-propyl)-ethanolamino]-2,7-bis(*cis*-2,6-dimethyl-morpholino)-6-phenylpteridine, is a dipyridamole derivative and, similar to the parent compound, a bifunctional modulator. BIBW22BS was reported to have a 10-fold higher potency than dipyridamole in the enhancement

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of the antiproliferative effects of 5-fluorouracil [4]. We have previously shown that BIBW22BS is 20- to 100-fold more potent than the parent compound in the reversal of multidrug resistance in cancer cells that express P-glycoprotein (Pgp) [5].

In the current experiments, we compared the modulating effects ofBIBW22BS and dipyridamole on 5-fluorouracil, methotrexate and gemcitabine in a series of human cancer cell lines *in vitro*. In addition, we measured cellular free fluorodeoxyuridine monophosphate (FdUMP) levels after cells were exposed to 5-fluorouracil with or without the modulators. The effect of BIBW22BS on the antitumour activity of 5-fluorouracil or gemcitabine was studied in human tumour xenografts.

MATERIALS AND METHODS

Cell lines and characteristics

Various human cancer cell lines were used in the *in vitro* experiments (Table 1). Cells were grown in Dulbecco's modified Eagle's medium (Gibco, Breda, The Netherlands) supplemented with 10% heat-inactivated fetal calf serum (FCS; Sanbio, Uden, The Netherlands), 50 IU/ml penicillin and 50 μg/ml streptomycin (Flow, Irvine, Scotland) in a humidified atmosphere containing 5% CO_2 at 37°C. Cells were screened for *Mycoplasma* contamination by using a rapid detection system with a ^{3}H-labelled DNA probe (Gen-Probe, San Diego, California, U.S.A.) and were found to be negative.

Cells were characterised for the presence of a variety of antigens. The polyclonal rabbit αCEA antibody (1 : 100), directed against the carcinoembryonic antigen, was obtained from Dakopatts (Glostrup, Denmark). Monoclonal antibody JSB-1 (1 : 100) was used to detect Pgp [6]. The monoclonal antibody E48 (10 μg/ml), directed against a M_r 22,000 desmosomal glycoprotein, was used as a negative control [7]. For immunocytochemistry, cells were spun on slides, air-dried for 1 h and then fixed for 15 min in cold (4°C) acetone. Immunostaining was done with the Histostain-SP kit (Zymed, Burlingame, California, U.S.A.). The intensity of staining was expressed as strong (++), moderate (+), weak (±) or negative (−). The percentage of stained cells was also determined.

Table 1. *Human cancer cell lines and characteristics*

			Antigen expression‡		
Cell line	T_D*	*MDR1*†	JSB-1	αCEA	E48
Colon cancer					
COLO 205	40	0	—	+ (100%)	—
COLO 320	39	2.97	+ (50%)	± (100%)	—
SW1398	59	1.91	++ (80%)	+ (90%)	—
SW1116	49	0.10	± (15%)	± (40%)	—
LS174T	31	0.50	± (5%)	++ (30%)	—
HT29	35	0	—	+ (95%)	—
LoVo	31	0.10	+ (10%)	+ (95%)	—
WiDr	32	0	—	± (95%)	—
Ovarian cancer					
A2780	55	0	—	—	—
IGROV-1	39	0	—	—	—
Breast cancer					
MCF-7	38	0	—	—	—

*T_D, population doubling time *in vitro* in h. †*MDR1* gene expression as determined by the RNAse protection assay; the expression of *MDR1* samples is relative to the KB-8-5 cell line set at 1.0. ‡Antigen expression as determined by immunocytochemistry; ++ strong, + moderate, ± weak and — no staining; numbers in parentheses are the percentage of stained cells.

The expression of the *MDR1* gene was measured with the RNAse protection assay. The RNA was isolated from subconfluent cells using a NP40 lysis mix followed by centrifugation to separate membranes and nuclei from cytosol. RNAse protection was performed as described earlier [5]. Briefly, 10 μg of total RNA was hybridised with a [^{32}P]CTP-labelled anti-sense RNA probe, specific for *MDR1*-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. The results were calculated as the expression of *MDR1* samples relative to the *MDR1* expression in the multidrug-resistant KB-8-5 cell line, which was set at 1.0.

Drugs

5-Fluorouracil (Multipharma, Weesp, The Netherlands) was purchased as a solution of 50 mg/ml and methotrexate (Pharmachemie, Haarlem, The Netherlands) as a solution of 2.5 mg/ml. Gemcitabine was donated by Eli Lilly (Indianapolis, Indiana, U.S.A.) as a powder and was dissolved in 0.9% NaCl to a final concentration of 10 mg/ml. Doxorubicin (Farmitalia Carlo Erba, Nivelles, Belgium) was dissolved in water at a concentration of 2 mg/ml. Vincristine (Eli Lilly, Amsterdam, The Netherlands) was purchased as a solution of 1 mg/ml. BIBW22BS and dipyridamole (both from Dr Karl Thomae GmbH, Biberach, Germany) 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. Drugs were further diluted in tissue culture medium when investigated for the antiproliferative effects *in vitro*.

Drug sensitivity in vitro

Cellular drug sensitivities were measured with the MTT assay [8]. Cells were harvested with 0.2% EDTA or 0.25% trypsin plus 0.2% EDTA to obtain a single cell suspension. Cells were seeded in 100 μl tissue culture medium in 96-well microtitre plates at 5000 cells/well, except for A2780 at 3000 cells/well. The plates were incubated under standard conditions for 24 h. Drugs were added to achieve a final volume of 200 μl. The cells were then cultured for another 96 h. Thereafter, the medium was removed and 50 μl MTT (Sigma) in phosphate buffered saline (0.4 mg/ml) were added. The plates were incubated for an additional 2–3 h and 200 μl dimethylsulphoxide (DMSO) with 0.5% FCS were added to dissolve the formazan crystals. The optical density was read on a Labsystems Multiskan Bichromatic plate reader (Labsystems OY, Helsinki, Finland) at 540 nm. All drug concentrations were tested in four replicate wells and each experiment was performed in triplicate. The antiproliferative effects were expressed as the IC_{50}, which is the concentration of the drug inducing 50% growth inhibition when compared to the growth of control cells. The results were expressed in a modulation factor (potentiation or inhibition) based on the IC_{50} of the cells treated with the drug alone and that of cells treated with the drug and the modulator.

The influence of protein binding of both modulators on the antiproliferative effects of 5-fluorouracil was analysed in the COLO 205 cell line. Various concentrations of human serum albumin (final concentration 25 or 35 g/l) or normal human serum (final concentration 10 or 40%) were added to tissue

culture medium not containing FCS. The assay was carried out as described above.

Determination of free FdUMP

The determination of free FdUMP was based on an isotope-dilution assay of [6-^{3}H] FdUMP binding to *L. casei* thymidylate synthase [9] and modified by Van der Wilt and associates [10]. Cells in samples of 1×10^7/ml were incubated with 10 μM 5-fluorouracil with or without 1 μM BIBW22BS or dipyridamole for 4 h at 37°C. Thereafter, the cell pellets were denatured with the addition of trichloroacetic acid (final concentration of 8%) and subsequently neutralised with alamine/freon (1 : 2). The denatured suspensions (75 μl) were incubated with 10 μl 12 nM [6-^{3}H] FdUMP, 10 μl *L. casei* thymidylate synthase and 10 μl TRIS buffer in a final volume of 105 μl. To provide a standard curve, 10 μl FdUMP (concentration range from 0.01 to 0.5 μM) were added to a number of samples. After an incubation period of 2 h at 30°C, the samples were prepared for liquid scintillation counting. A ratio was calculated between the samples with mixed radiolabelled and cold FdUMP (standard curve) and the samples with only radiolabelled FdUMP. The standard curve of concentration against ratio provided a quantitation for the free FdUMP concentration (fmol/10^7 cells) in the samples.

Drug sensitivity in vivo

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. The COLO 205, WiDr and A2780 xenografts were established from cell lines grown in tissue culture medium. Mice were inoculated subcutaneously with 1×10^7 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 experiments were carried out in passage 2 or higher.

The COLO 205, WiDr and A2780 xenografts were measured twice a week in three dimensions with a slide caliper. The volume was calculated by the equation length × width × thickness × 0.5, and expressed in mm^3. At the start of treatment (day 0), groups of five–six tumour-bearing mice were formed to provide a mean tumour volume of approximately 100 mm^3 in each group. For *in vivo* use, BIBW22BS was dissolved in 0.1 N HCl at a concentration of 20 mg/ml and further diluted in 0.9% NaCl to 2.5 mg/ml at pH 2.7. The modulating capacity of BIBW22BS was determined by administration of BIBW22BS 50 mg/kg intravenously (i.v.) followed after 1 h by 5-fluorouracil 60 mg/kg intraperitoneally (i.p.) at days 0, 7, 14 and 21 or by gemcitabine 240 mg/kg i.p. at days 0 and 7. The maximum tolerated doses of 5-fluorouracil [11] and gemcitabine [12] were based on the occurrence of a mean weight loss of approximately 10% of the initial weight within 1 week after the first injection. The maximum tolerated dose of BIBW22BS of 50 mg/kg i.v. and the interval of 1 h between the modulator and the antimetabolites were deduced from earlier experiments in multidrug-resistant human tumour xenografts [5]. We selected the schedule because in the first 4 h after administration of BIBW22BS at a dose of 12.5 mg/kg i.v., mouse plasma levels were considered to be sufficient for modulation with reference to modulating concentrations *in vitro*. For the evaluation of drug efficacy, the tumour volume was expressed by the formula V_T/V_0, where V_T is the volume at any given day and V_0 is the volume at day 0. The ratio of the mean relative volume of treated tumours over that of control tumours multiplied by 100% (*T/C*%) was assessed at each day of measurement. Antitumour effects were expressed as the percentage of growth inhibition (100%−*T/C*%) and differences between groups were evaluated with Student's *t*-test.

RESULTS

First, the human cancer cell lines were characterised for growth, antigen expression and the presence of the *MDR1* gene. The results are summarised in Table 1. In five of the eight colon cancer cell lines, Pgp expression was detected with the JSB-1 antibody and the RNAse protection assay confirmed the presence of the *MDR1* gene.

The eight colon cancer cell lines were used to determine the efficacy of BIBW22BS in the modulation of 5-fluorouracil antiproliferative effects *in vitro*. For BIBW22BS, the IC_{50} in the colon cancer cell lines was well above 10 μM. A concentration range of 0.1 – 1.0 μM was used and at 1.0 μM of BIBW22BS, the antiproliferative effects of 5-fluorouracil were increased 2- to 6-fold in seven of the eight cell lines. The efficacy of BIBW22BS was clearly dose dependent (Table 2). Four human colon cancer cell lines (COLO 205, WiDr, SW1398 and COLO 320) with different responses to the combination of BIBW22BS plus 5-fluorouracil were selected for the comparison of the potency of BIBW22BS with that of dipyridamole. Again, a concentration range of 0.1 – 1.0 μM was studied. From Figure 1, it is clear that BIBW22BS enhanced the 5-fluorouracil antiproliferative effects to a similar extent to dipyridamole.

BIBW22BS was then compared with dipyridamole in the modulation of the antiproliferative effects of methotrexate and gemcitabine, and cell lines of tumours that may be responsive to these drugs were included. In MCF-7 and IGROV-1 cells the methotrexate antiproliferative effects were slightly increased upon the addition of BIBW22BS and dipyridamole at a concentration of 1.0 μM. However, a 3-fold increase in the methotrexate antiproliferative effects was observed in COLO 205 cells and the increase appeared to be dose dependent (Figure 1). BIBW22BS was no more effective than dipyridamole in the modulation of methotrexate efficacy. In the ovarian cancer cell line, A2780, and the two colon cancer cell lines, COLO 205 and WiDr, the antiproliferative effects of gemcitabine were

Table 2. BIBW22BS in the modulation of 5-fluorouracil antiproliferative effects in human colon cancer cell lines

	Potentiation factor ± S.D.*		
Cell line	BIBW22BS 0.1 μM	BIBW22BS 0.5 μM	BIBW22BS 1.0 μM
COLO 205	4.3 ± 2.0	5.9 ± 3.5	6.4 ± 2.9
WiDr	1.6 ± 0.2	2.0 ± 0.3	2.1 ± 0.9
SW1398	3.5 ± 1.0	4.5 ± 1.2	4.8 ± 1.8
LS174T	1.9 ± 0.7	2.8 ± 1.2	4.2 ± 1.8
COLO 320	1.0 ± 0.1	0.9 ± 0.1	0.9 ± 0.1
HT29	2.4 ± 0.5	3.2 ± 1.0	3.7 ± 1.2
LoVo	1.9 ± 0.3	2.2 ± 0.3	2.7 ± 0.4
SW1116	1.8 ± 0.4	3.5 ± 1.2	2.9 ± 0.7

*Significant concentration dependence ($P < 0.0007$, as calculated with $\sqrt{\epsilon z^2}$).

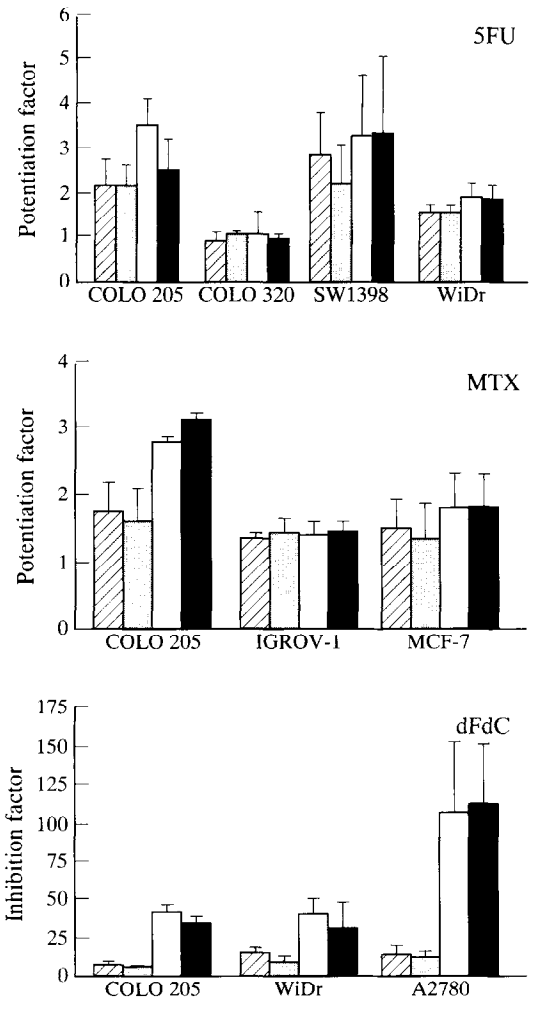

Figure 1. The modulating capacity of 0.1 μM BIBW22BS (□), 0.1 μM dipyridamole (□), 1.0 μM BIBW22BS (□) or 1.0 μM dipyridamole (■) on the antiproliferative effects of 5-fluorouracil (5FU), methotrexate (MTX) or gemcitabine (dFdC) in various human cancer cell lines. The results are means ± S.D. from at least three separate experiments and are expressed as a potentiation or an inhibition factor based on the relation between the IC_{50} of cells treated with drug alone and the IC_{50} of cells treated with drug and modulator.

extensively inhibited by BIBW22BS and dipyridamole in a dose-dependent manner. At concentrations of 0.5 and 1.0 μM, the antiproliferative effects of gemcitabine were reduced up to 30-fold in COLO 205 and WiDr cells and up to 100-fold in A2780 cells (Figure 1). Again, there was no difference in the modulating capacity between BIBW22BS and dipyridamole.

The COLO 205 cell line was selected to study the influence of protein binding because of its high sensitivity to modulation. The modulating capacity of BIBW22BS and dipyridamole was not reduced when human serum albumin (up to 35 g/l) was added to the medium. However, human serum albumin at 35 g/l resulted in an approximately 10-fold decrease in the antiproliferative effects of 5-fluorouracil alone or in combination with BIBW22BS or dipyridamole (Table 3). Normal human serum (up to 40%) added to tissue culture medium, which represents approximately 16 g/l human serum albumin, did not reduce the modulation capacity of BIBW22BS or dipyridamole (data not shown).

Dipyridamole has been demonstrated to increase the intracellular concentration of FdUMP [13]. Therefore, we measured intracellular free FdUMP concentrations in COLO 205, WiDr and COLO 320 cells, selected for differences in response to the inhibition of nucleoside transport. The assay was carried out five times, but levels of free FdUMP were not found to be increased after a 4 h incubation of 5-fluorouracil with either BIBW22BS or dipyridamole at 1.0 μM (data not shown).

Table 3. The influence of human serum albumin (HSA) on the antiproliferative effects of 5-fluorouracil in the presence of BIBW22BS or dipyridamole in COLO 205 cells

Protein	Modulator	PF*	IF†
Normal culture medium			
	1 μM BIBW22BS	3.9 ± 0.4	
	1 μM Dipyridamole	3.9 ± 0.2	
HSA 25 g/l			4.7 ± 0.8
	1 μM BIBW22BS	5.5 ± 0.9	3.6 ± 1.2
	1 μM Dipyridamole	4.6 ± 1.1	4.2 ± 0.7
HSA 35 g/l			9.3 ± 3.3
	1 μM BIBW22BS	5.8 ± 1.2	9.0 ± 6.0
	1 μM Dipyridamole	3.3 ± 0.8	12.3 ± 3.9

*Potentiation factor (± S.E.M.) determined by the ratio of the IC_{50} of cells treated with 5-fluorouracil and modulator versus the IC_{50} of cells treated with 5-fluorouracil alone. †Inhibition factor (± S.E.M.) determined by the ratio of the IC_{50} of cells treated in medium with HSA versus the IC_{50} of cells treated in regular medium.

In earlier experiments, we found BIBW22BS to be 20- to 100-fold more potent in the reversal of resistance in Pgp-positive human cancer cell lines [5]. Therefore, BIBW22BS was also compared with dipyridamole in the potentiation of the antiproliferative effects of vincristine and doxorubicin in two colon cancer cell lines, COLO 205 and COLO 320. Both cell lines showed a different response to the nucleoside-inhibiting effects of BIBW22BS. COLO 205 cells, which are Pgp-negative, were sensitive to the nucleoside-inhibiting effects of BIBW22BS whereas COLO 320 cells, which are Pgp-positive, were insensitive to the nucleoside-inhibiting effects of BIBW22BS. A concentration range of 0.1 – 1.0 μM of both modulators was tested in the presence of the anticancer drugs. At 1.0 μM of BIBW22BS, the antiproliferative effects of vincristine and doxorubicin in COLO 320 cells were increased 181- and 7.6-fold, respectively (Figure 2). Dipyridamole at the same concentration could only slightly increase the vincristine antiproliferative effects up to 7.1-fold (Figure 2). In the Pgp-negative cell line COLO 205, an insignificant increase in the antiproliferative effects of vincristine (2-fold), but no change with doxorubicin was observed with both modulators at 1.0 μM.

Since BIBW22BS potentiated the 5-fluorouracil antiproliferative effects in COLO 205 and WiDr cells *in vitro*, we determined the modulating capacity of BIBW22BS on the growth inhibition by 5-fluorouracil in corresponding xenografts. In these xenografts, BIBW22BS failed to enhance the antitumour effects of 5-fluorouracil (Table 4). However, 5-fluorouracil alone did not induce any notable tumour growth inhibition in these xenografts. Because of the significant inhibition of the antiproliferative effects of gemcitabine by BIBW22BS in A2780 cells, the modulator was studied in combination with gemcitabine in A2780 xenografts. Gemcitabine alone inhibited the tumour growth up to 99%. BIBW22BS failed to reduce the antitumour effects of gemcitabine. In a separate study, BIBW22BS was administered simultaneously with gemcitabine weekly × 2, but again no

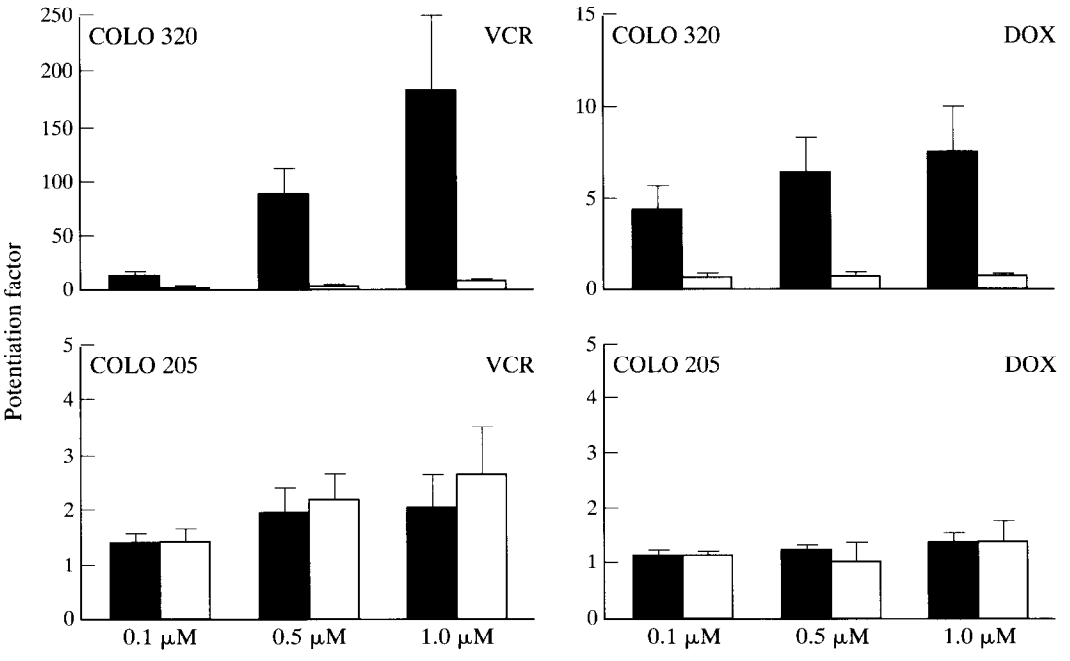

Figure 2. The influence of various concentrations of BIBW22BS (■) and dipyridamole (□) on the antiproliferative effects of vincristine (VCR) and doxorubicin (DOX) in COLO 320 (Pgp-positive) and COLO 205 (Pgp-negative) colon cancer cell lines. The results are means ± S.E.M. from at least three separate experiments and are expressed as a potentiation factor based on the relationship between the IC_{50} of cells treated with drug alone and the IC_{50} of cells treated with drug and modulator.

Table 4. The influence of BIBW22BS on the growth inhibition of 5-fluorouracil (5FU) or gemcitabine (dFdC) in human tumour xenografts

Xenograft	Drug* i.p.	BIBW22BS i.v.	Days	GI%†	R.W.L.%‡
COLO 205	none	50	q7d×4	13%	6.0 (± 3.5)
	5FU	—	q7d×4	28%	5.3 (± 2.5)
	5FU	50	q7d×4	14%	10.7 (± 1.2)
WiDr	none	50	q7d×4	5%	4.3 (± 3.8)
	5FU	—	q7d×4	0%	5.8 (± 1.6)
	5FU	50	q7d×4	0%	7.1 (± 3.5)
A2780	none	50	q7d×2	0%	0.0 (± 1.4)
	dFdC	—	q7d×2	99%§	17.8 (± 1.4)
	dFdC	50	q7d×2	99%§	15.7 (± 3.1)

*5FU 60 mg/kg weekly × 4 or dFdC 120 mg/kg weekly × 2 preceded or not by BIBW22BS 50 mg/kg with an interval of 1 h. †Growth inhibition expressed as 100%−*T/C*%, where *T/C*% is the optimal value of the mean volume in treated tumours/mean of relative volume in control tumours × 100%. ‡Mean relative weight loss when compared to day 0 (± S.E.M.). §Significant difference between treated and control tumours evaluated with Student's *t*-test. q7d, once every 7 days.

reduction of the tumour growth inhibition was observed (data not shown). The combinations of BIBW22BS and 5-fluorouracil or gemcitabine did not cause an increase in weight loss or other visible side-effects in the tumour-bearing mice.

DISCUSSION

BIBW22BS, a derivative of dipyridamole, was studied as a modulator of nucleoside transport in human cancer cell lines varying in the expression of particular drug transport characteristics. In seven of the eight colon cancer cell lines tested, an increase of the 5-fluorouracil antiproliferative effects was observed with BIBW22BS in a dose-dependent manner. BIBW22BS also enhanced the activity of methotrexate, but extensively reduced the gemcitabine antiproliferative effects. The efficacy of BIBW22BS as a nucleoside transport modulator was found to be similar to that of dipyridamole, but BIBW22BS was more potent in the reversal of multidrug resistance in a Pgp-positive colon cancer cell line. Despite the promising results obtained *in vitro*, BIBW22BS did not affect growth inhibition of either 5-fluorouracil or gemcitabine in human tumour xenografts in nude mice.

In the present experiments, BIBW22BS and dipyridamole were similarly effective in the modulation of the antiproliferative effects of 5-fluorouracil. Chen and associates [4] have reported that BIBW22BS at 1.0 μM can enhance the antiproliferative effects of 5-fluorouracil in KB cells by approximately 20-fold compared to approximately 2-fold in the case of dipyridamole. This finding does not agree with our data and this may possibly be explained by the higher sensitivity of KB cells to BIBW22BS at 1.0 μM, because in the presence of low, non-toxic concentrations of 5-fluorouracil, a 30% cell growth inhibition could already be measured.

The influence of BIBW22BS on the antiproliferative effects of 5-fluorouracil was dose dependent and varied between the eight colon cancer cell lines. Grem [1] has studied the possible mechanism by which dipyridamole augments the 5-fluorouracil antiproliferative effects in the HCT 116 colon cancer cell line. In this model cell line, the modulating capacity of the compound was found to be based on a selective increase in FdUMP levels by blocking the efflux of fluorodeoxyuridine from the cells [13]. Intracellular FdUMP in HCT 116 cells, after exposure to dipyridamole and 5-fluorouracil for 4 h, was 5-fold higher when

compared to 5-fluorouracil exposure alone. The addition of thymidine at 25 μM reduced the formation of FdUMP. Alternatively, deoxyuridine at 25 μM could enhance the antiproliferative effects of 5-fluorouracil in the absence or the presence of dipyridamole, which was probably caused by an increase in the amount of FdUMP formed [14]. We did not detect an alteration of FdUMP levels in the colon cancer cell lines COLO 205 and WiDr in which BIBW22BS at 1.0 μM could increase the 5-fluorouracil antiproliferative effects 6.4- and 2.1-fold, respectively. It is not likely that the assays used explain the difference, as Grem and Fischer [13] have detected [6-^{3}H] FdUMP by high-performance liquid chromatography (HPLC), while our assay is based on the measurement of free binding sites for *L. casei* thymidylate synthase. Both assays use a similar extraction procedure and measure levels far above the threshold level. Moreover, the enzyme assay is more specific since only one metabolite, FdUMP, can bind to thymidylate synthase with such high affinity, while HPLC detection of FdUMP is often hampered by co-elution of fluorodeoxyuridine [13]. It may well be possible that the inhibition of fluorodeoxyuridine efflux is not the only mechanism for the enhanced 5-fluorouracil action, as both 5-fluorouracil and dipyridamole were also found to produce alkaline labile sites in newly synthesised DNA [14, 15].

As with dipyridamole, BIBW22BS modulated the antiproliferative effects of other antimetabolites, such as methotrexate and gemcitabine. Methotrexate binds strongly to dihydrofolate reductase, and thereby produces a depletion of reduced folates, which are essential for the synthesis of thymidine monophosphate and purines [16]. *In vitro* studies have indicated that dipyridamole potentiates the antiproliferative effects of methotrexate through inhibition of thymidine salvage [17]. Indeed, both BIBW22BS and dipyridamole at 1.0 μM enhanced the antiproliferative effects of methotrexate in the three cell lines tested. In the case of gemcitabine, an analogue of deoxycytidine, both BIBW22BS and dipyridamole extensively inhibited its efficacy *in vitro*. Gemcitabine is phosphorylated by the enzyme deoxycytidine kinase into difluorodeoxycytidine triphosphate (dFdCTP). The cytotoxic effect of gemcitabine is associated with an inhibition of DNA synthesis by competitive incorporation of dFdCTP into the growing DNA strand [18]. The mechanism underlying the reduction of the gemcitabine efficacy is not yet known, but may be explained by the inhibition of the uptake of gemcitabine by facilitated diffusion-mediated membrane transport into the cells.

Unfortunately, the *in vivo* experiments of BIBW22BS did not result in modulation of the antitumour effects of 5-fluorouracil or gemcitabine. There are several reasons to explain this finding. Firstly, we are not aware of the concentrations and the retention time of BIBW22BS in the three human tumour xenografts studied. One may consider the dose and the schedule used in these experiments to be suboptimal for obtaining effective concentrations of BIBW22BS in tumour tissue. In addition, even in the presence of sufficient BIBW22BS concentrations for nucleoside transport inhibition, tumour growth delay by gemcitabine may also occur through passive diffusion. Secondly, in patients, dipyridamole is > 95% protein bound, particularly to albumin and α_1-acid glycoprotein [19, 20]. In the *in vitro* study, where human serum albumin (up to 35 g/l) or normal human serum (up to 40%) was added to the medium, we did not find a reduction in the modulating capacity of BIBW22BS or dipyridamole. Protein binding of dipyridamole in normal tissue culture medium is low as has been reported by Grem [1]. It may be that α_1-acid glycoprotein is the major determinant for dipyridamole binding, as the nucleoside-inhibiting activity of the compound was 10- to 200-fold reduced in the presence of a physiological concentration of that protein [21].

Dipyridamole is also known as a compound that can restore the sensitivity in Pgp-positive cells expressing the 'classical' multidrug resistance phenotype [4, 22, 23]. As an illustration of our earlier experiments [5], we again showed a higher potency of BIBW22BS in the modulation of vincristine and doxorubicin resistance in the Pgp-positive COLO 320 cell line when compared to dipyridamole at an equimolar concentration.

It can be concluded that BIBW22BS is more potent than dipyridamole in the modulation of multidrug resistance in Pgp-positive cells, but both compounds show a similar potency in the inhibition of nucleoside transport. Further studies are necessary to decide on the clinical usefulness of a bifunctional modulator to improve the efficacy of chemotherapy.

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