

# BIBW 22, a Dipyridamole Analogue, Acts as a Bifunctional Modulator on Tumor Cells by Influencing Both P-Glycoprotein and Nucleoside Transport<sup>1</sup>

Hong-xing Chen, Uwe Bamberger, Armin Heckel, Xin Guo, and Yung-chi Cheng<sup>2</sup>

Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06510 [H.-x. C., X. G., Y.-c. C.], and Departments of Chemical Research [A. H.] and Experimental Pathology [U. B.], Dr. Karl Thomae GmbH, D-7950, Biberach/Riss, Germany

## Abstract

BIBW 22, a phenylpteridine analogue of dipyridamole (DPM), enhanced vincristine cytotoxicity approximately 10 times more than DPM in a multidrug-resistant (MDR) KB V20C cell line. Using rhodamine 123 accumulation in KB V20C cells as an indicator of MDR phenotype, BIBW 22 was shown to be about 100 times more potent than DPM in inhibiting the MDR-associated efflux of rhodamine 123. Photolabeling of P-glycoprotein in KB V20C plasma membranes with 0.2  $\mu\text{M}$  [<sup>3</sup>H]jazidopine was strongly inhibited by 1  $\mu\text{M}$  BIBW 22, indicating that this compound reverses the MDR phenotype by interfering with MDR-associated P-glycoprotein.

In addition, BIBW 22 at 1  $\mu\text{M}$  could also enhance the cytotoxicity of 5-fluorouracil in KB cells about 20-fold. Its potency in inhibiting nucleoside transport is 7-fold more potent than that of DPM.

These results suggest that BIBW 22 is a potent bifunctional modulator which influences both P-glycoprotein and nucleoside transport in tumor cells. Potential use of this compound as a modulator of combination chemotherapy involving antimetabolites and drugs affected by MDR should be explored.

## Introduction

DPM<sup>3</sup> has been used therapeutically in humans for many years as a coronary vasodilator and platelet aggregation inhibitor (1). The major biochemical effects of DPM are inhibition of nucleoside membrane transport and cyclic AMP phosphodiesterase inhibition (1, 2). DPM has recently been reported to augment the activity of such anticancer agents as 5-fluorouracil, methotrexate, and cisplatin (3-5) and to sensitize MDR cells to MDR-associated drugs (6, 7).

Since 5-FUra is often used in combination with MDR-associated drugs, the inclusion of a bifunctional modulator, such as DPM, is postulated to improve the therapeutic potential of drug combinations by reversing the MDR phenotype as well as inhibiting nucleoside transport; the latter mechanism is known to decrease the cytotoxicity of such antimetabolites as 5-FUra and methotrexate (8, 9). The combined use of 5-FUra and DPM for cancer treatment has been studied before, but no therapeutic advantage has been reported (10). Whether the addition of another modulator of 5-FUra such as leucovorin may improve the therapeutic index remains to be determined (10). One of the possible reasons may be due to insufficient DPM concentrations; therefore, more potent agents with pharmacological properties similar to those of DPM are needed.

Recently a number of DPM analogues were evaluated with respect to their ability both to reverse MDR phenotype and to inhibit nucleoside transport. BIBW 22 (Fig. 1) was identified as the most effective DPM analogue in blocking P-glycoprotein function and inhibiting

nucleoside transport. It could also enhance the cytotoxicity of 5-FUra and sensitize MDR tumor cells to VCR.

## Materials and Methods

**Materials.** Pancreatin (Sigma Chemical Co., St. Louis, MO), VCR (Sigma), 5-FUra (Sigma), verapamil (Sigma), rhodamine 123 (Eastman Kodak Co., Rochester, NY), [<sup>14</sup>C]thymidine (56 mCi/mmol; ICN Radiochemicals, Irvine, CA), and [<sup>3</sup>H]jazidopine (56 Ci/mmol; Amersham, Arlington Heights, IL) were obtained from the sources indicated. Both DPM and BIBW 22 were synthesized by Dr. Karl Thomae GmbH, Biberach/Riss, Germany.

**Tumor Cell Lines.** The KB cells from the American Type Tissue Culture Collection (Rockville, MD) were maintained in RPMI 1640 supplemented with 5% fetal bovine serum and 0.1 mg/ml kanamycin and incubated under humidified air with 5% CO<sub>2</sub> at 37°C.

KB V20C cells, a subclone of the VCR-resistant cell line KB V20, were developed from the parental KB cells by stepwise selection for resistance with increasing concentrations of VCR. They were maintained under the same condition as outlined above with media supplemented with 20 nM VCR. These cells have been shown to overexpress P-glycoprotein by Western blot (data not shown).

**In Vitro Drug Sensitivity.** The KB V20C cells were maintained in drug-free media 3 days before determination of the concentration capable of inhibiting 50% of growth. Cells (10<sup>4</sup>) were seeded in 2 ml of culture media/well in 24-well plates (Corning Glass Works, Corning, NY). The cells were incubated with different concentrations of anticancer drugs in the absence or presence of either BIBW 22 or DPM at 5% CO<sub>2</sub> and 37°C for 72 h. Subsequently, the methylene blue cell staining method described by Finlay *et al.* (11) was used to measure the cytotoxic effect. All experiments were carried out in triplicate.

**Nucleoside Transport.** The transport of [<sup>14</sup>C]thymidine (56 mCi/mmol) at 37°C was used as an indicator of nucleoside transport. KB cells (4 × 10<sup>5</sup>) were seeded in a 35-mm plastic dish (Becton Dickinson, Lincoln Park, NJ) and incubated for 48 h at 37°C. The cells were preincubated for 15 min at 37°C with either BIBW 22 or DPM at appropriate concentrations, and then exposed to 1  $\mu\text{M}$  [<sup>14</sup>C]thymidine for 30 s. Each dish was placed on ice and washed 5 times with ice-cold phosphate-buffered saline containing 20  $\mu\text{M}$  DPM. Cells were harvested with 1 ml of 1% Sarkosyl and mixed thoroughly with 8 ml of scintillation solution (National Diagnostics, Manville, NJ); their radioactivity was then assayed (6).

**Rhodamine 123 Retention Assay.** KB or KB V20C cells (2 × 10<sup>5</sup>) were seeded in 1 ml of culture media/well in 24-well plates and incubated overnight at 37°C and then for 15 min at 37°C with serial concentrations of BIBW 22, DPM, or verapamil in fresh media. Subsequently, 5  $\mu\text{g}$  of rhodamine 123 were added, and the cells were further incubated at 37°C for 30 min. The cells were then rinsed once with media and incubated again in fresh media without rhodamine 123, but with BIBW 22, DPM, or verapamil at the indicated concentrations at 37°C for 20 min. Cells were then removed from the wells after pancreatin treatment and collected by centrifugation (500 × g). The cells were resuspended in fresh media and analyzed by flow cytometry with a FACS IV (Becton Dickinson, Mountain View, CA) at 488 nm and 360 mW. Green fluorescence of rhodamine 123 was measured using 530/30 nm and 580 band pass filters. A minimum of 10<sup>4</sup> cells/sample were analyzed and fluorescence was plotted on a 5-decade logarithmic scale. The median fluorescence was used as a quantitative measure of intracellular fluorochrome accumulation and used as a parameter for P-glycoprotein inhibition. The logarithmically measured fluorescence intensity was recalculated to its linear value (12).

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<sup>2</sup> To whom requests for reprints should be addressed.

<sup>3</sup> The abbreviations used are: DPM, dipyridamole; VCR, vincristine; MDR, multidrug resistance; 5-FUra, 5-fluorouracil.

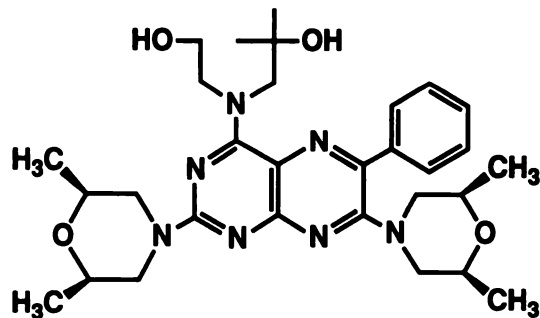


Fig. 1. Chemical structure of BIBW 22.

**Preparation and Photoaffinity Labeling of Plasma Membranes.** Preparation of the plasma membranes from KB or KB V20C cells and subsequent photolabeling with [ $^3\text{H}$ ]azidopine in the presence or absence of BIBW 22, DPM, or verapamil were performed as described previously (13). Briefly, the cells were washed and disrupted with a ground glass homogenizer (Fisher Scientific, Pittsburgh, PA). The homogenate was then centrifuged at  $1000 \times g$  for 10 min. The supernatant was overlaid on a 35% sucrose solution and centrifuged for 60 min at  $18,000 \times g$ . The membrane fraction at the interface was then centrifuged for another 60 min at  $100,000 \times g$ . Pellets were resuspended and stored at  $-70^\circ\text{C}$  until their use. The protein concentration was determined by the method of Bradford (14), using the Bio-Rad protein assay kit (Bio-Rad, Richmond, CA). Fifty  $\mu\text{g}$  of the plasma membrane protein were photolabeled in 40 mM Tris-HCl buffer (pH 7.4), containing 4% dimethyl sulfoxide and 200 nM [ $^3\text{H}$ ]azidopine in a final volume of 50  $\mu\text{l}$  in the absence or presence of BIBW 22, DPM, and verapamil, respectively. Photolabeled membrane proteins were then analyzed using 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis; a total of 25  $\mu\text{g}$  of protein was loaded onto each lane. The gel was fixed, treated with the fluorographic reagent Amplify (Amersham), then dried, and finally exposed for 7–10 days at  $-70^\circ\text{C}$  using Kodak XAR-5 film (Eastman Kodak).

## Results

**Potentiation of VCR by BIBW 22.** The sensitivity of both KB and KB V20C cells to VCR, as well as the potentiating effect of either BIBW 22 or DPM on VCR cytotoxicity are illustrated in Fig. 2. The 50% inhibitory concentration of VCR for KB cells was 2.8 nM and that of KB V20C was 80 nM. An approximate 30-fold difference in sensitivity was thus shown between the two cell lines. When different amounts of BIBW 22 or DPM were added, a concentration-dependent sensitization of KB V20C to VCR was observed. This sensitization to VCR was equivalent with 0.3  $\mu\text{M}$  BIBW 22 and 3.0  $\mu\text{M}$  DPM. An approximate 10-fold difference in potency was thus observed between the two compounds. Neither BIBW 22 nor DPM alone at similar concentrations had any cytotoxic effects on either tumor cell line.

**Modulation of Rhodamine 123 Accumulation.** It is well known that rhodamine 123 provides a good substrate for MDR-associated P-glycoprotein. Cellular retention of this dye can therefore be used to reflect the activity of P-glycoprotein in MDR cancer cells (15). Agents that block P-glycoprotein, such as verapamil, have been found to increase the retention of rhodamine 123 in MDR cells (12). Thus, we analyzed the accumulation of rhodamine 123 in sensitive KB and MDR KB V20C cells. When sensitive and resistant cells were exposed to rhodamine 123 at the concentration of 5  $\mu\text{g}/\text{ml}$ , KB V20C cells retained much less rhodamine 123 than sensitive KB cells (data not shown). This difference is to be expected because the KB V20C cells express higher levels of the MDR1 gene product, P-glycoprotein. Treatment with different concentrations of BIBW 22, DPM, or verapamil for 30 min did not change the amount of rhodamine 123 retained in sensitive KB cells (data not shown) but did cause a significantly increased accumulation of rhodamine 123 in KB V20C cells (Fig. 3A). Based on the dose-response curve (Fig. 3A), it is extrapolated that BIBW 22 at a concentration as low as 0.2  $\mu\text{M}$  could increase maximum rhodamine 123 retention in KB V20C cells by 50%. This compound is about 100- and 20-fold more potent than DPM and verapamil, respectively, in affecting MDR function, as reflected by rhodamine 123 retention in the resistant cell lines.

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**Inhibition of [ $^3\text{H}$ ]Azidopine Photolabeling of P-Glycoprotein by BIBW 22.** Azidopine, a photoactive analogue of dihydropyridine, can photolabel P-glycoprotein expressed in plasma membranes of MDR cells (16). Agents which reverse MDR, such as verapamil, interfere with this photoaffinity labeling (16). Asoh *et al.* (6) have reported that DPM could inhibit the photoaffinity labeling by [ $^3\text{H}$ ]azidopine at high concentrations of 10 to 100  $\mu\text{M}$ . Using this photolabeling technique, the effect of BIBW 22 on azidopine binding to P-glycoprotein was studied. As shown in Fig. 3B, the membrane protein of sensitive KB cells cannot be photolabeled by [ $^3\text{H}$ ]azidopine, whereas the P-glycoprotein of the KB V20C cells could be labeled. BIBW 22, at a concentration of 1  $\mu\text{M}$ , completely inhibited the photolabeling of [ $^3\text{H}$ ]azidopine in KB V20C cell membranes. In contrast, the presence of 10  $\mu\text{M}$  DPM resulted only in a partial inhibition

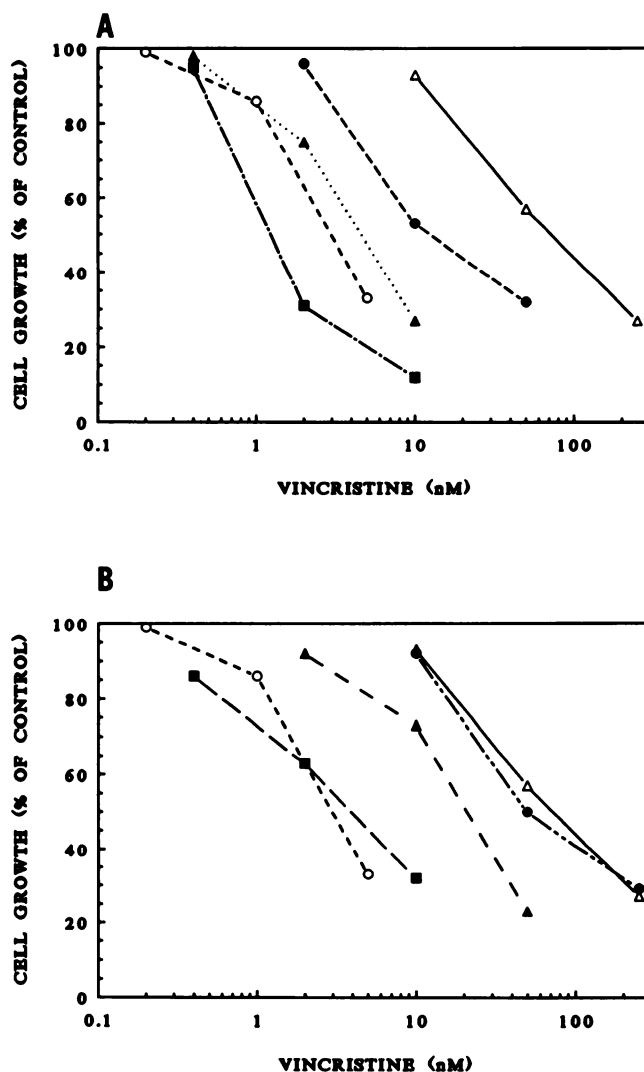
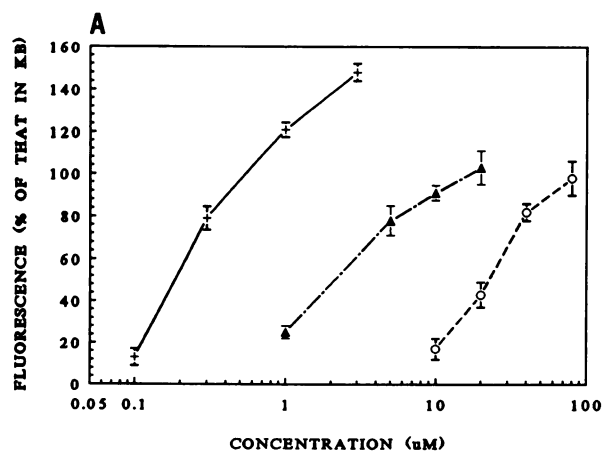


Fig. 2. Effect of BIBW 22 on the sensitivity of KB V20C cells to VCR. KB (○) and KB V20C (Δ) cells were exposed to 0–250 nM VCR for 72 h in the absence or presence of either BIBW 22 (A) or DPM (B). Drug concentrations of BIBW 22 were 0.1 (●), 0.3 (Δ), 1.0  $\mu\text{M}$  (■); that of DPM were 0.3 (●), 1.0 (Δ), 3.0  $\mu\text{M}$  (■). Cell growth was determined by a microtiter assay as described in "Materials and Methods." Each point represents the mean of triplicate determinations which differed by less than 5%.


**B**

CELL LINE	KB	KB V20C																				
		DRUGS (µM)	BIBW 22					DPM				VERAPAMIL										
			NONE	0	0.01	.1	1	.1	1	10	100	.1	1	10	100							

Fig. 3. (A) Effect of BIBW 22 on retention of rhodamine 123 fluorescence in KB V20C. KB V20C cells were incubated with the indicated concentrations of BIBW 22 (+), DPM (O), or verapamil (Δ) for 15 min before measuring rhodamine 123 retention by flow cytometry. Points, mean of three determinations; bars, SD. (B) Effect of BIBW 22 on photoaffinity labeling of P-glycoprotein with [<sup>3</sup>H]azidopine. KB V20C plasma membranes (50 µg of protein) were incubated with 0.2 µM [<sup>3</sup>H]azidopine in the absence or presence of BIBW 22, DPM, or verapamil at the indicated concentrations. After solubilization, photolabeled protein were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described in "Materials and Methods."

of the photolabeling. The inhibitory effect of BIBW 22 on [<sup>3</sup>H]-azidopine binding to P-glycoprotein is greater than that of DPM and verapamil.

**Effect of BIBW 22 on Thymidine Transport.** It was reported that DPM specifically inhibits the cellular transport of nucleoside compounds such as uridine or thymidine (1, 2). The effect of BIBW 22, compared with that of DPM on thymidine transport, was examined and the results are shown in Fig. 4A. BIBW 22 inhibited thymidine transport by KB cells in a concentration-dependent manner and its potency was about 7-fold more than that of DPM. BIBW 22 could also inhibit the cellular transport of 5-fluorodeoxyuridine but not that of 5-FUra (data not shown).

**Effect of BIBW 22 on the Sensitivity of KB Cells to 5-FUra.** In cell culture, DPM was shown to increase the cytotoxicity of 5-FUra in a dose-dependent manner (3). The influence of BIBW 22 on 5-FUra cytotoxicity towards KB cells was studied and results are shown in Fig. 4B. BIBW 22 at 1.0 µM could decrease the 50% inhibitory concentration of 5-FUra shifted from 10 µM to 0.45 µM. DPM at 1 µM could only enhance the 5-FUra cytotoxicity of KB cells about 2-fold.

## Discussion

In view of the continued problems of MDR to such antineoplastic compounds as VCR, etoposide, and Adriamycin (8), attempts have been made by many laboratories to develop modulators which could reverse the MDR phenotype. Compounds such as verapamil, phenothiazine inhibitors, and others have been found to reverse the MDR phenotype by inhibiting the efflux of MDR-associated drugs (17-19). Since MDR-associated drugs are often used clinically in combination

with such antimetabolites as 5-FUra for refractory diseases, an effective compound against both the MDR phenotype and antimetabolite resistance would be ideal.

One common feature of the cytotoxicity of all antimetabolites is that several naturally occurring nucleosides can reverse this desired cytotoxic effect. The presence of these nucleosides in serum or their release from surrounding cells could, therefore, render target cells resistant to such antimetabolites as 5-FUra (8, 9). Thus compounds which would reverse the MDR phenotype and inhibit nucleoside transport would have a unique modulating activity in combination chemotherapy. They could reverse the MDR phenotype by interfering P-glycoprotein function (17, 18) and enhance the cytotoxicity of 5-FUra, presumably by inhibiting thymidine uptake from surrounding dead cells as well as by producing a selective increase in intracellular 5-fluorodeoxyuridine monophosphate levels (20). DPM is a compound known to interfere with both mechanisms of tumor cell resistance. The effective concentration, however, required *in vitro* for this bifunctional modulation is difficult to achieve *in vivo* without severe side effects. In this study, BIBW 22 is characterized as a more potent bifunctional modulator than DPM. The compound is shown to be

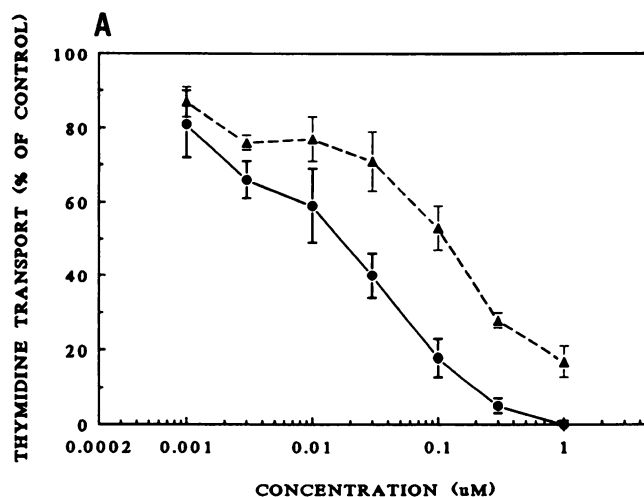
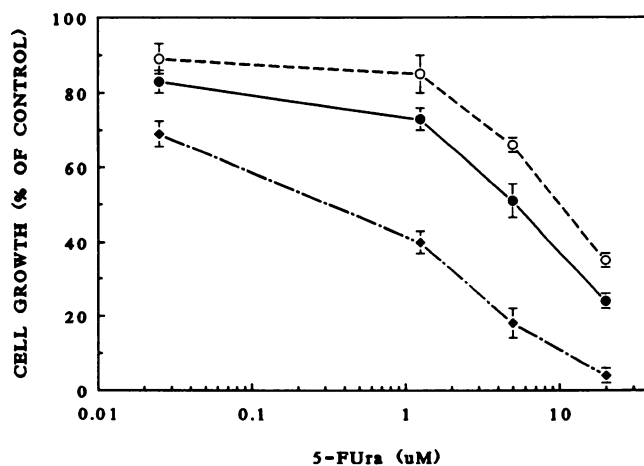

**B**


Fig. 4. (A) Effect of BIBW 22 on transport of [<sup>14</sup>C] thymidine by KB cells. KB cells were incubated with 1 µM [<sup>14</sup>C]thymidine in the absence or presence of either BIBW 22 (●) or DPM (Δ) at the indicated concentrations for 30 s. Radioactivity in cells was determined as described in methods. Points, mean of three determinations; bars, SD. (B) Effect of BIBW 22 on the sensitivity of KB cells to 5-FUra. KB cells were exposed to 0-20 µM 5-FUra for 72 h in the absence (○) or presence of either 1 µM BIBW 22 (◆) or DPM (●). Cell growth was determined by a microtiter assay as described in "Materials and Methods." Points, means of three determinations; bars, SD.

100-fold more potent than DPM in reversing the MDR phenotype as examined by rhodamine 123 retention in a P-glycoprotein overexpressing cell line. Furthermore, it is 7-fold more potent than DPM in preventing nucleoside transport, as reflected by [<sup>14</sup>C]thymidine transport. It is thus reasonable to expect that clinically acceptable doses of BIBW 22 would be sufficient to enhance the effect of MDR-associated drugs and antimetabolites. This hypothesis is under current experimental investigation.

In terms of the possible molecular mechanism of action by which DPM or BIBW 22 can modulate MDR phenotype and nucleoside transport, an initially attractive hypothesis would be that these agents interact via a single receptor, which in turn plays a key role in modulating both activities. Based on our observation on the activities of DPM on modulating MDR phenotype and nucleoside transport,<sup>4</sup> however, we do not favor this simple explanation. Further studies are necessary to understand the molecular mechanisms responsible for the pharmacological activities of DPM and BIBW 22.

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<sup>4</sup> Unpublished results.