Biochemical Pharmacology, Vol. 46, No 8, pp 1421–1424, 1993
Printed in Great Britain

0006–2952/93 $6.00 + 0.00

CHLOROQUINE RESISTANCE IN *PLASMODIUM FALCIPARUM* IS NOT REVERSED BY BIBW-22, A COMPOUND REVERSING THE MULTIDRUG RESISTANCE PHENOTYPE IN MAMMALIAN CANCER CELLS

ANGELA DIECKMANN-SCHUPPERT,* UWE BAMBERGER† and RALPH T. SCHWARZ
Zentrum für Hygiene und Medizinische Mikrobiologie, University of Marburg, Robert-Koch-Str. 17, 35037 Marburg; and †Dr Karl Thomae GmbH, 88397 Biberach, Germany

(*Received* 31 *March* 1993; *accepted* 7 *July* 1993)

Abstract—The pteridine derivative BIBW-22 (4-[*N*-(2-hydroxy-2-methyl-propyl)-ethanolamino]-2,7-bis(*cis*-2,6-dimethyl-morpholino)-6-phenylpteridine), which had been developed for the treatment of multidrug-resistant cancer and binds to P-glycoprotein, was tested against chloroquine resistant *Plasmodium falciparum* strains in culture. Based on the result that BIBW-22 enhanced rather than lowered chloroquine resistance *in vitro*, it is concluded that chloroquine resistance in malaria parasites may not be mechanistically linked to the multidrug-resistant phenotype of chloroquine resistant *P. falciparum*.

The antimalarial drug chloroquine has, due to its efficacy and low toxicity, for a long time been the mainstay of antimalarial chemotherapy. Because of the rapid spreading of chloroquine-resistant malaria since the early 1960s, the chemotherapy of malaria had since then to rely on the use of comparably less efficient and more toxic drugs, hampering effective malaria control. The need for new antimalarials to overcome chloroquine resistance has not only led to some substitutes for chloroquine but also initiated a search for substances that could reverse chloroquine resistance to sensitivity. Use was made of the fact that chloroquine resistance in *Plasmodium falciparum* exhibits some similarities with multidrug-resistant mammalian tumor cells. The calcium channel blocker verapamil was the first compound demonstrated to relieve chloroquine resistance in *P. falciparum in vitro* [1]. Due to its cardiodepressive effects, the clinical usefulness of verapamil in malaria therapy has, however, been questioned [2]. Therefore, less toxic substitutes suited for clinical use are highly desired. The substance BIBW-22‡ (4-[*N*-(2-hydroxy-2-methyl-propyl)-ethanolamino]-2,7-bis(*cis*-2,6-dimethyl-morpholino)-6-phenylpteridine), developed as a multidrug resistance-reversing compound for use in cancer therapy [3], has no calcium channel blocking activity, but is a much more potent inhibitor of multidrug resistance than verapamil. Because chloroquine resistance in *P. falciparum* has been linked to transport mechanisms comparable to those underlying multidrug resistance, we investigated the effect of BIBW-22 on this biological phenomenon. The results obtained led us to conclude that chloroquine resistance in malaria parasites is independent of their eventual multidrug-resistant phenotype.

* Corresponding author Tel. (49) 6421-285493; FAX (49) 6421-285482.

‡ Abbreviations: BIBW-22, 4-[*N*-(2-hydroxy-2-methyl-propyl) - ethanolamino] - 2,7 - bis(*cis* - 2,6 - dimethyl - morpholino)-6-phenylpteridine; mdr, multidrug resistance; Σ FIC, sum of the fractional inhibition concentrations.

MATERIALS AND METHODS

Parasites and culture conditions. The investigations reported here were performed using the *P. falciparum* strains FCBR ([4], obtained from Dr B. Enders, Behring AG, Marburg, F.R.G.), T9/94, and T9/96 ([5], obtained from Dr A. A. Holder, National Institute for Medical Research, London, U.K.). The asexual intra-erythrocytic stages were maintained in a suspension of 5% hematocrit human A^+ erythrocytes in RPMI 1640 medium according to Trager and Jensen [6], and supplemented with 21.5 mM Hepes, 0.37 mM hypoxanthine, 100 mg/L neomycin base and 10% (v/v) human A^+ serum. The cultures were incubated at 37° in modular incubation chambers (Flow) at 5% oxygen, 5% carbon dioxide and 90% nitrogen. Synchronous development of the cultures was achieved by repeated sorbitol treatment [7, 8]. Parasitemia was determined by counting of Giemsa-stained thin slides.

Inhibition tests. The following stock solutions were prepared in double distilled water: 1 mM chloroquine diphosphate (Sigma), 1 mM verapamil hydrochloride (Sigma), 1.7 and 177 mM (1 and 100 mg/mL, respectively) BIBW-22. All stock solutions were kept frozen at −20° and not used after more than 1 week. Working dilutions were prepared freshly each time by diluting with culture medium, and were filter sterilized immediately before use. For dose–response studies, cultures of 2.5% hematocrit harbouring 1–1.5% young ring stage parasites were incubated in duplicate in microtitration plates with serial 2-fold dilutions of the substance to be tested. Slides were prepared from each well after 50–54 hr, the number of newly formed rings being regarded as an inverse measure of toxicity. Unlike the test of [^{3}H]-

Table 1. *In vitro* sensitivity (IC_{50} values) of the three *P. falciparum* strains suggest the compounds used in this study

Compound	Strain FCBR	T9/94 (μM)	T9/96
Chloroquine	0.270	0.172	0.016
Verapamil	5.3	5.2	6.3
BIBW-22	9.9	13.5	11.1

All values are arithmetic means from three to five determinations.

hypoxanthine incorporation into trophozoites, direct counting of parasites additionally measures the reinvasion process and results are less likely to depend on the specific mode of action of the respective drug tested. IC_{50} values and approximate summarized fractional inhibition constants (Σ FIC, [9]) were determined graphically from semi-logarithmic plots of the dose–response data.

RESULTS

The strains FCBR and T9/94 proved to be highly resistant to chloroquine, whereas T9/96 was sensitive to chloroquine. Verapamil as well as BIBW-22 inhibited equally all three strains, the IC_{50} for BIBW-22 ranging between 10 and 13 μM, which was about twice as much as for verapamil (summarized in Table 1).

The combination of chloroquine and 1 μM verapamil proved to be synergistic in all three strains investigated, resulting in a shift of the dose–response curve by a factor 2–10 to the left, which is in the range reported for other strains [1, 10–14].

The combination of chloroquine and BIBW-22, however, was found to be antagonistic for the two chloroquine-resistant strains. In the case of the FCBR strain, combining chloroquine with 1.7 μM BIBW-22 did not markedly affect the position of the dose–response curve obtained with chloroquine alone (not shown), but upon raising the concentration of BIBW-22 to 3.5 μM significant antagonism was seen, reflected in a shift of the dose–response curve to the right by a factor of 1.3 (Fig. 1a). This became even more pronounced at concentrations up to 8.8 μM BIBW-22. The Σ FIC was roughly estimated as 2.

For the T9/94 clone, 1.7 μM BIBW-22 in combination with chloroquine produced marked antagonism, which was consistently enhanced at 3.5 μM and above, and reflected in a shift of the dose–response curve by a factor 2 to the right (Fig. 1b) and an approximate Σ FIC of 2.

In contrast to these two strains, the chloroquine-sensitive clone T9/96, once generated from the same field isolate as T9/94, showed a synergistic effect when chloroquine was combined with 1.7–8.8 μM BIBW-22, resulting in a shift of the dose–response curve to the left by a factor of 1.6 (Fig. 1c) at 3.5 μM.

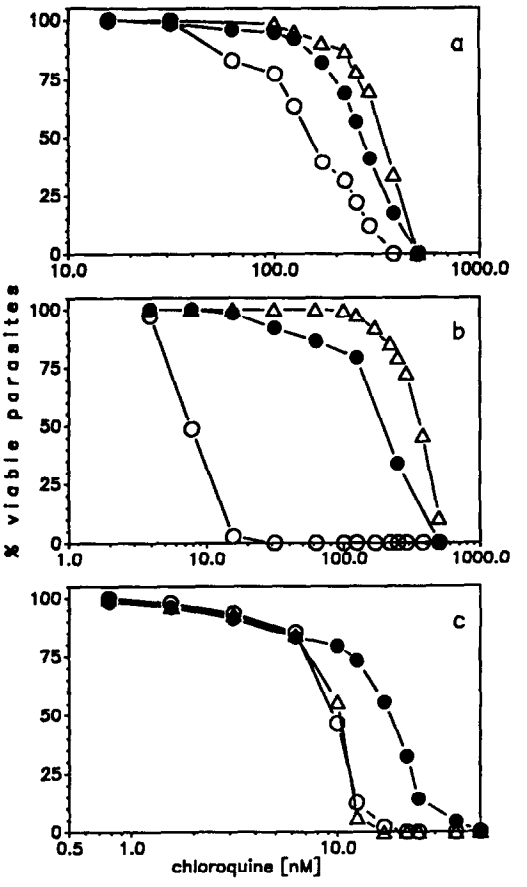

Fig. 1. Dose–response curves obtained *in vitro* for *P. falciparum* strains (a) FCBR, (b) T9/94 and (c) T9/96, exposed for 50 hr to chloroquine alone (●), chloroquine plus 1 μM verapamil (○) or chloroquine plus 3.5 μM BIBW-22 (△). All values are averages from three to five determinations.

DISCUSSION

In *P. falciparum*, chloroquine accumulates in acidic compartments of the parasite [15, 16], which is supposed to be the basis for its antimalarial action. A 40–50-fold lower accumulation is seen in resistant parasites [10, 17] and regarded as the basis for chloroquine resistance in malaria. This decreased accumulation has been attributed to more rapid export from the resistant parasites. In this respect, chloroquine resistance in *P. falciparum* resembles mechanisms underlying multidrug resistance in mammalian tumor cells.

In malignant diseases, the expression of P-glycoprotein, a membrane-associated, ATP-dependent pump known to transport lipophilic substances out of the cell, is the best studied and clinically most relevant reason for multidrug resistance [18, 19].

Two genes named *Pfmdr* have been identified in *P. falciparum*, one of which bears homologies with the human *mdr*1 gene [20]. The amplification of the *Pfmdr* gene(s) in some resistant strains has been

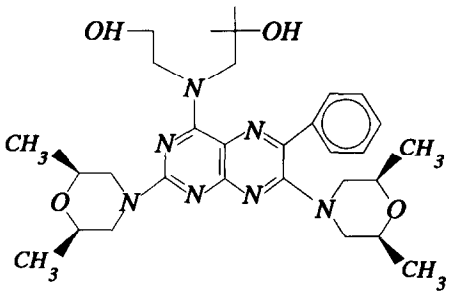

Fig. 2. Chemical structure of BIBW-22.

related to quicker export of chloroquine from resistant parasites and thereby to chloroquine resistance [21].

In mammalian tumor cell lines, the P-glycoprotein-mediated multiple drug resistance can be modulated by verapamil and other calcium channel blockers as well as by calmodulin antagonists [22]. Furthermore, verapamil and related compounds were shown to relieve chloroquine resistance in *P. falciparum in vitro* [1, 11–14]. However, not only the *S*(−)-isomer of verapamil, which binds to calcium channels, but also the *R*(+)-isomer which does not bind, shows this effect, suggesting that the verapamil effect is at least partially independent of calcium channels [23]. Therefore, it was of interest to investigate whether a substance that reversed the mdr phenotype in mammalian tumor cells without binding to calcium channels would mimick the verapamil effect on *P. falciparum in vitro*.

BIBW-22, a highly substituted pteridine, whose chemical structure is shown in Fig. 2, was patented [24] as a multiple drug resistance-reversing substance. This compound was shown to inhibit the binding of the specific label azidopine to P-glycoprotein. In addition, BIBW-22 restores intracellular drug accumulation in resistant tumor cells and, thereby, is able to totally reverse multidrug resistance [3]. Because BIBW-22 is not a calcium channel blocker, it was regarded as a model substance to test whether chloroquine resistance in *P. falciparum* is mechanistically related to the multidrug resistance phenotype. While BIBW-22 alone is less inhibitory than verapamil towards the three strains included in this study, its combination with chloroquine proved to be antagonistic against the chloroquine resistant strains, rather than synergistic as seen with verapamil and chloroquine. Interestingly, slight synergism was found for the strain T9/96, which is sensitive to chloroquine despite being resistant to the chemically related drug mefloquine. Both T9 clones were equally resistant to mefloquine (IC_{50} = 200 nM), suggesting chloroquine resistance and the possibility to influence it not to be linked to mefloquine resistance in these strains.

Our results underline an earlier report by Wellems *et al.* [25], who, after a genetic cross between resistant and sensitive *P. falciparum*, found independent segregation of chloroquine resistance and mdr phenotype. Moreover, mefloquine resistance of the *P. falciparum* strain W2-mef, reported to be accompanied by amplification of the *Pfmdr*1 gene, was by the same authors found not to be associated with an increase in chloroquine resistance in this strain. The recent genetic mapping of chloroquine resistance to the *P. falciparum* chlorosome No. 7 further supports the idea of the *Pfmdr*1 gene or its gene product not being linked to chloroquine resistance in *P. falciparum*, as the *Pfmdr*1 gene maps to chromosome 5 [26]. In conclusion, the putative reversal of the mdr phenotype alone does not seem to affect chloroquine resistance in *P. falciparum* in the strains investigated here. This was shown *in vitro* using the model compound BIBW-22, known to reverse multidrug resistance in mammalian tumor cells, which rather enhances chloroquine resistance in two of the *P. falciparum* strains tested.

Acknowledgements—*Plasmodium falciparum* strain FCBR was obtained from Dr B. Enders, Behring Co., Marburg, F.R.G. *P. falciparum* cloned strains T9/94 and T9/96 were obtained from Dr A. A. Holder, National Institute for Medical Research, London, U.K. Prof. V Kretschmer (University Blood Bank, Marburg) kindly provided human erythrocytes for the *in vitro* cultivation of *P falciparum*. The authors thank Mr Heinrich Kaiser for skilful technical help.

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