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Pleiotropic Resistance to Diverse Antimalarials in Actinomycin D-Resistant *Plasmodium falciparum*

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ABSTRACT. The development and spread of multidrug-resistant *Plasmodium falciparum* are major health concerns. The molecular mechanisms of multidrug resistance, including resistance to many quinoline-based antimalarials, are largely unknown. In this study, we report on the isolation and partial characterization of actinomycin D (actD)-resistant *P. falciparum* (3D7^R/actD2.3) from a chloroquine-susceptible strain, 3D7. The stepwise selection of an actD-resistant clone (3D7^R/actD2.3) led to the isolation and cloning of *P. falciparum* that grew in the presence of 2 ng/mL of actD. The parental isolate (3D7) did not grow in the presence of a 10-fold lower drug concentration (0.2 ng/mL). The latter estimate of parasite growth was determined by direct counting of parasites in infected red blood cells. Estimates of drug resistance levels to actD, using a [^{3}H]hypoxanthine uptake and incorporation method, showed a 3-fold difference in the IC_{50} between 3D7 and 3D7^R/actD2.3. Interestingly, 3D7^R/actD2.3 *P. falciparum* parasites were less sensitive to several antimalarials (chloroquine, mefloquine, quinidine, and artemisinin) and to the mitochondrial specific dye Rhodamine 123. Drug transport studies using [^{3}H]actD showed that 3D7^R/actD2.3 accumulated less drug than 3D7. Moreover, the accumulation of [^{3}H]actD was energy dependent. To determine if *Pfmdr1* expression, previously implicated in drug resistance to certain antimalarials, mediated the resistance phenotype of 3D7^R/actD2.3, *Pfmdr1* levels in 3D7 and 3D7^R/actD2.3 were compared by Southern and northern blot analyses. Our results revealed no differences in *Pfmdr1* copy number or mRNA levels between 3D7 and 3D7^R/actD2.3. Furthermore, comparison of *Pfmdr1* sequences between 3D7 and 3D7^R/actD2.3 showed no differences. In addition, verapamil, which reverses P-glycoprotein-mediated drug resistance in mammalian cells, did not reverse the resistance of 3D7^R/actD2.3 to actD or chloroquine. Taken together, the findings of this study demonstrated that *in vitro* selection of *P. falciparum* for resistance to actD leads to decreased sensitivity to diverse drugs and that this pleiotropic drug resistance is associated with reduced drug accumulation not mediated by *Pfmdr1*. BIOCHEM PHARMACOL **59**;9:1123–1132, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. malaria; *P. falciparum*; *Pfmdr1*; actinomycin D; chloroquine resistance; multidrug resistance

The evolution and spread of chloroquine-resistant *Plasmodium falciparum* have limited the use of chloroquine in many regions where malaria is endemic [1, 2]. Resistance to other antimalarials (mefloquine, halofantrine, and quinine) and multidrug-resistant *P. falciparum* could threaten the use of existing and new antimalarials [3–5]. Earlier studies have implicated *Pfmdr1*, an *mdr1*-like gene, in chloroquine or mefloquine and halofantrine resistance in *P. falciparum* [6–8]. Furthermore, transfection of *Pfmdr1* in Chinese hamster ovary (CHO) cells increased the susceptibility of cells to chloroquine, consistent with the intracellular localization of the *Pfmdr1* gene product [9]. However, genetic studies showed no correlation between the inheritance of the *Pfmdr1* gene and chloroquine resistance [10, 11]. Similarly, no correlation was seen between chloroquine resistance and *Pfmdr1* expression or point mutations in field isolates of *P. falciparum* [12]. More recently, *in vitro* selection of *P. falciparum* resistant to chloroquine, mefloquine, or halofantrine showed no change in *Pfmdr1* expression [13–15].

P-glycoprotein homologs or *mdr1*-like genes have been cloned from several parasites, which include *Leishmania* (*ltpgpA*, *ldmdr1*, and *lemdr1*; [16–18]), *Entamoeba histolytica* (Eh*pgp1*; [19]), *Schistosoma mansoni* (SMDR1; [20]), *Trypanosoma cruzi* (tc*pgp1*; [21]), *Trichomonas vaginalis* (Tv*pgp1*; [22]), and *Giardia duodenalis* (Gd*pgp1*; [23]). However, few P-glycoprotein homologs have been implicated directly in the multidrug resistance phenotype of these parasites [24, 25]. Thus, although a direct role for *Pfmdr1* in *P. falciparum* drug resistance remains controversial, there is now mounting evidence that points to multiple mechanisms and/or changes leading to the rise of resistance to antimalarials. Recently, using linkage and haplotype analyses of resistant and susceptible parasites, Su *et al.* [26] identified the expression of a 330-kDa protein that correlates with chloroquine resistance in *P. falciparum* isolates from Southeast Asia and Africa. Other studies [27, 28], using amiloride derivatives to inhibit chloroquine uptake,

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identified the plasmodial Na^+/H^+ exchanger as a chloroquine importer that is differentially stimulated in chloroquine-susceptible and -resistant parasites. More recently, using a quinidine-susceptible strain of *Saccharomyces cerevisiae*, four *S. cerevisiae* genes were shown to confer resistance to quinoline-containing antimalarials [29]. Taken together, the mechanisms of resistance to chloroquine or quinoline-based drugs in *P. falciparum* are not clear. However, given the grievousness of the situation, with incidences of malaria as high as 500 million cases per year (World Health Organization, 1996), and the rapid spread of quinoline-resistant *P. falciparum*, better understanding of the molecular mechanisms of drug resistance in this parasite is essential.

In this study, it was of interest to select clones of *P. falciparum* resistant to drugs that have established mechanisms of toxicity and do not interact with targets in red blood cells. One such drug is actD,* with a well-established function as an inhibitor of DNA replication and transcription through binding to GC sequences [30]. Furthermore, the rationale for using actD was based on earlier studies where the overexpression of mammalian mdr-1 and reduced drug efflux were tightly associated with the use of actD in the selection of resistant tumor cells (for a review, see Ref. 31). In addition, to allow for a direct comparison between resistant and susceptible parasites, it was important to derive resistant clones from a known susceptible strain of *P. falciparum*. In this report, we describe the isolation and partial characterization of actD-resistant *P. falciparum*.

MATERIALS AND METHODS

Parasite Culture

Parasites were cultured *in vitro* according to the method of Trager and Jenson [32]. Parasites were maintained on a 5% suspension of type O erythrocytes in RPMI 1640 medium supplemented with 28 mM $NaHCO_3$, 32 mM hypoxanthine, 2 mM glutamine, 25 mM HEPES, pH 7.4, and 10% human plasma. Ten milliliters of the above parasite suspension was added to a T-25 tissue culture flask and flushed with 3% O_2, 4% CO_2, and 93% N_2. Flasks were incubated at 37°, and the medium was replaced every 24 hr. The chloroquine-susceptible (3D7) and -resistant (FAC8–15) clones of *P. falciparum* (gifts from Dr. A. F. Cowman at Walter and Eliza Hall Institute in Australia) were grown continuously without and with 15 ng/mL of chloroquine, respectively. The $3D7^R$/actD2.3 parasite was cultured as above but in the presence of 2 ng/mL of actD.

Selection and Cloning of Drug-Resistant Parasites

Parasites resistant to actD were selected *in vitro* from 3D7 chloroquine-susceptible isolates as outlined in Fig. 1.

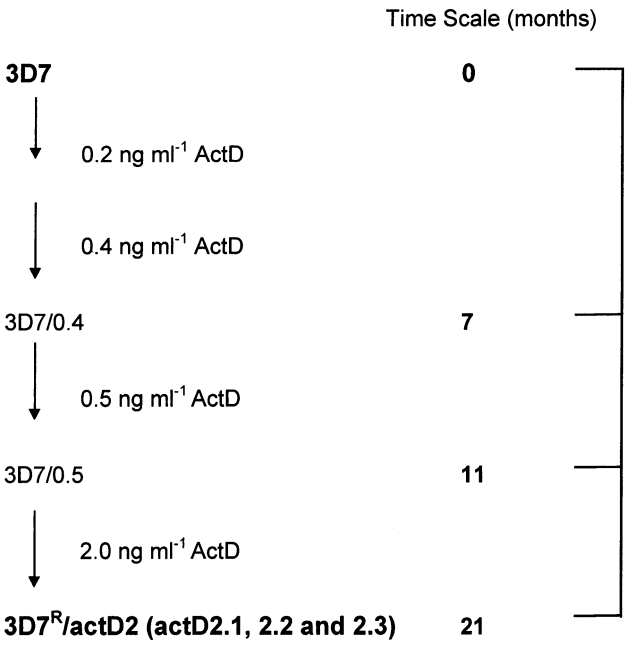

FIG. 1. Selection of actD-resistant *P. falciparum*. Chloroquine-susceptible *P. falciparum*, 3D7, was grown *in vitro* in the presence of increasing concentrations of actD (0.2, 0.4, 0.5, and 2.0 ng/mL). *P. falciparum* parasites that grew in 2 ng/mL of actD were cloned by limiting dilution.

Briefly, actD was added to 5% cultures and allowed to incubate at 37° for 48 hr, at which point the parasitemia was checked and the growth of the parasite was scored. The drug pressure was removed for 48 hr when the parasitemia fell below 0.2%. This cycle of drug selection was continued until a parasite culture that was able to withstand 2 ng/mL of actD ($3D7^R$/actD2.3) was obtained. As indicated in Fig. 1, several actD-resistant isolates were obtained over a period of 21 months and cloned by limiting dilution in 96-well plates. The cloning procedure was repeated two times, and three clones that grew continuously in 2 ng/mL of actD were isolated ($3D7^R$/actD2.1, 2.2, and 2.3). All three clones showed similar resistance profiles, and, therefore, the $3D7^R$/actD2.3 clone was used throughout this study. The actD-resistant clones have been in culture for more than a year and were shown to be stably resistant to the drug even when the drug was withdrawn for several months.

Cytotoxicity Assay

Parasite cultures were washed three times with 5 mL of RPMI 1640 (Gibco) and synchronized twice with 15% sorbitol in RPMI 1640 for 5 min at 37° [33]. After synchronization, parasitized RBCs were resuspended in an equal volume of 10% serum in RPMI and washed two times by centrifugation at 200 *g* for 5 min. For cytotoxicity assays, the parasite cultures were diluted to 0.1% parasitemia in 2% haematocrit. One hundred microliters of synchronized parasitized RBCs was aliquoted into each well of the 96-well

* *Abbreviations*: actD, actinomycin D; RBC, red blood cell; Rh123, Rhodamine 123; SSC, 0.9 M sodium chloride, 0.09 M sodium citrate, pH 7.0; and PCR, polymerase chain reaction.

culture plates, containing 100 μL of cytotoxic drugs (chloroquine, actD, mefloquine, quinine, quinidine, artemisinin, or Rh123) or the calcium channel blocker verapamil. Plates were incubated in a gassed chamber at 37° for 3 days. Following day 3, 100 μL of the top medium was removed carefully and replaced with fresh medium with or without drugs. The latter step was repeated for days 5 and 6 of the cytotoxicity assay. On day 7, 50 μL of [^{3}H]hypoxanthine monohydrochloride (0.1 mCi/mL; New England Nuclear-Dupont, Inc.) was added to each well of the 96-well plates and allowed to incubate for an additional 17 hr at 37°. Parasitized RBCs were harvested on Millipore glass fiber sheets (Skatron, Cat. No. 11731) pre-saturated with 3% BSA in PBS using a multi-well cell harvester (Skatron, Inc.). The Millipore membranes were allowed to dry and placed in tubes containing scintillation fluid (New England Nuclear-Dupont, Inc.). The amount of incorporated [^{3}H]hypoxanthine was determined by fluorometry using a liquid scintillation counter (LBK Wallac, 1219 Rack Beta, Inc.). Parasitemia values are expressed as a percentage of control in the absence of drugs (mean ± SEM of a representative of three experiments carried out in triplicate). Alternatively, the effects of drugs were determined by direct counting of parasites in 1000 RBCs.

Drug Uptake

Parasitized RBCs were washed several times in PBS and incubated with 10 nM [^{3}H]actD (20 mCi/mmol; New England Nuclear-Dupont, Inc.) plus 10 mM D-glucose for 30–60 min at 37° before the cell suspension was centrifuged through 0.5 mL of ice-cold dibutylphthalate and processed for scintillation counting as described previously [34]. For ATP depletion experiments, parasites were washed as above and preincubated in PBS containing 100 nM sodium azide and 10 mM 2-deoxyglucose for 30 min prior to the addition of 10 nM [^{3}H]actD. The amount of [^{3}H]actD uptake was determined thereafter as indicated above.

Southern and Northern Blots

Southern and northern blot analyses were performed using standard procedures [35]. For Southern blots, 10-μg samples of DNA from susceptible and resistant clones were digested with *Eco*RI and electrophoresed through a 0.7% agarose gel. The DNA was transferred to a Hybond-N nylon membrane (Amersham, Inc.), and the membrane was pre-hybridized in a solution containing 6x SSC, 5x Denhardt's reagent, 0.5% SDS, 100 mg/mL of salmon sperm DNA, and 50% formamide at 42°. ^{32}P nick-translated probes coding for parts of the *Pfmdr1* gene (3331–3744 bp [36]) or the α-tubulin gene (690–950 bp [37]) were added separately and hybridized for 18 hr. The membrane was washed with 2x SSC with 0.5% SDS at room temperature for 10 min and then with 1x SSC with 0.1% SDS at 65°. For northern blot analysis, 10 μg of total RNA from susceptible and resistant parasites previously synchronized in sorbitol was fractionated with glyoxal/DMSO, electrophoresed on a 1% agarose gel, transferred to a nylon membrane, and probed with a *Pfmdr1*-specific probe at 42°. The membrane was washed for 30 min at room temperature in 0.5x SSC, and the signal was developed by exposing the membrane to a Kodak x-ray film with an intensifying screen.

PCR and DNA Sequencing

The nucleotide sequences of two regions in *Pfmdr1*, from 3D7^R/actD2.3 and 3D7, were determined by sequencing the two fragments (135–610 bp and 2980–3850 bp; [36]) obtained from PCR of genomic DNA [9]. PCR primers (24–30 nucleotides) were designed to cover the two regions (Asn86 to Tyr184 and Ser1034 to Asp1246) in *Pfmdr1* associated with drug resistance in certain isolates of *P. falciparum* [38]. Briefly, the reaction was performed in 50 μL of PCR buffer [100 mM Tris–HCl (pH 9), 500 mM KCl, 15 mM $MgCl_2$] containing 200 mM of each dNTP, 100 pmol each of forward and reverse primers, 200 ng of template DNA (3D7 and 3D7^R/actD2.3) and 5 U of *Taq* DNA polymerase. PCR conditions were as follows: one cycle of 94° × 1 min, 40 cycles of 94° × 1 min (denaturation), 45° × 2 min (annealing), and 72° × 3 min (extension), and 1 cycle of 72° × 7 min [39]. The PCR fragments were purified from gel slices and cloned into a TA-cloning vector™, PCRII, according to the manufacturer's protocol (Invitrogen, Inc.). Plasmid DNA was prepared and used for double-stranded DNA sequencing (dideoxy method) [40].

RESULTS AND DISCUSSION

Selection of ActD-Resistant P. falciparum

ActD is an extremely toxic antibiotic presently used in the treatment of cancer. The mechanism of actD toxicity is mediated by its binding to GC sequences, leading to the inhibition of DNA replication and transcription [30]. Resistance to actD, in mammalian cells, has been accomplished by changes in drug accumulation. Indeed, actD-resistant tumor cells have consistently shown the overexpression of the *mdr-1* gene product (or P-glycoprotein), which in turn confers a multidrug resistance phenotype [31]. To study the mechanism of multidrug resistance in *P. falciparum*, it was of interest to develop an *in vitro* model system whereby resistant parasites were derived from a parental drug-susceptible strain using actD. The selection of drug-resistant parasites from a chloroquine-susceptible strain of *P. falciparum* (3D7) was achieved by stepwise drug pressure with increasing concentrations of actD (Fig. 1). RBCs infected with 3D7 were grown in the presence of sublethal concentrations of actD. Following a given drug pressure, the surviving parasites were transferred to fresh medium and allowed to recover for 48 hr in the absence of drug. The drug pressure was applied multiple times until no decrease in parasitemia was observed at a given drug concentration (Fig. 1). Using this protocol, several clones

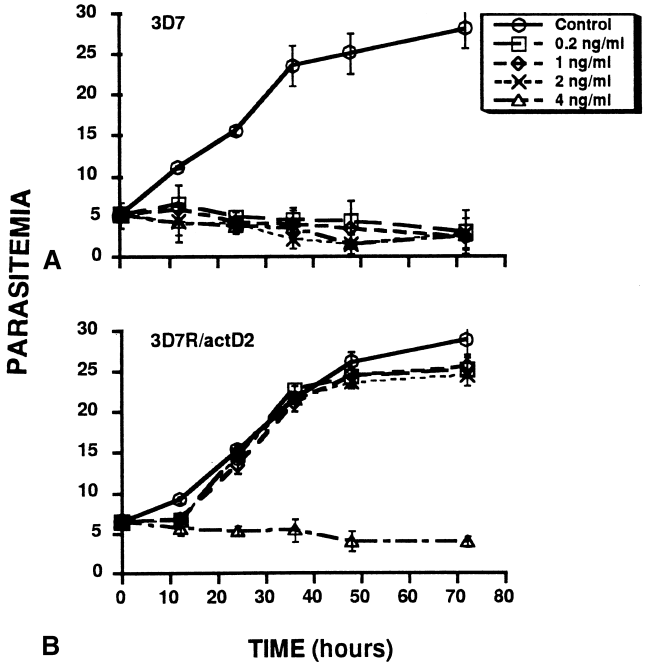

FIG. 2. Effects of actD on the growth of 3D7 and 3D7^R/actD2.3 *P. falciparum*. Drug-susceptible (3D7) and actD-resistant (3D7^R/actD2.3) *P. falciparum* were incubated in the absence and in the presence of increasing concentrations of actD (0.2 to 4 ng). The growth of the parasite was determined by direct counting of parasites in 1000 RBCs every 12 hr for a total of 72 hr. All different stages of the parasite were counted and compared with the number of parasites in the absence of actD (as control). The above values are means ± SEM of a representative experiment carried out in triplicate.

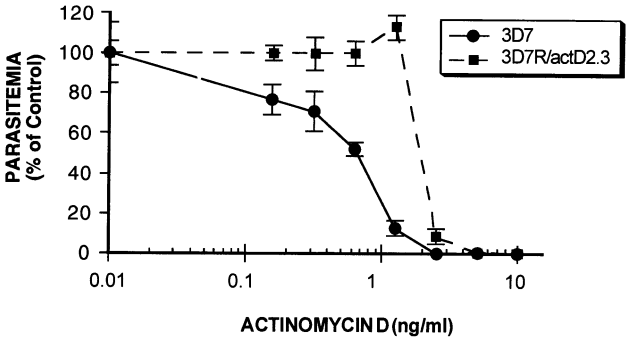

FIG. 3. Concentration–response curves of drug-susceptible and -resistant *P. falciparum*. 3D7 and 3D7^R/actD2.3 *P. falciparum* were incubated without and with increasing concentrations of actD. Parasite growth was determined by measuring [^{3}H]hypoxanthine incorporation. The effect of actD on the growth of parasites is expressed as percent of control (without drug). Each point on the curve represents the mean ± SEM of a representative of four experiments carried out in triplicate.

were isolated by limiting dilution and designated as 3D7^R/actD2 (clone 2.1, 2.2, or 2.3). Figure 2 shows the growth of 3D7 and one of the latter actD-resistant clones (3D7^R/actD2.3) over a 72-hr period in the absence and presence of actD. The effect of actD on the growth of the parasite was determined by direct counting of parasites in 1000 RBCs. Figure 2 shows that actD at 2 ng/mL did not inhibit the growth of 3D7^R/actD2.3 parasites, whereas 4 ng/mL inhibited their growth. By contrast, 3D7 did not grow in the presence of 0.2 ng/mL of actD (a 10-fold lower drug concentration) (Fig. 2). The extreme sensitivity of 3D7 *P. falciparum* to actD is consistent with the mode of action of the drug [30] and the high AT base composition of the genome of this parasite [41]. Moreover, as RBCs are void of nuclear DNA, intracellular host target will not deplete the actD drug concentration.

To determine the concentration of actD that inhibits the growth of 3D7 and 3D7^R/actD2.3 by 50% (IC_{50}), parasitized RBCs were grown in the absence and presence of increasing concentrations of actD, and the relative growth of parasites was determined from the incorporation of [^{3}H]hypoxanthine (see Materials and Methods). The results in Fig. 3 show a 3-fold difference in the IC_{50} of actD toward 3D7 and 3D7^R/actD2.3 (0.6 and 2 ng/mL, respectively). Differences in levels of resistance between 3D7 and 3D7^R/actD2.3 were dependent on the assay used to estimate the growth of the parasite. For example, direct counting of the parasite showed consistently higher folds of resistance between 3D7 and 3D7^R/actD2.3 than the radiolabeled hypoxanthine incorporation method (10- vs 3-fold, respectively). Thus, although it is presently not clear why these two methods of estimating the effects of this drug on the growth of *P. falciparum* yield different results, similar observations have been reported previously with other antimalarials [42, 43]. In spite of the differences between the two methods in estimating the level of resistance, *in vitro* selected *P. falciparum* display low levels of drug resistance. Chloroquine- or mefloquine-resistant *P. falciparum* showed a 2- to 3-fold increase in their IC_{50} compared with that of parental drug-susceptible parasites [13–15]. These findings are in contrast with similar studies using tumor cell lines, in which resistant clones display 10- to 400-fold resistance to the selecting drug [31]. Although the differences between the two systems are not clear and are somewhat difficult to compare, we speculate that resistance in *P. falciparum* to actD and chloroquine may involve multiple changes. This possibility is supported by the fact that chloroquine resistance appeared after many years of drug pressure [2]. However, once developed, resistant parasites appear to require less time to acquire resistance to other drugs or to display multiple drug resistance.

Reduced Sensitivity of 3D7^R/ActD2.3 to Antimalarials

To characterize the resistance profile of the 3D7^R/actD2.3 clone further, it was of interest to examine the sensitivity of 3D7^R/actD2.3 to several antimalarials (e.g. chloroquine, mefloquine, quinine, quinidine, and artemisinin) and to Rh123. Figure 4 shows the effects of increasing concentrations of chloroquine, mefloquine, quinine, quinidine, artemisinin, and Rh123 on the growth of 3D7 and 3D7^R/actD2.3 parasites. The results in Fig. 4 show 3D7^R/actD2.3 to be less sensitive than 3D7 to chloroquine, mefloquine, or

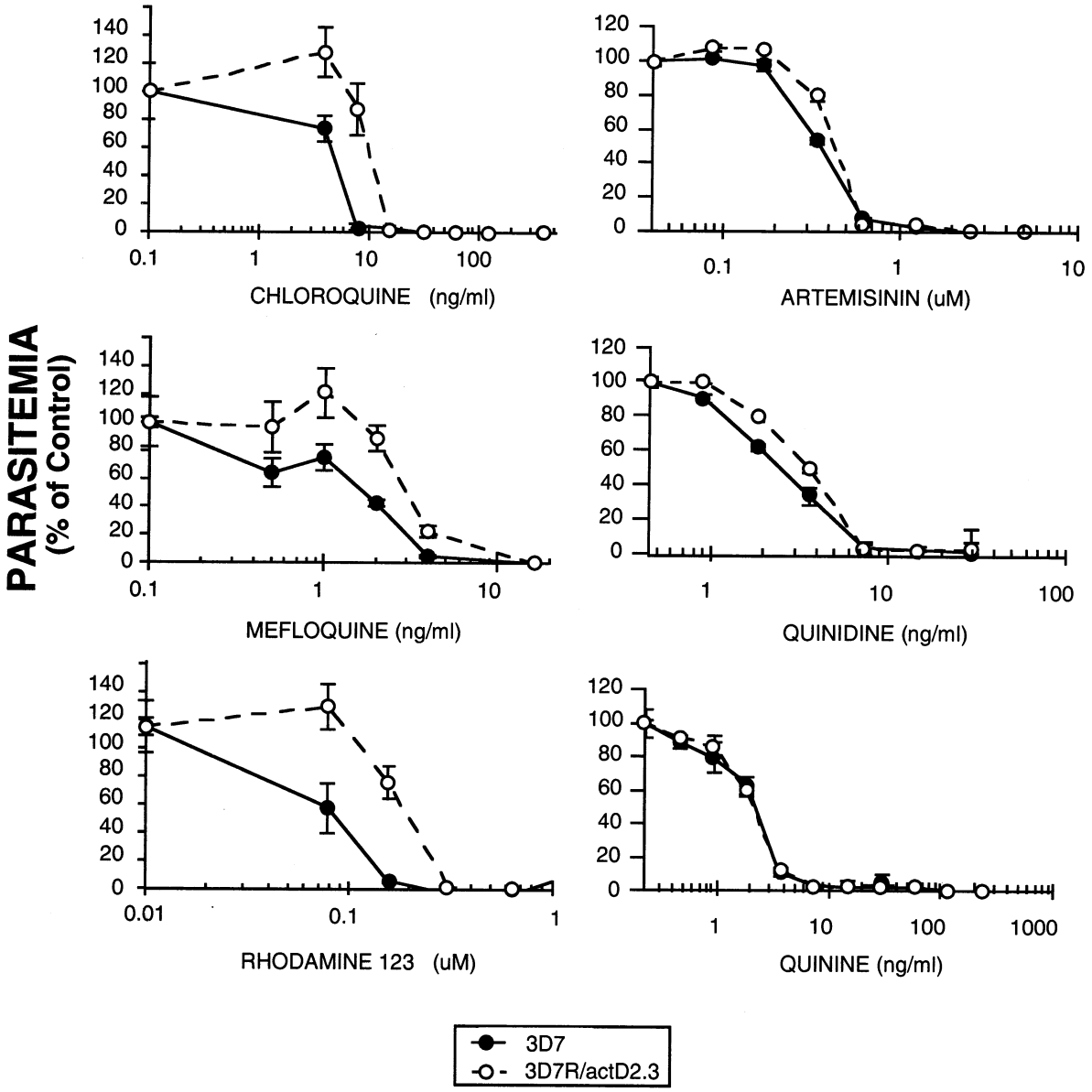

FIG. 4. Concentration–response curves for 3D7 and 3D7^R/actD2.3 in the presence of increasing concentrations of chloroquine, mefloquine, Rh123, artemisinin, quinidine, and quinine. The effects of drugs on the growth of the parasites were determined as in Fig. 3. The above values are means ± SEM of a representative of four experiments carried out in triplicate.

Rh123, and to a lesser extent resistant to quinidine or artemisinin, but not to quinine. The IC_{50} values of 3D7^R/actD2.3 for chloroquine, mefloquine, and Rh123 were 2-fold higher than those of 3D7, as measured by the radiolabelled hypoxanthine incorporation method (Fig. 4). The measured decrease in sensitivity to quinidine and artemisinin was less than 2-fold, but was observed consistently. Given the structural and functional differences between actD and the various antimalarials, the decreased sensitivity of 3D7^R/actD2 is unlikely to be due to changes in a receptor binding site. Several mechanisms have been proposed to account for the rise of pleiotropic drug resistance, such as altered drug accumulation [44], dysregulation of ion trafficking [45], and changes in the apoptotic threshold of cells [46]. The finding that resistance to actD is associated with decreased sensitivity to structurally and functionally diverse drugs, including Rh123, a model substrate for the P-glycoprotein drug efflux pump [47], is consistent with altered drug accumulation. Moreover, Rh123 has been shown to interact directly with mammalian P-glycoprotein [48] and the multidrug resistance-associated protein (MRP1*). Furthermore, *Leishmania donovani* selected for resistance to vinblastine was shown to overexpress a P-glycoprotein homologue and to mediate the transport of Rh123 [18].

Previous studies have shown that verapamil, a calcium channel blocker, reverses mdr-1-mediated multidrug resistance in tumor cells [49]. Although the mechanism of

* Daoud R, Scheper RJ and Georges E, Manuscript in preparation.

multidrug resistance reversal by verapamil is not entirely clear, verapamil has been shown to compete with cytotoxic drugs for binding to P-glycoprotein [50, 51]. The early findings that verapamil reverses chloroquine resistance have presumed a P-glycoprotein-mediated multidrug resistance in *P. falciparum* [52]. However, since different strains of *P. falciparum* (chloroquine-susceptible and -resistant) were compared in those studies, the observed effect of verapamil may have been due to differences in sensitivity between different strains of *P. falciparum* [53]. Furthermore, verapamil did not increase the sensitivity of mefloquine-resistant *P. falciparum* [6], nor did it reverse the multidrug resistance phenotype of other parasites that overexpress a P-glycoprotein homologue (e.g. *L. donovani*) [17, 18]. In this study, 3D7- or 3D7^R/actD2.3-parasitized RBCs were grown in the presence of increasing concentrations of actD or chloroquine with and without 2 or 8 μM verapamil. Interestingly, no differences were observed in the IC_{50} of actD or chloroquine toward 3D7 or 3D7R/actD2.3 in the presence of 2 μM verapamil (data not shown). Higher concentrations of verapamil (8 μM) were toxic to both susceptible and resistant parasites. Taken together, although these results do not rule out a P-glycoprotein-like mechanism in 3D7^R/actD2 *P. falciparum*, nonetheless they are consistent with mounting evidence for the ineffectiveness of verapamil as a reversing drug for resistant *P. falciparum* [1, 9, 53].

Uptake of [^{3}H]ActD in 3D7^R/ActD2.3

Although the results of this study showed that *in vitro* selection of actD-resistant *P. falciparum* revealed pleiotropic drug resistance not reversible by verapamil, it was of interest to determine if the observed decrease in drug sensitivity of 3D7^R/actD2.3 was due to decreased drug accumulation. Drug transport studies were performed comparing drug uptake between 3D7 and 3D7^R/actD2.3 parasites, using [^{3}H]actD. Figure 5A shows the steady-state accumulation of [^{3}H]actD (10 nM) in 3D7, 3D7^R/actD2.3, and uninfected RBCs following a 30-min incubation at 37° (see Materials and Methods). The accumulation of [^{3}H]actD in 3D7 increased to almost double the drug concentration present at 0-min incubation. In contrast, the actD-resistant clone, 3D7^R/actD2.3, accumulated much less [^{3}H]actD. Uninfected RBCs did not show a significant difference in [^{3}H]actD accumulation (Fig. 5A). To determine if the reduced drug accumulation in 3D7^R/actD2.3 was due to enhanced efflux, *P. falciparum*-infected RBCs were loaded with [^{3}H]actD, and drug efflux was determined following 30 min of incubation. The results in Fig. 5B show a greater decrease of [^{3}H]actD levels in 3D7^R/actD2.3 than in 3D7. Uninfected RBCs showed a similar decrease in [^{3}H]actD levels as in 3D7^R/actD2.3-infected RBCs (Fig. 5B). Interestingly, depletion of ATP pools by incubation with 10 mM 2-deoxyglucose and 100 nM sodium azide potentiated the accumulation of [^{3}H]actD in 3D7^R/actD2.3 to the same level as that in 3D7. Taken together, these results showed that 3D7^R/actD2.3 parasites accumulated less [^{3}H]actD than the parental 3D7 parasites and that the accumulation of drug was energy-dependent. Ongoing studies to address the drug transport mechanism in 3D7^R/actD2.3 will examine the initial rates of drug accumulation and efflux, in addition to the effects of exogenously added drugs on [^{3}H]actD transport. The identification of another energy-dependent multidrug efflux mechanism in tumor cells that is mediated by multidrug resistance-associated protein (MRP1; [54]), which is not reversed by verapamil [55], may be responsible for the observed energy-dependent transport of actD in 3D7^R/actD2.3. Moreover, we have demonstrated recently that chloroquine binds to and is exported from tumor cell lines that overexpress MRP1 [56].

Pfmdr1 Expression in ActD-Resistant P. falciparum

As indicated earlier, results from several studies have demonstrated a correlation between *Pfmdr1* (an *mdr1* homologue) expression and resistance to mefloquine, halofantrine, and quinine in *P. falciparum* [6, 13, 57]. To determine if *Pfmdr1* is involved in the resistance phenotype of 3D7^R/actD2.3, *Pfmdr1* copy number and level of expression were compared between 3D7^R/actD2.3 and the parental 3D7 *P. falciparum*. The chloroquine-resistant isolate, FAC8–15, was used as a positive control, since it was shown previously to contain three copies of the *Pfmdr1* gene and to express high levels of its mRNA [7]. Genomic DNA from 3D7, FAC8–15, and 3D7^R/actD2.3 were digested with *Eco*RI, separated by agarose gel electrophoresis, transferred, and probed with a 413-bp cDNA fragment that encodes sequences between transmembranes 11 and 12 (3331–3744 bp) [36] of *Pfmdr1*. Figure 6A shows a representative Southern blot whereby a 9-kb *Eco*RI fragment that hybridized to the *Pfmdr1* probe was found in all three cell lines. FAC8–15 showed an extra *Eco*RI fragment of approximately 6 kb, not present in either 3D7 or 3D7^R/actD2.3 (Fig. 6A). This fragment corresponds to the internal junction of the *Pfmdr1* amplicon [36]. Although some differences in the intensity of the 9-kb fragment were seen in 3D7 and 3D7^R/actD2.3, they were not reproducible. Furthermore, similar results were also obtained using another cDNA probe that encodes sequences in the ATP binding domain of *Pfmdr1* (from 1732 to 2280 bp; data not shown). Equal DNA loading was confirmed by subsequent reprobing of the same Southern blot with an α-tubulin-specific probe from *P. falciparum* [37].

To compare the levels of *Pfmdr1* expression among the above three cell lines of *P. falciparum*, northern blot analysis was performed using 10 μg of total RNA from 3D7, FAC8–15, and 3D7^R/actD2.3 trophozoites isolated from synchronized cultures. Figure 6B shows a northern blot probed with the *Pfmdr1* specific probe used earlier (transmembrane 11 to 12). These results showed high levels of *Pfmdr1* expression in FAC8–15 but not in 3D7 or 3D7^R/actD2.3. Figure 6B shows an ethidium bromide-stained blot with ribosomal RNA, indicating equal loading of RNA.

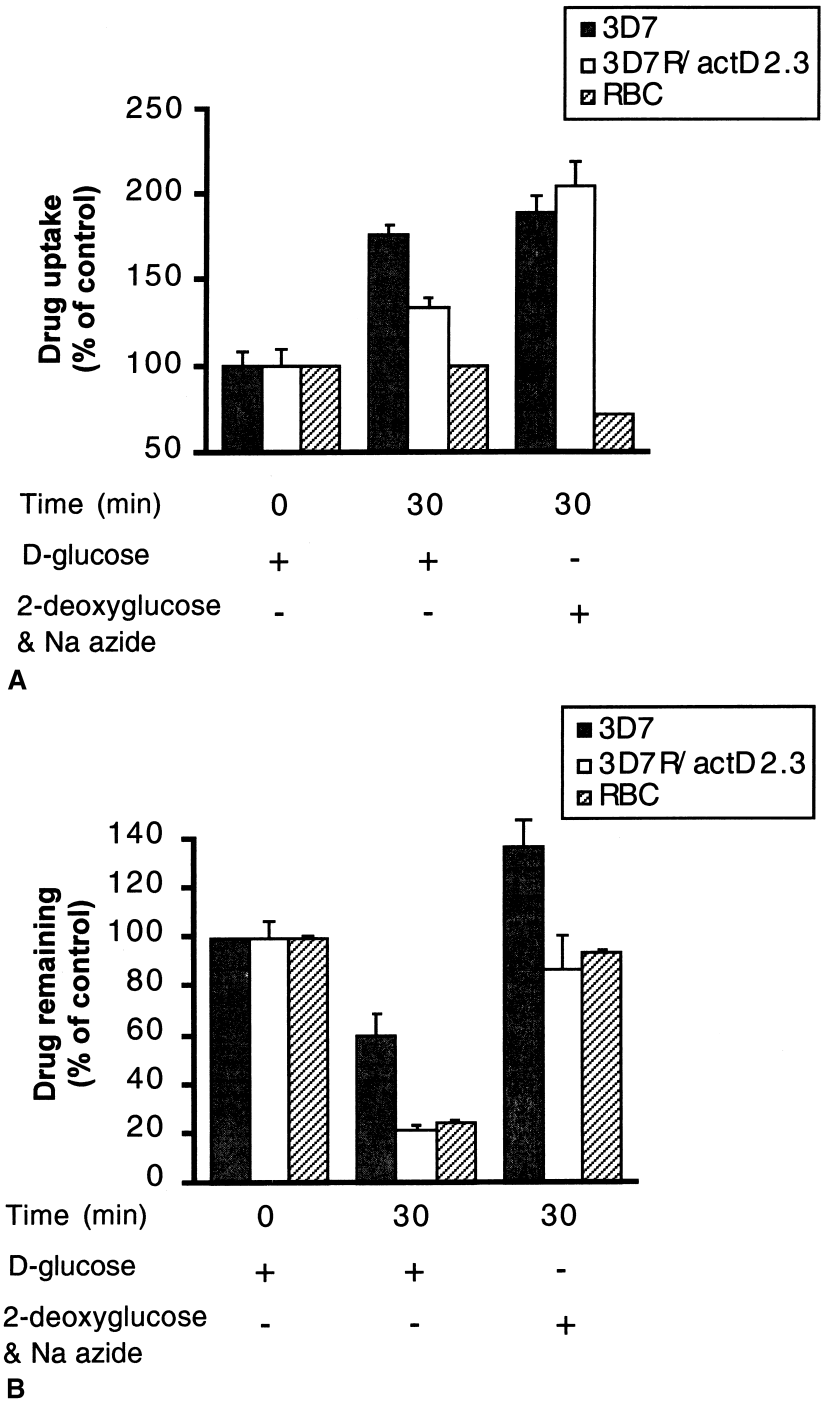

FIG. 5. Drug transport with 3D7 and 3D7^R/actD2.3 *P. falciparum*. Parasite cultures of 3D7 and 3D7^R/actD2.3 were incubated at 37° for 30 min with 10 nM [^{3}H]actD in PBS in the presence of 10 mM D-glucose or 10 mM 2-deoxyglucose and 100 nM sodium azide. Drug accumulation in 3D7, 3D7^R/actD2.3, and RBCs is expressed as percent of control (3460, 2910, and 2505 cpm, respectively). For drug efflux, 3D7 and 3D7^R/actD2.3 infected cultures were preincubated with 10 nM [^{3}H]actD in the presence of 10 mM 2-deoxyglucose and 100 nM sodium azide for 30 min prior to washing the parasites with ice-cold PBS. The amounts of [^{3}H]actD remaining in 3D7, 3D7^R/actD2.3, and RBCs were determined after a 30-min incubation at 37° in PBS containing 10 mM D-glucose and expressed as percent of control (5450, 5980, and 1790 cpm, respectively). [^{3}H]ActD accumulation values are the means ± SEM of a representative of three experiments carried out in triplicate.

The absence of increased mRNA levels of *Pfmdr1* in 3D7^R/actD2.3 suggests that the latter gene product is unlikely to mediate the resistance to actD. Although these results point to the lack of involvement of *Pfmdr1* in actD resistance, earlier reports have suggested that several point mutations in *Pfmdr1* (Asn86Tyr, Ser1034Cys, Asn1042Asp, and Asp1246Tyr) may be important to chloroquine or mefloquine resistance [38]. To address this possibility, the regions

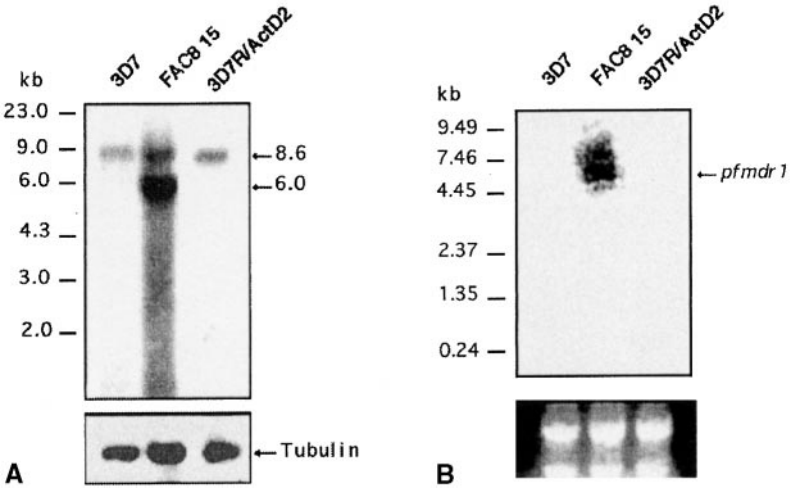

FIG. 6. Pfmdr1 in 3D7, 3D7^R/actD2.3, and FAC8–15 *P. falciparum*. For Southern blot analysis, 10 μg of DNA from 3D7, FAC8–15, or 3D7^R/actD2.3 *P. falciparum* was digested with *Eco*RI, transferred to Hybond-N nylon membrane, and probed separately with a *Pfmdr1*-specific probe (A) or an α-tubulin-specific probe. For northern blot analysis, 10 μg of total RNA from synchronized 3D7, FAC8–15, and 3D7^R/actD2.3 was fractionated, electrophoresed, and transferred to a membrane, followed by hybridization with a *Pfmdr1* probe (B). The ethidum bromide-stained gel is shown as a control for loading. The migration of the DNA or RNA marker bands is indicated to the left of panels A and B.

encoding these amino acid changes were cloned from 3D7 and 3D7^R/actD2.3 by PCR, and their nucleotide sequences were compared. Our results showed identical sequences for 3D7 and 3D7^R/actD2.3 (Asn86, Tyr184, Ser1034, Asn1042, and Asp1246; data not shown). In fact, the relevance of point mutations in *Pfmdr1* to drug resistance is controversial [57–60]. Taken together, our results demonstrated that *Pfmdr1* was not involved in the expression of the drug-resistance phenotype of 3D7^R/actD2.3.

In conclusion, *in vitro* selection of *P. falciparum* resistant to actD has led to the isolation of parasites that display pleiotropic resistance to several drugs, including many antimalarials. Moreover, although the mechanism of drug resistance in 3D7^R/actD2.3 is presently not clear, drug transport studies showed that 3D7^R/actD2.3 accumulated less [^{3}H]actD and that this reduced accumulation was reversed by agents that deplete ATP pools. The reduced sensitivity of 3D7^R/actD2.3 to chloroquine and other quinoline-based drugs is interesting and may be due to an ATP-dependent transporter. However, it is equally possible that other mechanisms mediate the resistance to actD and/or quinoline-based antimalarials. For example, it will be of interest to determine if recently identified proteins associated with chloroquine [26] or quinoline-drug resistance [29] are altered between 3D7 and 3D7^R/actD2.3. Work is in progress to identify the molecular changes that confer the multidrug resistance phenotype on 3D7^R/actD2.3.

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