

Antimalarial Effects of Macrolactones Related to FK520 (Ascomycin) Are Independent of the Immunosuppressive Properties of the Compounds

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The polyketide macrolactone FK506 inhibits the growth of *Plasmodium falciparum* in culture and the enzymatic (peptidyl-prolyl *cis-trans* isomerase [PPIase]) and chaperone activities of a recently identified *P. falciparum* FK506-binding protein (PffFKBP35). However, the potent immunosuppressive properties of FK506 exclude it from consideration as an antimalarial drug. We describe the antimalarial actions of the related compound FK520 and a number of its nonimmunosuppressive analogues. All compounds were shown to be strong inhibitors of parasite growth, regardless of their immunosuppressive potency. Although some of the compounds inhibited the PPIase activity of recombinant PffFKBP35, they all inhibited the chaperone activity of this bifunctional protein. These findings suggest that the antimalarial effects of this class of drug may be mediated via inhibition of the chaperone activity rather than via the enzymatic activity of PffFKBP35. Elucidating the precise intracellular functions of PffFKBP35 may facilitate the design of more potent inhibitors that retain their specificity for parasite target protein.

Malaria remains one of the most significant human diseases worldwide. It is estimated that ~40% of the world's population is currently at risk of contracting the disease, resulting in an estimated 500 million clinical cases annually [1]. This number has been predicted to double in 20 years unless new methods of control are devised and implemented [2]. The causative agent of malaria in humans is 1 of 4 species of *Plasmodium*, with *P. falciparum* causing both the most severe and most common forms of the disease [3]. Of the limited number of drugs available, more and more are becoming obsolete as the parasites rapidly develop resistance

[4]. Much hope has been placed in the development of vaccines [5], but the high degree of antigenic variation exhibited by the parasites is a difficult hurdle to surmount. In the absence of effective, long-term vaccines, drug development remains fundamental in the fight against malaria.

It has previously been shown that FK506 (tacrolimus) and rapamycin (sirolimus), drugs that are commonly used because of their potent immunosuppressive activities [6], have activity against *P. falciparum* in culture [7]. Little is known about the antimalarial mode of action of these drugs, but their mechanisms of immunosuppression in humans have been described in some detail [8, 9], and it is possible that an analogous pathway in *P. falciparum* may result in growth inhibition. This idea has formed the basis for the study of the antimalarial activities of these drugs.

Central to FK506/rapamycin-induced immunosuppression is a 12-kDa cytosolic protein termed FK506-binding protein (hFKBP12) [10]. This is the archetypal member of the growing FKBP family of proteins. The natural function(s) of FKBP remain unclear, but the finding that they possess peptidyl-prolyl *cis-trans* isom-

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erase (PPIase) activity in vitro [11, 12] is strongly suggestive of a role in protein folding. The isomerization of specific peptidyl-prolyl bonds is, together with the formation of disulfide bonds between cysteine residues, one of the slowest steps in protein folding [10]. Certain FKBP also exhibit chaperone-like activity in vitro, which suggests that they may have a more general role in protein folding. For example, the ribosome-associated *Escherichia coli* FKBP homologue known as trigger factor exhibits both PPIase and chaperone activities and is thought to act as a cotranslational chaperone that assists in the folding of nascent polypeptides as they emerge from the peptide exit channel of the ribosome [13]. A number of FKBP have been implicated in the regulation of signal transduction mechanisms because of their association with proteins involved in signaling pathways [14–20].

Although hFKBP12 is not the only FKBP present in T lymphocytes, and although other FKBP isoforms can bind these drugs with varying affinities, hFKBP12 is the principal mediator of FK506- and rapamycin-induced immunosuppression [21]. Both FK506 and rapamycin inhibit the PPIase activity of hFKBP12, but this is apparently not their mechanism of immunosuppression. Rather, both drugs inhibit distinct secondary targets through mechanisms that are dependent on combined structural characteristics of the drug-hFKBP12 complex: the binary complex formed between FK506 and hFKBP12 within the cytosol of T lymphocytes subsequently binds to the serine/threonine protein phosphatase calcineurin [22], whereas the ultimate target of the hFKBP12-rapamycin complex is the protein serine/threonine kinase target of rapamycin (TOR) [23]. By inhibiting the phosphatase activity of calcineurin, FK506 disrupts an important signal transduction pathway that ordinarily results in the activation of T lymphocytes (i.e., the $G_0 \rightarrow G_1$ transition) on antigen presentation, whereas the rapamycin-mediated inhibition of TOR disrupts distinct signal transduction pathways involved in the proliferation (i.e., the $G_1 \rightarrow S$ transition) of activated T cells.

Both of these immunosuppressive drugs belong to the polyketide family of molecules. They are secreted secondary metabolites from the soil bacteria *Streptomyces tsukabensis* (FK506) [24] and *S. hygroscopicus* (rapamycin) [25]. A crucial consideration for the development of therapeutically useful antimalarials on the basis of the effects of these drugs is that they should lack immunosuppressive capability. FK520 is a natural analogue of FK506 produced by *S. hygroscopicus ascomyceticus* [26, 27]. FK520 differs from FK506 only by an allyl-to-ethyl change at position C-21, but this modification has little effect on the immunosuppressive nature of the compound, given that it retains the ability to bind hFKBP12, and the resulting hFKBP12-FK520 complex inhibits calcineurin. Analysis of the 3-dimensional structure of the hFKBP12-FK520 complex shows that approximately one-half of the FK520 molecule is in contact with hFKBP12, with

the remainder of the drug being solvent exposed [28]. These solvent-exposed portions of the drug, together with regions of the hFKBP12 molecule, make up a composite binding surface for calcineurin. Modifications of this solvent-exposed region, through both chemical [29] and genetic [30, 31] methods, have facilitated the design of nonimmunosuppressive analogues of FK520 that retain the ability to bind hFKBP12 but are incapable of subsequently inhibiting calcineurin.

We have recently characterized the first FKBP from *P. falciparum*, PffFKBP35 [32]. PffFKBP35 is composed of an N-terminal FKBP domain and 3 tetratricopeptide repeats (TPRs)—degenerate sequences of 34 aa that are involved in protein-protein interactions [33, 34]—and exhibits both PPIase and chaperone activities. Both of these activities of the FKBP domain of recombinant PffFKBP35 were inhibited by FK506, which suggests that the antimalarial action of FK506 may be mediated through this protein. We describe the antimalarial nature of FK520 and a number of its synthetic, nonimmunosuppressive analogues. Two of the compounds that we tested did not inhibit PffFKBP35 PPIase activity but inhibited parasite growth and the chaperone activity of the FKBP domain of recombinant PffFKBP35, which suggests that the antimalarial action may result from inhibition of the protein's chaperone activity. Because PffFKBP35 appears to be the only FKBP in the parasite and shows significant differences from any human FKBP, it may represent a valid antimalarial drug target.

MATERIALS AND METHODS

Compounds and reagents. FK520 and its analogues were prepared as described elsewhere [31]. 18-ene-20-oxa-FK520 and its 13-desmethoxy-13-methyl analogue were isolated by treatment of the C-18 hydroxylation product with acid, according to the procedure of Kawai et al. [35]. Stock solutions of each compound were prepared in dimethyl sulfoxide (DMSO). All other reagents were from Sigma Aldrich, unless otherwise stated.

Parasite culture. *P. falciparum* FCH5C.2, a clone of a chloroquine-sensitive strain, was maintained in continuous culture according to the method of Trager and Jensen [36]. The cells were maintained in human erythrocytes (Irish Blood Transfusion Services) at 5% hematocrit in RPMI 1640 medium supplemented with 25 mmol/L HEPES, 1% neomycin sulfate, 0.18% sodium bicarbonate, 50 μ g/mL hypoxanthine, and 10% horse serum that had been previously heat inactivated at 56°C for 30 min (all reagents were cell-culture grade). Parasites were cultured at 37°C in a candle jar with reduced O_2 tension.

Growth-inhibition assays. To assess the effects of FK520 and its analogues on cultured *P. falciparum*, asynchronous parasitized human erythrocytes at 0.8% parasitemia and 2% hematocrit were grown, for 72 h, in RPMI 1640 culture medium supplemented with the appropriate compound in 96-well flat-bottom microtiter plates. Drugs were diluted from stock solutions in DMSO into

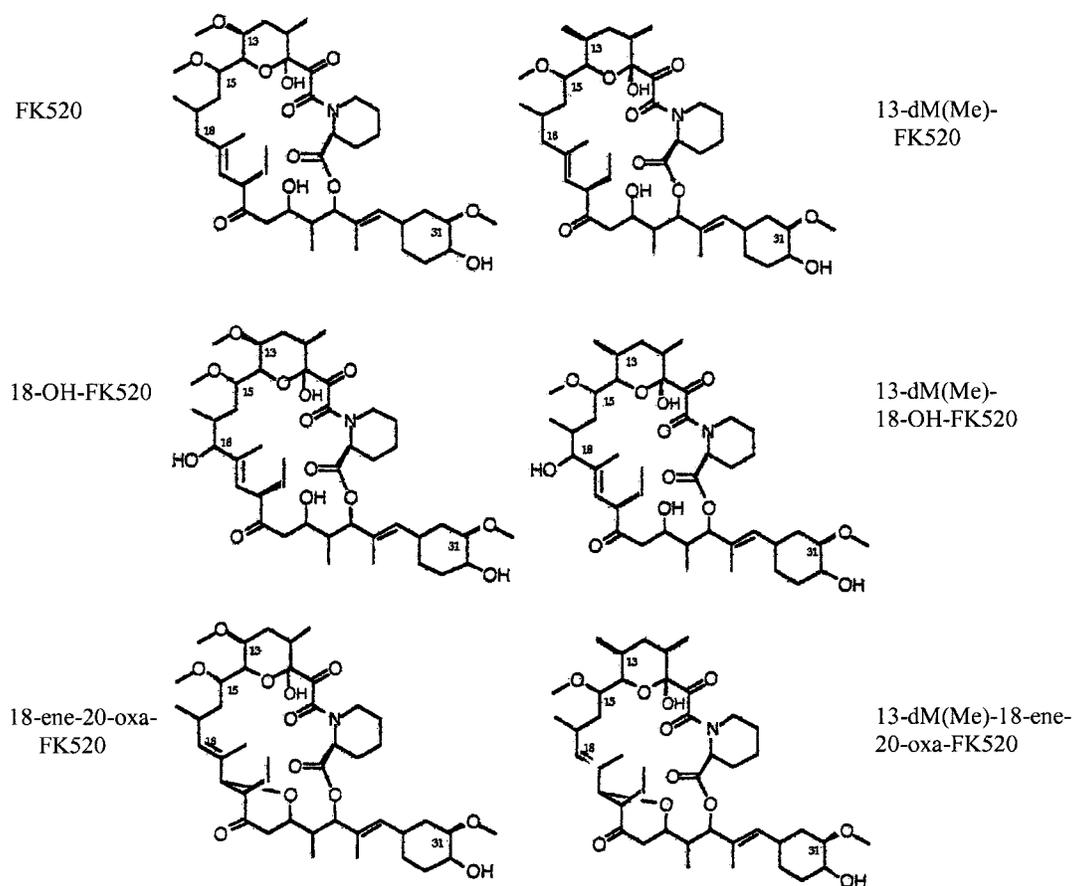


Figure 1. Structures of FK520 and its synthetic, nonimmunosuppressive analogues: 13dM(Me)-13-desmethoxy-13-methyl

culture medium and then serially diluted 2-fold, in wells of the microtiter plates, down to subinhibitory concentrations. After incubation, the effect of the compounds on parasite growth was determined by use of the parasite lactate dehydrogenase-based assay of Makler et al. [37]. Dose-response curves were constructed for each drug. The IC_{50} values were determined graphically from the respective dose-response curves.

PPIase assay. Recombinant maltose binding protein (MBP)-PfkFBP35-His₆ was produced in *E. coli* and purified to homogeneity by sequential nickel-chelate and ion-exchange chromatographies, as described elsewhere [32]. Its PPIase activity in the absence or presence of drugs was assessed by use of a standard protease-coupled assay [32]. Briefly, the *cis-trans* conversion of a chromogenic peptide substrate, which is cleaved by chymotrypsin only in its *trans* conformation, was measured spectrophotometrically. The concentration of enzyme was 0.25 $\mu\text{mol/L}$, the assay buffer consisted of 50 mmol/L HEPES and 100 mmol/L NaCl (pH 8.0), and the assay temperature was 0°C (to minimize the nonenzymic background isomerization). In assays in which drugs were included, they were added as 1- μL volumes of 1000 times the desired concentration (final concentration, 0.05–5 $\mu\text{mol/L}$), prepared in DMSO. One microliter

of solvent alone served as a control. The IC_{50} values of PPIase activity were determined graphically from the respective dose-response curves.

Metabolism of compounds by cultured parasites. The ability of parasites to metabolize 18-ene-20-oxa-FK520 and 13-dM(Me)-18-ene-20-oxa-FK520 into other forms was assessed by treating *P. falciparum* cultures with 5 $\mu\text{mol/L}$ of these compounds for 48 h ($\sim IC_{50}$). Cultures were transferred to microfuge tubes and centrifuged, and the pellets were frozen at -70°C in preparation for analysis by high-resolution mass spectrometry, as described elsewhere [31]. Cells treated identically but exposed to FK520 or 13-dM(Me)-FK520 served as controls.

Chaperone assays. MBP-FKBP-His₆ was produced and purified as described elsewhere [32]. The thermal denaturation of pig heart mitochondrial citrate synthase and bovine liver rhodanase was achieved essentially as described elsewhere [32]. Briefly, citrate synthase (1.5 $\mu\text{mol/L}$ monomer) was incubated at 43°C in 40 mmol/L HEPES (pH 7.5) for 30 min, and aggregation during the denaturation process was measured by monitoring the increase in absorbance at 360 nm in a Shimadzu UV-1601PC spectrophotometer with a thermostatted cuvette holder, by use of a quartz microcuvette. Rhodanase (4.4 $\mu\text{mol/}$

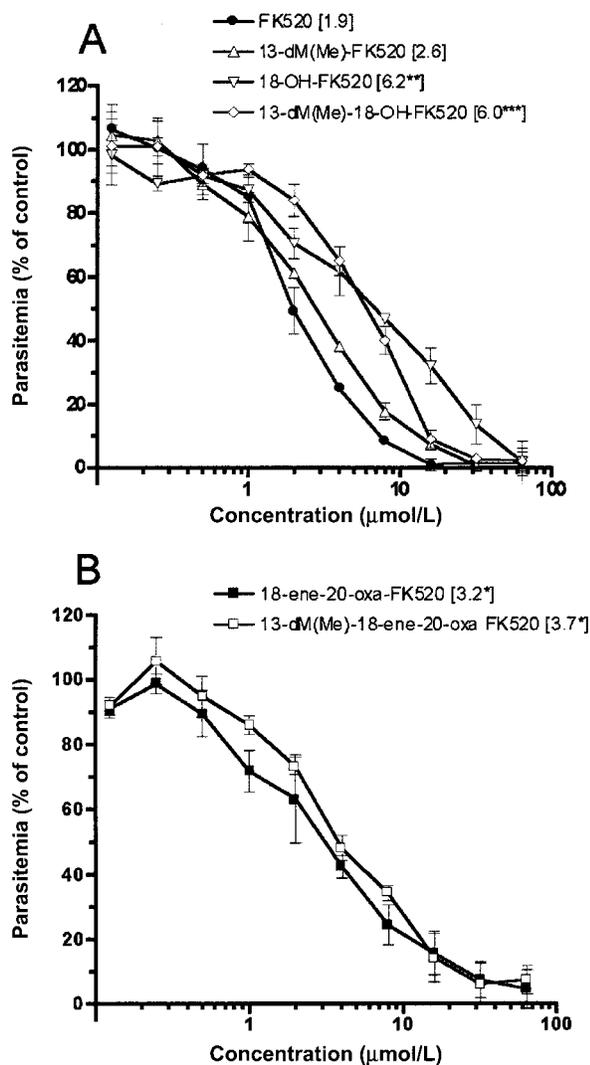


Figure 2. Inhibition of *Plasmodium falciparum* growth in culture by FK520 analogues. Cells were treated for 72 h with varying concentrations of the indicated compounds, and cell growth was assessed as described in Materials and Methods. The parasitemia for cells grown in the absence of drugs was set as 100%. *A*, Effects of compounds with high affinity for human FK506-binding protein (hFKBP12). *B*, Effects of compounds with low affinity for hFKBP12. Drug vehicle (dimethyl sulfoxide) had no effect on cell growth at concentrations used in the assay (data not shown). Values in square brackets represent the geometric mean IC_{50} (in $\mu\text{mol/L}$) for cells in culture ($n = 6$). Bars, SE. Data for each of the compounds were compared with those for FK520 by use of unpaired Student's *t* test: * $P < .05$; ** $P < .01$; and *** $P < .001$.

L) was incubated at 44°C in 40 mmol/L sodium phosphate (pH 8.0) for 30 min, and aggregation was monitored as for citrate synthase. The effects of additional components on aggregation were assessed as described in Results.

RESULTS

Five nonimmunosuppressive analogues of FK520 (figure 1) were tested for their antimalarial properties. Three of these—

13-dM(Me)-FK520, 18-OH-FK520, and 13-dM(Me)-18-OH-FK520—can bind human FKBP12, but the resulting binary complexes have low affinity for calcineurin [29, 31]. In contrast, neither 18-ene-20-oxa-FK520 nor 13-dM(Me)-18-ene-20-oxa-FK520 had measurable affinity for hFKBP12 ($K_d > 25 \text{ nmol/L}$). However, all of these compounds exhibited significant, dose-dependent inhibitory effects against *P. falciparum* growth in culture (figure 2). Compounds with the 18-OH or 18-ene-20-oxa substitutions were slightly less active than FK520, by ~3- and ~2-fold, respectively.

To investigate whether this growth inhibition was mediated by an effect on the enzymatic activity of PfFKBP35, the effects of these compounds on the PPIase activity of a recombinant form of PfFKBP35, fused at the N-terminus to MBP and at the C-terminus to oligo(His)₆ (MBP-PfFKBP35-His₆) [32] were measured. All of the compounds known to interact with hFKBP12 were shown to bind to and inhibit the PPIase activity of MBP-PfFKBP35-His₆ (figure 3). The 2 compounds that do not interact with hFKBP12, by contrast, caused only a minor reduction in the PPIase activity of PfFKBP35.

The lack of PPIase inhibitory action by the 18-ene-20-oxa compounds did not correlate with their antimalarial activity. We postulated that these compounds might be incapable of interfering with PfFKBP35 function until they are metabolized into “active,” FKBP-binding forms (e.g., by cleavage of the ether bridge between C-20 and C-24) within the parasite. Therefore, lysed parasites from cultures that had been treated with sublethal concentrations of 18-ene-20-oxa-FK520 or 13-dM(Me)-

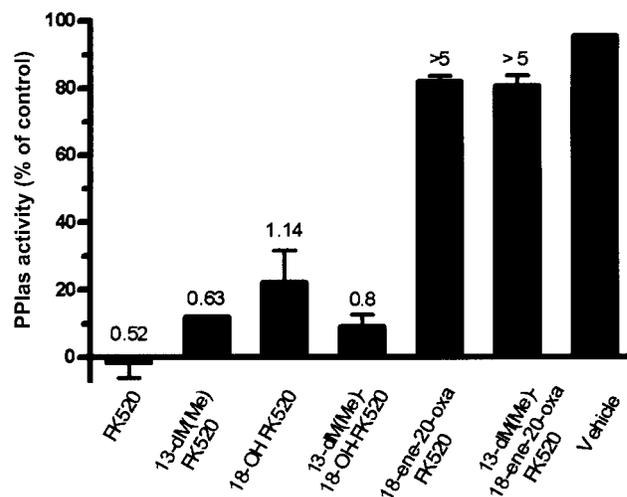


Figure 3. Inhibition of peptidyl-prolyl *cis-trans* isomerase (PPIase) activity of maltose binding protein (MBP)-PfFKBP35-His₆ by FK520 and its analogues. The activity of 0.25 $\mu\text{mol/L}$ MBP-PfFKBP35-His₆ was assayed at a fixed concentration of inhibitor (5 $\mu\text{mol/L}$). The activity of 0.25 $\mu\text{mol/L}$ MBP-PfFKBP35-His₆ in the absence of inhibitor was set to 100%. IC_{50} values (in $\mu\text{mol/L}$) for PPIase activity, determined as described in Materials and Methods, are given above the respective columns. Bars, SEs of 3 replicates.

18-ene-20-oxa-FK520 were analyzed by mass spectrometry. The starting compounds were detected at concentrations comparable to those of the *P. falciparum*-free control, and no metabolites were detected down to the lower limit of detection (5 ng/mL; data not shown).

PfFKBP35 is a bifunctional protein that displays PPIase activity and chaperone activity [32]. This chaperone activity was shown by the protein's ability to inhibit the thermal aggregation of 2 model substrates, citrate synthase and rhodanese. This activity is conferred by both the N-terminal (FKBP) and C-terminal (TPR) domains of PfFKBP35. FK506 was shown to have no measurable effects on the chaperone activity of either the full-length protein or the isolated C-terminal domain but was shown to have significantly inhibited the chaperone activity of the isolated N-terminal domain. The effects of the FK520 analogues on the chaperone activity of both full-length PfFKBP35 (MBP-PfFKBP35-His₆) and the isolated FKBP domain (MBP-FKBP-His₆) were assessed in both model systems (figures 4 and 5). None of the FK520 analogues had any effect on the ability of the full-length protein MBP-PfFKBP35-His₆ to prevent the thermal aggregation of either citrate synthase or rhodanese. However, as was the case with FK506 [32], all compounds reduced the chaperone activity of the truncated MBP-FKBP-His₆. In the absence of inhibitors, MBP-FKBP-His₆ reduced the aggregation of citrate synthase by ~70%, whereas only a ~20% reduction in aggregation was achieved in the presence of 10-

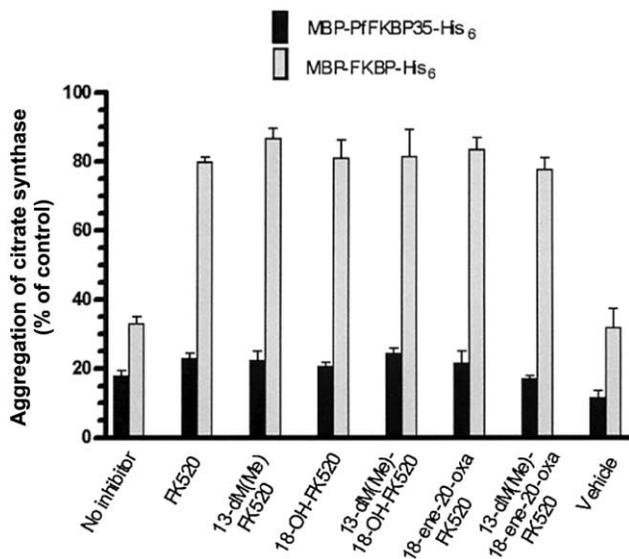


Figure 4. Inhibition of thermal aggregation of citrate synthase (1.5 $\mu\text{mol/L}$ monomer) in the presence of equimolar amounts of either full-length *P. falciparum* FK506-binding protein (PfFKBP35) (maltose binding protein [MBP]-PfFKBP35-His₆) or an isolated FKBP domain (MBP-FKBP-His₆) and effects of inhibitors (15 $\mu\text{mol/L}$) on chaperone activities. The maximum absorbance at 360 nm for citrate synthase in the absence of additional components was set to represent 100% aggregation. Bars, SEs of 3 replicates.

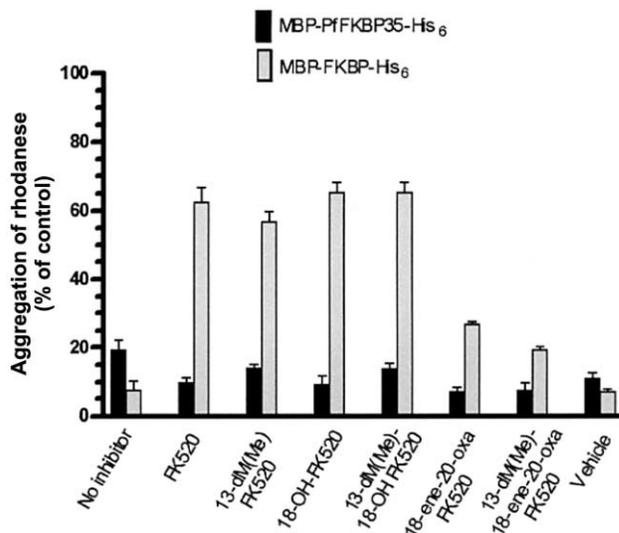


Figure 5. Inhibition of thermal aggregation of rhodanese (4.4 $\mu\text{mol/L}$) in the presence of equimolar amounts of either full-length *P. falciparum* FK506-binding protein (PfFKBP35) (maltose binding protein [MBP]-PfFKBP35-His₆) or an isolated FKBP domain (MBP-FKBP-His₆) and effects of inhibitors (22 $\mu\text{mol/L}$) on chaperone activities. The maximum absorbance at 360 nm for rhodanese in the absence of additional components was set to represent 100% aggregation. Bars, SEs of 3 replicates.

fold molar excesses of any of the inhibitors (figure 4). For rhodanese, 5-fold molar excesses of FK520, 13-dM(Me)-FK520, 18-OH-FK520, and 13-dM(Me)-18-OH-FK520 allowed MBP-FKBP-His₆ to reduce aggregation by only ~40%, compared with >90% in the absence of inhibitors (figure 5). The effects of 18-ene-20-oxa-FK520 and 13-dM(Me)-18-ene-20-oxa-FK520 were intermediate between these extremes (figure 5). None of the inhibitors alone had any effect on the aggregation of either citrate synthase or rhodanese (data not shown).

DISCUSSION

The previously reported antimalarial properties of FK506 [7] prompted us to test nonimmunosuppressive derivatives for their inhibitory effects on parasite growth and PfFKBP35 activity. Five nonimmunosuppressive analogues of FK520 (a natural analogue of FK506, with an allyl-to-ethyl substitution at C21 but similar immunosuppressive properties) were tested, and all exhibited potent inhibitory effects against *P. falciparum* in culture.

On the basis of the hypothesis that PfFKBP35, the only FKBP identified in the proteome of *P. falciparum*, is the target for FK506 and its nonimmunosuppressive analogues, there are at least 2 possible models by which these drugs could exert their antimalarial effects. One such model, by analogy with the current models of the immunosuppressive and antifungal actions of FK506/FK520 and rapamycin, is that the compounds bind first to PfFKBP35, then the FKBP-ligand binary complex in-

hibits an essential parasite target. For FK506/FK520-induced immunosuppression, the target of this pathway in T lymphocytes is calcineurin [22], whereas the hFKBP12/rapamycin complex inhibits the kinase TOR [23]. The antifungal mechanisms of FK506 and rapamycin are likewise mediated via FKBP-ligand-calcineurin or FKBP-ligand-TOR ternary complexes, respectively [38–41]. A calcineurin-like sequence is present in the *P. falciparum* genome database (<http://www.plasmodb.org>), and activity attributable to a calcineurin-like phosphatase has been identified in parasite extracts [42]. However, we have shown that recombinant PffFKBP35 inhibits the phosphatase activity of bovine calcineurin in vitro in the absence of FK506 [32]. Furthermore, it seems unlikely that the *P. falciparum* calcineurin is involved in mediating the antimalarial effects of these drugs, because each analogue has a different structural modification in the effector domain, yet all retain antimalarial potency of approximately the same order.

A second possible model by which these inhibitors could exert their antimalarial effects is through direct inhibition of PffFKBP35's cellular activity. The obvious mechanism for such a model is that the polyketides inhibit the protein's PPIase activity. However, 2 of the compounds tested showed very little inhibition of the PPIase activity of recombinant PffFKBP35. These 2 compounds, 18-ene-20-oxa-FK520 and 13-dM(Me)-18-ene-20-oxa-FK520, were the only ones tested that were unable to bind hFKBP12. This led us to conclude that the antimalarial action of all the analogues is not mediated through inhibition of PffFKBP35's PPIase activity. A similar conclusion was drawn for the antimalarial action of cyclosporin A (CsA), another immunosuppressive drug that inhibits the activity of a distinct class of PPIase enzymes, the cyclophilins. The finding that certain nonimmunosuppressive derivatives of CsA are potent antimalarials, even though they lack anti-PPIase action, showed that the antimalarial mechanism of this class of drugs is probably not a direct result of PPIase inhibition [43]. This was also shown for the antischistosomal effects of cyclosporins [44].

We have recently reported a second activity of PffFKBP35 [32]. PffFKBP35 exhibits chaperone activity, in that it inhibits the thermal aggregation in vitro of 2 model substrates, citrate synthase and rhodanese. Such activity has been shown for certain FKBP's from other organisms [45–48]. Both the N-terminal (FKBP domain) and C-terminal (TPR domain) portions of the PffFKBP35, produced as isolated polypeptides, were shown to contribute to this activity. None of the FK520 analogues had any significant effects on the chaperone activity of the full-length PffFKBP35. However, when the isolated FKBP domain was used in place of the full-length protein, all compounds showed significant inhibitory effects. All compounds were equi-potent inhibitors of the chaperone activity of the FKBP domain when citrate synthase served as the model substrate, but, when rhodanese was used, the effects of the 18-ene-20-oxa com-

pounds were less pronounced than those of the other analogues. The difference in the inhibition profiles obtained with these 2 systems may be a result of experimental differences—for example, in the citrate synthase assays, the ratio of compound to FKBP was 10:1, but it was only 5:1 for rhodanese assays—or a result of differences in the intrinsic properties of the 2 substrates. The finding that the compounds had no effect on the chaperone activity of the full-length protein is most probably due, as in the case of FK506 [32], to the chaperone activity of the C-terminal portion of PffFKBP35 being unaffected. This indicates that these compounds interact with PffFKBP35 at the N-terminal FKBP domain, as expected.

The data presented here suggest that the antimalarial mode of action of this class of compounds may be mediated through an effect on PffFKBP35 but probably not via the inhibition of the protein's PPIase activity. Our findings indicate that inhibiting the ability of PffFKBP35 to act as a chaperone may be the primary mechanism of action of the FK506/FK520 class of drugs against *P. falciparum*, and this may, in fact, represent the primary intracellular function of PffFKBP35. The mechanism by which trigger factor functions in the folding of newly synthesized polypeptides in *E. coli* is not well understood, but Kramer et al. [49] have recently shown that it is independent of its intrinsic PPIase activity. Indeed, the biological significance of the PPIase activity of certain other FKBP's remains controversial, because their functions appear to be independent of enzymatic activity [16, 47, 50–54].

It is possible, however, that the effects of the compounds are not mediated directly through inhibition of the chaperone activity of PffFKBP35 per se but, rather, through an indirect effect caused by loss of activity of a specific, essential parasite protein whose activity is dependent on PffFKBP35. For example, hFKBP52 has been implicated in the targeted movement of steroid receptors to their sites of action in the nucleus [53]. The C-terminal region of hFKBP52, which contains 3 TPR motifs, directs its association with the steroid receptor heterocomplex, whereas its N-terminal PPIase domain directs its interaction with dynein, the microtubule-associated motor protein involved in retrograde transport. Interestingly, the interaction of the hFKBP52 PPIase domain with dynein is independent of hFKBP52's intrinsic PPIase activity. This arrangement of an N-terminal PPIase domain followed by a C-terminal tripartite TPR domain is strikingly similar to the domain architecture of PffFKBP35. Work in our laboratory is currently focused on identifying intracellular binding partners for PffFKBP35, because this may well hold the key to elucidating the role of the protein in the parasite and the precise mechanism of action of these drugs. By further dissecting the mode of action of this class of drugs (18-ene-20-oxa-FK520 and 13-dM(Me)-18-ene-20-oxa-FK520 in particular), more potent derivatives could be designed that retain their specificity for the parasite protein.

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