The antimalarial action of FK506 and rapamycin: evidence for a direct effect on FK506-binding protein PfFKBP35

PAUL MONAGHAN†, DARREN B. LENEGHAN‡, WESLEY SHAW *and* ANGUS BELL*

Department of Microbiology, School of Genetics & Microbiology, Moyne Institute, Trinity College Dublin, Ireland

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SUMMARY

FK506 and rapamycin (Rap) are immunosuppressive drugs that act principally on T-lymphocytes. The receptors for both drugs are FK506-binding proteins (FKBPs), but the molecular mechanisms of immunosuppression differ. An FK506–FKBP complex inhibits the protein phosphatase calcineurin, blocking a key step in T-cell activation, while the Rap – FKBP complex binds to the protein kinase target of rapamycin (TOR), which is involved in a subsequent signalling pathway. Both drugs, and certain non-immunosuppressive compounds related to FK506, have potent antimalarial activity. There is however conflicting evidence on the involvement of *Plasmodium* calcineurin in the action of FK506, and the parasite lacks an apparent TOR homologue. We therefore set out to establish whether inhibition of the *Plasmodium falciparum* FKBP PfFKBP35 itself might be responsible for the antimalarial effects of FK506 and Rap. Similarities in the antiparasitic actions of FK506 and Rap would constitute indirect evidence for this hypothesis. FK506 and Rap acted indistinguishably on: (i) specificity for different intra-erythrocytic stages in culture, (ii) kinetics of killing or irreversible growth arrest of parasites and (iii) interactions with other antimalarial agents. Furthermore, PfFKBP35's inhibitory effect on calcineurin was independent of FK506 under a range of conditions, suggesting that calcineurin is unlikely to be involved in the antimalarial action of FK506.

Key words: malaria, *Plasmodium*, antimalarial, FK506, rapamycin, FKBP, calcineurin, drug interaction.

INTRODUCTION

Of more than 5000 proteins encoded by the genome of the most lethal human malarial parasite *Plasmodium falciparum*, perhaps several hundred have been suggested to be potential chemotherapeutic targets; yet the number of fully validated targets remains much smaller (Flannery *et al*. 2013). Even when known inhibitors of a given protein have demonstrated antimalarial activity, this observation is insufficient basis for concluding that it is the effect on that protein that leads to the death of the parasite or the arrest of its growth or development. Full validation is usually achieved only when it has been shown that genetic ablation of the proposed target achieves the same effect as the inhibitor (e.g. knock-out is lethal) and that mutations giving rise to changes in amino acid sequence of the proposed target are (in an otherwise identical genetic background) sufficient to confer resistance to the inhibitor. The limited number of well-validated targets is to some extent a consequence of the time consuming and difficult nature of carrying out the necessary genetic manipulations in *Plasmodium* parasites. In the case of the *P. falciparum* FK506-binding protein PfFKBP35 (Monaghan & Bell, 2005), we lack transgenic parasite data, but in this paper take a different approach to the question of whether inhibitors of this protein should make good antimalarial drugs.

FK506-binding proteins (FKBPs) were discovered in 1989 as the major receptors of the immunosuppressive macrolactones FK506 (tacrolimus) and rapamycin (Rap, sirolimus) (Siekierka *et al*. 1989; Bierer *et al*. 1990). They have since been found widely in biology and attributed numerous functional roles related to protein folding, regulation and signal transduction (Galat, 2003). FK506 and Rap block the characteristic peptidyl-prolyl *cis–trans* isomerase (PPIase) activity displayed by most FKBPs, but their immunosuppressive effects are not a direct result of this inhibition; rather, the drug–FKBP complexes target separate proteins involved in signalling, namely the phosphoprotein phosphatase calcineurin (PPP3) in the case of FK506 and the protein kinase mammalian target of rapamycin (mTOR) in the case of Rap (Bierer *et al*. 1990; Galat, 2003). Uncannily, the lipophilic, cyclic undecapeptide cyclosporin A (CsA) affects the same target as FK506, also in the form of a complex with its receptor, cyclophilin, which in spite of being unrelated in structure to the FKBPs is also a PPIase (Galat, 2003). FK506 and CsA are therefore commonly referred to as calcineurin inhibitors, but in some cases their pharmacological activities are unrelated to calcineurin (Galat & Bua, 2010; Harikishore & Yoon, 2015). In fact,

* Corresponding author: Institute of Cancer Research, 123 Old Brompton Rd., London SW7 3RP, UK. E-mail: angus.bell@icr.ac.uk
† Present address: Evidence Action, Hanoi, Vietnam.
‡ Present address: Jenner Institute, ORCRB, Roosevelt Drive, Headington, Oxford OX3 7DQ, UK.

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non-immunosuppressive congeners of FK506 and CsA have been under investigation as potential therapies for diseases as diverse as cancer, neurodegeneration, hepatitis C infection and malaria (Bell *et al*. 2006; Cao & Konsolaki, 2011; Gaali *et al*. 2011; Frausto *et al*. 2013; Harikishore & Yoon, 2015).

FK506, Rap and CsA, all of which were originally discovered as antimicrobial agents, have high nano- to low micromolar potency against cultured blood-stage *P. falciparum* parasites (Bell *et al*. 1994). The parasite possesses an FKBP (PfFKBP35) (Braun *et al*. 2003; Kumar *et al*. 2005; Monaghan & Bell, 2005), a number of cyclophilins and cyclophilin-like proteins (Hirtzlin *et al*. 1995; Reddy, 1995; Berriman & Fairlamb, 1998; Gavigan *et al*. 2003; Marín-Menéndez & Bell, 2011), and a calcineurin (Dobson *et al*. 1999; Kumar *et al*. 2004). Some researchers have assumed that these drugs exert their antimalarial actions via calcineurin (Kotaka *et al*. 2008; Singh *et al*. 2014). This appears to be the case for certain other antimicrobial activities such as that on *Cryptococcus neoformans* (Juvvadi *et al*. 2016). There are however several problems with this idea, including (i) the potent antimalarial activities of non-immunosuppressive and non-calcineurin-binding congeners of FK506 and CsA (Bell *et al*. 1994; Monaghan *et al*. 2005) and (ii) the unusual, FK506-independent calcineurin-binding property of PfFKBP35 (Kumar *et al*. 2005; Monaghan & Bell, 2005) (not found by Yoon *et al*. (2007)). Moreover, *Plasmodium* lacks an obvious mTOR homologue (although it does have related kinases), suggesting that macrolactones target this parasite by another route.

In view of these reservations, and of recent articles describing the design and evaluation of non-immunosuppressive, non-macrolactone PfFKBP35 inhibitors with potent activity on blood-stage parasites (Harikishore *et al*. 2013*a*, *b*), we decided to explore a simpler hypothesis – namely that the relevant target of FK506, Rap, and other FKBP ligands is PfFKBP35 itself. We reasoned that if PfFKBP35 were the antimalarial target, the pharmacological actions of these two drugs on cultured parasites should be similar, relative to their potencies. If on the other hand calcineurin were the target of FK506 and a hitherto unknown protein (e.g. kinase) the target of Rap, then it should be possible to distinguish the effects of the two drugs. In assays of specificity for different developmental stages, kinetics of killing (or irreversible growth arrest), and interaction with other antimalarial agents, we found it impossible to differentiate between FK506 and Rap. Our data are therefore consistent with the notion of PfFKBP35 as the primary mediator of antimalarial action and underscore the need for genetic validation of this possible drug target.

MATERIALS AND METHODS

Drugs, experimental compounds and reagents

Drugs and chemicals were obtained from Sigma Aldrich (Dublin) unless otherwise stated.

Calcineurin phosphatase assay

Binding of PfFKBP35 to calcineurin was measured via protein serine/threoine phosphatase assay of the latter, basically as described by Monaghan & Bell (2005) except that the concentrations of some of the reagents were varied as described under ‘Results’. Calcineurin (recombinant human) was supplied by Enzo Life Sciences (Exeter, U.K.) and the assay kit by Promega (MyBio Ltd., Kilkenny, Ireland). The recombinant FK506-binding domain of PfFKBP35, PfFKBD, was produced in *Escherichia coli* as described in Monaghan & Bell (2005) with the following modifications. After overexpression, *E. coli* lysis and binding steps performed as described previously, the nickel chelate affinity chromatography column (HisTrap® HP 5 mL, GE Life Sciences) was washed with metal chelate affinity chromatography (MCAC) buffer (25 mM sodium phosphate, 500 mM NaCl, pH 7·4) supplemented with 75 mM imidazole and recombinant protein was eluted with MCAC buffer supplemented with 200 mM imidazole.

Parasite culture and susceptibility testing

Plasmodium falciparum line 3D7 was cultured in human erythrocytes using standard methods as previously described (Fennell *et al*. 2006). Drug susceptibility measurement using the parasite lactate dehydrogenase (pLDH) method was done as by Cunningham *et al*. (2008) and drug combinations tested using a chequerboard arrangement according to Gavigan *et al*. (2007). At least two to three replicate determinations were made. Combination data analysis was generated by the Horizon Chalice™ Analyzer software, available online at http://cwr.horizondiscovery.com. For assays of specificity for different stages, cultures were age-selected by two treatments with sorbitol (Lambros & Vanderberg, 1979) 36 h apart then grown to the appropriate age range before measuring drug susceptibility. To assess whether the inhibitory effects of the drugs were reversible and to examine the kinetics of killing (or irreversible arrest of growth), mature trophozoite [24–30 h post-invasion (p.i.)] cultures of starting parasitaemia 0·8% were grown with 30 μM FK506, 30 μM Rap or drug-free solvent at an equivalent concentration. After different time periods the cultures were centrifuged, washed twice with pre-warmed wash medium (growth medium lacking serum substitute and antibiotic) and recultured. After 48 h of drug-free culture,

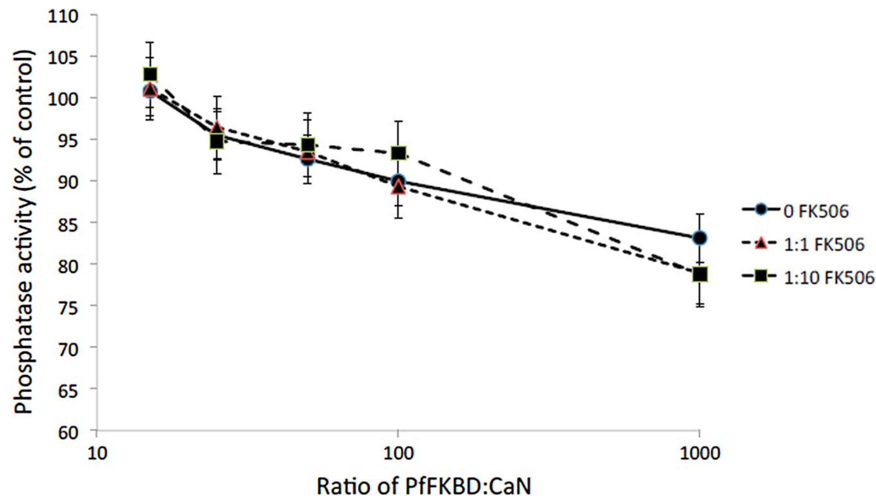

Fig. 1. Effects of different reagent ratios on calcineurin (CaN) binding by PfFKBD in the presence and absence of FK506. Activity of calcineurin was measured by the fluorescence of a rhodamine-conjugated peptide substrate that, upon dephosphorylation by calcineurin, is digested by a protease, releasing highly fluorescent rhodamine. The fluorescence attributable to 40 nM calcineurin was set as 100%. The effects of increasing molar ratio of PfFKBD to calcineurin (*x*-axis) and of PfFKBD to FK506 (circles, no FK506; triangles, 1:1 ratio; squares, 1 PfFKBD:10 FK506) on the activity of calcineurin were assessed. Bars show the SEM of values from three replicate experiments. To ensure that reduced fluorescence was not due to inhibition of the protease, a control peptide whose fluorescence is independent of phosphorylated state was incorporated into each reaction. No inhibition of protease occurred (data not shown).

Giemsa-stained thin blood smears were counted microscopically.

RESULTS

Calcineurin binding by PfFKBP35 in the absence of FK506

The classical molecular model of immunosuppressive action of FK506 involves the modulation of a protein–protein interaction. FKBP has low or no affinity for the phosphatase calcineurin unless the small-molecule modulator FK506 is present, in which case a complex forms in which the phosphatase activity is inhibited (Galat, 2003). This effect is highly specific because it does not occur with Rap or with certain FK506 congeners with minor chemical modifications. The unusual finding of Monaghan & Bell (2005) that PfFKBP35 could interact with calcineurin irrespective of the presence or absence of FK506 therefore suggested that inhibition of calcineurin was unlikely to be the source of antimalarial activity of FK506. This finding was independently confirmed by Kumar *et al*. (2005) using the related compound ascomycin (FK520). Yoon *et al*. (2007) by contrast found calcineurin inhibition by PfFKBP35 to be somewhat FK506-dependent. Consideration of the different experimental conditions in the three papers suggested that the discrepancy could not be accounted for by the type of recombinant PfFKBP, the sources of the calcineurin and other reagents, or the type of phosphatase assay employed. There were however differences in the relative concentrations of the major reagents that might have accounted for the different results. We therefore repeated the experiment of Monaghan & Bell (2005), varying the ratios of PfFKBP35:calcineurin and FK506:PfFKBP35 to cover all those used in the three studies. Since it gave the same effect as PfFKBP35, the recombinant protein containing the PfFKBD alone was used (Monaghan & Bell, 2005). The PfFKBP35:calcineurin ratio was varied from 20:1 to 1000:1 in the absence of FK506 or with FK506 added at an equimolar or 10-fold higher concentration relative to PfFKBD (Fig. 1). As expected, calcineurin was inhibited more at higher PfFKBP35:calcineurin ratios, though the degree of inhibition was less than recorded previously. The presence of FK506 had no effect, even at a 10-fold molar excess (relative to PfFKBD). We therefore concluded that the FK506-independent interaction of PfFKBP35 with calcineurin is maintained over a range of experimental conditions.

Antimalarial action of FK506 and Rap on different blood stages

Different drugs active on asexual, blood-stage malarial parasites often differ in the part of the intraerythrocytic cycle that they most affect (Le Manach *et al*. 2013). For example, drugs that disrupt haemoglobin digestion tend to affect mainly the trophozoite stage, during which the rate of this process is maximal, while those affecting cell division tend to be most potent on schizonts. In an attempt to differentiate the actions of FK506 and Rap, parasites were age selected, grown to different stages, and tested for

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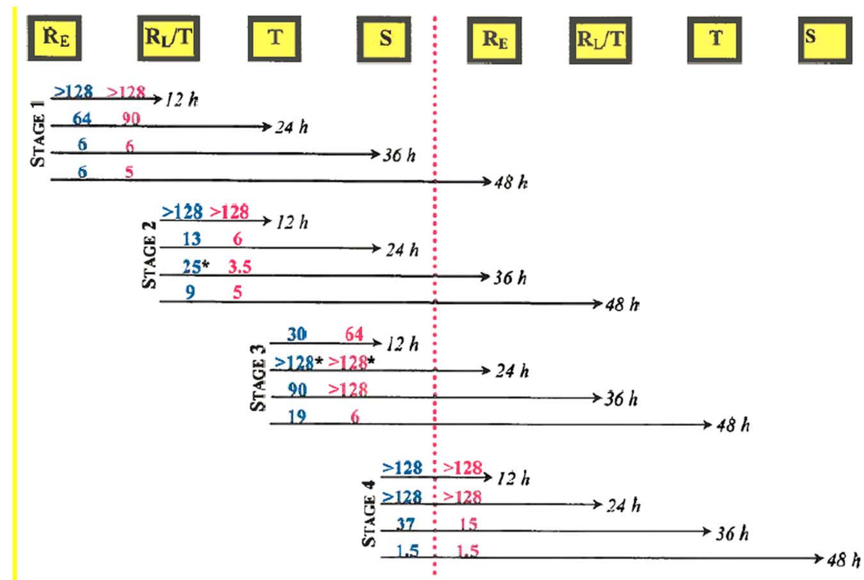

Fig. 2. Stage-dependent effects of FK506 and rapamycin on cultured blood-stage *P. falciparum*. Parasite cultures were age-selected by sorbitol treatment and grown to early rings (R_E, 0–6 h p.i.), late rings/early trophozoites (R_L/T, 12–18 h p.i.), mature trophozoites (T, 24–30 h p.i.) or schizonts (S, 36–42 h p.i.). Median inhibitory concentrations (IC_{50}) were determined for cultures commencing at R_E (stage 1), R_L/T (stage 2), T (stage 3) and S (stage 4) over periods of 12, 24, 36 and 48 h using the pLDH method. IC_{50} values indicated in blue (FK506) and red (rapamycin) are averages of two determinations, each titrated in duplicate. The arrows indicate the progression of untreated parasites in the same experiment. *, IC_{50} values are higher than those from preceding time points, presumably because surviving parasites multiplied on entering a second intra-erythrocytic cycle (dotted red line).

susceptibility by a standard, microplate-based antimalarial test after 12 h (Fig. 2). Of four 12-h stages, only stage 3, in which control parasites developed from trophozoite forms with an average age around 27 h p.i. to early schizonts (~39 h p.i.), was susceptible to FK506 with an IC_{50} of 30 μM. All other stages were resistant up to 128 μM. When the period was extended to 24, 36 or 48 h, the results also indicated that parasites passing through the trophozoite stage were most susceptible, though it was necessary to account for multiplication of surviving parasites during the assay and the fact that the amount of LDH per parasite increases through the cycle from ring to schizont. The results for Rap were broadly very similar to those for FK506 except that Rap appeared to be slightly more potent on average.

Kinetics of killing (or irreversible growth arrest) by FK506 and Rap

Antimicrobial (including antimalarial) agents with different mechanisms of action also commonly differ in whether they are static or cidal and the time taken for their effects to become irreversible. FK506 and Rap (both at 30 μM) were >90% cidal (or at least had effects that could not be reversed by washing the drug away and reculturing for 48 h) to trophozoites within 6–9 h (Fig. 3). The surviving parasites in the FK506 or Rap treated cultures contained higher proportions of rings, indicating that either there was some delay to development in survivors or that trophozites were killed more rapidly than rings. As with the stage-dependent susceptibility experiment, there was no clear difference between FK506 and Rap except that the latter was slightly more potent.

Antimalarial interactions of FK506 and Rap with other compounds

Antimicrobial drugs are said to interact when the potency of two or more drugs combined in an assay of antimicrobial activity is either higher (synergism) or lower (antagonism) than would be expected from the individual activities simply added together (additivity) (Greco *et al*. 1995; Bell, 2005). The activity of a drug A is affected by drug B and/or *vice versa* usually as a result of some connectivity between the components and pathways of the cell that are targetted by the drugs (Zimmermann *et al*. 2007). Drugs with similar mechanisms of action should therefore have similar profiles of (pharmacodynamic) interaction with other drugs, and drugs with different mechanisms should have distinct interaction profiles; this prediction has been confirmed, for example in *E. coli* (Yeh *et al*. 2006). We therefore compared the interactions of FK506 and Rap with other antimalarial compounds using a chequerboard arrangement and a pLDH read-out as described before (Gavigan *et al*. 2007). We have previously used this method to demonstrate

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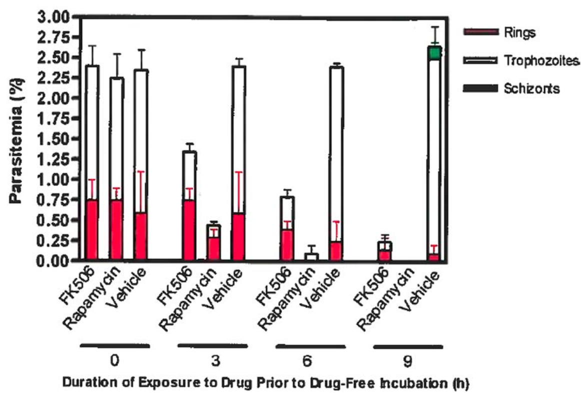

Fig. 3. Kinetics of killing or irreversible growth arrest of trophozoite-stage *P. falciparum* by FK506 and rapamycin. Cultures were age selected (24–30 h post-invasion) by sorbitol treatment and exposed for 0, 3, 6 or 9 h to 30 μM FK506, rapamycin or to an equivalent concentration of solvent as a control. Parasitized erythrocytes were then washed free of drug and recultured for 48 h before counting on Giemsa-stained thin smears. Experiments were done in duplicate with >1000 erythrocytes counted on each slide; vertical bars show SEM.

synergistic and antagonistic interactions among various drugs, including CsA, quinolines and peptidase inhibitors (Gavigan *et al*. 2001, 2007). The compounds chosen included CsA, the standard antimalarial drugs artemisinin and chloroquine, the Hsp90 inhibitor geldanamycin and the Hsp70 inhibitor pifithrin-μ. We choose the last two because both heat-shock proteins interact with PfFKBP35 (Kumar *et al*. 2005; Leneghan & Bell, 2015) and there was a possibility that this might lead to interactions of Hsp90 or Hsp70 inhibitors with FK506 and/or Rap. Pifithrin-μ had not previously been tested on *Plasmodium* but we found it to be antimalarial in culture with an IC_{50} of 15 μM.

FK506 and Rap were first tested in combination (Fig. 4 panels A–D). Based on the negligible deviation ('Loewe volume' = −0·263: see Fig. 4 legend) of the observed parasite growth inhibition from that predicted for no interaction [Loewe additivity (Greco *et al*. 1995)] (Fig. 4D) it was clear that there was no interaction between these two. By contrast, some of the combinations of FK506 and Rap with other compounds indicated substantial deviation from additivity: for example, FK506 and geldanamycin (Loewe volume = −4·53, Fig. 4H) appeared antagonistic. Looking at the pattern of interaction (Loewe volume values, Table 1), the data for FK506 and Rap were essentially indistinguishable: both showed apparent antagonism with chloroquine, geldanamycin, pifithrin-μ and (to a lesser extent) with CsA, and apparent (marginal) synergism with artemisinin. By contrast, CsA had some apparent interactions that were similar to those of FK506 and Rap and some that were different [e.g. (marginal) antagonism to artemisinin]. This analysis does not determine the statistical significance of the deviation from additivity, which is a separate (and highly specialized) analysis (Greco *et al*. 1995) that we have not attempted here. As a point of comparison, the combination of CsA and chloroquine (Loewe volume = −4·02 in the present study) was previously shown to be antagonistic at a significance level of $P < 0{\cdot}001$ using the same parasite line and experimental conditions (Gavigan *et al*. 2007). Taken together, the data indicate that the interactions of FK506 and Rap with a limited number of antimalarial compounds were very similar.

DISCUSSION

PfFKBP35 and its *Plasmodium vivax* homologue PvFKBP35 have received attention as potential antimalarial drug targets, with reports of ligands with potent antimalarial activity (Monaghan *et al*. 2005; Harikishore *et al*. 2013*a*, *b*) and atomic-level structures in complex with ligands (Kotaka *et al*. 2008; Alag *et al*. 2009; Bianchin *et al*. 2015). Our understanding of the function of the protein in the parasite has however lagged behind. The gene is conserved among several *Plasmodium* strains and species whose genomes have been sequenced (www.plasmodb.org) but we do not yet know the consequences of genetic ablation. The function of PfFKBP35 can be guessed at based on data from FKBPs of other organisms (Bell *et al*. 2006) – we expect it to be involved in folding, transport and/or regulation of other proteins and perhaps in the response to stresses such as the temperature shocks encountered on transfer between hosts (Acharya *et al*. 2007). Its interacting partner proteins are now known to include heat-shock proteins and histones (Leneghan & Bell, 2015) but the physiological/biochemical consequences of these molecular interactions have not yet been extensively investigated. In this paper, we have shown that the pharmacodynamic actions of two PfFKBP35 ligands, FK506 and Rap, were essentially indistinguishable. They had similar stage-dependent effects (Fig. 2), kinetics of killing or irreversible growth arrest (Fig. 3), and interaction with other antimalarial agents (Fig. 4, Table 1). Given that the downstream effects of FKBP–FK506 and FKBP–Rap complexes in other systems are utterly different, our data are consistent with the idea that it is the action of these ligands on PfFKBP35 itself that is likely to be relevant to antimalarial activity. To be confident of this conclusion however it will be necessary to provide further evidence, such as large-scale comparisons of the pharmacodynamic properties of FK506 and Rap with numerous other inhibitors or analysis of the phenotypes of transgenic PfFKBP35 knock-out and/or knock-down parasites.

An alternative hypothesis for the antimalarial activity of FK506 suggests that (by analogy with

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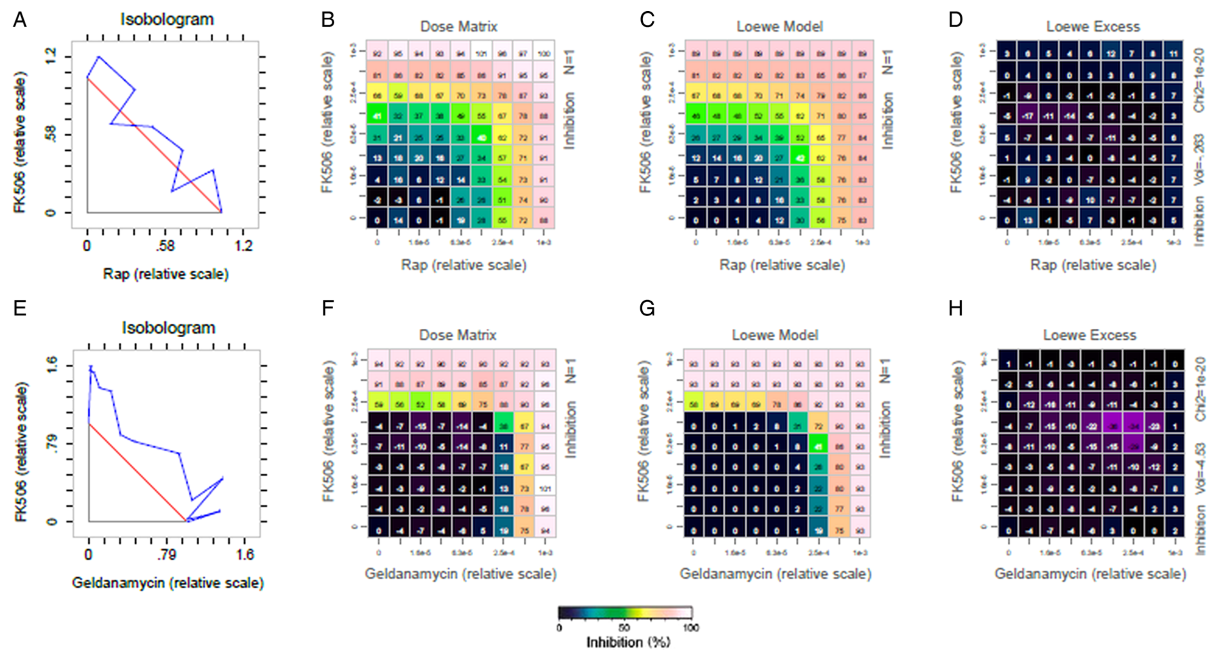

Fig. 4. Examples of antimalarial drug interaction analysis using the Horizon Chalice™ Analyzer software. (A–D) combination of FK506 and Rap; E–H: combination of FK506 and geldanamycin. (A, E) Isobolograms showing 50% inhibitory concentrations (IC_{50}) of the two compounds alone and in the presence of the other. Each compound was titrated in 8 2-fold steps alone and in combination. Susceptibility was measured on parasite line 3D7 after 72 h using the pLDH method and at least three independent determinations were done. Concentrations are shown on a relative scale where $IC_{50} = 1$. Points around the diagonal line are suggestive of additivity; points below left suggest synergism, and above right, antagonism. (B, F) 'Observed' inhibition of parasite growth (%) by each compound. For the purposes of the analysis, concentrations are shown on a relative scale (starting at around $4 \times IC_{50}$). (C, G) 'Expected' inhibition of parasite growth by the various combinations based on inhibition determined using the compounds alone and assuming no interaction (i.e. Loewe additivity). (D, H) 'Deviation' of the observed inhibition values from those expected from the Loewe additivity model. Positive values (blue) indicate that inhibition was more than expected and negative values (purple) less than expected. The volume (Vol.) is a measure of the overall deviation of the data from those expected under Loewe additivity: a large positive value indicates synergism, a large negative value indicates antagonism, and values close to 0 indicate additivity.

its immunosuppressive and certain other pharmacological actions) the drug works via inhibition (in a complex with PfFKBP35) of the parasite calcineurin (Bell *et al*. 2006). One observation that appears to support this notion is that the patterns of gene transcription in parasites exposed to FK506 and CsA (also, in complex with cyclophilin, a known calcineurin inhibitor) were virtually identical (Kotaka *et al*. 2008). Since the only known common target of the chemically dissimilar FK506 and CsA is calcineurin, this finding suggests a role for calcineurin in global gene regulation in parasites. It does not however show that the effect on this regulatory programme is responsible for the lethality of FK506 and/or CsA. Moreover, the experiment was performed in schizonts, which in our study were not the stage most susceptible to the action of FK506 (Fig. 2). Recently published studies by Paul *et al*. (2015) and Philip & Waters (2015) provide evidence that calcineurin mediates attachment of *Plasmodium* merozoites to host erythrocytes, in addition to having other roles across the life cycle. These findings suggest that an inhibitor targetting calcineurin should be most effective when applied at the very end of the intraerythrocytic cycle, in contrast to what was found with FK506 in this study, in the study of Kotaka *et al*. (2008) and by Paul *et al*. (2015).

Also arguing against the calcineurin hypothesis was the observation that PfFKBP35 or its FKBD alone could bind to calcineurin in the absence of FK506 *in vitro* (Monaghan *et al*. 2005; Kumar *et al*. 2005). In view of the conflicting result reported by Yoon *et al*. (2007), we explored the effect of different experimental conditions on this outcome. FK506-independent calcineurin inhibition by PfFKBD was consistent across a range of reagent ratios (Fig. 1). The reason for this difference from human FKBP12, which requires FK506 for calcineurin binding, may be related to one or more regions in which the two FKBD sequences diverge substantially; one possibility is that the N-terminal 'extension' of PfFKBP35 is somehow responsible. Whether this interaction is relevant in intact parasites is not known, but our observations make it hard to imagine a scenario in which FK506 targets

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Table 1. Interactions between FK506, Rap and CsA based on analysis using the Horizon Chalice™ Analyzer software (see Fig. 4)

'Volume' of deviation from additivity[a]	FK506	Rap	CsA
Rap	−0·263		
CsA	−2·85	−2·51	
Artemisinin	1·22	1·84	−1·31
Chloroquine	−4·63	−3·58	−4·02
Geldanamycin	−4·53	−5·07	−5·27
Pifithrin-μ	−3·34	−3·82	−4·24

[a] Positive values indicate a positive interaction (synergism) and negative values a negative interaction (antagonism). Larger 'volumes' indicate greater deviation from additivity, but the software does not test the statistical significance of the deviation.

Plasmodium calcineurin to produce its antimalarial effect.

Yet, another argument against calcineurin and in favour of PfFKBP35 as the target of the FKBP ligands is that non-immunosuppressive, non-calcineurin binding congeners of FK506 retained antimalarial activity (Monaghan *et al*. 2005). Non-macrolactone PfFKBP35 ligands designed using structure-aided methods have achieved even greater antimalarial potency (Harikishore *et al*. 2013*a*, *b*). These observations suggest that designing or discovering potent PfFKBP35 ligands that have low binding to mammalian FKBPs (Blackburn & Walkinshaw, 2011; Bianchin *et al*. 2016) may be a productive approach to antimalarial chemotherapy. Before devoting large resources to this enterprise, it would however be wise to obtain genetic evidence of the validity of PfFKBP35 as a therapeutic target.

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