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Adamantyl Derivative As a Potent Inhibitor of *Plasmodium* FK506 Binding Protein 35

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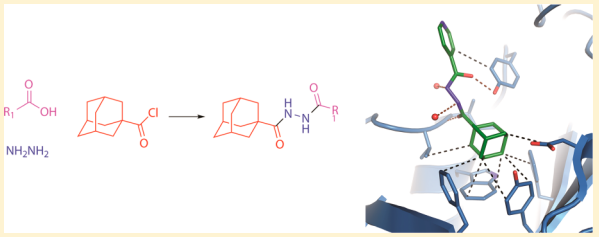
Supporting Information

ABSTRACT: FKBP35, FK506 binding protein family member, in *Plasmodium* species displays a canonical peptidyl-prolyl isomerase (PPIase) activity and is intricately involved in the protein folding process. Inhibition of *Pf*FKBP35 by FK506 or its analogues were shown to interfere with the in vitro growth of *Plasmodium falciparum*. In this study, we have synthesized adamantyl derivatives, Supradamal (SRA/4a) and its analogues SRA1/4b and SRA2/4c, which demonstrate submicromolar inhibition of *Plasmodium falciparum* FK506 binding domain 35 (FKBD35) PPIase activity. SRA and its analogues not only inhibit the in vitro growth of *Plasmodium falciparum* 3D7 strain but also show stage specific activity by inhibiting the trophozoite stage of the parasite. SRA/4a also inhibits the *Plasmodium vivax* FKBD35 PPIase activity and our crystal structure of *Pv*FKBD35 in complex with the SRA provides structural insights in achieving selective inhibition against *Plasmodium* FKBPs.



KEYWORDS: *FK506 binding protein, FKBP35, peptidyl-prolyl-isomerase, immunophilin, chaperone*

Malaria is one of the most dreadful diseases causing an estimated 800,000 deaths annually in the tropical and subtropical regions like India, Africa, Southeast Asia, and South America.[1,2] Recent incidence of parasite resistance to most effective drug treatment regimens-artemisinin and chloroquine pose a serious challenge for combating malaria and its clinical treatment. FK506 or its nonimmunosuppressive derivatives bind to *Plasmodium* FK506 binding protein 35 (hereafter referred as FKBP35), inhibit its enzymatic activity, and show in vitro growth inhibition of *Plasmodium*.[3−5] FKBP35 is a well characterized FKBP in *Plasmodium falciparum* and *vivax* species possessesing an N-terminal FK506 binding domain, a tripartite tetratricopeptide repeat (TPR) domain and a calmodulin binding domain at its C-terminus. FKBP35 is highly homologous to human FKBP family members like FKBP12, FKBP51, and FKBP52 in possessing peptidyl-prolyl-isomerase (PPIase) and chaperone activities.[4,6] FKBP family members mediate protein−protein interactions regulating various physiological processes. FKBP12-FK506 binary complex interacts with calcineurin and inhibits the dephosphorylation of transcription factor, nuclear factor of activated T cells (NFATc). This prevents the nuclear translocation of NFATc and subsequent stimulation of IL-2 synthesis leading to suppression of immune responses.[7] FKBP family members show a canonical PPIase or rotamase activity. FKBPs through their rotamase activity play an important role in various physiological activities like protein stability,[8−10] chaperonic activity,[11,12] receptor signaling,[13−15] protein trafficking,[16,17] transcription,[18,19] calcium homeostasis,[15,20] neuroprotective, neurotrophic activities,[21] and malaria.[2,22,23] These physiological roles of FKBPs represent them as a potential target for pharmacological intervention.

Previous study by Braun et al., showed that a synthetic ligand of FK506 (SLF) binds tightly to FKBP12 but lacks cellular toxicity.[5] The physiological levels of human FKBP12 levels (5 μM) are much higher in comparison to *Plasmodium* FKBP35 (100 nM) providing a therapeutic window to achieve selectivity toward parasite FKBP35.[5] Furthermore, lack of cellular toxicity has further been exploited to increase the bioavailability of HIV protease inhibitor by tethering FKBP12 ligand, SLF (Chart 1), to amprenavir.[24] Babine et al., has shown that an adamantyl-like fragment bonded to pyridine via a sulfur atom (5, Chart 1) binds and inhibits the enzymatic activity of FKBP12 at micromolar range. The crystal structure of FKBP12 with this compound revealed that the adamantyl-like fragment docks onto the base of Trp58, whereas the distal pyridine ring interacts with His87 residue at the β4−β5 loop.[25] In this study, we demonstrate that adamantyl derivative 4a/SRA and its analogues bind to *Plasmodium* FKBD35 inhibiting its PPIase activity as well as *Plasmodium falciparum* 3D7 strain growth in vitro.

A three-step synthesis for adamantine building blocks (4a−c) was set up starting from the corresponding acids (1a−c,

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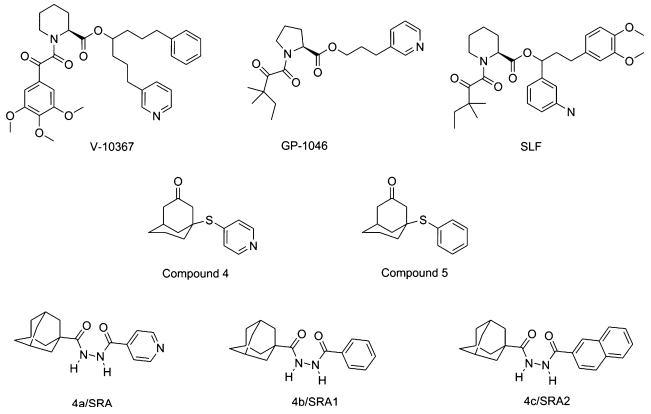
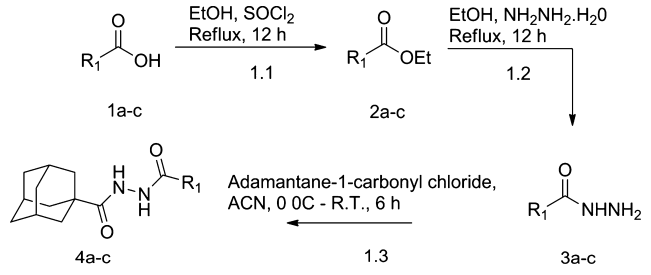
Chart 1. Chemical Structures of Known FKBP Ligands and 4a/Supradamal (SRA)



Scheme 1). The acid was treated with thionyl chloride in the presence of ethanol to yield its ethyl esters (2a–c). Further,

Scheme 1. Synthesis of Adamantyl Derivatives



ethyl esters (2a–c) were converted into its hydrazides (3a–c) by using hydrazine in the presence of ethanol. Finally, hydrazides were treated with adamantane-1-carbonyl chloride to obtain the target molecules (4a–c). (Scheme 1).

Interaction analysis of FK506 with human and *Plasmodium* FKBPs reveal a strikingly similar pattern in both the species albeit with a few variations at the $\beta4-\beta5$ loop. Conserved interaction pattern of FK506 with *Plasmodium* FKBP35 and human FKBP12 further bolsters the utility of small molecule inhibitors of FKBP12 ligands as scaffolds for achieving the parasite FKBP35 inhibition.[26−28] Our molecular modeling studies suggested that the adamantyl derivative (Supradamal, SRA or 4a) had a reasonably good fitness score of 52.66 when compared to FK506 (79.77). Similar to the pipecolinyl moiety of FK506, the adamantyl fragment is predicted to dock on to the canonical binding site Trp78 (S1) and the linker can form hydrogen bonding interactions with Ile75 and Tyr101 and hydrophobic contacts with Tyr101. Thus, SRA could favor the S2 site between $\alpha1-\beta5$ loop and orient away from the $\beta4-\beta5$ loop (S3), which is involved in a number of protein−protein interactions such as calcineurin, Inositol triphosphate IP3 receptor, and TGF-beta signaling (Supporting Information Figure S1a). Therefore, we chose to increase the linker length by 4-atoms in order to induce steric clashes with His87 of human FKBP12 and to enhance the probability of binding to *Plasmodium* FKBP35.

Supradamal (SRA) and its derivatives SRA-1 and SRA-2 inhibits the *Plasmodium falciparum* FK506 binding domain (FKBD35) activity significantly at nanomolar range (Table 1). Our in vitro enzymatic assay results suggest that SRA is unlikely to inhibit calcineurin phosphatase activity (Figure 1) signifying

Table 1. Effect of Adamantyl Derivatives on *Plasmodium* FKBD35 Activity

compd	R_1	*Pf*FKBP35 IC_{50} (nM)
4a/SRA	4-pyridyl	83 ± 9.32 (75 ± 6.32)[a]
4b/SRA1	phenyl	94.75 ± 11.8
4c/SRA2	1-napthyl	49.3 ± 6.2

[a]Refers to IC_{50} against *Plasmodium vivax* FKBP35. SD (standard deviation) represents the error from two independent experiments performed in duplicate (n = 2).

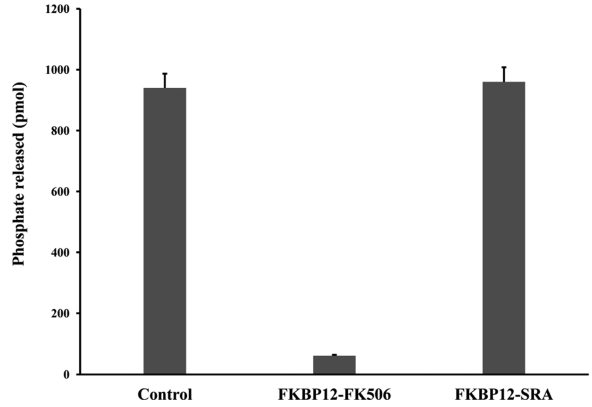


Figure 1. Calcineurin phosphatase assay. Compound 4a (SRA)/FK506 complexes with human FKBP had no effect on calcineurin phosphatase activity, while FK506 complex with human FKBP12 inhibited calcineurin phosphatase activity. Calcineurin alone served as control.

that these novel ligands do not possess the ability to inhibit calcineurin phosphatase activity and therefore do not suppress immune responses, unlike FK506, which inhibits the phosphatase activity of calcineurin leading to immune suppression.

To investigate whether SRAs inhibit parasites maturation, growth inhibition assays were carried out using 3D7 parasites. The maturation of highly synchronized ring-infected erythrocytes was monitored for an entire intraerythrocytic developmental cycle (IDC) (48 h) in the presence of SRAs and known *P. falciparum* inhibitors (chloroquine (CQ) and artemisinin (ART)) or in absence of treatment (control). Parasitaemia and parasite morphology were determined at 8 h time interval. During the first 8 h (ring stage development), no parasitaemia (Figure 2a) and morphological changes (Figure 2b) were observed between the SRA-treated, CQ-treated, and untreated parasites, whereas a rapid decrease in parasitaemia was observed with ART-treated parasites, in line with the known effect of ART on ring parasites stages. Subsequently, control parasites mature successively to trophozoite (16–24 h), then to schizont (32–40 h) with further invasion (48 h) (Figure 2b) leading to increased parasitaemia at 40 and 48 h (Figure 2a). In contrast, parasitaemia of SRAs and CQ-treated parasites decrease in a similar pattern during trophozoite development (16–24 h) (Figure 2a) where the parasites died and disintegrated (Figure 2b). Taken together, these results suggest that SRA inhibitors interfere with the trophozoite stage development.

To confirm the specificity of the inhibitory activity of SRAs on trophozoite stages, we investigated the direct effect of SRAs on highly synchronized ring, trophozoite, and schizont stage maturation by incubating parasites with 500 nM (2× IC_{50},

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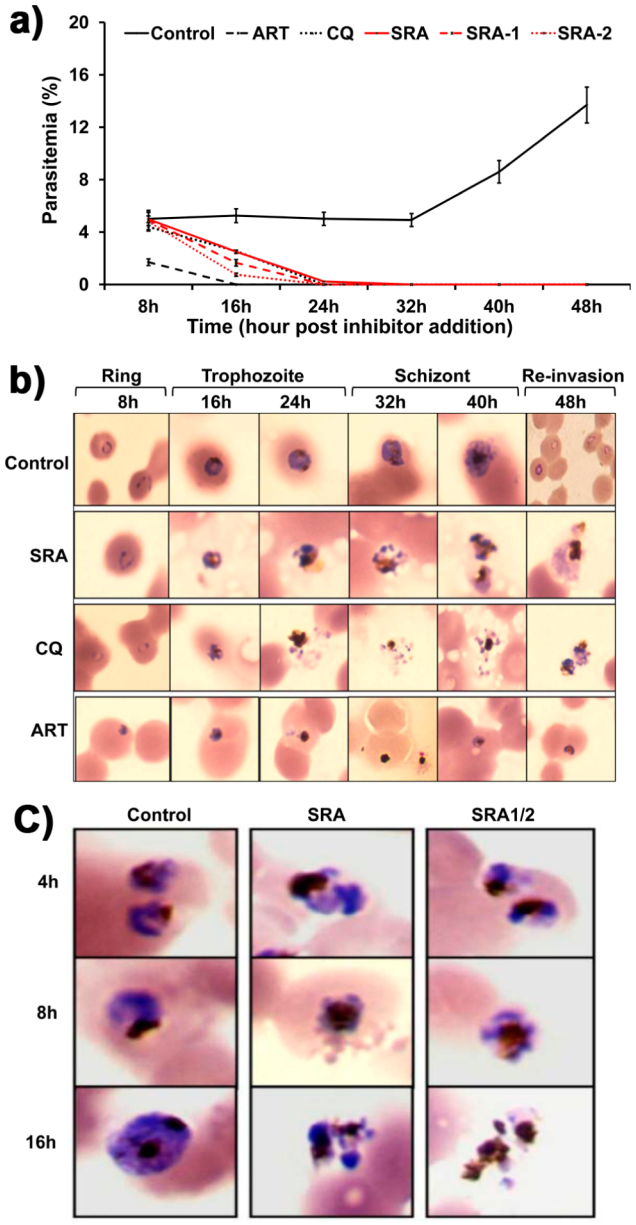

Figure 2. Effects of SRA analogues on *Plasmodium falciparum* IDC. (A) Effect of SRA on parasitemia (%) was monitored every 8 h upon the addition of inhibitor(s) and were compared to untreated control parasites. (B) Parasite morphology was monitored by Giemsa stain at each time point. Illustrations of parasite morphology at 8, 16, 24, 32, 40, and 48 h after addition of inhibitor are shown. (C) Effects of SRA/SRA1 and SRA2 on trophozoite development. Parasite morphology was monitored by Giemsa stain at 4, 8, and 16 h after the addition of inhibitor. During the first 4 h, no significant morphological differences were observed between the treated and control parasites. During the subsequent development, the control parasites progress to the next generation (formation of mature schizont and ring), while the SRA treated cells remain arrested at the trophozoite stage. The results show that SRA and its derivatives mainly interfere with trophozoite stage development.

Supporting Information Figure 2) of each SRA analogue. No effect was seen on ring and schizont maturation (data not shown), while control trophozoite parasites successfully matured to schizont stages (Figure 2c, control); the development of SRA-treated trophozoites was impaired after 8 h of SRA addition followed by disintegration after 16 h (Figure 2c). While this finding confirms that SRA inhibitors directly interfere with the trophozoite development. Further studies using chemical biology approaches with SRA may provide further insights into the protein−protein interactions that might be involved in its mode of action.

To understand the molecular basis of SRA interaction, we attempted to crystallize SRA and its analogues with FKBD of both *Plasmodium falciparum* and *vivax* FKBP35.[28] Crystals with *Pf*FKBD35 did not yield good quality crystals for structure elucidation. However, the crystal screening of *Pv*FKBD35 with SRA gave good quality crystals. The crystallographic structure of *Pv*FKBD35 in complex with SRA was determined to a resolution of 1.72 Å (atomic coordinates have been deposited to protein data bank with PDB code 4MGV). Refined crystallographic structure revealed a topology consisting of seven β-stranded sheets and a short α-helix similar to the previous FKBD35 structures.[26,28,29] The electron density for SRA (Figure 3a) was traced unequivocally in the hydrophobic binding pocket. The hydroxyl oxygen atoms in carbonyl hydrazide linker region of SRA make critical hydrogen bonding contacts (Supporting Information Table S1) with the catalytic

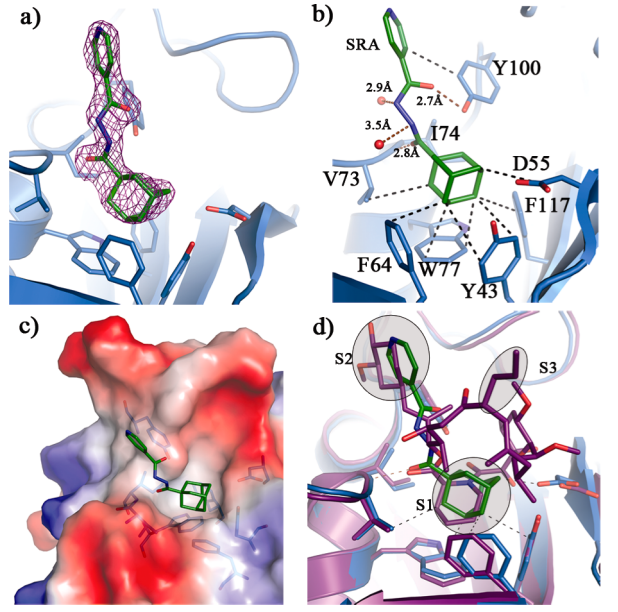

Figure 3. Comparison of SRA-FKBD35 interactions. (A) The 2Fo-Fc electron density map contoured at 1σ cutoff for the SRA inhibitor (green sticks); also shown are the interacting active site residues (blue sticks). (b) The hydrogen bonds (brown dashes) formed by SRA with Tyr100, Ile74, and two water molecules are shown with their distances labeled. It could be clearly seen that a network of hydrogen bonds are made by the four atoms of SRA. The nonbonded contacts (black dashes) are primarily made by the adamantyl ring of SRA. (c) The electrostatic surface diagram showing the docking of SRA in the active site pocket, with the adamantyl ring sitting deep inside the base of the pocket with Trp77 acting like a platform. (d) Superposition of the FK506 complex (purple) on the SRA complex, indicating three key features (shown within circle) of SRA interactions. The adamantyl ring overlies on the pipecolinyl ring (S1) of FK506 and the pyridine ring on the cyclohexyl ring (S2). In addition, the allyl group (S3) of FK506 responsible for the calcineurin inhibition is seen to be completely away from SRA. This explains the nonimmunosuppressive nature of SRA at a molecular perspective. All the figures are in a similar orientation, with the interacting residues labeled in panel b alone. The figure has been generated using PyMOL.[32]

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residues Tyr100 and Ile74 (Figure 3b). In addition, the two nitrogen atoms in the linker also form hydrogen bonds with the nearby water molecules (Figure 3b). Thus, a network of hydrogen bonds with atoms from the trunk of the inhibitor holds it strongly in the active site pocket. Further nonbonded contacts (Supporting Information Table S1) predominantly by the adamantyl ring of SRA with residues like Tyr43, Asp55, Phe64, Val73, Trp77, Tyr100, and Phe117 lock the ligand deep inside the pocket (Figure 3c) where Trp77 forms a platform-like architecture (Figure 3b,c). Crystallographic complex structure of human FKBP12-FK506-calcineurin[7] reveals that the allyl group of FK506 (the effector domain) docks into Ca^{2+}-calmodulin activated calcineurin and that modification of this allyl group to either ethyl (FK520)[30] or acetyl (FK1706)[31] moieties had markedly hampered the inhibition of calcineurin phosphatase activity. Superposition with the FK506 complex of *Pv*FKBD35 (PDB ID 3IHZ) revealed some key features of SRA orientation and interaction. The adamantyl and pyridine rings of SRA overlay on to the pipecolinyl and cyclohexyl rings of FK506, respectively (Figure 3d). As SRA lacks substituent groups onto the allyl group of FK506 (the effector domain), it adopts an orientation that does not interfere with the calcineurin binding region (Figure 3d). Further, the four atom linker stabilized by a network of hydrogen bonds is responsible for orienting the pyridine ring toward the cyclohexyl ring of FK506. Therefore, it is evident that SRA does not impinge the calcineurin activity, rendering it to be a nonimmunosuppressive inhibitor (Figure 2). Additionally it possesses stronger affinity toward the hydrophobic pocket of FKBP due to its adamantyl group. Thus, on the basis of the crystal structure, the molecular basis of the nonimmunosuppressive nature of SRA has been explained.

In conclusion, the present study presents adamantyl derivatives as potent inhibitors of *Plasmodium* FKBD35 enzymatic activity as well as trophozoite stage growth inhibition of *Plasmodium falciparum* 3D7 strain. Further, our crystallographic structure complex of *Plasmodium vivax* with SRA further provides insights into its mode of action and lack of immune suppression.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures for the synthesis and characterization of the compounds, the in vitro PPIase assay, growth inhibition assays, crystallization, X-ray data collection, structure determination, refinement, and the ^{1}H NMR and ^{13}C NMR spectra of the reported compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

H.S.Y. designed the research. A.H., M.L.L., M.N., S.R., and K.K.P. performed the research. A.H., K.K.P., M.N., S.R., P.R.P., X.L., and H.S.Y. analyzed the data. A.H., M.L.L., K.K.P., M.N., S.R., P.R.P., and H.S.Y. drafted the manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

FKBP, FK506 binding protein; FKBD, FK506 binding domain; PPIase, peptidyl-prolyl-isomerase; CaN, calcineurin; *Pf*, *Plasmodium falciparum*; *Pv*, *Plasmodium vivax*; TLC, thin layer chromatography

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