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Inhibition properties of collagen hydrolysates against FKBP35 of *Plasmodium knowlesi*

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ABSTRACT

Aims: FK506-binding protein 35 from *Plasmodium knowlesi* (Pk-FKBP35), a member of peptidyl-prolyl cis-trans isomerase (PPIase), is considered a viable target for the development of the novel antimalarial drug targeting zoonotic malaria in Malaysia. While FK506 effectively inhibits this protein, this drug is not applicable due to its immunosuppressive effects. This study aims to assess the inhibitory potential of different collagen hydrolysates (CH) against Pk-FKBP35, as FK506 replacers.
Methodology and results: Recombinant full-length Pk-FKBP35 was initially over-expressed using *Escherichia coli* (BL21) host cells and subsequently purified via affinity chromatography coupled with size-exclusion chromatography. In this study, four distinct CH were employed, originating from bovine, bone broth, fish and swine. The results revealed that all CH inhibited PPIase catalytic activity of Pk-FKBP35 with IC_{50} values 1.63 mg/mL (bovine CH), 2.97 mg/mL (fish CH), 33.01 mg/mL (swine CH) and 13.91 mg/mL (bone broth CH), which were much higher than that of FK506. Furthermore, these CHs retained the ability of Pk-FKBP35 to inhibit calcineurin phosphatase activity, yet not as extreme as FK506.
Conclusion, significance and impact of study: The inhibition is predicted due to the presence of proline-rich peptides in CH, which were able to block the substrate binding cavity of Pk-FKBP35. This study suggested that CH might have no serious immunosuppressant effect and is promising for further harnessing for antimalarial compounds.

Keywords: Collagen hydrolysate, peptidyl-prolyl cis-trans isomerase (PPIase), *Plasmodium knowlesi*, FKBP35, zoonotic malaria

INTRODUCTION

Malaria, which is a life-threatening vector-borne disease caused by *Plasmodium* parasites, continues to become a burden in public health in Malaysia. The progressive increase of zoonotic malaria caused by *Plasmodium knowlesi* in Malaysia, particularly in the Borneo region, poses a significant challenge to eradicate this infection (Chin *et al*., 2020; Muhammad *et al*., 2022). Moreover, *P. knowlesi's* short asexual replication cycle leads to severe infections that are chronic, symptomatic and even lead to death (Daneshvar *et al*., 2009; Singh and Daneshvar, 2013). Additionally, *Plasmodium* has shown resistance to available antimalarial drugs in some regions (Menard and Dondorp, 2017). Hence, it poses further threats and challenges in eliminating malaria entirely. Moreover, there is still limited study for antimalarial drugs targeting FKBP35 of *P. knowlesi* (Pk-FKBP35). Thus, it is necessary to explore novel therapeutic targets for developing effective antimalarial drugs against Pk-FKBP35.

Plasmodium's FK506-binding protein 35 (FKBP35) is considered a viable target to develop a novel antimalarial drug. This protein is a member of peptidyl-prolyl cis-trans isomerase (PPIase) which has the capability to catalyse a slow isomerisation of cis-prolyl peptide bond during protein folding (Fanghanel and Fischer, 2004). Accordingly, Goh *et al.* (2018) suggested that FKBP35 of malaria parasites plays critical roles in protein folding and cellular events in parasite cells. The immunosuppressive drug, FK506, which is also known as a specific inhibitor of the FKBP family protein, has been demonstrated to exhibit antimalarial activity (Bell *et al.*, 1994; Harikishore *et al*., 2013).

Pk-FKBP35 comprises the FK506-binding domain (FKBD) and the tetratricopeptide repeated domain (TPRD) (Goh *et al*., 2018). FKBD is responsible for the catalytic activity of this protein which is crucial for the regulation of PPIase activity (Alag *et al*., 2013). Conversely, the TPRD is regarded as a key for facilitating the dimerization of the protein which is important to provide binding to the protein substrates (Goh *et al.*, 2018). Earlier, Yoon *et al.* (2007) and Goh *et al.* (2018) demonstrated that calcineurin is one of the protein substrates that is functionally regulated by *Plasmodium* FKBP35. Stie and Fox (2008) described calcineurin as a Ca^{2+}/calmodulin-dependent serine-threonine protein phosphatase, which is a key component in the T-cell

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receptor-mediated signalling that is required for T-cell activation (Liu *et al.*, 1991; Jain *et al.*, 1993). The activation of T-cells is related to the dephosphorylation of the nuclear factor of activated T cells (NFAT). The binding of the FK506-FKBP12 complex to calcineurin obstructs NFAT's access to its catalytic site. This inhibition prevents NFAT dephosphorylation, subsequently hindering T-cell activation (Jain *et al*., 1993). Interestingly, FKBP35 of *P. falciparum* and *P. vivax* were reported to be able to block calcineurin phosphatase activity even in the absence of FK506, albeit low. The presence of FK506, nevertheless, enhances the inhibition properties. (Yoon *et al*., 2007; Goh *et al.*, 2018). In addition, studies revealed that calcineurin is essential during the blood stage development of the parasite, where inhibition of this protein leads to the lethal effect of the parasite (Dobson *et al.*, 1999; Singh *et al.*, 2014; Park *et al.*, 2019).

FK506 inhibits both PPIase and calcineurin activities, blocking parasite cell replication and calcium-mediated cellular events in the life cycle (Philip and Waters, 2015; Goh *et al.*, 2018). Nevertheless, FK506 is not feasible since it triggers the suppression of the immune system (Feske *et al.,* 2003; Stie and Fox, 2018). Alternatively, therapeutic peptides are promising compounds that could be explored for replacing FK506. In malaria, antimicrobial peptides were widely explored to discover novel peptide-based antimalarial drugs (Bell, 2011). Additionally, a study done by Karthik *et al.* (2014), proved that peptides obtained from the marine actinobacteria exhibited effectiveness against the *Plasmodium* parasite responsible for causing malaria.

Given that FKBP35 is a member of PPIase, this protein has a high preference to bind to prolyl bonds which leads to an assumption that proline-rich peptides should exhibit high binding affinity to this protein. Indeed, a synthetic substrate of the FKBP family protein was also designed on the basis of the presence of a prolyl bond in its sequence (Fanghanel and Fischer, 2004). Earlier, the ability of FK506 to bind to FKBP family proteins was also due to the presence of a pipecolinyl ring in its structure that mimics a prolyl bond to be recognized by FKBP (Van Duyne *et al.,* 1991). One of the proline-rich proteins is collagen which is mainly formed by the amino acid glycine (33%), proline and hydroxyproline (22%) (Sorushanova *et al.*, 2019). Hydrolysis of collagen yielded a library of peptides with the size of 3-6 kDa and may contain a high number of proline residues (León-López *et al.*, 2019). The collagen hydrolysate (CH), however, has never been tested in its ability to inhibit any member of FKBP family proteins.

This study describes the ability of CH from various sources to modulate FKBP35 functions, pertaining to its PPIase catalytic activity and inhibition of calcineurin phosphatase activity. For the first time, the current study demonstrated the promising use of CH for further exploration in the development of antimalarial peptides.

MATERIALS AND METHODS

Preparation of collagen hydrolysates

CH used in this study were purchased from the local market, which originated from bone broth (Vital Proteins®, USA), bovine (Halavet, Turkey), fish and swine (Tamachan, Japan). The CH was prepared by dissolving them in autoclaved distilled water into desired concentrations.

Over-expression and purification of Pk-FKBP35

The overexpression and purification of Pk-FKBP35 were carried out following the methods described in a previous report (Goh *et al.*, 2018). A plasmid containing the gene encoding Pk-FKBP35 was transformed into *Escherichia coli* BL21(DE3) cells and inserted into pET29b between *Nde*I and *Xho*I sites. The transformed cells were grown in Luria-Bertani (LB) broth medium supplemented with 35 µg/mL of kanamycin at 37 °C for 16 h. Protein expression was then induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) once the OD600 reached between 0.6 and 0.7. The culture was further incubated at 25 °C for 16 h and harvested by centrifugation at 8,000× *g* for 10 min. The resulting pellet was resuspended in 20 mM phosphate buffer (pH 8.0) with 150 mM NaCl which subsequently was disrupted by sonication followed by centrifugation at 35,000× *g* for 30 min at 4 °C to collect the soluble fraction.

Purification of Pk-FKBP35 was performed from the soluble fraction using affinity chromatography coupled with gel-filtration chromatography. HiTrap TALON crude (Cytiva, Marlborough, USA) 1 mL column was used for the affinity chromatography, with an equilibration buffer consisting of 20 mM phosphate buffer (pH 8.0), 150 mM NaCl and 5 mM imidazole. The elution was performed using a linear gradient of 300 mM imidazole. The eluted fraction was subsequently concentrated and loaded onto a Superdex 200 (GE Healthcare, Amersham, UK) column using 20 mM phosphate buffer (pH 8.0) and 100 mM NaCl. The expression and purity of protein were analysed using 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). The protein concentration was determined through UV absorption (NanoDrop^{TM} 2000/2000c Spectrophotometer), where the absorbance at 280 nm of 0.1% (1 mg/mL) is 0.73, calculated according to the method of Goodwin and Morton (1946).

PPIase catalytic activity

PPIase catalytic activity was conducted using a protease coupling assay according to Fischer *et al.* (1989). To determine the inhibition properties of CH, the assay was conducted in the absence or presence of various concentrations of CH. The assay used chymotrypsin and the Suc-Ala-Leu-Pro-Phe-*p*Na (Suc-ALPF-*p*Na) as the protease and substrate, respectively. Briefly, a reaction mixture containing 35 mM HEPES buffer (pH 7.8), 25 µM substrate and 20 nM Pk-FKBP35 was prepared in the absence or presence of various concentrations of the CH. The mixture was then incubated for 3 min followed by the immediate addition of chymotrypsin to a final concentration of 11.4 µM. The rate of isomerisation was monitored by the changes in concentration of *p*-nitroanilide (*p*Na) at 390 nm absorption using a microplate reader Infinite® 200 PRO (Tecan Life Sciences, Männedorf, Switzerland). The inhibitory activity of the compounds was observed, and the IC_{50} value was calculated from the dose-response curve using SigmaPlotv10.

Calcineurin inhibition assay

The experimental procedure for this assay was adapted from Mondragon *et al.* (1997). The calcineurin assay involved measuring the phosphatase activity of 40 nM bovine brain calcineurin with Pk-FKBP35 in the presence or absence of CH with or without FK506. To determine the enzymatic activity, the ProFluor serine/threonine phosphatase assay was employed. This assay utilizes the fluorescence detection of a rhodamine-conjugated peptide substrate, which, upon dephosphorylation by calcineurin, is cleaved by the protease. All reaction mixtures were prepared in 100 µL volumes according to the instructions provided by the manufacturers and supplemented with calmodulin. The release of phosphate was monitored using an Agilent Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies, California, USA).

RESULTS AND DISCUSSION

The Pk-FKBP35 were expressed in *E. coli* in soluble forms and were successfully purified using affinity chromatography coupled with size exclusion chromatography (Figure 1). The apparent size of Pk-FKBP35 was approximately 37 kDa when evaluated in SDS-PAGE. This is in agreement with its theoretical size of 36.6 kDa when estimated based on its amino acid sequence. The discrepancy of about 1 kDa is due to the presence of 6His-tag at the N-terminal of this protein. This result is also in good agreement with Goh *et al.* (2018) who successfully expressed and purified this protein using the *E. coli* system. Notably, the gene encoding Pk-FKBP35 is a synthetic gene that was optimised in its codon. The success of expression and purification of this protein in the current study confirmed that the approach of the synthetic codon-optimised gene is an excellent approach to produce parasite recombinant protein.

Further, when the PPIase catalytic activity of Pk-FKBP35 was measured against Suc-Ala-Leu-Pro-Phe-*p*NA, the protein exhibited remarkable PPIase activity with the catalytic efficiency (k_{cat}/K_M) of 1.0×10^5 M^{-1} s^{-1}. Nevertheless, this value is about 5-fold lower compared to the previous report by Goh *et al.* (2018). The discrepancies might be due to the differences in the type of substrate used for the assay. Suc-ALPF-*p*NA was used in the current study, which is a chromogenic substrate. Meanwhile, Goh *et al.* (2018) used a fluorogenic substrate of Suc-ALPF-AMC in the study. According to Ikura and Ito (2007), the AMC peptide substrate is more sensitive compared to the substrate detected under visible light. However, the use of AMC substrate is quite costly and yields more noise as compared to *p*NA substrate. On the other hand, the catalytic activity of this protein from the current study was found to be comparable to FKBP35 of *P. vivax* and *P. falciparum* as earlier reported by Alag *et al.* (2010). To note the studies on FKBP35 from *P. vivax* and *P. falciparum* also used a chromogenic substrate rather than a fluorogenic one. This validation established that the discrepancy from Goh *et al.* (2018) was indeed attributable to substrate-related concerns. Noteworthy, the detectable activity of Pk-FKBP35 in this study also confirmed that this purified protein is in its active form.

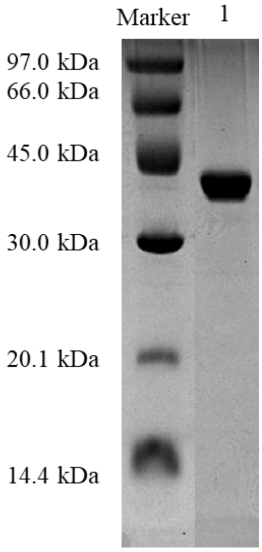

Figure 1: SDS-PAGE of purified Pk-FKBP35 on 15% SDS-PAGE stained with Coomassie Brilliant Blue. Marker refers to a low molecular weight marker kit (Cytiva, Marlborough, USA), while lane 1 corresponds to the elution fraction after double-step purification.

Further, when PPIase catalytic activity was determined in the presence of CH, the changes in the activity were observed, as shown in Figure 2. It is obvious that the changes are concentration-dependent, which implies that CH exhibited apparent inhibition activity against Pk-FKBP35. As a control, FK506 was also used to inhibit the PPIase activity of Pk-FKBP35 which also demonstrated inhibition properties in a concentration-dependent manner. The calculated IC_{50} value of FK506 was found to be 2.35×10^{-4} mg/mL (Table 1), which is comparable to the previous report by Goh *et al.* (2018). Accordingly, it suggested that even though the peptide substrate used in this study is different, it is still feasible to use the chromogenic peptide substrate to investigate the PPIase activity of Pk-FKBP35. This is supported by Stepaniak (2000), who observed that the sensitivity of fluorescence substrates does not offer any obvious

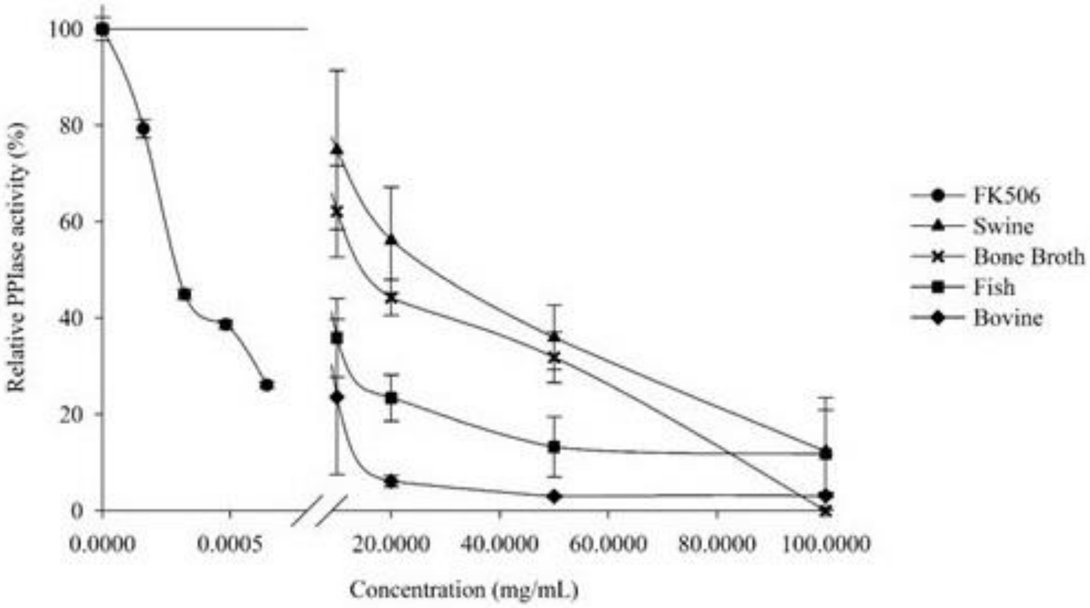

Figure 2: The relative PPIase activity (%) against the concentration of collagen hydrolysates (mg/mL).

Table 1: IC_{50} values of different CH in mg/mL in comparison with the control (FK506).

Collagen hydrolysates (CH)	IC_{50} value (mg/mL)
Bovine	1.63 ± 0.0072
Fish	2.97 ± 0.0012
Swine	33.01 ± 0.188
Bone broth	13.91 ± 0.619
FK506	0.000235 ± 0.0000047

advantage over the chromogenic substrates for evaluation in the study.

Further, the IC_{50} value of each CH against Pk-FKBP35 was also calculated which indicated that bovine and fish CH inhibition activity was better than swine and bone broth CH (Table 1). Bovine and fish inhibited Pk-FKBP35 with IC_{50} values of 1.63 mg/mL and 2.97 mg/mL, respectively (Table 1). Although the IC_{50} value of the control inhibitor, FK506, was better than CH, these results displayed the possibility and capability of CH to inhibit the PPIase activity of Pk-FKBP35. As collagen is rich with proline, the CH might contain proline-rich peptides which are plausible to exhibit the capability to dock into the substrate binding cavity of Pk-FKBP35 and block its activity. Accordingly, it is believed that the mechanism behind the inhibition properties of CH is associated with the proline residues in the hydrolysate. These CH may compete with the substrate ALPF to the binding cavity of Pk-FKBP35 and inhibit the subsequent activity of the protein. In suppressing the PPIase activity of Pk-FKBP35, it is hypothesised that the fish and bovine CH may occupy the FKBD catalytic domain of the Pk-FKBP35. This is because FKBD is considered the catalytic site that is vital for the regulation of PPIase activity (Alag *et al.*, 2013; Goh *et al.*, 2018). Disrupting the function of Pk-FKBP35 can result in its inability to catalyse the folding process of proteins containing cis-proline bonds within *Plasmodium* cells. Consequently, this would ultimately hinder the replication of the parasite's cells. However, further isolation of the proline-rich peptides from CH is still required for further investigation of their inhibitory properties against Pk-FKBP35 to validate this hypothesis. Earlier, Ishikawa and Bächinger (2014) indicated that FKBP22 indeed was able to interact with collagen through proline residues and catalyse its folding rate during the synthesis event. This, at least, supports the possibility of peptide fragments from collagens that are rich in proline to be able to interact with Pk-FKBP35. Notably, different CH may consist of diverse proline components, which accounts for the variation observed in PPIase inhibition against Pk-FKBP35.

Further, a calcineurin assay was done to investigate the effect of the CH on the calcineurin phosphatase activity. As shown in Figure 3, the presence of Pk-FKBP35 alone, without FK506, decreased the activity of calcineurin almost by half and the inhibition was further depleted with the presence of FK506. This is supported by a study done by Goh *et al.* (2018), where Pk-FKBP35 alone may inhibit the calcineurin activity, and the addition of FK506 enhances these inhibitory properties. Further, when Pk-FKBP35 with one of CH was added into calcineurin (without FK506), the activity of calcineurin was slightly suppressed to 75-80% residual activity. This is much higher than the residual activity of calcineurin in the presence of Pk-FKBP35 and FK506 (<20%). Interestingly, when FK506 was added to the mixture containing Pk-FKBP35 and CH, the residual activity of calcineurin was dropped to about 60%, which was still much higher than the residual activity of calcineurin in the presence of Pk-FKBP35 and FK506. This indicated that

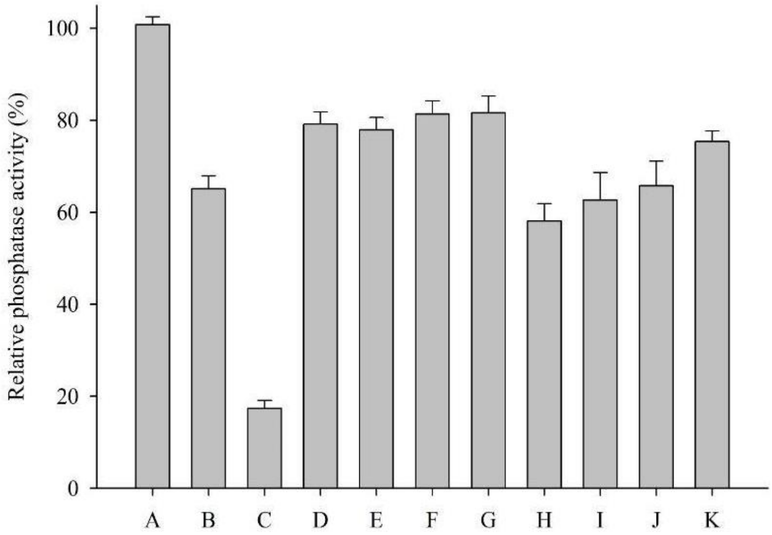

Figure 3: Inhibition of calcineurin by Pk-FKBP35, Pk-FKBP35-CH complex with and without FK506. (A) Calcineurin, (B) Calcineurin+Pk-FKBP35, (C) Calcineurin+Pk-FKBP35+FK506, (D) Calcineurin+Pk-FKBP35+Swine CH, (E) Calcineurin+Pk-FKBP35+Bovine CH, (F) Calcineurin+Pk-FKBP35+Fish CH, (G) Calcineurin+Pk-FKBP35+Bone broth CH, (H) Calcineurin+Pk-FKBP35+Swine CH+FK506, (I) Calcineurin+Pk-FKBP35+Bovine CH+FK506, (J) Calcineurin+Pk-FKBP35+Fish CH+FK506 and (K) Calcineurin+Pk-FKBP35+Bone broth CH+FK506.

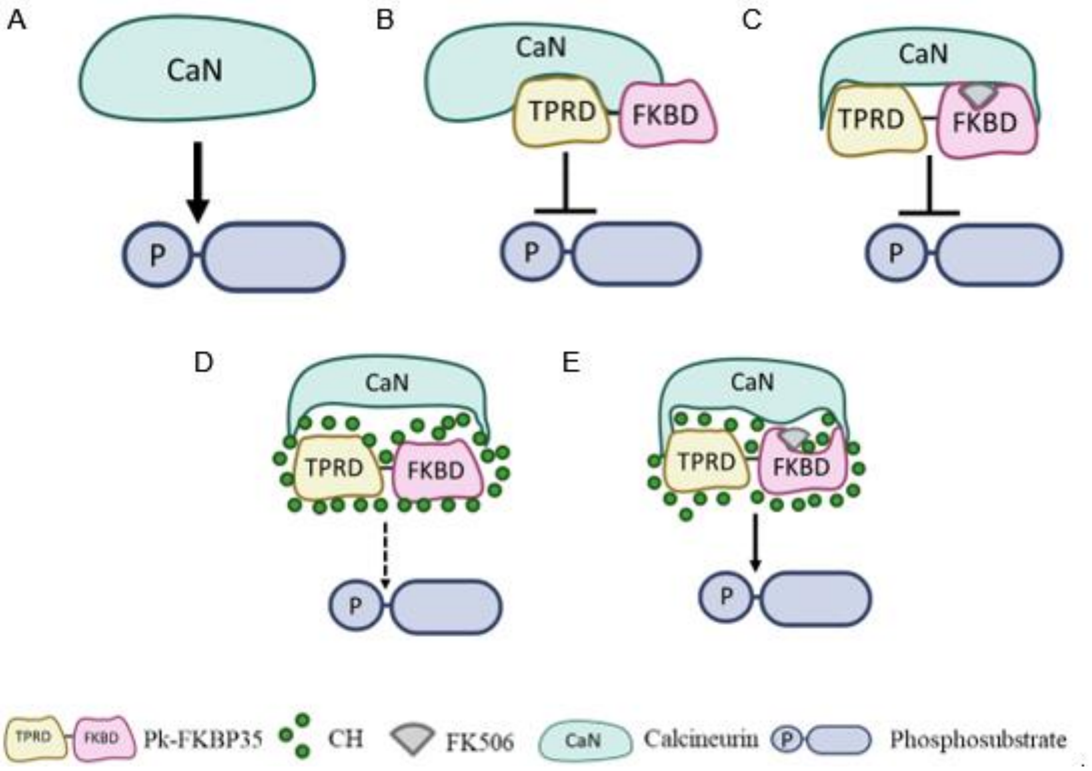

Figure 4: Illustration of the effect of Pk-FKBP35, FK506 and CH in the calcineurin phosphatase assay. (A) Calcineurin, (B) Calcineurin+Pk-FKBP35, (C) Calcineurin+Pk-FKBP35+FK506, (D) Calcineurin+Pk-FKBP35+CH, (E) Calcineurin+Pk-FKBP35+CH+FK506. Thick arrow-headed line: Complete calcineurin phosphatase activity; bar-headed line: Inhibition of calcineurin phosphatase activity; dotted arrow-headed line: Partial restoration of calcineurin phosphatase activity; thin arrow-headed line: Partial calcineurin phosphatase activity.

the presence of CH might restore the activity of calcineurin, but not fully. This also suggested that CH may inhibit the catalytic activity of Pk-FKBP35 without severe immunosuppressive effect as in FK506. However, further investigation should be done to validate this assumption. This suggested that CH may compete with the formation of the FKBP-FK506 complex which leads to the inability of the complex to inhibit the calcineurin phosphatase activity.

Earlier, Goh *et al.* (2018) proposed that the catalytic domain (FKBD) of Pk-FKBP35, might serve as the primary site for binding of calcineurin. This assertion finds support in Monaghan and Bell (2005), who observed that the inhibition of calcineurin by FKBP35 from *P. falciparum,* was facilitated through its catalytic domain. Notably, Goh *et al.* (2018) reported that apart from FKBD, the non-catalytic domain of Pk-FKBP35, namely TPRD, might also engage in partial interaction with calcineurin and potentially influence its phosphatase activity to some extent. The involvement of the non-catalytic domain in the interaction with calcineurin was also reported for human FKBP51 (Li *et al.*, 2002). Accordingly, the involvement of CH in the complex formation of Pk-FKBP35 and calcineurin is then proposed in Figure 4. In the absence of Pk-FKBP35, FK506 and CH, calcineurin is in a free form and therefore able to access the substrate for its phosphatase activity (Figure 4a). The reduction of phosphatase activity of calcineurin in the presence of Pk-FKBP35 and FK506 is associated with the binding of FK506 to FKBD, which then form a complex with calcineurin and suppress its activity (Figure 4b and 4c). However, in the presence of CH, suppression of phosphatase activity of calcineurin by Pk-FKBP35 was not as extreme as FK506. This indicated that, in the presence of CH, calcineurin was not fully in the complex form with Pk-FKBP35. The proline-rich peptides of CH might occupy FKBD and inhibit its PPIase catalytic activity, but this seems insufficient for strong binding to calcineurin and extreme suppression of its phosphatase activity. Alternatively, CH peptides interfere with the binding between Pk-FKBP35 and calcineurin, resulting in a weak interaction and partial restoration of calcineurin phosphatase activity (Figure 4d). Meanwhile, when FK506 was added to the mixture of Pk-FKBP35 and CH, the phosphatase activity of calcineurin was further decreased, although to a lesser extent. This indicated that FK506 might replace some proline-rich peptides of CH from FKBD and partly facilitate the interaction with calcineurin to block its phosphatase activity (Figure 4e). Notably, the IC_{50} value of FK506 for PPIase catalytic activity of Pk-FKBP35 was extremely lower than that of CH (Table 1), which implied that FK506 might exhibit stronger binding affinity to FKBD than CH. It is therefore plausible to assume that FK506 is able to replace CH peptides from FKBD, which should restore the interaction with calcineurin and inhibit its phosphatase activity. Nevertheless, the mixture of Pk-FKBP35+FK506+CH retained the phosphatase activity of calcineurin much higher than that of Pk-FKBP35+FK506. This implied that FK506 was not able to completely restore the ability of Pk-FKBP35 to interact with calcineurin for inhibition of phosphatase activity. Alternatively, CH peptides did not only occupy FKBD but also TPRD, which further weakened the interaction with calcineurin. Hence, even though FK506 was able to dock into FKBD, the presence of CH peptide in the other region, including TPRD, interferes with the proper interaction of calcineurin. Altogether, this might suggest that CH only exhibited serious inhibition against the PPIase catalytic activity of Pk-FKBP35 but was not really able to facilitate proper complex with calcineurin for its phosphatase activity inhibition. This may lead to possible unique properties of CH in not interfering immune response of the patient pertaining to calcineurin activity yet remain to be experimentally proven.

CONCLUSION

Fish and bovine CH carry the potential as new drug targets to be developed as antimalarial drugs targeting Pk-FKBP35. These CH inhibited the PPIase catalytic activity of Pk-FKBP35 and did not affect the calcineurin phosphatase activity. The suppression of the catalytic activity of the Pk-FKBP35 protein may inhibit the catalysing of new synthesis proteins and disturb the parasite's replication cycle. Additionally, as CH disrupts the formation of the FKBP-FK506 complex, thereby hindering Pk-FKBP35's ability to suppress calcineurin, it is hypothesised that these CH will not interfere with the immune response of the parasite's host. Hence, regarded as a safer option. However, further analysis still needs to be done to verify the assumptions.

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