Article

FIKK Kinase, a Ser/Thr Kinase Important to Malaria Parasites, Is Inhibited by Tyrosine Kinase Inhibitors

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ABSTRACT: A relatively high-affinity inhibitor of FIKK kinase from the malaria parasite *Plasmodium vivax* was identified by in vitro assay of recombinant kinase. The FIKK kinase family is unique to parasitic organisms of the Apicomplexan order and has been shown to be critical in malaria parasites. The recombinant kinase domain was expressed and screened against a small molecule library, revealing a number of tyrosine kinase inhibitors that block FIKK kinase activity. A family of tyrphostins was further investigated, to begin exploring the FIKK kinase pharmacophore. Finally, emodin was identified as a relatively high-affinity FIKK kinase inhibitor, identifying this family of anthraquinones as potential lead compounds for the development of antimalarials targeting the FIKK kinase.
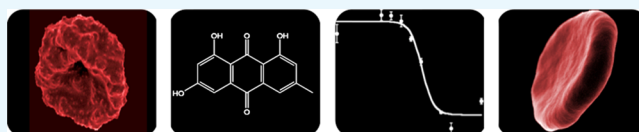
INTRODUCTION

All members of the phylum Apicomplexa, which includes malaria parasites, have at least one FIKK kinase.[1] Indeed, FIKK kinases are a rather prominent feature of the genome of malaria parasites.[2−4] However, their function in the complex life cycle of the malaria parasite is unclear. *Plasmodium falciparum*, the species that causes the most deadly form of human malaria, secretes 17 different FIKK kinases into its host red blood cell, suggesting a role in the blood stages of infection. In vitro secreted FIKK kinases have been shown to phosphorylate human dematin, a cytoskeletal protein of the red blood cell.[5] Likewise, knockout of individual secreted kinases affects erythrocyte rigidity, further implying a role in cytoskeletal alteration.[6,7] However, in *Plasmodium berghei*, a species of malaria parasite that infects rodents, knockout of the FIKK kinase resulted in dramatically slowed growth in the intra-hepatocytic stage, along with impaired sporozoite production.[8] *Plasmodium vivax*, the parasite responsible for most cases of human malaria worldwide, has a single FIKK kinase but is not amenable to genetic manipulation. For this reason, development of a pharmacological inhibitor could provide an important tool for probing its importance to this species of malaria parasite. Because other members of the Apicomplexan family, including the disease-causing parasite *Cryptosporidium parvum*, have a FIKK kinase homolog, inhibitors of this enzyme have the potential to shed light on the physiology of a variety of human pathogens.

In addition to their role in parasite biology, FIKK kinases are unusual members of the protein kinase superfamily. As a class, protein kinases have a number of highly conserved sequence motifs related to their function.[9] However, FIKK kinases lack a number of these conserved sequence features. The FIKK kinase from *P. vivax* (PvFIKK) is a non-RD kinase, lacking the Arg-Asp sequence that typically responds to phosphorylation of the kinase activation loop.[10] In addition, although most FIKK

kinases share the metal-binding DFG loop, this signature is absent in the PvFIKK sequence, with only the Asp itself seemingly conserved. FIKK kinases in general lack the GxGxφG motif, a stretch of amino acids that typically makes up the back of the ATP-binding site.[11,12] Finally, a comparison of the amino acid sequences of the FIKK kinase family from *P. falciparum*, as well as the single FIKK kinases from *P. vivax*, *P. berghei*, and *C. parvum*, shows that they mostly have a small, polar amino acid side chain as the "gatekeeper" residue, controlling access to the ATP-binding site (Figure 1). The typical Ser/Thr gatekeeper residue is a large, hydrophobic residue, and only about 10% of eukaryotic Ser/Thr kinases have a similarly small, polar residue in this position.[13] This stands in contrast to tyrosine kinases, which typically do have small, polar gatekeeper residues. One reason this distinction is important is that the identity of the gatekeeper residue has been shown to affect the types of molecules that can inhibit a given kinase.[14,15] Individually, none of these deviations from canonical kinase motifs is unique to the FIKK kinase, but the fact that they are all seen in a single protein suggests that this is an unusual member of the kinase family.

This work identifies small molecule inhibitors of the FIKK kinase from *P. vivax*, the parasite responsible for most human malaria. The potency of these inhibitors is relatively low, rendering them primarily useful as lead compounds that provide a scaffold for further development into truly potent and selective inhibitors. The identification of potent and selective FIKK kinase inhibitors would provide a tool for studying this enzyme's role in parasite physiology, through pharmacological manipulation. In addition, characterizing the pharmacophore of these unusual Ser/Thr kinases may expand our knowledge of

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vivax|PvFk       ISGNIVIVSEFFSEDILDFIDRR
berghei|PbFk     INGNIVLVSEFFSEDILDFIDRR
cparvum|CsFk     DTTHIILVSEHYGEDLLDYLDKC
PFA0130c|PfFk1   KKGYVVMVCEFFGEDIFDYTISE
PFC0060c|PfFk3   VNGYIVMISEYYGEDIFDFILRE
PFD1165w|PfFk4.1 KKGYVVMISELYGEDVFKYVKNK
PFD1175w|PfFk4.2 VGGNIVLVTELFGEDLFQYINKR
PFE0045c|PfFk5   KKGNVVLIYELFGESLFTNLVKS
MAL7P1.144|PfFk7.1 NCGYVVMVSEYYGEDIFDFIIKR
MAL8P1.203|PfFk8 MNGYIIIVSEFFNEDILDFIDRR
PFI0095c|PfFk9.1 INGNIVIVSELYGDDIKNYKKKM
PFI0100c|PfFk9.2 NKANVIMIWEFFGQNLKEFLHSE
PFI0105c|PfFk9.3 ISGNIVIVSELFGKDIKKFLFTE
PFI0110c|PfFk9.4 KKGYVVMVSEYYGKDTYKYLRKK
PFI0115c|PfFk9.5 MIGYVVMISEFFGEDLENYIYNI
PFI0120c|PfFk9.6 MSGYILMVSESFGEDLETYLCTV
PFI0125c|PfFk9.7 MKGYVVMVFEFFGEDLDCFMNRM
PF10_0160|PfFk10.1 INGNIVIISELFGEDIYTYLTKQ
PF10_0380|PfFk10.2 KDGYVVFISELYGQDLFQYINNK
PF11_0510|PfFk11 SIGNIVIVSELYGQNVFEYIEKR
PFL0040c|PfFk12  MDGYIVMVSELFGEDLFDFNKRF
PF14_0733|PfFk14 MNGYIV--CEEPDKNIMDYLLKN
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Figure 1. Sequence alignment of a variety of FIKK kinases, showing conservation of a small gatekeeper residue (shaded), more typical of eukaryotic tyrosine than serine/threonine kinases.

the kinds of molecules that bind and inhibit eukaryotic kinases in general, particularly those with unusual structural features.

RESULTS

Recombinant expression of some plasmodial FIKK kinases has been previously demonstrated, namely, active full-length PfFIKK4.1, along with kinase domains from PfFIKK4.1 and PfFIKK8 and the nonsecreted FIKK kinase from *P. berghei*.[2,5,8,16] Here, the kinase domain of *P. vivax* FIKK kinase (PvFIKK) was expressed and purified for the first time. The recombinant protein is an active kinase, phosphorylating both dematin (a human red blood cell cytoskeletal protein previously identified as a PfFIKK4.1 substrate) and itself. Its activity was monitored through the use of the phospho-specific protein stain, Pro-Q Diamond (Invitrogen, Carlsbad, CA) (Figure 2).

The recent discovery of a peptide motif that can be phosphorylated by FIKK kinases allowed the development of a plate reader-based enzyme-coupled optical assay, providing continuous kinetic monitoring.[16] Because PvFIKK has a
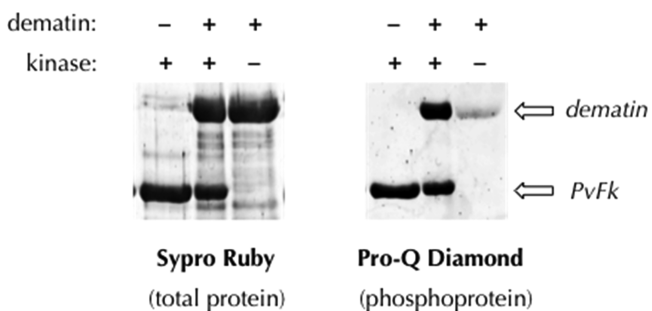

Figure 2. Protein gel of the kinase domain from *P. vivax* FIKK kinase (PvFIKK) heterologously expressed in *Escherichia coli*. Staining with both Sypro Ruby (stains all proteins) and Pro-Q Diamond (stains phosphoproteins) reveals that the recombinant kinase domain is active and phosphorylates itself (autophosphorylation), as well as recombinant human dematin, a known substrate of FIKK kinases.

tyrosine kinase-like gatekeeper residue, we theorized that Tyr kinase inhibitors may be effective against the enzyme. We used the enzyme-coupled assay to test a small family of tyrphostins, known as Tyr kinase inhibitors[17] (Figure 3). This assay identified compounds with the ability to inhibit PvFIKK. The dose−response curves showed clear differences among the family members, where tyrphostin 47 was the most potent of the compounds tested. This compound is significantly more effective than tyrphostin 46, in spite of their great chemical similarity. It was not possible, in this assay, to achieve saturating concentrations of tyrphostins because they absorb significantly at the analyte wavelength of 340 nm. In the absence of these saturating concentrations, IC_{50} for tyrphostin 47 can only be estimated to be approximately 100 μM.

The ultraviolet absorption of potential inhibitors at 340 nm makes a non-absorbance-based assay attractive for screening of inhibitors that need to be applied at relatively high concentration. Using human dematin as the substrate, a radioisotope-based filter-binding assay was adapted to quantify phosphorylation of the protein target via liquid scintillation counting. This endpoint assay was used to assess the ability of compounds from an 84-compound library of known kinase inhibitors to inhibit PvFIKK at a fixed concentration of 1 mM. Assigning an arbitrary cutoff of 70% inhibition for both replicates revealed three compounds (emodin, CGP 57380, and bisindolylmaleimide VII) with apparent ability to block PvFIKK activity, noting that this collection of compounds does not include tyrphostins (Figure 4). These three compounds are all known to inhibit tyrosine kinases (emodin has been shown to inhibit both classes of kinase).[18,19] Returning to the enzyme-coupled assay for validation of the results of the screen, selected compounds were retested (Figure 5). To rule out false positives, the three compounds emerging from the screen were tested. To test for false negatives, a selection of compounds that were not effective in the filter-binding assay were tested. Generally, the enzyme-coupled assay validated the results of the radioisotope assay in that the three compounds emerging from the screen reduced residual PvFIKK activity to less than 50%, at a concentration 10-fold less than that of the filter-binding assay. Likewise, compounds that were ineffective in the filter-binding assay left PvFIKK activity within 90% of dimethyl sulfoxide (DMSO)-treated enzyme. In addition to these compounds from the screen, the tyrphostins were included, along with a bisindolylmaleimide not included in the initial screen. Of the compounds validated as potential PvFIKK inhibitors, emodin appeared the most effective at concentrations below 100 μM. A dose−response curve for emodin showed that it inhibits PvFIKK at a concentration of 1.9 ± 0.7 mM (Figure 6). Data for this dose−response curve were collected over six independent trials, performed by different individuals with different preparations of recombinant PvFIKK.

DISCUSSION

The kinases of pathogens have often been proposed as potential drug targets.[20] Interfering with signal transduction has strong potential to render parasites harmless, either killing them outright, inhibiting their growth, or interfering with their ability to infect. Of course, our own cells also rely on kinases for signal transduction, so any drug that targets a parasite kinase must be specific, to avoid affecting its human host. Malaria parasites are eukaryotes, so their set of kinases has a great deal of overlap with our own. However, one family of kinases shared
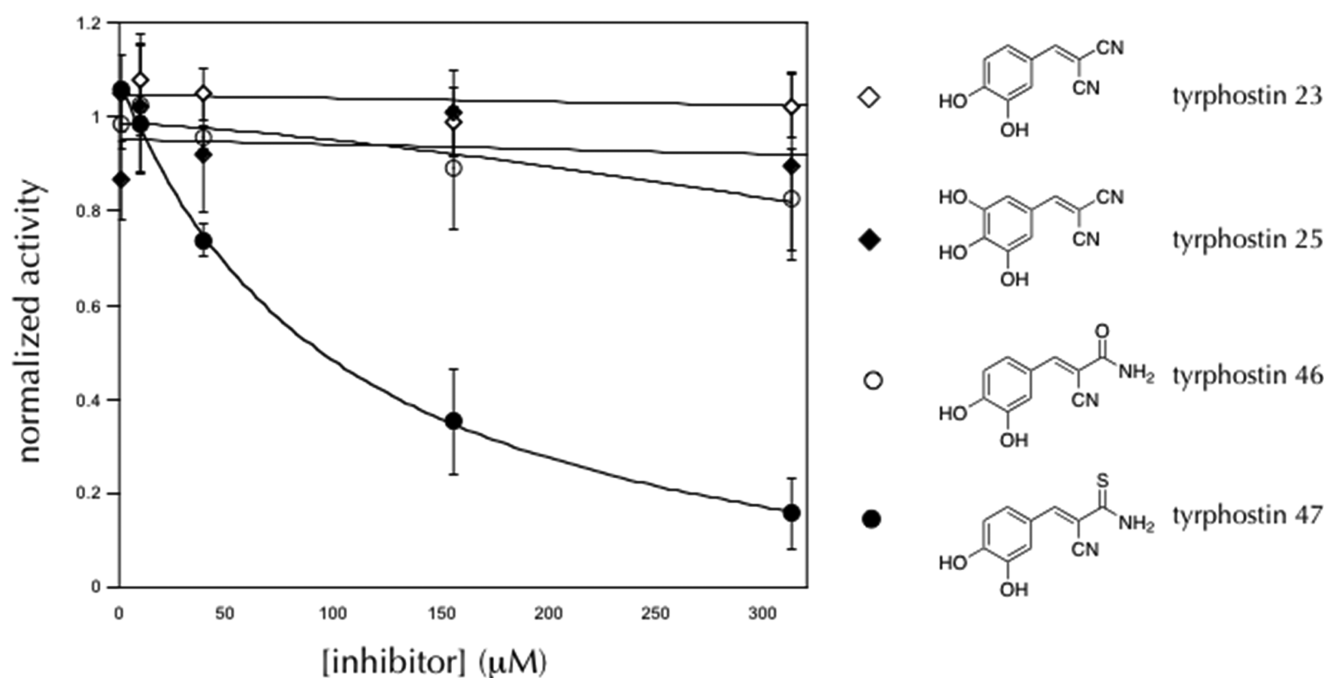
Figure 3. Continuous, colorimetric enzyme-coupled assay demonstrates differential inhibition of recombinant PvFIKK by various tyrphostins, a class of tyrosine kinase inhibitor.
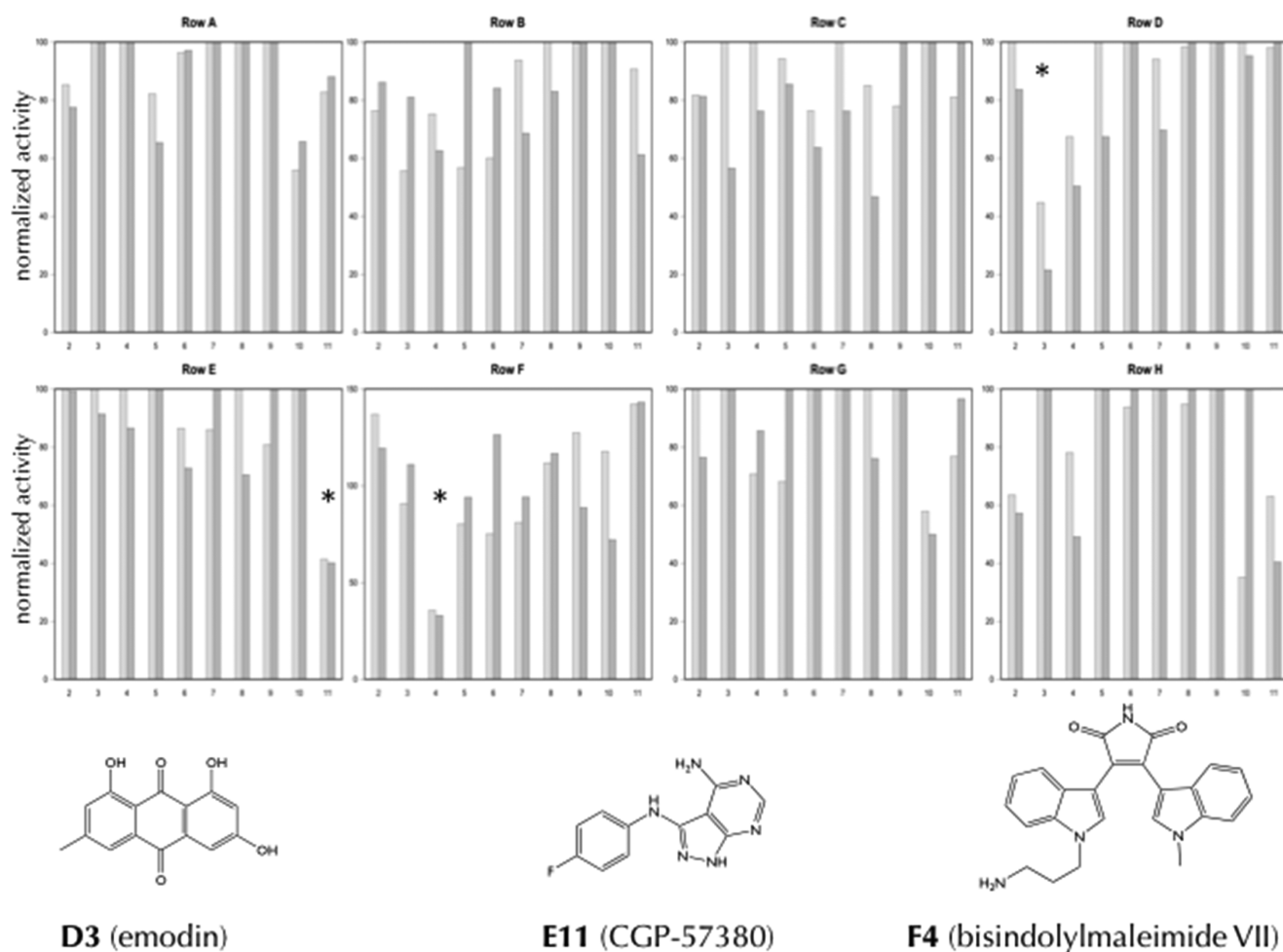

Figure 4. Screening of a library of known kinase inhibitors against PvFIKK, using a filter-binding radioisotope assay to monitor dematin phosphorylation.
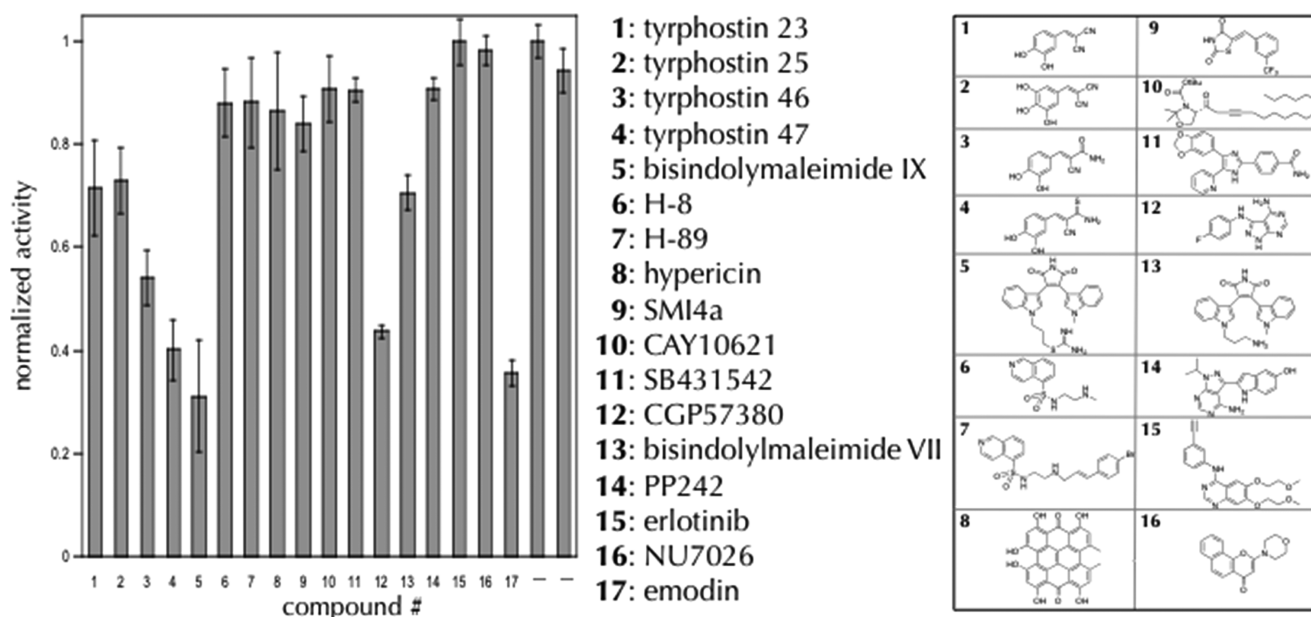
Figure 5. Validation of compounds emerging from library screen by measuring inhibition of PvFIKK by 100 μM compound in an enzyme-coupled assay.
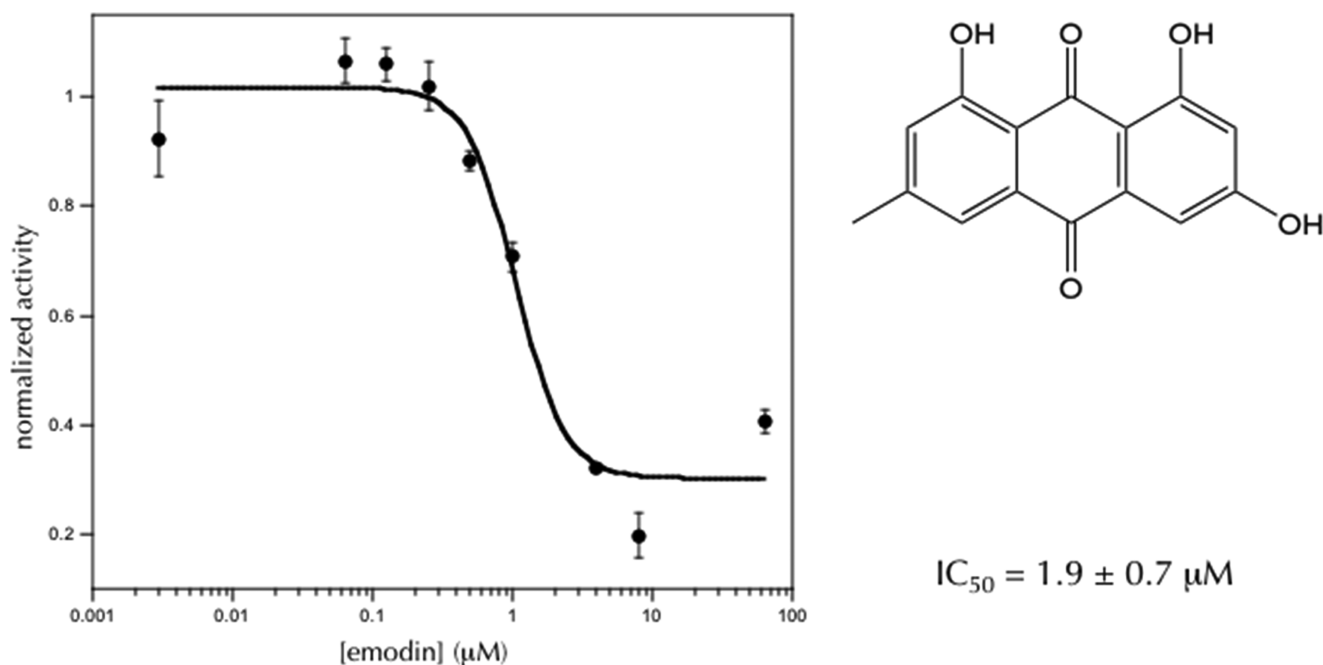

$$IC_{50} = 1.9 \pm 0.7 \ \mu M$$

Figure 6. Typical dose–response curve for inhibition of recombinant PvFIKK by emodin. IC_{50} value determined as an average of six independent trials.

by all malaria parasites has rather distinctive features. These kinases are referred to as FIKK kinases, after a four amino acid Phe-Ile-Lys-Lys sequence motif that they hold in common.[2−4] Most malaria parasites have a single FIKK kinase, and it has been shown to be functionally important in *P. berghei*, a rodent malaria that serves as a genetically manipulable model for the genus.[8] On the other hand, knockout of the FIKK kinase from the related parasite *Toxoplasma gondii* does not affect parasite growth.[21] Likewise, the recent discovery of an inhibitor for *C. parvum* FIKK kinase shows that parasites treated with this inhibitor survive.[22] Pharmacological tools to block the activity of the plasmodial FIKK kinase may then be helpful in resolving

the question of which plasmodial FIKK kinases are essential. Globally, most human malaria cases result from infection by the species *P. vivax*.[23] We report here the identification of emodin, a plant-derived anthraquinone, as a relatively high-affinity inhibitor of the FIKK kinase from *P. vivax* (PvFIKK).[24]

All protein kinases must bind two substrates, ATP and their peptide target of phosphorylation. A typical kinase ATP-binding site is characterized by a glycine-containing motif (GxGxxG) that makes backbone hydrogen bonds to the β- and γ-phosphates of ATP and a residue at the back of the binding site that interacts with the exocyclic amine (N6) of the adenine nucleobase.[9,11] The identity of the latter residue has been

shown to control the inhibitors that are able to bind in the ATP-binding site, so it is referred to as the gatekeeper.[15] This gatekeeper residue is a large, hydrophobic residue in ~90% of eukaryotic Ser/Thr kinases and a small, polar residue in most tyrosine kinases.[13] The FIKK kinase family is unusual in both regards. First, it lacks a recognizable GxGxxG motif. Second, it has a tyrosine kinase-like gatekeeper residue, despite being a Ser/Thr kinase (Figure 1). In addition to these two alterations to the ATP-binding site, FIKK kinases are unusual Ser/Thr kinases in that they lack two signatures associated with activation by phosphorylation. They are the so-called non-RD kinases, lacking the conserved Arg residue that would typically stabilize a phosphoserine or phosphothreonine in the activation loop.[10] Second, the family of FIKK kinases lacks a strong consensus activation loop.[12] Because of these differences between typical eukaryotic Ser/Thr kinases, it may be possible to develop inhibitors that specifically target the FIKK kinase, while not interfering with host kinases.

Within the genus Plasmodium, the kinase domains of FIKK kinases are fairly highly conserved.[1−4] There is 84.5% sequence identity between *P. vivax* FIKK kinase and its closest homolog *P. falciparum* FIKK8. Across the Order Apicomplexa, there is somewhat less conservation. For example, the *Cryptosporidium* and *P. vivax* FIKK kinase domains are 38% identical. The FIKK kinases also have a large N-terminal domain of unknown function that is much less highly conserved. Another feature of the plasmodial FIKK kinases is that the family has undergone tremendous expansion in *P. falciparum* and some related species that infect higher primates so that there are 21 different PfFIKK kinases, almost all of which have an export signal.

The experimental approach taken here to identify potential FIKK inhibitors was to purify a recombinant version of the kinase domain of PvFIKK and then screen a library of small molecule kinase inhibitors. There is precedent for the recombinant expression of full-length FIKK kinases from *P. falciparum* and FIKK kinase domains from *P. falciparum, P. berghei*, and *C. parvum*, another disease-causing member of the Apicomplexan order.[5,8,16] This strategy has been effective in identifying inhibitors for the CpFIKK and PbFIKK kinases.[8,16] However, this is the first reported expression of *P. vivax* FIKK kinase. The PvFIKK kinase domain expressed and purified from *E. coli* was shown to be active against itself (autophosphorylation) and against a known FIKK kinase substrate, human dematin (Figure 2). Almost all Ser/Thr kinases autophosphorylate, but almost all Ser/Thr kinases have the RD motif and a recognizable activation loop.[25] The physiological role of autophosphorylation in a kinase like PvFIKK that lacks these features is not clear. Autophosphorylation does not appear to have an effect on PvFIKK activity in our assay. Osman et al. noted that they coexpressed FIKK8 from *P. falciparum* with phosphatase, to avoid autophosphorylation, and then preincubated the kinase with ATP prior to substrate addition.[16] In these experiments, we did not observe a noticeable difference in activity before and after incubation. It is possible that the observed autophosphorylation is not physiologically relevant, but it could also be the case that it has a role that would not be evident in these experiments, such as involvement in subcellular localization. The use of human dematin as a PvFIKK substrate is based on the observation that, like other FIKK, it will readily phosphorylate this protein.[5] Dematin is a protein of the red blood cell cytoskeleton, and there is no evidence that PvFIKK is exported. So, there is no a priori reason to believe that

dematin is a physiologically important substrate of PvFIKK. It was used here as a convenient protein substrate.

Because of PvFIKK's tyrosine kinase-like gatekeeper residue, we wondered whether tyrosine kinase inhibitors might potentially bind. We chose a small family of tyrphostins, known as Tyr kinase inhibitors, for assessment.[17] The assay was an enzyme-coupled determination of PvFIKK's ability to phosphorylate a Ser residue in a small peptide previously identified as a PfFIKK8 substrate.[18] As with dematin, PvFIKK is able to use this substrate, but no physiological relevance is imputed to it. A range of dose−response was observed among this small family of tyrphostins (Figure 3). The α-cyano nitriles had practically no effect in druglike concentrations (under 250 μM), regardless of the pattern of cinnamyl ring hydroxylation. However, α-amido nitriles were much more effective. Because these compounds are rather strongly colored, their absorption at 340 nm greatly complicates their use in the enzyme-coupled optical assay, particularly at the high concentrations needed to achieve saturation. Thus, IC_{50} can only be estimated, but the thioamide is clearly the most effective inhibitor in this family, with an apparent IC_{50} of roughly 100 μM.

Encouraged by the identification of tyrphostin 47 as a reasonably effective PvFIKK inhibitor, we obtained a library of small molecule kinase inhibitors containing both Ser/Thr and Tyr kinase inhibitors. Using a filter-binding assay with human dematin as substrate and with radioisotopes providing signal, we screened this 84-compound library for compounds with the ability to inhibit PvFIKK (Figure 4). To be as inclusive as possible, the screening was carried out at an inhibitor concentration of 1 mM. The three most effective compounds in this screen were annotated as tyrosine kinase inhibitors, with the exception of emodin, which has been reported to inhibit both Ser/Thr and Tyr kinases.[18,19] To validate the results of this screen, we retested a number of compounds from the screen using an enzyme-coupled assay.

At an inhibitor concentration of 100 μM, the results of the filter-binding screen were generally validated (Figure 5). Compounds identified as active in the filter-binding assay reduced the activity of PvFIKK to less than 50% of the uninhibited value. Those compounds judged not to be active in the filter-binding assay served as a negative control in this assay, leaving the treated activity within 90% of the DMSO-treated activity. The tyrphostin family and a bisindolylmaleimide not found in the screen were also assessed in this assay. In another validation step, the three compounds emerging from the screen were assessed for their ability to inhibit PvFIKK at concentrations closer to 10 μM. In this screen, emodin appeared to be most active.

A dose−response curve for PvFIKK activity in the presence of emodin gives an IC_{50} of 1.9 ± 0.7 μM (Figure 6). Although not sufficiently potent for use as an inhibitor immediately, the emodin architecture may constitute a useful starting point for synthesizing analogues with increased potency and selectivity. Emodin (CAS 518-82-1, PubChem CID: 3220) is a hydroxylated anthraquinone produced by a number of plant species.[24] Its physiological role in plants is not known, but plants that contain the compound have found their way into a number of traditional pharmacopeias. As the name suggests, emodin has traditionally been used as an emetic. More recent investigations into the clinical efficacy of emodin have shown it to inhibit both Ser/Thr and Tyr kinases and to act as a phytoestrogen.[18,19,26] Indeed, emodin's bioactivity led to its inclusion in the LOPAC[1280] library of pharmacologically active

compounds. This library has been screened against seven strains of *P. falciparum* in human blood culture, with the finding that emodin's efficacy against *P. falciparum* strains was mixed.[27] In three of the seven, emodin showed antimalarial activity, but results from the other four strains were inconclusive. Although the effectiveness of emodin against some strains of *P. falciparum* is suggestive, it is important to note that the ability of *P. falciparum* to compensate for the inhibition of FIKK kinase activity may be much greater than that of *P. vivax*. Unlike *P. vivax* and most other malaria parasite species, which have a single FIKK kinase, the more virulent *P. falciparum* species has 19 different FIKK kinases. Because *P. vivax* cannot be cultured in the laboratory setting, the effect of emodin on parasite survival is difficult to gauge.

The single-celled organisms of the Apicomplexan order include all malaria parasites. The FIKK kinase specific to these organisms has a number of distinctive features, making it a plausible drug target. We expressed the kinase domain of the FIKK kinase from *P. vivax* and screened it against a small molecule library, identifying the first reported inhibitors of the FIKK kinase family. The anthraquinone emodin inhibits PvFIKK kinase with relatively good (micromolar) affinity, establishing it as a potential lead compound for the development of selective inhibitors targeting the FIKK kinase.

■ MATERIALS AND METHODS

Expression and Purification of Recombinant FIKKPv-FIKK Kinase Domain in *E. coli*. The sequence of the *P. vivax* FIKK kinase was obtained from the PlasmoDB database (http://plasmodb.org, University of Pennsylvania, Philadelphia, PA).[28] An *E. coli*-harmonized gene corresponding to the sequence between residues EKQIK... and ...SCKLQ was synthesized (Biomatik, Wilmington, DE) and subcloned into the pHAT2 bacterial expression vector (M. Hyvönen, Cambridge University, Cambridge, U.K.) using NcoI/XhoI. This expression vector encodes an N-terminal 6His tag.[29]

The T7 Express strain (NEB, Beverly, MA) was chosen for the production of recombinant protein in *E. coli*. A 20 mL overnight culture of T7 Express cells transformed with the kinase domain of PvFIKK kinase in pHAT2 was inoculated from a glycerol stock in LB media containing ampicillin (100 mg/mL). This overnight culture was used to inoculate 1 L of medium (LB with 100 mg/mL ampicillin in both cases). The culture was grown at 37 °C with shaking at 250 rpm until $OD_{600} = 0.375$. The temperature of the incubator was reduced to 20 °C. After 10−20 min, IPTG was added to a final concentration of 0.7 mM. Overnight induction at 20 °C with shaking was followed by harvesting of the cells as above.

The frozen cell pellet was thawed in lysis buffer (20 mM HEPES pH 7.0, 1 M NaCl, 10 mM imidazole). Cell lysis was facilitated by probe-tip sonication, followed by clarification of the lysate by centrifugation at 34 000*g*, 4 °C, 50 min. The lysate was either loaded onto a 5 mL Profinity IMAC column (BioRad, Hercules, CA), typically at a flow rate of 0.5 mL/min, or bound in batches to the same resin. Nonspecifically bound proteins were removed with wash buffer (20 mM HEPES pH 7.0, 500 mM NaCl, 20 mM imidazole) and the protein eluted in high imidazole (20 mM HEPES pH 7.0, 500 mM NaCl, 250 mM imidazole). The fractions containing the highest protein concentration were pooled, where the concentration was determined by absorbance at 280 nm, measured by NanoDrop UV−vis spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). The pooled eluant from the nickel column was

loaded onto a preparative gel filtration column (Sephacryl S-200 16/60, GE Biosciences) and eluted at a flow rate of 0.25 mL/min with 20 mM HEPES pH 7.0, 500 mM NaCl, 1 mM dithiothreitol. The fractions corresponding to the appropriate monomeric molecular weight (50 kDa) were pooled and concentrated with a 4 mL capacity, 10 kDa cutoff centrifugal concentrator (Vivaspin 6, Sartorius, Göttingen, Germany). To provide a negative control, a mutant predicted to be inactive was prepared by changing a conserved active site Asp to Asn (..SHLDLTPENIL.. to..SHLNLTPENIL..). This kinase-dead mutant was expressed and purified under the same conditions as the wild-type kinase domain. A plasmid for bacterial expression of a modified dematin was the kind gift of Dr. James McKnight (Boston University School of Medicine, Boston, MA).[30]

Assessment of Kinase Activity by Phospho-Specific Staining. The homogeneity of the purified PvFIKK kinase domain was assessed by a sensitive total protein stain (Sypro Ruby, Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. In addition, ratiometric analysis of staining by the phospho-specific dye Pro-Q relative to Sypro Ruby staining provides a means of assessing the extent of phosphorylation catalyzed by recombinant PvFIKK kinase. The kinase domain alone or with recombinant human dematin at 1 mg/mL each (approximately 20 mM for PvFIKK and 15 mM for dematin) was incubated at 30 °C for 30 min in a 10 mL volume. The reaction was quenched by the addition of 2× sodium dodecyl sulfate (SDS) gel-loading buffer, and 15 mL was loaded onto a polyacrylamide gel for SDS-polyacrylamide gel electrophoresis. According to the manufacturer's long protocol, the gel was treated and then stained with Pro-Q Diamond and imaged (Typhoon FLA 7000 IP, GE Biosciences). The gel was then further stained with Sypro Ruby.

Analysis of Kinase Activity by Enzyme-Coupled Assay. Following Osman et al., an enzyme-coupled assay for PvFIKK was set up using a PK/LDH couple and a 10-mer peptide shown to be a PfFIKK8 substrate (RRRAPSFYRK, Biomatik, Wilmington, DE).[16] The kinase buffer was 50 mM HEPES pH 7.5, 120 mM NaCl, and 10 mM $MgCl_2$. In a 50 μL reaction, 3 μL of coupling enzyme was added (pyruvate kinase/lactate dehydrogenase P0294, Sigma-Aldrich, St. Louis, MO). An enzyme concentration of 1 μM was determined to be in the linear response range at fixed ATP, NADH, PEP, and peptide concentrations (1, 200, 2, and 500 mM, respectively). Where desired, 5 μL of inhibitor was added at the indicated concentration, in up to 50% DMSO, for a final concentration of up to 5% of the total reaction volume. A small effect on enzyme activity was observed at 10% DMSO, but not at 5% and below. For medium throughput, a Sunrise microplate reader was employed, set to a constant temperature of 37 °C (Tecan, Männedorf, Switzerland). Reaction conditions were established that produced slopes that were reproducible to within 5%, using 50 μL of reactions in half-area UV-transmitting 96-well plates (Greiner Bio-One, Monroe, NC). As a negative control, the kinase-dead version of the PvFIKK kinase domain was used at the same concentration as the wild-type domain. No activity above background hydrolysis of NADH was observed when using the kinase-inactive mutant. With some inhibitors, elevated concentrations interfered with the assay by absorbing strongly at 340 nm. For these inhibitors, the enzyme-coupled assay was not able to be used to accurately assess enzyme behavior at saturating inhibitor concentrations.

Filter-Binding Assay. To screen an entire compound library (Kinase Screening Library 10505, Cayman Chemical Company, Ann Arbor MI), a filter-binding assay was designed, closely following the recommendations of Cohen et al.[31] In a 10 mL volume, 1 mM purified PvFIKK was mixed with 40 mM purified human dematin−GST fusion protein in a kinase buffer (50 mM HEPES pH 7.5, 120 mM NaCl, 10 mM $MgCl_2$). To initiate the reaction, ATP was added to a final concentration of 100 mM, from a 250 mM stock solution that was supplemented with γ-^{32}P-ATP to approximately 1×10^5 cpm (EasyTide, 3000 Ci/mmol, PerkinElmer). When desired, 1 mL of inhibitor was added (in up to 50% DMSO) to a final concentration of 1 mM. The reaction was incubated at 30 °C for 10 min and then quenched by pipetting onto 2.5 cm radius filter circles of either P81 phosphocellulose or nitrocellulose paper (Whatman, GE Life Sciences), followed by immediate immersion in 75 mM phosphoric acid. After two 5 min washes in a large volume of fresh 75 mM phosphoric acid, the filter circles were treated with acetone and then air-dried. For scintillation counting, the dried filter circles were placed into scintillation vials without scintillation fluid and counted directly (Beckman GS2500, GMI). Each condition was replicated, and the background-corrected reading in counts per minute was normalized to the inhibitor-free condition.

Validation of Screening by Enzyme-Coupled Assay. Compounds identified as potential inhibitors of PvFIKK protein kinase activity from the filter-binding assay were retested against peptide in the previously described enzyme-coupled assay. In addition, a selection of compounds (H-89, hypericin, SMI4a, CAY10621, PP242, erlotinib, and NU7026) with no apparent activity in the filter-binding assay was assessed, to screen for false negatives. Whereas compounds were screened in the filter-binding assay at a 1 mM concentration, validation was carried out at a concentration of 100 μM. Results were generally quite consistent between the two screens, despite differences in methodology and substrate (the recombinant human cytoskeletal protein dematin versus a 10 amino acid peptide optimized as a PfFIKK8 substrate).

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Notes
The authors declare no competing financial interest.

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ABBREVIATIONS

FIKK, FIKK kinase; PvFIKK, *Plasmodium vivax* FIKK kinase; PfFIKK4.1, *Plasmodium falciparum* FIKK kinase 4.1

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