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Development of a Plasmepsin II Fluorescence Polarization Assay Suitable for High Throughput Antimalarial Drug Discovery

HORST FLOTOW, CHUNG-YAN LEONG, and ANTONY D. BUSS

ABSTRACT

Despite decades of research, malaria remains the world's most deadly parasitic disease. New treatments with novel mechanisms of action are urgently needed. Plasmepsin II is an aspartyl protease that has been validated as an antimalarial therapeutic target enzyme. Although natural products form the basis of most modern antimalarial drugs, no systematic high-throughput screening has been reported against this target. We have designed an effective strategy for carrying out high-throughput screening of an extensive library of natural products that uses a fluorescence resonance energy transfer primary screening assay in tandem with a fluorescence polarization assay. This strategy allows rapid screening of the library coupled with effective discrimination and elimination of false-positive samples and selection of true hits for chemical isolation of inhibitors of plasmepsin II.

INTRODUCTION

Nearly 40% of the world's population lives in areas at risk for malaria. Three hundred to 500 million new cases of malaria occur each year, resulting in an estimated 0.5–2.5 million deaths each year, the majority being children under 5 years of age. The emergence of (multi)drug-resistant malaria parasites and insecticide-resistant mosquitoes has triggered an alarming increase in the incidence and spread of the disease. No effective vaccine is available, and few new antimalarial drugs are undergoing clinical trials, so there is an urgent medical need for therapies based on novel mechanisms of action.[1,2]

Hemoglobin degradation (Fig. 1) inside infected red blood cells is essential for the growth and survival of the erythrocytic malaria parasite, providing the amino acids required for parasite protein synthesis. A number of unique parasitic proteases have been identified that are utilized to digest hemoglobin in the parasite food vacuole, and these represent valid targets for the development of novel antimalarial drugs (reviewed by Francis et al.[3]). Plasmepsins are unique parasite-encoded aspartic proteases that appear to be indispensable for parasite survival. Aspartic protease inhibitors have a profound effect on parasite multiplication *in vitro*, suggesting that plasmepsins are valid drug targets with a novel mechanism of action for antimalarial therapy.[4,5] Plasmepsin II was selected for initial studies because both the native and recombinant forms are well characterized, a crystal structure has been determined, and sufficient quantities of the expressed enzyme are available for assay development and high-throughput screening.[6–8]

To screen our library of natural product extracts, we have implemented a fluorescence resonance energy transfer (FRET) assay similar to those described in the literature.[9,10] To effectively and quickly select true hits and thus overcome some of the difficulties associated with autofluorescence, color interference, and the like, we have also developed a fluorescence polarization (FP) assay for plasmepsin II (see Owicki[11] for a review on the principles and applications of FP measurements).

MATERIALS

Proplasmepsin II was generously provided by Dr. Richard Moon from Roche Basel (Switzerland). The substrate peptides for the FRET (dabsyl-GABA-ERJFLSFPE-edansyl, where GABA = γ-aminobutyric acid and J = L-norleucine) and FP (biotin-LERJFLSFPK-fluorescein, where J = L-norleucine) assays were synthesized by Mimotopes (Melbourne, Australia). Pepstatin A, sanguinarine chloride, esculin, scopoletin, and harmaline were from Sigma-Aldrich Corp. (St. Louis, MO). All compounds and the peptide substrates described in this study were made up as stock solutions in dimethylsulfoxide (DMSO); compound dilutions were also carried out in DMSO. Avidin and streptavidin were obtained from ProZyme (San Leandro, CA).

Merlion Pharmaceuticals Pte. Ltd., The Fleming, Singapore.

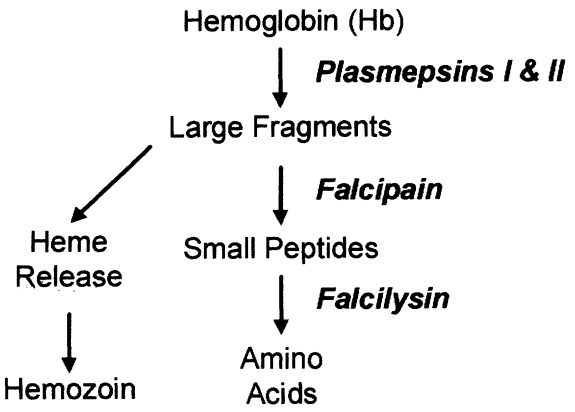

FIG. 1. Hemoglobin metabolism in the malaria parasite *Plasmodium falciparum*.

METHODS

Pro-plasmepsin II activation

Proplasmepsin II was activated by diluting the protein to 0.3 mg/ml in 0.1 M citrate (pH 4.5) containing 0.1% Tween-20 and 50 mM dithiothreitol and incubating at room temperature for 40 min. The activated enzyme was diluted in 50 mM sodium acetate buffer (pH 4.7) plus 0.01% Tween-20 and added to the substrate to start the reaction.

FRET assay

The FRET assays contained enzyme at 75 ng/ml, 10 μM substrate peptide, and samples at varying concentrations. These homogeneous continuous assays were carried out in a 50-μl final volume in 384-well black microplates and read in either an ULTRA or SPECTRAFLUOR PLUS (Tecan Group, Ltd., Männedorf, Switzerland) using 360/535-nm excitation/emission filters. Under these conditions, the assay was linear over at least 2 h.

FP assay

The optimized assay conditions for the FP assay were enzyme at 75 ng/ml, 1 μM FP-substrate peptide, and inhibitors or samples where appropriate. The reaction was initiated by the addition of enzyme to the reaction mixture containing the peptide and samples in a 50-μl final volume in 384-well black microplates. After 30 min, the assay was stopped by the addition of 25 μl of 75 mM borate (pH 10) containing 0.05% Tween-20 and streptavidin (83.3 μg/ml) and read in a Tecan ULTRA using 485/535-nm excitation/emission filters.

RESULTS

A FRET assay for plasmepsin II activity similar to ones described in the literature was set up first to screen the natural product extract library. This proved to be a fast and simple yet

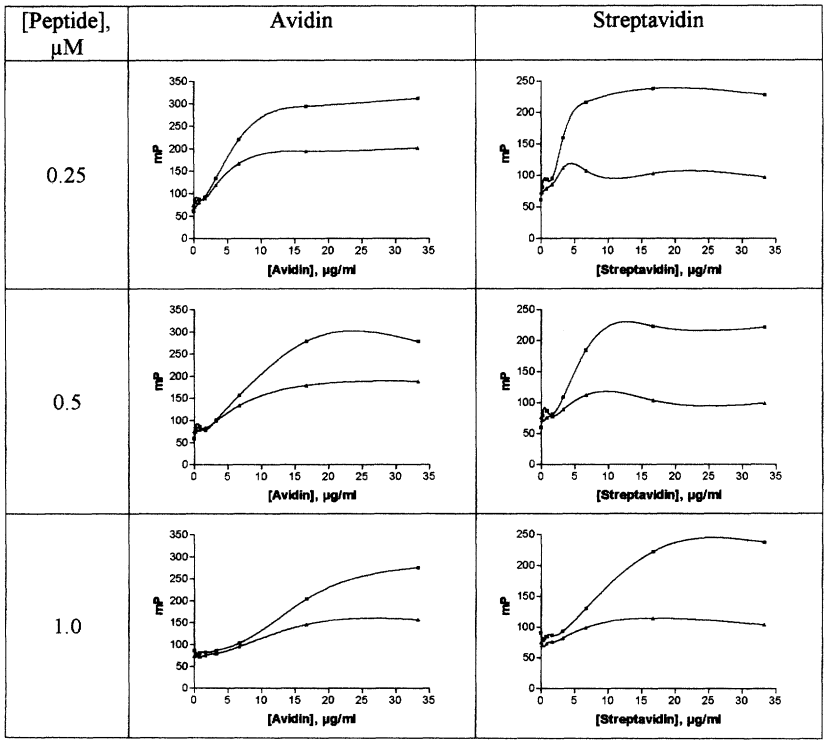

FIG. 2. Titration of avidin and streptavidin. Assays were carried out as described in *Methods*, with the addition of avidin and streptavidin at the concentrations indicated.

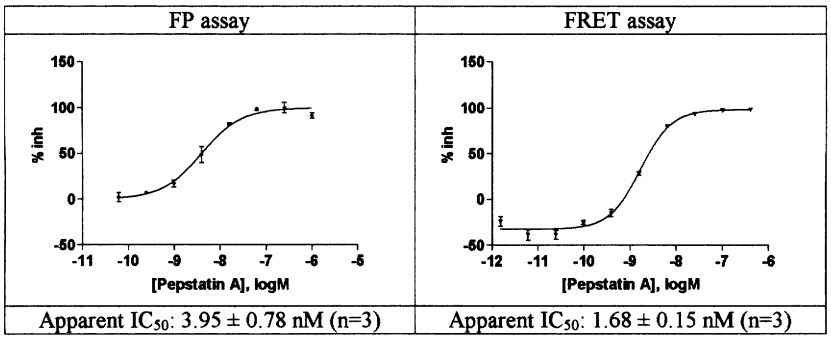

FIG. 3. Pepstatin A dose response. Assay were carried out as described in *Methods*, with the addition pepstatin A at the appropriate concentration.

very reproducible and robust screening assay, costing around 8 cents per well. To discriminate false positives and facilitate selection of hits for chemical isolation of inhibitory compounds, a FP assay was developed and employed alongside the FRET assay during retesting of hits from the primary screening.

The substrate used for the FP assay has the same cleavage site as the one used in the FRET assay, and is labeled with biotin and fluorescein at the amino- and carboxyl-termini, respectively. Two potential "anchors" for this biotinylated peptide were tested. Streptavidin gave better signal:noise ratios than avidin (Fig. 2). To ensure the robustness and extend the linear range of the time course of the FP assay, a 1 μM peptide substrate concentration was chosen and streptavidin added to 20 μg/ml for all subsequent assays. Under these conditions, the assay was linear for at least 60 min (results not shown). The reaction was stopped by adding the streptavidin in 75 mM borate buffer (pH 10), which effectively terminates the reaction and increases the pH for optimal fluorescein fluorescence. The FP signal of the stopped reaction was stable for at least 24 h at room temperature, potentially allowing unattended operation of this assay overnight. Although the cost of this assay is nearly the same as the FRET assay (around 10 cents per well), it should be noted that measuring FP requires more sophisticated instrumentation.

Both assays were highly reproducible and produced consistent IC_{50} values for pepstatin A of 2–4 nM (apparent IC50: FRET 1.68 ± 0.15 nM [n = 3], FP 3.95 ± 0.78 nM [n = 3]; Fig. 3). The quality control results of both assays showed excellent day-to-day reproducibility, with Z′ values[12] consistently better than 0.8 (Table 1). Because the samples were dissolved and added in DMSO, the DMSO tolerance of plasmepsin II and the assay components was investigated. Both the FP and the FRET assay could tolerate in excess of 10% DMSO in the assay (results not shown).

Natural product extracts and isolated natural products are often highly colored, and many of them are fluorescent. Figure 4 shows the utility of having both assay formats available to select screening hits for further chemical isolation work. Sanguinarine chloride and pepstatin A were picked up as hits in the FRET assay. The FP assay was able to eliminate sanguinarine chloride as a false positive, while clearly showing activity with pepstatin A, a true-positive screening hit. Esculin (a coumarin derivative), scopoletin, and harmaline showed high fluorescence in the FRET assay (wavelength of 360/535 nm [excitation/emission]), registering as false negatives and potentially masking any inhibitory activity found in those samples. Using the FP assay, this interference could be completely eliminated because all three samples showed baseline activity.

DISCUSSION

Although most antimalarial drugs are based on natural products, few high-throughput screening programs against extensive natural product extract collections have been reported. The main reason has been that very few validated antimalarial target enzymes with novel mechanisms of action are available in the quality and quantity required for assay development and high-throughput screening. In addition, the assay formats employed for carrying out small-scale screening of inhibitors of many potentially novel antimalarial targets are not amenable for reformatting to make them tractable for high-throughput screening.

We have chosen plasmepsin II, a protease unique to the parasite, which is involved in hemoglobin catabolism and essential for parasite survival and propagation. The activity of plasmepsins can be assayed using peptide substrates in a FRET format, which can be adapted for high-throughput screening. Plasmepsin II has been cloned and expressed in an active form by several groups,[13,14] and a structure in complex with isovaleryl pepstatin has been determined.[8] Although plasmepsin II has been used to screen combinatorial protease inhibitor li-

TABLE 1. QUALITY CONTROL PARAMETERS OF THE FP AND FRET ASSAYS[a]

	FP	*FRET*
Average signal ± SD	245 ± 7	2239 ± 124
Average noise ± SD	67 ± 4	82 ± 4
Signal:noise	3.66	27.3
Z′	0.81	0.82

[a]Assay were carried out as described in *Methods*, and the results shown represent the average of 8 experiments.

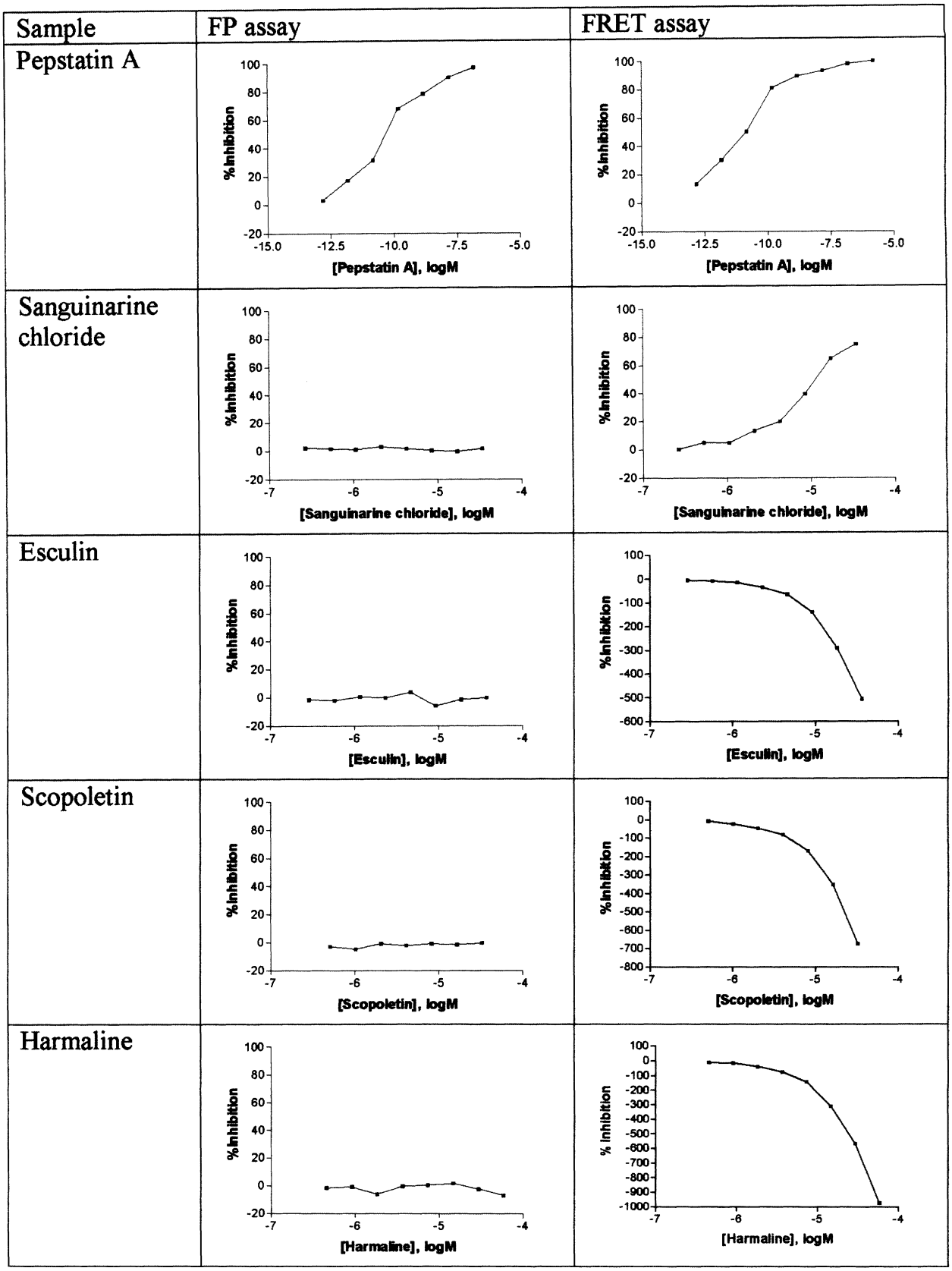

FIG. 4. Comparison of plasmepsin II FRET and FP assays to eliminate color and fluorescence interference (false positive/negative hits). Assay were carried out as described in *Methods*, with the addition of the compounds at the appropriate concentration.

braries with some success,[14,15] no natural product screening has been reported.

Screening natural product extracts requires special considerations in the selection of assay formats and assay development. Many natural product extracts (samples) are highly colored and may contain fluorescent compounds that interfere with the readout of a number of commonly used chromophores and fluorophores. Furthermore, the samples are complex mixtures of compounds, and the isolation of the active one may require substantial effort. It is therefore critical to develop a screening assay that is fast, simple, and very robust, along with relevant secondary assays for effectively and rapidly selecting the true hits. For plasmepsin II, we have therefore chosen a simple and economical FRET assay to carry out the screening. This kinetics assay is simple and highly reproducible, with very little day-to-day variation. By carrying out this screen in 384-well plates, we have further minimized the reagent consumption and the time taken to carry out the primary screening. Although the hit rate from this assay is acceptably low, our preliminary testing revealed a few natural products that could interfere with the readout, producing false positive (hits) and false negatives (which could mask hits).

To allow rapid selection of true hits and unmask any potential inhibitors in strongly fluorescing samples, we have developed a plasmepsin II FP assay using a small peptide substrate that is fluorescein labeled at one end and biotinylated at the other. When streptavidin is added to stop the reaction, proteolytic activity is thus measured as a decrease in polarized fluorescence. Fluorescein is a cost-effective, widely available fluorophore that has excitation and emission wavelengths that are quite distinct from the ones used for EDANS (5-((2-aminoethyl)amino)naphthalene-1-sulfonic acid). Although the time taken to read each 384-well plate is increased approximately 4-fold over the FRET assay, this is a simple homogeneous assay format that is suitable for high-throughput screening using robotic plate handling for unattended operation. FP is a ratiometric measurement, and it appears to be highly suitable for screening natural product extracts. As shown in Figure 4, it effectively eliminates interference from endogenous color and fluorescence often found in these samples that can produce false-positive and false-negative results.

The application of the FP assay in tandem with a FRET-based screening assay can therefore be translated into significant savings in time and resources in deciding on which hits (samples) to process for chemical isolation and characterization. We are now using this approach in our plasmepsin II screen for new antimalarial drug leads.

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