Biochimie 152 (2018) 159–173

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Research paper

In vitro and *in silico* studies of naphthoquinones and peptidomimetics toward *Plasmodium falciparum* plasmepsin V

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ARTICLE INFO

Article history:
Received 21 February 2018
Accepted 4 July 2018
Available online 6 July 2018

Keywords:
Malaria
Naphthoquinone
Peptidomimetic
PEXEL
Plasmepsin V

ABSTRACT

Plasmodium proteases play both regulatory and effector roles in essential biological processes in this important pathogen and have long been investigated as drug targets. Plasmepsin V from *P. falciparum* (*Pf*PMV) is an essential protease that processes proteins for export into the host erythrocyte and is a focus of ongoing drug development efforts. In the present study, recombinant protein production, inhibition assays, binding studies as well as molecular docking and molecular dynamics simulation studies were used to investigate the mode of binding of a PEXEL-based peptidomimetic and naphthoquinone compounds to *Pf*PMV. Consistent with our previous study, refolded *Pf*PMVs were produced with functional characteristics similar to the soluble counterpart. Naphthoquinone compounds inhibited *Pf*PMV activity by 50% at 50 μM but did not affect pepsin activity. The IC_{50} values of compounds 31 and 37 against *Pf*PMV were 22.25 and 68.94 μM, respectively. Molecular dynamics simulations revealed that PEXEL peptide interacted with *Pf*PMV active site residues via electrostatic interactions while naphthoquinone binding preferred van der Waal interactions. P_1'-Ser of the *Pf*EMP2 substrate formed an additional H-bond with Asp365 promoting the catalytic efficiency. Additionally, the effect of metal ions on the secondary structure of *Pf*PMV was examined. Our results confirmed that Hg^{2+} ions reversibly induced the changes in secondary structure of the protein whereas Fe^{3+} ions induced irreversibly. No change was observed in the presence of Ca^{2+} ions. Overall, the results here suggested that naphthoquinone derivatives may represent another source of antimalarial inhibitors targeting aspartic proteases but further chemical modifications are required.

1. Introduction

Malaria in human is caused by parasites of the genus *Plasmodium* including *P. falciparum, P. vivax, P. ovale, P. malariae and P. knowlesi*. Of these five species, *P. falciparum* is responsible for the majority of deaths associated with malaria infections. *P. vivax* is less dangerous but accounts for considerable morbidity, and the others are found less frequently [1]. Artemisinin, the most potent antimalarial drug, has been identified as *P. falciparum* resistance drug in five countries of the Greater Mekong Subregion [1]. Chemotherapeutic control faces significant challenges due to the emergence of artemisinin and multidrug resistance. Therefore, there is an urgent need to identify new drug targets. The *P. falciparum* genome

Abbreviations: DABCYL, 4-((4-(dimethylamino)phenyl)azo)benzoic acid; DMSO, dimethyl sulfoxide; EDANS, 5-((2-aminoethyl)amino)naphthalene-1-sulfonic acid; FRET, fluorescence resonance energy transfer; HIV-1, human immunodeficiency virus-1; HRPII, histidine-rich protein II; *Pf*EMP2, *Plasmodium falciparum* erythrocyte membrane protein 2.

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https://doi.org/10.1016/j.biochi.2018.07.002
0300-9084/

encodes 10 aspartic proteases called plasmepsins (PMs), which are involved in the diverse cellular process. *Pf*PMI, *Pf*PMII, *Pf*PMIV and histo-aspartic protease (*Pf*HAP), are involved in hemoglobin degradation in the acidic food vacuole with available crystal structures [2,3]. *Pf*PMV is responsible for the cleavage of the *Plasmodium* export element (PEXEL) motif [4,5]. *Pf*PMVII is expressed in zygotes and ookinetes and may play a role in *Plasmodium* mosquito midgut invasion [6]. *Pf*PMIX and *Pf*PMX have essential functions for invasion and egress [7,8]. To survive within human red blood cells, malaria parasites have to extensively remodel the host cell through exporting hundreds of proteins that have a broad range of functions. The majority of exported proteins contain a pentameric motif called the PEXEL motif (RxLxE/Q/D) at the N-terminus downstream of the secretory signal peptide [9,10]. The PEXEL sequence is cleaved by *Pf*PMV on the C-terminal side of the conserved Leu to leave the exported protein with xE/Q/D at the N-terminus, which is then acetylated [11,12]. A second group of exported proteins which do not contain the PEXEL motif is called PEXEL negative exported proteins (PNEPs). Unlike PEXEL proteins, PNEPs lack a signal peptide but instead possess a single hydrophobic region downstream of the N-terminus to promote protein export [13,14].

*Pf*PMV is an aspartic protease anchored in ER via a transmembrane domain. The C-terminus of *Pf*PMV resides within the parasite cytosol, and the catalytic protease domain locates within the ER lumen [15]. *Pf*PMV has 590 amino acid residues (68 kDa), containing 25 hydrophobic amino acids at the C-terminus that putatively function as a membrane anchor, and a 15-residue hydrophobic sequence at the N-terminus that may serve as a signal sequence for entry into the secretory system [16]. The *Pf*PMV gene is refractory to disruption, suggesting that *Pf*PMV has an essential function in the parasite [4,5,17,18]. Recently, the crystal structure of *Pv*PMV was solved, revealing a canonical aspartyl protease fold with three unusual features [19]. The first of these is the nepenthesin (NAP) insertion at the N-terminal part of the enzyme, which may control substrate entry into the active site and influence enzyme specificity. The second atypical feature is a helix-turn-helix (HTH) motif near the C-terminus of the enzyme, which is well conserved in PMV from other *Plasmodium* species but not found in vacuolar plasmepsins. The third unusual feature is an unpaired cysteine residue (C140 for *P. vivax* and C178 for *P. falciparum*) located in the flap of the structure. This cysteine residue is also atypical and restricted to *Plasmodium* species. A few PEXEL-mimetic inhibitors have been developed toward PMV. Although the unpaired cysteine points into the active site, it does not appear to make any contact with the PEXEL-mimetic inhibitor, WEHI-842 [19,20]. A second PEXEL-mimetic, WEHI-916, inhibits the activity of purified PMV isolated from both *P. falciparum* and *P. vivax,* but higher concentrations of WEHI-916 are required to kill parasites engineered to overexpress *Pf*PMV [18,21]. Compound 1, another PEXEL-mimetic, can inhibit *Pf*PMV activity *in vitro* at the picomolar range but fails to block parasite growth due to poor stability and trans-membrane permeability [17]. Recently, PEXEL peptidomimetics with P_2 variation have been developed [22] and the analogues with either cyclohexylglycine or phenylglycine in the P_2 position are the most potent inhibitors of PMV. In addition to peptidomimetics, aminohydantoins were reported to moderately inhibit *Pf*PMV ($IC_{50} = 977$ nM) [23].

Quinones have long been known to display antimalarial activities. Atovaquone, a hydroxy-1, 4-naphthoquinone, shows excellent anti-malarial activity but poor pharmaceutical properties [24]. Several other quinone derivatives such as hydroxynaphthoquinones have been developed and tested against *P. falciparum*. Compound N3, a potent inhibitor of mitochondrial electron transport, has an IC_{50} value of 433 nM against cultured *P. falciparum* with little toxicity against human cell lines [24]. Quinones are also widely distributed among natural products. Rhinacanthin-N (Rhi-N) and Rhinacanthin-Q (Rhi-Q), isolated from leaves and roots of *Rhinacanthus nasutus,* and used in Thailand for the treatment of cancer, have been reported to inhibit the mosquito cytochrome P_{450} enzymes (CYP6AA3 and CYP6P7) [25]. Some naphthoquinone aliphatic esters exhibit significant antimalarial activities with IC_{50} values in the submicromolar range. Naphthoquinone aliphatic esters, compounds 31 and 37 (Fig. 1), show potent inhibition against *P. falciparum* with no cytotoxicity toward Vero cell lines [26]. These two compounds also display potent inhibition against *P. falciparum* 3D7 *cyt* bc_1, but not against rat *cyt* bc_1 [26]. Several aryl quinones have also been reported to inhibit β-secretase (BACE1), β-amyloid aggregation and disaggregation of β-amyloid fibrils, suggesting these compounds are candidates for treating Alzheimer's disease [27]. Hinnuliquinone inhibits the activity of both wild-type and mutants of HIV-I protease with IC_{50} values of 2.5 and 1.8 μM, respectively [28]. Thus, Rhi-N, Rhi-M, and NKPSL4 (Fig. 1) are also selected for our study.

To elucidate structure-activity relationships for the development of potent and specific inhibitors, here we investigate kinetic parameters of recombinant *Pf*PMVs and enzyme inhibition by five naphthoquinone compounds. Our results reveal that naphthoquinone compounds 31 and 37 exhibit IC_{50} values against *Pf*PMV in the micromolar range. Molecular dynamics simulations of *Pf*PMV and naphthoquinone compounds, as well as a PEXEL peptide, confirms that the PEXEL peptide interacts with *Pf*PMV via electrostatic interactions while the naphthoquinone compounds employ van der Waals interactions. C3-OH of the naphthoquinone ring forms several interactions with critical amino acid residues in the active site which may highlight a critical moiety of the molecule. Collectively, our study has reported non-peptidomimetic compounds that could potentially block *Pf*PMV activity.

2. Materials and methods

2.1. Expression and inclusion body preparation

E. coli BL21 (DE3) cells harboring pET32a-*Pf*PMVp37 and pET32a-*Pf*PMVm84 plasmids were employed for overexpressing Trx-*Pf*PMVp37 and Trx-*Pf*PMVm84 in *E. coli* following the previously described protocol [29]. Cell culture was harvested by centrifugation at 3020x*g* for 20 min at 4 °C. Cells were resuspended in extraction buffer (50 mM Tris-HCl pH 8.0, 0.5 M NaCl, and 5 mM imidazole) supplemented with 1.6 mM β-mercaptoethanol and 1% Triton X-100. The cell suspension was lysed by sonication (Sonics Vibracell VCX750). Following clarification of the lysate by centrifugation at 13,000x*g* for 45 min at 4 °C, inclusion bodies were pelleted and kept at −20 °C. Proteins used for unfolding and refolding experiment were prepared according to previously published protocol with some modifications [30]. Briefly, 1 g of inclusion bodies were washed with 10 mL of buffer A (50 mM Tris−HCl pH 8.0, 5 mM EDTA and 0.15 M NaCl) and sonicated for 30 min of sonication programmed as 5-sec pulse on and 5-sec pulse off at 4 °C. After washing, the inclusion bodies were recovered by centrifugation at 13,000x*g* for 30 min. The inclusion bodies were washed and sonicated in buffer B (50 mM Tris−HCl pH 8.0, 5 mM EDTA, 0.15 M NaCl and 2 M urea) and centrifuged at 13,000x*g* for 30 min. This step was repeated three times. The inclusion bodies were finally washed and sonicated in buffer C (50 mM Tris−HCl pH 8.0, 5 mM EDTA, 0.15 M NaCl and 0.5% Triton X-100). After washing, the inclusion bodies were clarified by centrifugation at 13,000x*g* for 30 min. The washed inclusion bodies were solubilized in solubilization buffer (50 mM Tris-HCl pH 8.0, 0.5 M NaCl, 8 M urea and 20 mM DTT) for overnight at room temperature with gentle stirring. The denatured protein was kept at −20 °C for further use in a