International Journal of Antimicrobial Agents 50 (2017) 287–302

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

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

Review

Targeting the active sites of malarial proteases for antimalarial drug discovery: approaches, progress and challenges

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ARTICLE INFO

Article history:
Received 19 September 2016
Accepted 27 April 2017

Keywords:
Malaria
Protease
Multidrug resistance
Inhibitor design
Plasmodium

ABSTRACT

Malaria is an infectious disease causing vast mortality and morbidity worldwide. Although antimalarial drugs are effective in several parts of the world, there is a serious threat to malaria control as malaria parasites are continuously developing widespread resistance against currently available antimalarial drugs, including artemisinin. Such widespread antimalarial drug resistance confirms the need to improve the efficacy of existing or new drugs as well as to develop alternative treatments through the identification of novel drug targets and the development of candidate drugs. Similar to proteases in other parasitic diseases such as leishmaniasis, schistosomiasis, Chagas disease and African sleeping sickness, malarial proteases constitute the major virulence factors in malaria. Malarial proteases belong to several classes and many of them have been targeted for the design and discovery of antimalarial agents. This review summarises the approaches, progress and challenges in the design of small-molecule inhibitors as antimalarial drugs targeting the inhibition of various malarial proteases.

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1. Introduction

Malaria is a mosquito-borne infectious disease claiming the lives of hundreds of thousands of young children every year [1,2]. Several malaria-endemic countries are facing loss of their developmental potential and economic growth as well as a huge impact on their healthcare systems [3–5]. *Plasmodium falciparum* is the most common species causing malaria in humans [6]; other species causing human malaria include *Plasmodium ovale*, *Plasmodium malariae*, *Plasmodium knowlesi* [7,8] and *Plasmodium vivax* [6,9–13]. The complete of life cycle of the malaria parasite is well described in the literature [14–17].

Drug-resistant malaria remains a huge challenge for humans. Based on the full *P. falciparum* genome [18], new antimalarial targets for drug development can be identified and validated. Based on their roles in the parasite's life cycle, antimalarial targets can be generally classified into three groups: (i) biosynthesis; (ii) membrane transport and signalling; and (iii) haemoglobin catabolism. The first group of targets are malarial enzymes involved in various biosynthesis pathways, such as the folate biosynthesis pathway [19–21], thus generating nutrients required for growth of the malaria parasite [22–24]. The second group of targets are those involved in several pathways such as (i) uptake/transport of nutrients into cells, (ii) generation and maintenance of transmembrane electrochemical gradients [e.g. the plasmodial surface anion channel (PSAC)] [25–27] and (iii) enzymes whose substrates are involved in intracellular signal transduction, such as farnesyltransferase [28–30]. Finally, the third group of targets participates in haemoglobin digestion and plays a vital role in the development of intraerythrocytic malaria parasites.

Given that there are various protease families with extensive functional redundancy, modulation of protease function has remained challenging to date. In addition, various evidence indicates that knock-down or inhibition of specific proteases leads to the upregulation of others [31,32]. In the area of protease-targeted discovery and development of antimalarial drugs, the malarial proteases involved in the haemoglobin digestion pathway have been targeted in the past decades [33–35]. This review will mainly focus on the design of various classes of small-molecule inhibitors as antimalarial agents/drugs targeting the active sites of malarial proteases.

2. Malarial proteases involved in haemoglobin degradation/catabolism

Given that malarial parasites have limited capacity for de novo amino acid biosynthesis, the majority of amino acids required for the parasite's own protein biosynthesis and energy metabolism are supplied by haemoglobin degradation products [36,37]. Malaria parasites express a number of proteases in the food vacuole for the degradation of haemoglobin imported from the host's red blood cells [36,38]. The haemoglobin degradation process [37] follows a well-ordered pathway as outlined in Fig. 1.

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http://dx.doi.org/10.1016/j.ijantimicag.2017.04.006
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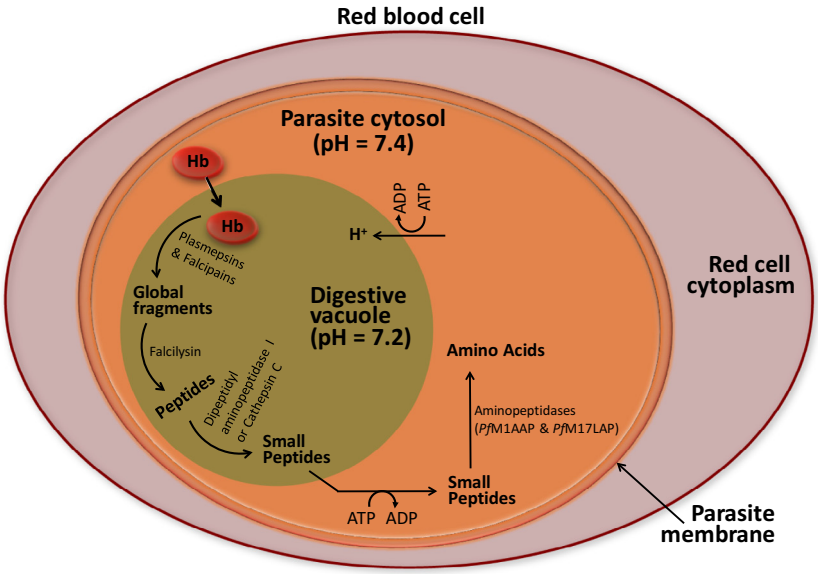

Fig. 1. Haemoglobin degradation process in the *Plasmodium falciparum* food vacuole.

Malarial proteases can be classified into two functional classes: (i) proteases involved in invasion and rupture of erythrocytes; and (ii) proteases involved in haemoglobin degradation. Malarial proteases that play crucial roles in haemoglobin degradation include: plasmepsins, which are aspartic proteases [39]; falcipains, which are cysteine proteases; dipeptidyl aminopeptidase 1 (DPAP1); the metalloprotease falcilysin; and proplasmepsin convertase, which activates proplasmepsin to plasmepsins [37]. Although it is still unknown whether plasmepsins or falcipains catalyse the first cleavage of haemoglobin [40], it has been proposed that an initial cleavage occurs between amino acids Phe33 and Leu34 located in the hinge region of the domain responsible for holding together the oxygen-bound tetramer; the cleavage exposes haemoglobin to undergo further sequential cleavage into smaller peptides both by plasmepsins [39] and falcipains [37]. The metalloproteases falcilysin [41,42] and DPAP1 [43,44] cleave the resulting small peptides to even shorter oligopeptides that are finally hydrolysed to free amino acids by aminopeptidases. With the increasing availability of high-resolution X-ray structures, recent efforts of rational drug discovery have shifted towards targeting these crucial malarial proteases using novel small molecule [45].

3. Aspartic proteases or plasmepsins

Aspartic proteases are a highly specific family of protease enzymes comprising two highly conserved aspartates in the active site [46,47]. These enzymes are optimally active in an acidic pH range and use an activated water molecule bound to one or more aspartate residues for catalysis of their peptide substrates [47]. Plasmepsins are categorised into 10 subtypes [Plm-I, II and IV–X and histidine aspartic protease (HAP)] based on encoding of 10 different genes with high intergenomic similarity [39,47]. HAP is unique plasmepsin that has a histidine in the place of the first canonical aspartic acid. The precise role of the majority of plasmepsins in haemoglobin degradation is not well defined to date. Selected plasmepsins, such as Plm-I, II, IV and HAP, are widely studied malarial plasmepsins because of their crucial role in haemoglobin degradation and to the availability of their crystal structures [46]. Plm-II is the first such plasmepsin whose X-ray crystal structure was extensively used to discover novel protease inhibitors as potential antimalarials [48]. The crystal structure of Plm-II revealed the presence of a catalytic water molecule exhibiting bidentate H-bond interaction with two aspartates in its catalytic site. Using Plm-II as a template structure, computational models of other plasmepsins were developed in view of the fact that the binding sites of Plm-I, IV and HAP share 84%, 68% and 39% identity, respectively, with that of Plm-II [39,49]. To catalyse proteolysis, the water molecule, activated by aspartates, makes a nucleophilic attack on the carbonyl carbon of the peptide substrate leading to a tetrahedral intermediate, which in turn, upon protonation of the nitrogen, results in the degradation products (Fig. 2A) [39]. As discussed above, Plm-I and Plm-II possibly catalyse the initial step in the breakdown of haemoglobin between Phe33 and Leu34 (α-chain) to generate globin and free heme. In addition to the involvement of plasmepsins in haemoglobin degradation, the importance of Plm-IV also emerged recently owing to the fact that it is the only plasmepsin localised in the digestive vacuole of *P. falciparum*, which has orthologues in other *Plasmodium* spp. infecting humans.

3.1. Structural perspective of Plm-II

Plm-II contains 329 amino acids and a single chain folded into two topologically similar C- and N-terminal domains. Like other aspartic proteases, the active site of apo Plm-II consists of a catalytic water molecule bridged with Asp34 and Asp214 to form a catalytic dyad [50]. A unique N-terminal flap that adapts an open conformation during inhibitor uptake and a closed conformation after inhibitor uptake, in co-operation with Leu292 located at the proline-rich loop (Ile290–Pro297), is an important structural feature of Plm-II that should be considered during inhibitor design [39,47,51]. It has been concluded based on inhibitor-bound Plm-II X-ray structures (PDB-IDs: 1LEE and 1LF2) as well as molecular docking simulations that the side chain of Met75, located at the N-terminal flap region, drifts away from the active site in order to accommodate a bulkier inhibitor [52]. Altogether, the active site of Plm-II is quite flexible and is able to accommodate inhibitors of variable size; such flexibility is governed by the N-terminal flap and proline-rich loop. The events of flap opening and dynamics have recently been described [53,54]. Plm-II shares 73% sequence homology with Plm-I, suggesting that both enzymes may be inhibited by closely related molecules [55]. Fig. 3 depicts superimposed X-ray structures of plasmepsins. These plasmepsins also have high structural

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