ABSTRACT  Several microbial metalo-aminopeptidases are emerging as novel targets for the treatment of human infectious diseases. Some of them are well validated as targets and some are not; some are essential enzymes and others are important for virulence and pathogenesis. For another group, it is not clear if their enzymatic activity is involved in the critical functions that they mediate. But one aspect has been established: they display relevant roles in bacteria and protozoa that could be targeted for therapeutic purposes. This work aims to describe these biological functions for several microbial metalo-aminopeptidases.

INTRODUCTION  Several microbial metalo-aminopeptidases are emerging as novel targets for the treatment of human infectious diseases. Some of them are well validated as targets and some are not; some are essential enzymes and others are important for virulence and pathogenesis. For another group, it is not clear if their enzymatic activity is involved in the critical functions that they mediate. But one aspect has been established: they display relevant roles in bacteria and protozoa that could be targeted for therapeutic purposes. This work aims to describe these biological functions for several microbial metalo-aminopeptidases. The main biological functions and molecular properties of these enzymes that support them as targets are presented in Table 1.
| Protease family | Metalo-aminopeptidase (source) | Molecular activity or property that determines their main functions | Main functions | Essentiality or involvement in virulence | Experimental evidences that support their relevance |
|----------------|--------------------------------|---------------------------------------------------------------|---------------|-----------------------------------------|--------------------------------------------------|
| M1             | PfA-M1 (P. falciparum parasite) | Alanyl-aminopeptidase activity                                 | Hemoglobin degradation | Essential | - Bestatin and specific inhibitors block parasite growth  
- Their toxicity is reduced in parasites overexpressing PfA-M1  
- Knockout is lethal |
|                | MtLAP (M. tuberculosis bacterium) | Leucyl-aminopeptidase activity                                 | Unknown       | Essentiality not demonstrated | Bestatin inhibits bacterial growth in vitro and during macrophage infection  
- Knockout is lethal |
|                | PfA-M17 (P. falciparum parasite) | Leucyl-aminopeptidase activity                                 | - Hemoglobin degradation  
- Erythrocyte invasion (probably)  
- Other housekeeping functions | Essential | - Bestatin and a specific inhibitor block parasite growth  
- Knockout is lethal |
|                | TbLAP-B (T. brucei parasite)     | Leucyl-aminopeptidase activity (probably)                       | Kinetoplast DNA segregation | Not essential, involved in virulence | Down-regulation induces a delay in cytokinesis |
| M17            | LAP-B (Leishmania spp. parasite) | Leucyl-aminopeptidase activity (probably)                       | - Leucine supply during host infection  
- Intracellular protein degradation and turnover  
- Host cell invasion (all probably) | Essentiality not demonstrated | Selective inhibition may interfere with parasite viability |
|                | AcLAP (A. castellani parasite)   | Leucyl-aminopeptidase activity                                 | Encystation    | Not essential, involved in virulence | Knockdown and bestatin produce encystation inhibition |
|                | TgLAP (T. gondii parasite)       | Leucyl-aminopeptidase activity (probably)                       | Hydrolysis of dipeptides produced by cathepsin Cs and proteasoma (probably) | Not essential, involved in virulence | Knockout inhibits the parasite ability to attach and/or invade cultured cells, attenuating virulence in a mouse model |
|                | SaM17-LAP (S. aureus bacterium)  | Cysteinyl-glycinase activity (probably)                         | - Bioactivates / inactivates key cellular proteins involved in metabolism, cell wall biosynthesis or signaling  
- Sulfur metabolism (all probably) | Not essential, involved in virulence | - Required in vitro for bacterial survival inside human macrophages  
- Knockout attenuates virulence in in vivo mouse models |
TABLE 1 (continued). Main biological functions and molecular properties of microbial metalo-aminopeptidases that support their essentiality or involvement in virulence.

| Protease family | Metalo-aminopeptidase (source) | Molecular activity or property that determines their main functions | Main functions | Essentiality or involvement in virulence | Experimental evidences that support their relevance |
|-----------------|--------------------------------|---------------------------------------------------------------|----------------|----------------------------------------|--------------------------------------------------|
| TdM17-LAP       | *(T. denticola* bacterium)     | Cysteinyl-glycinase activity                                 | Glutathione catabolic pathway | Essentiality not demonstrated          | Glutathione and Cys-Gly protect the cellular components from oxidative damage |
| HpM17AP         | *(H. pylori* bacterium)        | Cysteinyl-glycinase and arginyl-aminopeptidase activities    | - Defense in human macrophages | Essentiality not demonstrated          | - Upregulated in response to the anti- *H. pylori* agent, NE-2001, and oxidative stress caused by nitric oxide and metronidazole - Bestatin inhibits bacterial growth |
| MtMetAP1a       | *(M. tuberculosis* bacterium)  | Methionyl-aminopeptidase activity                            | Removal of N-terminal methionine from newly synthesized peptides | Essentiality not demonstrated          | - Knockdown inhibits bacterial growth - Overexpression confers resistance to the antibacterial effect of enzymatic inhibitors |
| MtMetAP1c       | *(M. tuberculosis* bacterium)  | Methionyl-aminopeptidase activity                            | - Removal of N-terminal methionine from newly synthesized peptides - A major role in the host macrophage phagosome | Essentiality not demonstrated          | - Overexpression confers resistance to the antibacterial effect of enzymatic inhibitors |
| LdMetAP2        | *(L. donovani* parasite)      | Methionyl-aminopeptidase activity                            | - Apoptosis | Essentiality not demonstrated          | - Overexpression and apoptosis are associated - Inhibitors prevent the induction of apoptosis, but do not prevent parasite death |

*Note: All functions and properties are based on experimental evidence and biological implications.*
dases, and compound 4, a synthetic PfA-M1 inhibitor [13], in the murine malaria model Plasmodium chabaudi [14].

(2) The toxicity of these compounds is reduced in transgenic parasites overexpressing PfA-M1 [13]. (3) The PfA-M1 specific inhibitors inhibit the in vitro parasite growth [4].

(4) The absence of this enzyme in knockout parasites is lethal [2]. All of these results are indicative of the target character of PfA-M1 for the search of a new class of antimalarials [3, 4].

AMINOPEPTIDASES BELONGING TO THE M17 FAMILY OF PROTEASES

M17 aminopeptidases have leucyl-aminopeptidase (LAP) activity, responsible in most cases for the biological functions related with their target character. But other M17 enzymes, mainly from bacteria, exhibit also cysteinyl-glycinase activity, which is involved in their critical cellular functions. Another group of M17 LAPs have roles that do not depend on their enzymatic activity, but on their quaternary structure (transcriptional regulation, for example).

M17 aminopeptidases whose main function depends on LAP activity

M17 LAP from the bacterium Mycobacterium tuberculosis

The growth inhibition of the bacterium M. tuberculosis by bestatin, in vitro and during macrophage infection, supports the involvement of the M17 leucyl-aminopeptidase (MtLAP) in physiological and pathogenic processes in tuberculosis. This enzyme is probably essential for in vivo bacterial survival and pathogenesis [15].

M17 LAP from P. falciparum

The M17 LAP from P. falciparum (PfA-M17) is involved in the hemoglobin digestion, with the functions described above. The blockade of this LAP activity is toxic in vitro for P. falciparum and P. chabaudi chabaudi [16, 17]. In contrast to PfA-M1, PfA-M17 may have additional functions, since its specific inhibition in parasite cultures causes growth retardation early in the erythrocytic stages, before hemoglobin digestion begins [4]. PfA-M17 could participate in red cell invasion process, since bestatin diminishes the rings number 24 h after addition of schizont-infected erythrocytes to uninfected cells [17]. This enzyme is essential for parasite viability, since PfA-M17 gene knockout has been unsuccessful [2].

Basic M17 LAP from the parasite Trypanosoma brucei

In host infection processes, the basic M17 LAP from the parasite T. brucei (TbLAP-B) could have some of the following functions: provide an essential amino acid (leucine is a precursor for sterol biosynthesis [18, 19]), being involved in infectivity [20], regulate stress responses and signal transduction [21], act as protein chaperones [22], be required for glutathione metabolism [23], and participate in host cell invasion [24, 25].

Interference RNA-mediated down-regulation of TbLAP-B induces a nonlethal growth defect, causing a delay in cytokinesis. Ectopic expression of the TbLAP-B-hemagglutinin fusion in procyclic T. brucei causes the loss of kinetoplast DNA, failure of the mitochondrial membrane potential and related growth defects. Parasites expressing TbLAP-B-hemagglutinin can duplicate their kinetoplast DNA, but correct separation fails. The enzyme down-regulation and ectopic expression indicate its clear involvement in kinetoplast DNA segregation [26].

Basic M17 LAP from the parasite Leishmania spp.

The LAP activity of the soluble extracts of the parasite Leishmania spp. was almost completely (90-95%) inhibited by anti-porcine LAP IgG (this antibody inhibits the basic M17 LAPs from Leishmania spp. -LAP-Bs-), indicating that LAP-Bs are responsible for the bulk of this activity in parasite extracts. The selective inhibition of LAP-B may interfere with parasite viability [27], because Leishmania spp. are auxotrophic for branched-chain amino acids [28, 29]. Therefore, LAP-B could provide an essential amino acid in host infection processes (leucine is a precursor for fatty acids and sterol biosynthesis [18]). Furthermore, LAP-B could participate in intracellular protein degradation and turnover [30], and host cell invasion [24, 25].

M17 LAP from the parasite Acanthamoeba castellanii

The cysts of the parasite A. castellanii, knocked-down for M17 LAP (AcLAP), do not show separated ectocyst and endocyst, discernible by transmission electronic microscopy, indicating cell wall rupture. A similar morphology exhibits cells treated with bestatin, suggesting that decreased AcLAP activity causes parasite cell wall ultrastructural changes, closely related with encystation inhibition. It is possible that the affectation in protein turnover blocks the cyst wall synthesis or produces the cell breakdown by oligopeptide accumulation [20]. However, a selective M17 LAP inhibitor is required to confirm that this phenotype is only the result of the AcLAP inhibition [31].

M17 LAP from the parasite Toxoplasma gondii

The M17 LAP from the parasite T. gondii (TgLAP) could be involved in the hydrolysis of dipeptides produced by cathepsin Cs in parasitophorous vacuole [32]. Alternatively, the TgLAP substrates could be peptides generated in the proteasomal protein degradation pathway [33]. Knockout of TgLAP inhibits the parasite’s ability to attach and/or invade cultured cells, and this reduces replication and attenuates virulence in a mouse model [34]. However, this phenotype has not been directly associated with the enzyme LAP activity, and could be related to other unknown protein functions [31].

M17 aminopeptidases whose main function depends on cysteinyl-glycinase activity

M17 LAP from the bacterium Staphylococcus aureus

Despite not being essential for the bacterium S. aureus, its M17 LAP (SmA-M17-LAP) plays an important role in virulence. This enzyme is required in vitro for bacterial survival inside human macrophages. Further, S. aureus with a disrupted SmA-M17-LAP gene had severely attenuated virulence in both...
localized and systemic infections in in vivo mouse models. It has been proposed that SoM17-LAP bioactivates/innactivates key cellular proteins involved in crucial functions, such as metabolism, cell wall biosynthesis or signaling. This proteolysis would confer any advantage for the bacterium in the harsh host environment [35].

*S. aureus* produces the low-molecular-weight thiol bacillithiol (Cys-GlcN-mal) instead of glutathione [36]. Cysteine-containing molecules are cysteine sources during nutrient restriction [37], and are important in cellular defense against low pH, oxidative and osmotic stress. In addition, sulfur metabolism has been linked to virulence [38, 39]. For this reason, the cysteinyl-glycinase activity of SoM17-LAP suggests its importance for *S. aureus* virulence [40].

**M17 LAP from the bacterium Treponema denticola**
The M17 LAP from the bacterium *T. denticola* (TdM17-LAP) was identified as the probably only cysteinyl-glycinase involved in the glutathione catabolic pathway, by immunodepletion of the most cysteinyl-glycinase activity in the soluble fraction of sonicated *T. denticola* cells, when the bacterium was grown under standard conditions. Hydrogen sulfide, ammonium, pyruvate, glutamate and glycine are produced in equimolar amounts by this pathway [23, 41]. Both glutathione and Cys-Gly can play critical roles in maintaining cellular redox status, protecting the cellular components from oxidative damage. These two thiol-containing molecules can also modify the cysteine residues of some proteins, regulating their activities [42].

**M17 LAP from the bacterium Helicobacter pylori**
The M17 LAP from the bacterium *H. pylori* (HpM17AP) is upregulated in response to the anti-*H. pylori* agent, NE-2001 [43], and oxidative stress caused by nitric oxide [44] and metronidazole [45]. These evidences, together with the enzyme allosteric nature and high efficiency, suggest that HpM17AP may play a relevant role in the *H. pylori* life cycle [46]. The response against nitric oxide [44] suggests a role in defense in human macrophages [47]. In addition, the response against metronidazole suggests an involvement in drug resistance mechanisms, in addition to a relevant housekeeping role [45]. These HpM17AP functionalities in response to cellular oxidative stress could potentially result from the cysteinyl-glycinase activity of the protein [45, 47].

*H. pylori* utilizes the stomach’s mucosal glutathione, produced as the major defense mechanism against low pH, oxidative and osmotic stress [38], as a glutamate source [48]. The resultant Cys-Gly dipetide produced by the glutathione catabolism is cleaved to salvage cysteine [47]. On the other hand, high activity of HpM17AP on peptides with essential N-terminal arginine [49] may contribute to maintain an adequate cytoplasmic pool of free arginine, which could be used for synthesis of polyamines required for optimal *H. pylori* growth [47]. Bestatin inhibits the growth of *H. pylori* in culture [46], an effect probably caused by HpM17AP inhibition [31].

M17 aminopeptidases whose main function depends on their quaternary structure

**M17 LAP from the bacterium Pseudomonas aeruginosa**
The hexameric M17 LAP from the bacterium *P. aeruginosa* (PhpA) transcriptionally regulates the virulence-associated *algD* gene, encoding an enzyme of the alginate biosynthetic pathway [50]. Alginate is involved in biofilm formation, and its overproduction characterizes the highly-mucoid phenotype of cystic fibrosis in the lung [51]. By mutating one of the PhpA metal-binding residues, but not by bestatin inhibition, the transcription of the *algD* gene is increased and a slow growth phenotype is generated in vivo. This suggests that the aminopeptidase activity is not required for transcriptional regulation [50], and mutations could result in hexamer disruption [31], as observed for tomato M17 LAP [52].

**M17 LAP from the bacterium Vibrio cholera**
In the bacterium *V. cholerae* the expression of virulence factors, such as cholera toxin, are mediated by a complex regulatory circuit, highly dependent on environmental temperature and pH. Disruption of the gene encoding the M17 LAP from *V. cholerae* (VcPepA) resulted in increased levels of cholera toxin under non-inducing conditions (pH 8.4 and 37°C), under which toxins would normally not be observed. In contrast, under inducing conditions (pH 6.5 and 30°C), the absence of VcPepA has no effect on toxin levels [53]. Behari et al. [53] identified a potential target sequence in the *V. cholerae* genome to which VcPepA might bind, and therefore propose that the protein modulates transcription of the toxin gene under different environmental conditions. Enzymatic activity of VcPepA would not be involved in this function.

**AMINOPEPTIDASES BELONGING TO THE M18 FAMILY OF PROTEASES**
The M18 aspartyl-aminopeptidase from *P. falciparum* (PFA-M18) could be involved in protein catabolism, including the turnover of parasite proteins and hemoglobin degradation. The parasitophorous vacuole location (in addition to cytosolic) suggests that, like PfA-M17, PFA-M18 may have other relevant functions in addition to hemoglobin digestion [54]. For example, the enzyme could have a role in erythrocyte membrane rupture during merozoite release or reinvasion, since it binds the membrane protein spectrin [55].

PFA-M18 knockdown results in a lethal phenotype with relevant morphological alterations, as was observed by electron microscopy [54]. Other gene disruption/truncation experiments, resulting in ~10 % aspartyl-aminopeptidase activity compared to wild-type parasites, indicate that the enzyme is dispensable for the erythrocytic cycle but this generates negative consequences for the parasite [2].
AMINOPEPTIDASES BELONGING TO THE M24 FAMILY OF PROTEASES

M24 methionyl-aminopeptidases (MetAP) from M. tuberculosis

Bacterial protein synthesis is initiated with an N-formylmethionine, whose N-formyl group is removed by peptide deformylase. Thereafter, M24 methionyl-aminopeptidases (MetAPs) remove the N-terminal methionine. Since this essential process is required for protein post-translational modifications, activity, stability, localization or degradation, the excision pathway is a potential drug target in tuberculosis [56].

M. tuberculosis MetAP1a (MtMetAP1a) antisense-RNA knockdown, and not MtMetAP1c, inhibits bacterial growth in vitro [57]. MtMetAP1c is inhibited at high methionine concentrations and it could not be essential. In contrast, MtMetAP1a is not inhibited by methionine and it could have an essential role in methionine salvage [58]. On the other hand, in contrast to MtMetAP1a, MtMetAP1c retains 60% activity at pH 5.5, suggesting a major role in acidic environments, like the host macrophage phagosome [59]. Overexpressed MtMetAP1a and MtMetAP1c in M. tuberculosis confer resistance to the antibacterial effect of MetAP inhibitors [57], indicating that MtMetAPs may be promising targets for the development of antituberculosis agents.

M24 MetAP from the parasite Leishmania donovani

The treatment of L. donovani promastigotes with miltefosine (an oral drug against the parasite) induces the overexpression of the parasite M24 MetAP (LdMetAP2) by 3.5 times [60]. This treatment produces an apoptotic programme cell death with activation of caspase 3/7 protease like activity [61-63]. However, the treatment with the MetAP2 inhibitor TNP-470, or miltefosine and TNP-470, or miltefosine and the caspase-3 inhibitor N-Acetyl-Asp-Glu-Val-Asp-al, do not show activation of this activity. Moreover, MetAP2 inhibitors prevent the induction of nuclear apoptosis in L. donovani, as was confirmed by flow cytometry, and analysis of DNA fragmentation, translocation of phosphatidyl serine from the inner to the outer side of plasma membrane, mitochondrial membrane damage and concentration of cytosolic calcium. However, LdMetAP2 inhibition does not prevent parasite cell death, since this aminopeptidase is also involved in the removal of N-terminal methionine from the nascent polypeptides [63].

The main biological functions and molecular properties of these enzymes that support them as targets are presented in Table 1.

CONCLUSION

Some metalo-aminopeptidases, as MtMetAP1a, PfA-M17 and PfA-M18, are essential enzymes for their microorganisms and, therefore, they have been well validated as targets. Others, as MtMetAP1c, SoM17-LAP, PhPA, VcPepA, TbLAP-B, AcLAP and TgLAP, are not essential but are required for virulence and pathogenesis, or their activities confer some advantage for microbial growth under given conditions. For another group, formed by MtLAP, TdM17-LAP, HpM17AP, LAP-B and LdMetAP2, their biological functions are predicted as crucial for microorganism survival in the human host, although they are not yet validated as targets. Some bacterial LAPs, such as SoM17-LAP, TdM17-LAP and HpM17AP, have also cysteinyl-glycinase activity. The roles of several metalo-aminopeptidases, as PhpA and VcPepA, do not depend on their enzymatic activity. More work with potent and specific inhibitors or gene knockout experiments are required to elucidate the essential roles or not of these enzymes inside microbial cells. As a group, these enzymes are novel drug targets for the treatment of human infectious diseases.

ACKNOWLEDGMENTS

This work was supported by the International Foundation for Sciences (grant F/4730-2), and the project assigned to J. González-Bacerio and associated to the Cuban National Program of Basic Sciences.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Please cite this article as: Jorge González-Bacerio, Maikel Izquierdo, Mirtha Elisa Aguado, Ana C. Varela, Maikel González-Matos and Maday Alonso del Rivero (2021). Several microbial metalo-aminopeptidases as targets in human infectious diseases. Microbial Cell 8(10): 239-246. doi: 10.15698/mic2021.10.761

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