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Development of protease inhibitors for protozoan infections

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Abstract

Purpose of review—To highlight the promise of parasite proteases as novel targets for development of new antiparasitic chemotherapy. Proteolytic enzymes play key roles in the life cycle of protozoan parasites or the pathogenesis of diseases they produce. These include processing of host or parasite's surface proteins for invasion of host cells, digestion of host proteins for nutrition, and inactivation of host immune defense mediators.

Recent findings—Drug development for other markets has shown that proteases are druggable targets, and protease inhibitors are now licensed or in clinical development to treat hypertension, diabetes, thrombosis, osteoporosis, infectious diseases, and cancer. Several protease targets have been validated by genetic or chemical knockout in protozoan parasites. Many other parasite proteases appear promising as targets, but require more work for validation, or to identify viable drug leads. Because homologous proteases function as key enzymes in several parasites, targeting these proteases may allow development of a single compound, or a set of similar compounds, that target multiple diseases including malaria, trypanosomiasis, leishmaniasis, toxoplasmosis, cryptosporidiosis, and amebiasis.

Summary—Proteases have been validated as targets in a number of parasitic infections. Proteases are druggable targets as evidenced by effective antiprotease drugs for the treatment of many human diseases including hypertension and AIDS. Future drug development targeting parasite proteases will be aided by the strong foundation of biochemical, structural, and computational databases already published or available online.

Keywords

drug; inhibitor; parasite; protease; protozoa

Introduction

Proteases are a large, diverse, and ubiquitous group of enzymes that play key roles in almost every biological phenomenon. As potential drug targets, they have the advantage of years of biochemical analysis, including detailed depiction of their mechanism of catalysis, substrate specificity, and structural correlates of function (MEROPS, <http://merops-sanger.ac.uk>). Arguably, more is known about the biochemistry and structure of proteases than any other enzyme family. Furthermore, proteases have been validated as druggable targets. Protease inhibitors are currently used as drugs to treat hypertension and HIV infection, and intensive programs to target proteases for the treatment of diabetes, cancer, and osteoporosis have reached clinical trials [1]. Knowledge of structural or functional relationships and substrate

specificity of proteases makes them ideal candidates for computational-assisted drug design, and the availability of large libraries of protease inhibitors produced in industry provides a foundation for discovery of new leads for antiparasitic chemotherapy.

Proteases are divided into four major families. Trypsin family (SA) serine proteases represent the most abundant group in vertebrates, where they function in blood coagulation, the complement cascade, intestinal digestion, and many other physiologic processes. In general, serine proteases of protozoan parasites are of the subtilisin (SB), not trypsin type. The best characterized of these are the subtilisin-like proteases of *Plasmodium falciparum*. PfSUB-1 processes parasitophorous vacuole serine repeat antigen protein (SERA) proteins (also predicted to be proteases) to facilitate erythrocyte rupture at the completion of the erythrocyte cycle [2]. PfSUB-2 is then responsible for the release of merozoite surface proteins required for erythrocyte invasion [3,4]. Relatively less work has focused on chemotherapeutic hits or leads against protozoan serine proteases, but interest in protozoan subtilisin-like targets is increasing.

Two subtilisin-like proteases have also been identified in *Toxoplasma gondii*, TgSUB1, and TgSUB2. Both are homologous to their respective *P. falciparum* subtilases. TgSUB1 is localized to the microneme, an apical secretory and adhesion organelle, and is hypothesized to be involved in the processing of several micronemal proteins. TgSUB2 is a putative maturase in the rhoptry organelles. This gene could not be disrupted in tachyzoites suggesting that it is essential [5]. Because both *T. gondii* subtilases may be involved in secretory organelle maturation and proteolytic processing, they represent potential chemotherapeutic targets, which are worth further investigation.

In the trypanosomatids, serine protease research has centered on the Clan SC proteases, oligopeptidase B (OpdB), and prolyl oligopeptidase (POP). During host cell entry, *Trypanosoma cruzi* OpdB is believed to generate a Ca^{2+} -signaling agonist that mediates parasite's entry into nonphagocytic cells [6]. Targeted deletion of OpdB impairs the ability of *T. cruzi* to invade host cells and attenuates virulence *in vivo* [7]. *T. cruzi* POP, which specifically hydrolyzes human collagen (types I and IV) and fibronectin, has been implicated in parasite's adhesion to host cells and cell entry [8]. The invasive capacity of *T. cruzi* is reduced *in vitro* in the presence of OpdB and POP inhibitors [7,9]. The *Leishmania OpdB* gene has also been cloned and a structural homology model has been produced [10]. The serine protease inhibitors L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK), benzamidine, and a sea anemone-derived Kunitz-type inhibitor (ShPI-I) were found to be leishmanicidal against *Leishmania amazonensis* and induced changes in the ultrastructure of the parasite's flagellar pocket [11].

Multiple serine proteinases' genes have been identified in *T. cruzi* including a carboxypeptidase that appears unique to parasite versus host [12], and an oligopeptidase B involved in Ca^{2+} -signaling during cell invasion [13].

Metalloproteases are key enzymes for vertebrate cell migration and cancer invasion, as well as a number of hormone-processing events. Metalloproteases are represented in the genomes of several protozoan parasites. In *P. falciparum*, two metallo-aminopeptidases are present in the parasite's food vacuole, and appear to contribute to the hydrolysis of globin-derived peptides into free amino acids [14]. Many metalloproteases are annotated in the *T. cruzi* genome [15]. A membrane-bound *T. cruzi* metalloprotease, similar to the leishmanial gp63, may modulate infection of host cells [16]. Two metallocarboxypeptidases are of interest because they are similar to primitive prokaryotic enzymes [17].

The proteasome is a multiprotease cytoplasmic organelle, key to protein turnover in both host and parasite. Because of promising clinical trials of a proteasome inhibitor for cancer

therapy, individual parasite proteasome components may be targeted for future chemotherapy as well.

Aspartyl proteases function primarily in the lysosomes of mammalian cells but may play a wider role in protozoan parasites. Notably, the sole protease of HIV is an aspartyl protease that is the target of highly active antiretroviral protease inhibitors. The plasmepsins of malarial parasites digest hemoglobin in the parasite's food vacuole to provide minor acids for parasite's protein synthesis [18]. Multiple potent inhibitors of plasmepsins have been synthesized, but relatively few compounds with reasonable activity against malarial parasites have been identified. *T. cruzi* also has two aspartyl proteases of unknown function [15].

Two metacaspase genes, TcMCA3 and TcMCA5, have been identified in *T. cruzi*. Metacaspases are also reported in *P. falciparum* and are sufficiently distinct from host proteases to be attractive targets [15].

Remarkably, the commonest proteases in protozoan parasites are members of the Clan CA, or papain family of cysteine proteases. Mammalian Clan CA cysteine protease homologues function primarily intracellularly, whereas those of protozoa may function extracellularly or within relatively accessible intracellular compartments. This biological selectivity has been exploited for the development of protease inhibitors targeting cysteine proteases in a number of parasites, including *Entamoeba*, *Toxoplasma*, *T. cruzi*, *Trypanosoma brucei*, and *P. falciparum* [5,19–21].

Leishmania has multiple cathepsin L-like cysteine proteases implicated in virulence including the CPA and CPB gene arrays. Disruption of *Leishmania mexicana* CPB reduces lesion development in BALB/c mice [22]. This reduced virulence is associated with the failure of CPB to induce IL-4 and to produce a Th2 response [23]. CPA/CPB double null mutant parasites show even further loss of virulence in macrophages and *in vivo*. Additionally, deletion of these genes or treatment of the parasites with the Clan CA peptidase inhibitor, K11777, interferes with autophagy and metacyclogenesis in *L. mexicana* [24]. In *P. falciparum*, falcipain cysteine proteases are required for the hydrolysis of hemoglobin in the parasite's food vacuole, in cooperation with the plasmepsin aspartic proteases and the metallo-aminopeptidases described above [19,20].

Depending upon the organism, drug-development projects targeting this family of proteases are at the hit-to-lead stage, or, in some cases at the lead optimization or Investigative New Drug (IND) enabling stages. In *T. cruzi*, the cysteine protease, cruzain (aka cruzipain), is a validated target of effective inhibitors, and a drug candidate, K777, is in late preclinical trials for Chagas' disease [25,26••].

Can one develop a specific enough parasite protease inhibitor to overcome selectivity issues with host homologues?

Given the extensive biochemical analysis of protozoan proteases, the multiple crystal structures that have been solved with small molecule leads, and the diversity of inhibitor chemistry, exquisite inhibitor specificity for parasite enzymes can be developed. For example, early SmithKline Beecham anticathepsin K protease inhibitor libraries were screened against the cysteine protease of *T. cruzi*, and hits were identified that had IC₅₀ or Ki values 100-fold different from those of homologous host proteases. Nevertheless, extreme biochemical selectivity may not be necessary for antiprotozoan drugs because of the inherent biologic selectivity in the function and location of protozoan proteases. Parasites such as *Plasmodium* and *T. brucei* reside in bloodstream. Therefore, protease inhibitors must only be targeted to the blood, and not to other tissue compartments for efficacy. Even in the

case of the intracellular *Leishmania* and *T. cruzi* parasites, the intracellular amastigote stage may selectively take up inhibitors. Small molecule protease inhibitors might mimic amino acids or purines for which the parasite has a specific mechanism for uptake. This observation has been exploited for inhibitor design [27]. In summary, the extracellular concentration of inhibitors, necessary to chemically knockout a parasitic enzyme, is likely much lower than predicted by assays of biochemical sensitivity. Furthermore, homologous host proteases are generally present in lysosomes, a less accessible subcompartment within mammalian cells where proteases are present in millimolar concentrations [28]. The difficulty in designing cathepsin S and cathepsin B inhibitors to effectively inhibit mammalian protease activity in anticancer and anti-inflammatory development programs underscores the difficulty in knocking out host protease activity, even when using an inhibitor that is effective biochemically. Gene knockouts of host Clan CA proteases demonstrate little or no phenotype unless multiple genes are deleted [29]. Telling data also comes from safety studies done on protease inhibitors in which compounds such as vinyl sulfones, which are excellent inhibitors of mammalian cathepsin L, yet showed no protease-related toxicity in dose escalation studies and 7-day dosing studies in rats, dogs, and primates [30••].

Is the cost of protease inhibitors not prohibitive for the developing world?

Drugs for protozoan infections must be very inexpensive for widespread use in resource-poor regions. There is a misconception that the cost of protease inhibitors is prohibitive, based upon the cost of protease inhibitors to treat HIV infection or other chronic diseases in the United States and Europe. Many protease inhibitors can be made cheaply. The chemistry of protease inhibitors is so diverse that inexpensive synthetic schemes using simple starting materials can be developed or selected. Second, the cost of HIV protease inhibitors is dependent on market considerations. HIV protease inhibitors have been distributed to economically poor regions of the world at a much lower cost [31]. Finally, most protozoan infections will require only short courses of treatment, leading to much cheaper therapeutic regimens compared with the chronic treatment required for hypertension, diabetes, or chronic viral infections.

Reversible versus irreversible protease inhibitors

In the context of the development of drugs targeting infectious diseases, an argument is always raised as to whether irreversible enzyme inhibitors can be suitable drugs. Irreversible inhibitors are filtered out of virtually all pharmaceutical industry drug-development programs. The concern is that of residual compound remaining in tissue, and the specter of autoimmune idiosyncratic drug reactions. This concern is certainly rational for the chronic therapies (e.g. to treat diabetes, high cholesterol, or hypertension). However, this strict filter may be inappropriately applied to anti-infective chemotherapy, particularly antiparasitic. If the ideal drug is one that would be taken for a period of 2 days to 3 weeks, then issues of long-term safety are less applicable. Furthermore, many established drugs are, in fact, irreversible enzyme inhibitors. These include penicillin and the other β -lactam antibiotics.

The controversy over irreversible inhibitors as drugs is also compounded by the definition of 'reversible'. Effective HIV protease inhibitors have K_i in the picomolar, sometimes approaching femtomolar range, with slow off-rates. If a femtomolar inhibitor has a slow off-rate, how 'reversible' is it in terms of tissue residue? Furthermore, other 'reversible' protease inhibitors developed in the pharmaceutical industry for markets such as osteoporosis contain nitrile groups that react with the active site of target proteases to form a transient covalent bond. It is unclear whether differences between these compounds and others that are truly irreversible have any biological significance. Perhaps most importantly in the context of antiparasitic chemotherapy, irreversible inhibitors are frequently more

effective in in-vivo models of parasitic disease than reversible inhibitors. The reason for this is that irreversible inhibitors may effectively arrest parasite's protease activity after a single bloodstream 'spike'. In contrast, reversible inhibitors must be maintained through continuous dosing and have longer half-lives to ensure arrest of target enzyme activity.

Are protease inhibitors not hepatotoxic?

No published work supports this assumption globally. Hepatotoxicity is not a significant issue for any of the protease inhibitors currently being used as drugs. Elevated transaminase [e.g. alanine transaminase (ALT)] levels noted at high drug doses should not be interpreted as 'hepatotoxicity'. ALT is a cytoplasmic enzyme of hepatocytes, which is leaked to bloodstream following even reversible hepatocyte injury. This occurs with a bewildering array of currently used drugs, including in up to 2% of individuals taking cholesterol-lowering drugs [32]. No such elevation of ALT, and certainly no histologic evidence of hepatotoxicity were seen at therapeutic doses (<100 mg/kg) either *in vitro* with cultured human hepatocytes, or in a published study of the cysteine protease inhibitor treatment of Chagas' disease in dogs [33]. Repeat dose escalation and chronic dosing studies currently being carried out at SRI International under the auspices of an NIAID contract should clarify these issues. Information on the pharmacokinetics and safety of one cysteine protease inhibitor series has been published [30••].

Current partnerships, funding support, and protease inhibitor drug leads

Chemical structures that have been released for protease inhibitor leads, currently being developed as antiparasitics, can be found in a recent review [19]. These include the vinyl sulfone protease inhibitor, K777, and purine-based inhibitors exploiting uptake pathways in parasites [27]. The purine derivative work is ongoing at St. Jude Children's Hospital and Research Center with support from that foundation. The preclinical development and IND-enabling work on K777 is currently being supported by the National Institutes of Allergy and Infectious Diseases. The other project far along in drug development is a collaboration between GSK and Dr Phil Rosenthal at the University of California, San Francisco/Sandler Center supported by the Medicines for Malaria Venture (MMV). Promising inhibitor leads targeting the falcipain protease for the treatment of *P. falciparum* infection have emerged from this collaboration, but their structures remain proprietary. There is also an ongoing development of protease inhibitors, modeled after the successful osteoporosis drug candidate [1•], in both industrial and academic medicinal chemistry groups. At present, the structures of these leads remain proprietary. Aside from MMV, the Sandler Foundation and DNDi (Drugs for Neglected Diseases Initiative) are taking active roles in funding support for protease inhibitor development targeting African trypanosomiasis and Chagas' disease.

Conclusion

Proteases have been validated as targets in a number of parasitic infections and ongoing research will likely broaden the target spectrum (Table 1 Table 1). A key factor is that proteases are 'druggable' targets as evidenced by the widespread use of protease inhibitors as effective therapy for hypertension and AIDS, and the current clinical development of protease inhibitors for diabetes, cancer, thrombosis, and osteoporosis. Drug development targeting parasite proteases is aided by a strong foundation of biochemical, structural, and computational studies and the diversity of potential chemotypes as drug leads.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

•of special interest

••of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (pp. 000–000).

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Table 1

Pipeline of protease targets in protozoan parasites

Parasite	Protease	Discovery	Hits	Leads	Validation	References
<i>Trypanosoma cruzi</i>	Cruzaïn	X	X	X	X	[12,16,17, 25, 26••, 33]
	Cathepsin B	X	X			
	OPB	X	X			
	Carboxypeptidas [fix break]	X				
<i>Trypanosoma brucei</i>	Brucipain	X	X	X	X	[27]
	TbCatB	X	X	X	X	
Leishmania	CpB	X	X		X	[10,11,22,24]
	OPB	X	X			
	Subtilisin	X				
	X		X	X		[5]
Toxoplasma	Falcipains	X	X	X	X	[4•,14]
	Plasmeppsins	X	X			
	Falcilysin	X	X			
<i>Entamoeba histolytica</i>	Cysteine protease	X	X			[21]

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