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Cysteine Protease Inhibitors as Chemotherapy for Parasitic Infections

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Abstract—Analysis of the evolution, localization and biologic function of papain family cysteine proteases in metazoan and protozoan parasites has provided important and often surprising insights into the biochemistry and cellular function of this diverse enzyme family. Furthermore, the relative lack of redundancy of cysteine proteases in parasites compared to their mammalian hosts makes them attractive targets for the development of new antiparasitic chemotherapy. The treatment of experimental models of parasitic diseases with cysteine protease inhibitors has provided an important 'proof of concept' for the use of cysteine protease inhibitors in vivo. Evidence has now accumulated that cysteine protease inhibitors can selectively arrest replication of a microbial pathogen without untoward toxicity to the host. Furthermore, this can be achieved with reasonable dosing schedules and oral administration of the drug. Initial studies have confirmed the efficacy of cysteine protease inhibitors in treatment of *Trypanosoma cruzi*, *Plasmodium falciparum* and *Leishmania major*. Work on *Trypanosoma brucei*, the agent of African trypanosomiasis, is preliminary but also promising. Target validation studies have shown that biotinylated or radiolabeled irreversible inhibitors specifically bind to the cysteine protease targets thought to represent the major activity within the parasite. In the case of *T. cruzi*, the effect of inhibitors appears to be predominantly in blocking protease processing. Transfection studies using variant constructs have supported this model. Finally, the generation of null mutants for the multiple protease genes in *Leishmania mexicana* has provided the first genetic support for the key role of this enzyme family in parasite virulence. Safety studies in rodents and analysis of uptake of inhibitors by parasites and host cells suggest that the selectivity of inhibitors for the parasite targets may reside in the lack of redundancy of parasite proteases, the higher concentration of host proteases in intracellular compartments, and differential uptake of inhibitors by parasites. Attempts to elicit resistance to cysteine protease inhibitors in parasite cultures suggest that mechanisms of induced resistance are independent of resistance to the traditional antiparasitic agents. This suggests that cysteine protease inhibitors may provide an alternative to traditional therapy in drug-resistant organisms.

Introduction

Papain family cysteine proteases are the focus of a resurgent interest in synthetic protease inhibitor development for chemotherapy of a number of diseases.[1] The conventional view of proteases like cathepsin B and cathepsin L held that these enzymes are predominantly, if not exclusively, localized to the lysosome-endosome compartment and function for intracellular protein degradation.[2] Exocytosis or secretion of these proteases was viewed as an 'abnormal' state characteristic of a number of diseases. For example, invasive tumor cells were found to express cathepsin B on their surface or in the extracellular milieu.[3] Cathepsin L was found to be the major excreted protein of transformed fibroblasts.[4] 'Lysosomal' enzymes were found to contribute to extracellular matrix degradation in inflammatory conditions like rheumatoid arthritis,[5] and degenerative conditions like osteoporosis.[6] In fact, this 'abnormal' or 'pathologic' extracellular action of papain family cysteine proteases in mammalian cells recapitulates 'normal' function in more primitive eukaryotic cells. Work on metazoan and protozoan parasites has provided important insights into the evolution, localization, and biologic function of this enzyme family.[7] The key role of cysteine proteases in parasitic infections, coupled with the relative lack of redundancy compared to mammalian systems, has made parasite proteases attractive targets for the development of new chemotherapy.[7,8]

Confirmation that cysteine protease inhibitors can selectively arrest infections by malaria, *Leishmania* and *Trypanosoma cruzi* parasites in vivo

Several studies confirmed the efficacy of cysteine protease inhibitors in arresting and killing parasites in tissue culture models of parasite replication or cell

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invasion.[9–12] The key remaining observation needed for 'proof of concept' in a program of drug development, would be the demonstration of selective killing of parasites by cysteine protease inhibitors in animal models of infection. Three issues had to be addressed: (1) Can sufficient blood and tissue levels of cysteine protease inhibitors be achieved to eliminate a parasitic infection? (2) Are therapeutic levels tolerated by the mammalian host? (3) Can oral bioavailability and practical dosing regimens be achieved?

Several studies have now demonstrated the efficacy of cysteine protease inhibitors against parasitic infections in vivo. The first of these was the demonstration that fluoromethyl ketone-derivatized dipeptides could cure a murine infection of malaria.[13] However, the doses required for cure resulted in significant, though reversible, toxic effects. Subsequent development of other cysteine protease inhibitor scaffolds and chemistries eliminated the problem associated with fluoromethyl ketone toxicity. Efficacy of vinyl sulfone-derivatized dipeptides has been shown in murine models of malaria, and is discussed in more detail in the article by Rosenthal and colleagues.[16] A therapeutic regimen of 100 mg/kg/day of Mu-Phe-hPhe-VS-phenyl was also shown to arrest replication of *Leishmania major* in an animal model of cutaneous leishmaniasis.[14] More extensive in vivo studies have been carried out in murine models of Chagas' disease, caused by *Trypanosoma cruzi*. Several independent experiments have confirmed the ability of vinyl sulfone-derivatized dipeptides to rescue mice from an acute lethal infection of *T. cruzi* in which mice are infected with 10^5–10^6 trypomastigote forms.[15] Recent pharmacokinetic analysis and identification of primary metabolites has allowed more rational dosing regimens to be established. The half-life of the vinyl sulfone peptidomimetics is approximately 30 to 40 min. Eight to twelve hour dosing regimens (100 mg/kg/day) for 20 days were effective not only at rescuing mice from lethal infections, but actually producing cure.[15]

Furthermore, the recent development of orally bioavailable cysteine protease inhibitors has led to studies confirming oral administration can also eliminate parasites.[16] Preliminary studies have also confirmed that cysteine protease inhibitors can arrest replication of *Trypanosoma brucei* in mice. The data are summarized in more detail below.

These initial in vivo studies with peptidomimetics provide an important 'proof of concept' that cysteine protease inhibitors can selectively arrest parasite infections without undue toxicity to the host. Further development of more effective and specific protease inhibitor scaffolds is an ongoing effort in both academic centers and industry (e.g. ref 17) and should yield even more suitable leads.

Target validation

Is the activity of specific cysteine protease inhibitors against parasitic organisms, observed in culture systems or in animal models of infection, due in fact to inhibition of target proteases, or to unrelated effects? Three different observations have produced strong circumstantial or, in some cases, direct support for the action of inhibitors against the perceived targets. First, in studies of the effect of cysteine protease inhibitors against *T. cruzi* and *Leishmania*, it was observed that regardless of the chemistry of the inhibitor, the morphologic abnormalities produced in the parasite were the same for all inhibitors that were effective against the protease target.[18,19] In the case of *T. cruzi*, inhibitors produced a characteristic abnormality in the Golgi apparatus due to accumulation of unprocessed protease, and subsequent osmotic shock to the intracellular compartment where processing would normally take place. Processing in *T. cruzi* is autoproteolytic, as confirmed by both direct analysis of recombinant protein as well as transfection studies with mutant protease constructs.[20,21] The unusual Golgi abnormality has been observed in parasites treated with fluoromethyl ketone- or vinyl sulfone-derivatized peptidomimetics, with peptides containing heterocyclic oxygen-containing leaving groups, and with reversible dihydrazides and acylamide inhibitors. In other words, regardless of the specific chemical structure or mechanism of inactivation, inhibitors which proved effective against the target enzyme 'cruzain' all produced the same intracellular abnormality.

Similar correlations were documented for the effect of inhibitors on *L. major*.[14,19] In this case, accumulation of undigested material in the megasome (lysosomal compartment) and flagellar pocket were observed with all inhibitors effective against the cathepsin B- or cathepsin L-like proteases. The situation in *Leishmania* is somewhat more complex because of the presence of three cysteine protease gene families,[22] but the effect of inhibitors on parasite cells appears consistent with their known activity against multiple *Leishmania* protease targets. Similar correlations have also been made with the effect of cysteine protease inhibitors on the malaria parasite food vacuole.[23,24]

The second type of study that supported target validation was the use of specific, irreversible peptidomimetic inhibitors 'tagged' with biotin or a radiolabel. In the case of both *T. cruzi* and *L. major*, incubation of these inhibitors with live parasites, and subsequent lysis, extraction, and electrophoretic separation, demonstrated exclusive binding of the inhibitor to specific cysteine protease targets within the parasite.[14,15]

The third type of validation has come from transfection and knockout studies targeting specific cysteine protease genes. For example, transfection and expression of mutant cruzain precursor, in which the proenzyme-catalytic domain processing site had been altered, resulted in the same Golgi abnormality observed with protease inhibitors.[21] Unfortunately, despite attempts for several years by several laboratories, a *T. cruzi* cysteine protease knockout has not been generated. This may reflect the key role this enzyme plays in parasite biology. On the other hand, the redundant protease systems in *Leishmania mexicana* have proven more

accessible to genetic analysis. The *cpa* gene, coding for a cathepsin L-like protease gene, was first eliminated but revealed no phenotype.[25] A single knockout of *cpc*, the cathepsin B-like cysteine protease, also yielded no observable phenotype.[26] However, when a cassette of several *cpb* cathepsin L-like protease genes was eliminated, there was decreased virulence (intracellular infection) similar to the effects of protease inhibitors.[27] A double knockout of *cpa* and *cpb* resulted in a complete absence of lesion development in immune-competent mice.[28] Key future studies will be the evaluation of triple knockouts in which all cysteine protease activity has been eliminated. Nevertheless, the observations on double knockouts support the contention that these enzymes play important roles in intracellular infection or amastigote replication. More recently, Mottram and colleagues have shown that transfection of an inactive mutant of *cpb* into a *cpa–cpb* double knockout results in accumulation of unprocessed precursor in the flagellar pocket, similar to the results seen with cysteine protease inhibitors that target several of the *Leishmania* papain family cysteine proteases (personal communication).

Preliminary work on the use of cysteine protease inhibitors in African trypanosomiasis

Compared to other trypanosmatids such as *Leishmania* and *T. cruzi*, research into the potential of synthetic cysteine protease inhibitors as chemotherapeutic agents of African trypanosomiasis is just beginning. There are only a few published reports describing in vitro trypanocidal activity by peptidyl diazo- and fluoromethyl ketones. Tests in vivo are at a very early stage (see below). The cysteine proteases themselves, however, are biochemically well-characterized and consist predominantly of a group of lysosomal cathepsin L-like enzymes[29–31] with a major form of 28 kDa in *T. brucei* (trypanopain-Tb)[32] and 32 kDa in *Trypanosoma congolense* (trypanopain-Tc).[33,34] The activity of trypanopains is developmentally regulated,[35] being 10 to 15-times higher in the bloodstream of short stumpy forms of *T. brucei* than in either the long slender or procyclic (insect) forms.[36] Trypanopains have been cloned and sequenced,[37–39] and trypanopain-Tb has been expressed in *Escherichia coli*.[40] Similar to cruzain and the Type I cysteine proteases of *Leishmania*, trypanopains have a C-terminal extension.[37–39]

In vitro killing of *T. brucei* has been shown for the peptidyl fluoromethyl ketones Z-Ala-ImNva(imidazolyl norvalyl)-CH_2F and Z-Ala-Phe-CH_2F.[11] After 3 h at 37 °C in the presence of 175 μM inhibitor all bloodstream forms had been killed. Interestingly, the procyclic stage, which contains less trypanopain activity (above) was refractory. Also, the corresponding diazomethyl ketone of Z-Ala-Phe-CH_2F was without effect, indicating the essential nature of the fluoromethyl ketone group. Robertson et al.[30] demonstrated killing of *T. brucei* procyclics with comparable concentrations of Z-Phe-Ala-CHN_2 and Z-Phe-Phe-CHN_2 after 72 h at 28 °C. With *T. brucei* and *T. congolense*, a trypanocidal effect of various diazomethyl ketones could only be observed at 100–1000-fold higher concentrations than those required to inhibit trypanopain. Thus, the possibility that these compounds inhibited proteases other than trypanopain was discussed.[33] More recently, Troeberg and Coetzer tested a number of fluoromethyl ketone and vinyl sulfone-derivatized dipeptides. They demonstrated labeling of the target cysteine protease by a biotinylated inhibitor for target validation and found *N*-methyl-pip-Phe-hPhe-VSphenyl had an ED_{50} of 110 nanomolar against live trypanosomes in culture.

Preliminary in vivo tests are encouraging. Treatment of mice infected with *T. brucei* from days 3 to 6 post-infection with daily ip doses of 250 mg/kg Z-Phe-Ala-CHN_2 decreased parasitemia to undetectable levels for the following 3 days. Though the parasitemia levels then rose, the mice so treated survived on average for 68 days post-infection whereas placebo-treated mice survived for 38 days. Administration of the inhibitor was associated with an almost complete inhibition of trypanopain activity. (Scory and Caffrey, unpublished results).

Troeberg and colleagues have screened a number of chalcones, dihydrazides and acylamides for inhibition of trypanopain-Tb, and the most potent of these were then tested in mice infected with *T. brucei*. The dihydrazide compound ZLIII43A ($K_i = 28$ nM), administered as a single 0.5 mg sc dose at the time of infection, prolonged the survival of mice by 6 days over control mice, which died at 8 days post-infection (Troeberg and Coetzer, personal communication).

The above experiments performed in vivo indicate that synthetic cysteine protease inhibitors could prove useful in the development of new chemotherapies of African trypanosomiasis by targeting trypanopain. It seems, therefore, only to be a matter of time before more extensive drug design strategies are employed.

Preliminary toxicology studies of cysteine protease inhibitors effective in experimental models of parasitic infections

In vivo studies have now confirmed that infections with *L. major*,[14] *T. cruzi*,[18] *Schistosoma mansoni*, and *Plasmodium falciparum*[13] can be arrested or cured with specific cysteine protease inhibitors. These studies have provided important 'proof of concept' that cysteine protease inhibitors can selectively eliminate parasitic organisms without undue toxicity to the host. The first cure of a parasitic infection by a cysteine protease inhibitor, that of *P. falciparum* by a fluoromethyl ketone-derivatized peptidomimetic, required Q.i.D. dosing at 1 mg/dose. This regimen resulted in reversible lethargy and hypothermia in the mice, most likely due to generation of toxic fluoride-containing metabolites. Subsequent studies used less toxic vinyl sulfone or heterocyclic oxygen-containing peptidomimetics, which are still irreversible inhibitors. Vinyl sulfone inhibitors were tolerated when given ip or orally at doses as high as 6 mg/day to 20 g mice.[15] A 45-day course of the therapeutic regimen of 100 mg/kg/day was completely tolerated (Engel, unpublished data). More recently, an NIH-sponsored range-finding study (N01-AI-6537)

suggested that one vinyl sulfone lead, *N*-methyl-piperazine-Phe-hPhe-vinyl sulfone phenyl, was tolerated by male mice at iv bolus doses of approximately 40 mg/kg, and by female mice up to 75 mg/kg. The plasma levels corresponding to these doses were 2–4 times plasma levels necessary to eliminate *T. cruzi* or *P. falciparum*. These results are very encouraging and suggest that there is no serious or unexpected toxicity that results from the administration of cysteine protease inhibitors, even if they are irreversible and not exquisitely specific.

The use of irreversible protease inhibitors to treat parasitic infections

Most of the work to date in animal models of parasitic infection has utilized irreversible cysteine protease inhibitors such as fluoromethyl ketones, heterocyclic oxygen-containing peptidomimetics, or vinyl sulfones. Despite concerns about the potential of using irreversible protease inhibitors, no toxic effects suggestive of autoimmune or inflammatory phenomena have been observed. The issue of irreversible versus reversible inhibitors is probably less important in the short courses of treatment required for microbial infections than would be the case in longer-term use of cysteine protease inhibitors in diseases such as cancer, osteoporosis or arthritis. Furthermore, previous articles raising the concern about generation of covalent adducts on proteins referred primarily to multiepitope addition (for example, a peptide bound at multiple sites to a carrier protein), and not specifically to protease inhibitors bound at a single active site.[41] The potential targets for protease inhibitors are also not long-lived, and the accumulation of the irreversible inhibitor at the enzyme active site may not be much greater than that observed for a tight binding inhibitor with a slow offrate, or a slow turnover inhibitor. Nevertheless, the 'holy grail' of protease inhibitor development, even for parasitic infections, would be a reversible, tight binding and very specific inhibitor.

What host enzyme systems might be 'accidentally' targeted by antiparasitic cysteine protease inhibitors?

Initial studies to date have utilized available inhibitors which were not entirely specific for their parasite targets. As noted above, despite this lack of specificity, little or no toxicity has been noted. This is probably in large part due to the fact that the parasite targets are in a more vulnerable location within the mammalian host. Malaria, schistosomes and *T. brucei*, for example, are bloodstream organisms not requiring effective inhibitors to reach tissue sites. *T. cruzi* resides free in the host cell cytoplasm and is therefore more easily targeted than the homologous host proteases that are in host cell lysosomes. Labeling studies have suggested that inhibitors which selectively kill parasites in vivo may indeed bind to one or two host cell targets, perhaps cathepsin S or B for the vinyl sulfones.[15] Why, then, is more toxicity not seen? In part this may be due to the redundancy and concentration of the host proteases relative to the parasite targets. Parasites generally have a limited number of cysteine protease genes, and the concentrations of host proteases within the lysosomal or other cellular compartments may far exceed the concentration of the comparable parasite targets. Parasites may also selectively take up and therefore concentrate small molecular weight inhibitors as part of their highly evolved adaptation to intracellular or bloodstream parasitism.

One important observation has been the lack of toxicity of vinyl sulfone dipeptides versus tripeptides if the three sidechains are hydrophobic (Roush and Engel, unpublished data). Recent observations that vinyl sulfones can react with threonine at the active site of proteasome subunits, suggests an explanation for this specificity of toxic effects.[42] Hydrophobic tripeptide inhibitors are excellent inhibitors of the proteasome, and proteasome inhibition has been associated with mammalian cell toxicity. The recognition of this phenomenon has therefore directed further development of antiparasitic cysteine protease inhibitors to the smaller molecular weight dipeptide or pseudodipeptide scaffolds.

Attempts to elicit drug resistance to cysteine protease inhibitors in vitro

Little information is available regarding mechanisms mediating drug resistance in *T. cruzi*. Drug resistance to a traditional therapy for Chagas' disease (i.e. nifurtimox and benznidazole) and to anticancer drugs has been induced in vitro.[43–47] Nifurtimox resistance in trypomastigotes and epimastigotes of several *T. cruzi* strains and clones showed no correlation with levels of trypanothionine reductase and heat shock proteins, or with karyotypic alterations.[43] The mechanism involved in nifurtimox resistance in these strains remains unknown. Transporters mediating drug influx and efflux may be responsible for differences in nifurtimox uptake by natural drug-resistant and susceptible strains.[48] P-glycoproteins have been characterized in *T. cruzi*, and a daunomycin-resistant strain which exhibits cross-resistance to nifurtimox and benznidazole has been generated.[45] In this strain daunomycin induced overexpression of transporters of the multidrug-resistance (MDR) family. These results suggest that resistance to these drugs in *T. cruzi* may be partly due to increased drug efflux mediated by P-glycoproteins.

A nifurtimox-resistant (NR) *T. cruzi* clone[43] is cross-resistant to benznidazole but susceptible to cysteine protease inhibitors (Engel, personal data). This observation suggests that cysteine protease inhibitors may be a valid alternative chemotherapy in cases of nifurtimox and/or benznidazole-resistant Chagas' disease. Nevertheless, several issues remain, such as the degree of susceptibility of natural *T. cruzi* populations to these inhibitors. To address these issues, Scharfstein and colleagues at the Federal University of Rio de Janeiro (UFRJ), and our group at UCSF, both have developed stable drug resistant *T. cruzi* populations to potent peptidyl-based cysteine protease inhibitors that target cruzain/cruzipain. In both cases, protease inhibitor-resistant *T. cruzi* were developed by exposing epimastigotes to increasing concentrations of the inhibitors for over a year. Resistant *T. cruzi* stocks are routinely

maintained at 10–20-fold the lethal concentration. It is important to emphasize that resistant parasites are still sensitive to the two traditional therapeutic drugs, nifurtimox and benznidazole (ref 49 and Engel, personal communication).

A comparative study of the parental *T. cruzi* epimastigote stock (PS), treated protease inhibitor (vinyl sulfone)-sensitive (KS) and protease inhibitor-resistant(KR) parasites showed morphological and biochemical differences. In sensitive organisms cysteine protease inhibitors induced accumulation of unprocessed cruzain precursors, and interfered with the normal autoproteolytic maturation of the enzyme. Inhibitor treatment also induced an alteration in the normal cellular location of the enzyme (i.e. a decrease in mature cruzain within lysosomes and on cell surface membranes). Unprocessed cruzain molecules accumulated in peripheral dilations of Golgi cisternae in treated cells. The vinyl sulfone protease inhibitor induced major alterations in the Golgi apparatus and disrupted normal vesicle formation and trafficking. Later ultrastructural alterations were noted in the nuclear membrane, endoplasmic reticulum, and mitochondria.[18] Similarly to KS, KR parasites had a decreased concentration of lysosomal cruzain than PS organisms. In contrast to KS, KR cells had a normal subcellular morphology except for a hypertrophic Golgi complex and a markedly increased number of secretory vesicles, suggesting they have enhanced exocytosis/secretion of inactive cruzain-inhibitor complexes and unprocessed cruzain precursors (Engel, unpublished results). Comparative Western blot analyses of the susceptible KS strain with and without low concentrations of the *N*-Pip-F-hF-VSphenyl inhibitor showed accumulation of membrane-bound precursors of cruzain. This pattern differed for treated KR parasites where high levels of unprocessed cruzain precursors were present in the soluble fraction and in the culture media, suggesting that KR parasites are using a default secretory pathway to secrete high molecular weight cruzain-protease inhibitor complexes. Scharfstein and colleagues, on the other hand, have suggested that decreased drug internalization and reduced expression of cruzain may mediate resistance to peptidyl diazomethanes, and that this may be accompanied by upregulation of a different cysteine protease.[49] In summary, two possible resistance mechanisms have been reported in in vitro studies. It is noteworthy, however, that resistant organisms were difficult to select for and this may reflect unfavorable cellular alterations that must be made to overcome the lethal effects of protease inhibitors. Further analysis of the mechanism of resistance should help to direct future protease inhibitor development in order to synthesize inhibitors that might bypass specific resistance mechanisms.

Summary

Analysis of the effect of cysteine protease inhibitors in parasitic infections has led to an important 'proof of concept' for the use of cysteine protease inhibitors as therapy in a variety of diseases. The parasite studies have confirmed that cysteine proteases can be given to an animal host without undue toxicity to selectively eliminate an infectious organism. The selectivity of these inhibitors has (to date) not resided in their specificity against a particular parasite protease (versus host) target, but more in the vulnerability of the parasite enzyme due to localization or lack of redundancy. Similar situations may apply to other therapeutic uses of cysteine protease inhibitors. For example, cysteine proteases of transformed cells or tumor cells may reside on the cell surface or be released into the extracellular milieu. In inflammatory diseases, cysteine proteases are released extracellularly; and in osteoporosis, cathepsin K may operate in an extracellular microenvironment. In each case, the protease target would be more accessible than in its 'normal' intracellular compartment.

Recent advances in the development of new cysteine protease inhibitor scaffolds suggest that specificity to target proteases can also be achieved.[17] Therefore, the combination of serendipitous biologic selectivity with target-directed chemical specificity suggests an optimistic future for the use of cysteine protease inhibitors as therapy in a number of disease processes.

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