Cysteine Proteinase Inhibitors as Therapy for Parasitic Diseases: Advances in Inhibitor Design

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Abstract: Clan CA (papain-like) cysteine proteinases of protozoan parasites are validated targets for the rational design of new anti-parasitic chemotherapies. Peptidyl and peptidomimetic proteinase inhibitors of differing chemistries limit parasite survival *in vitro* and *in vivo*. Also, the development of activity-based affinity labels has enabled the identification and characterization of potential cysteine proteinase targets *in situ.* This article reviews the biology and physicochemistry of parasite proteinases and the ongoing design of peptidyl and non-peptidyl inhibitors to generate anti-parasitic compounds of greater efficacy with decreased toxicity to the host.

Key Words: Brucipain, rhodesain, cruzain, falcipain, vivapain, *Trypanosoma* spp., *Leishmania* spp., *Plasmodium* spp.

INTRODUCTION

Vector-borne protozoan diseases are responsible for morbidity and mortality in millions of people in tropical and subtropical areas of the world. Today approximately 40% of the world's population is threatened with malaria with an estimated 350 – 500 million clinical episodes and 1 – 2 million deaths each year, mainly children under the age of five [1, 2]. A total of 160 million people are at risk from American (Chagas' disease) and African (sleeping sickness) trypanosomiases with 18 million cases and 63,000 deaths annually [3]. Leishmaniasis threatens 350 million people with about 1.5 – 2.0 million new infections and 57,000 deaths reported yearly [4, 5]. As these parasitic diseases do not induce a pronounced protective immune response, the chances of developing vaccines are rather small. Therefore, chemotherapy remains the only practicable alternative option but few drugs are available for most of these diseases. In addition, many of the current medications are decades old and suffer from poor efficacies, toxic side-effects and emerging drug resistance. Thus, the development of novel drugs is urgently required for treatment of protozoal diseases [6].

Research in recent years has demonstrated that parasite cysteine proteinases of the papain family (clan CA; http://merops.sanger.ac.uk/) are promising chemotherapeutic targets. In particular, synthetic peptidyl and peptidomimetic compounds have been shown to display encouraging anti-parasitic activities *in vitro* and *in vivo* [7-11]. This article focuses on the recent development of cysteine proteinase inhibitors for the treatment of infections by *Plasmodium* spp., *Leishmania* spp. and *Trypanosoma* spp.

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PAPAIN-LIKE CYSTEINE PROTEINASES OF PROTOZOAN PARASITES

Both cathepsin B- and L-like cysteine proteinases have been characterized in many protozoan parasites [12]. In common with other papain-like cysteine proteinases, the parasite enzymes consist of a signal peptide, a propeptide and a catalytic domain [13]. After cleavage of the signal peptide and propeptide, the catalytic domain forms the mature proteolytically active enzyme of between 220 and 260 amino acids in length. Exceptions are the mature cathepsin L-like proteinases of trypanosome and *Leishmania* parasites which contain an 11 – 13 kDa C-terminal extension of unknown function.

The catalytic mechanism of parasite cysteine proteinases is typical of Clan CA proteinases [13-15] (Fig. **1**). Conserved cysteine and histidine residues located on opposite sides of the enzyme's active site cleft are spatially orientated to form a catalytic ion pair (Fig. **1**, ①). During substrate hydrolysis, the polarised nucleophilic thiolate attacks the carbonyl carbon of the sissile peptide bond and a transient tetrahedral intermediate is formed (Fig. **1**, ②). The oxyanion that is generated is stabilized by the so-called oxyanion hole, which comprises a conserved glutamine residue in clan CA enzymes. In the next step, a free amine is formed and the C-terminal part of the substrate is released (acylation) with simultaneous transformation of the tetrahedral intermediate into an acyl-enzyme (Fig. **1**, ③). Subsequently the electrophilic acyl-enzyme is attacked by the nucleophilic oxygen of water and a second tetrahedral intermediate is produced (Fig. **1**, ④). Finally, collapse of the tetrahedral intermediate generates the free acid and liberates the N-terminal part of the substrate (deacylation), and the enzyme is regenerated (Fig. **1**, ⑤).

The substrate binding region of papain-like cysteine proteinases has a number of binding pockets or subsites for substrate amino acid residues either side of the scissile peptide

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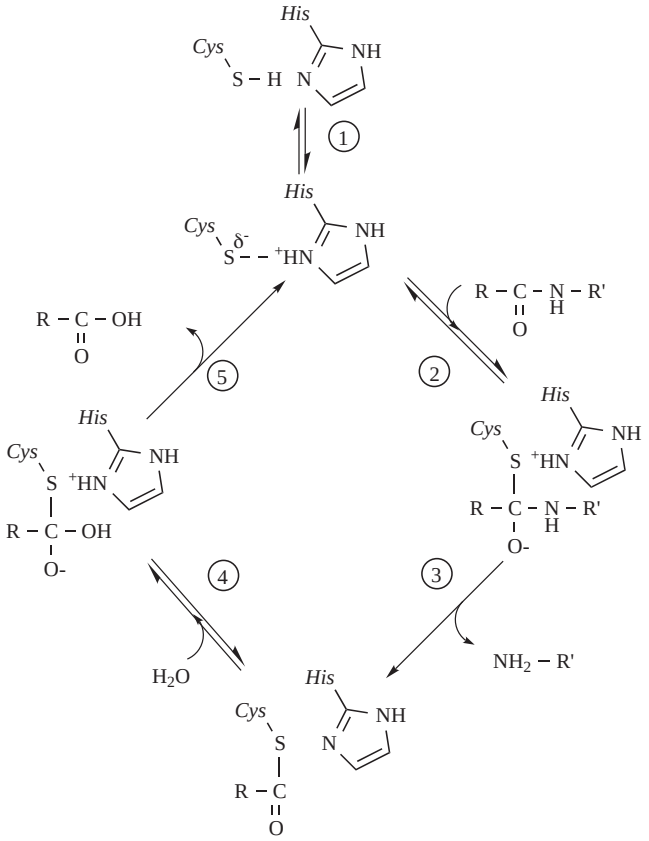


Fig. (1). Catalytic mechanism of proteolysis by papain-like cysteine proteinases.

bond. According to the terminology of Berger and Schechter [16], the subsites towards the N-terminus (non-prime side) are termed S_1, S_2, etc., and those towards the C-terminus (prime side), $S_1´$, $S_2´$, etc. Correspondingly, the substrate or inhibitor amino acids associating with each subsite are termed P_1, P_2, etc. and $P_1´$, $P_2´$, etc.

The expression and localization of the cysteine proteinases in protozoan parasites is dependent on the life-cycle stage. For example, even though cruzain, the cysteine proteinase of *T. cruzi*, is expressed in all life-cycle stages [17], in intracellular amastigotes, it is localized on the cell surface, whereas in epimastigotes which multiply in reduvid bugs, it is found in the endosomal/lysosomal system [18-20]. Falcipain-2 and -3, and vivapain-2 and -3, the respective cysteine proteinases of the malaria parasites, *P. falciparum* and *P. vivax*, are predominantly expressed in the erythrocytic stages and localized in the acidic food vacuole that is analogous to the lysosome [21-23]. Brucipain and rhodesain, from *T. brucei brucei* and *T. brucei rhodesiense*, respectively, are expressed in all life-cycle stages [24, 25]. The localization of brucipain and rhodesain in bloodstream forms is lysosomal [25, 26], whereas that in procyclic insect forms remains to be determined. Several isoforms of cathepsin L-like and one cathepsin B-like cysteine proteinases are found in *Leishmania* parasites [27, 28]. Most of these enzymes are confined to large lysosomes, so-called megasomes, in the intracellular amastigote life-cycle stage [28].

DIPEPTIDYL COMPOUNDS

Over the last decade numerous dipeptidyl substrate analogues have been investigated as inhibitors of parasite cysteine proteinases [25, 29-38]. Compounds tested included peptide aldehydes (CHO), halomethyl ketones (CH_2F, CH_2Cl), diazomethyl ketones (CHN_2), epoxy ketones (Exp), vinyl sulfones (VSR), vinyl sulfonate esters (VSOR) and vinyl sulfonamides (VSNH). Whereas the peptide segment is responsible for recognition of the inhibitor by the enzyme subsites, the electrophilic group reacts with the cysteine residue of the active site (Fig. **2**).

The structure-activity relationships of dipeptidyl compounds against brucipain, rhodesain, cruzain, falcipain and vivapain is summarized in Fig. (**3**) [25, 29-38]. On the non-prime side, homo-phenylalanine (hPhe) is a favoured substituent at the P_1 position. At P_2, brucipain, rhodesain and cruzain prefer phenylalanine (Phe) whereas falcipain and vivapain favour the smaller leucine (Leu). In fact, compounds with phenylalanine at P_2 are less effective inhibitors for the malarial cysteine proteinases. No significant difference in activity against the enzymes is observed for substituents at R_1: carbobenzoxy (Cbz), morpholine urea (Mu) and *N*-methyl piperazine urea (MePip) are all accepted. On the prime side, a favourable substituent at R_2 is a phenyl vinyl sulfonate ester (VSOPh) for the trypanosome enzymes and a fluoromethyl ketone (CH_2F) or *N*-phenyl vinyl sulfonamide (VSNHBn) for the malarial enzymes. In general, the structure-activity relationships observed for the compounds with the parasites' enzymes carry over when working with live parasites [9, 26, 29, 34, 36, 37, 39-46]. One notable exception, however, concerns trypanosomes at the R_2 position: the phenyl vinyl sulfonate ester (VSOPh) is favourable with respect to the enzymes whereas the phenyl vinyl sulfone (VSPh) shows greater trypanocidal activity.

Several dipeptidyl compounds have been tested for their efficacy in animal models of parasitic infections. Treatment of *T. brucei*-infected mice with Cbz-Phe-Ala-CHN_2 (Fig. **4**) (250 mg/kg once daily intra-peritoneally (i.p.) from days 3 to 6 post-infection (p.i.)) decreased the parasitemia to undetectable levels [26]. Importantly, the trypanocidal action of the compound was associated with an almost complete inhibition of brucipain in the trypanosomes. However, upon discontinuation of the treatment, parasitemia returned to control levels [26]. A similar result was obtained with MePip-Phe-hPhe-VSPh (Fig. **4**; 50 mg/kg twice daily i.p. from days 3 to 7 p.i.) [9]. On the other hand, the same compound rescued and cured mice from lethal and chronic infections with *T. cruzi* using 35 mg/kg thrice daily i.p. for 24 and 21 days, respectively [43]. MePip-Phe-hPhe-VSPh and the related compound Mu-Phe-hPhe-VSPh (Fig. **4**) delayed the development of footpad lesions in *Leishmania*-infected mice (100 mg/kg once daily i.p. for 28 days) [47, 48]. However, the effect was not permanent and after cessation of treatment, lesions were similar to those of control mice. MePip-Leu-hPhe-VS2Np (Fig. **4**; VS2Np = naphthalene vinyl sulfone) cured 40% of mice infected with *P. vinckei* (100 – 200 mg/kg twice daily orally for 4 days) [44]. The aldehyde compound Mu-Leu-hPhe-CHO (Fig. **4**) delayed the progression of murine *P. vinckei* malaria when infused continuously

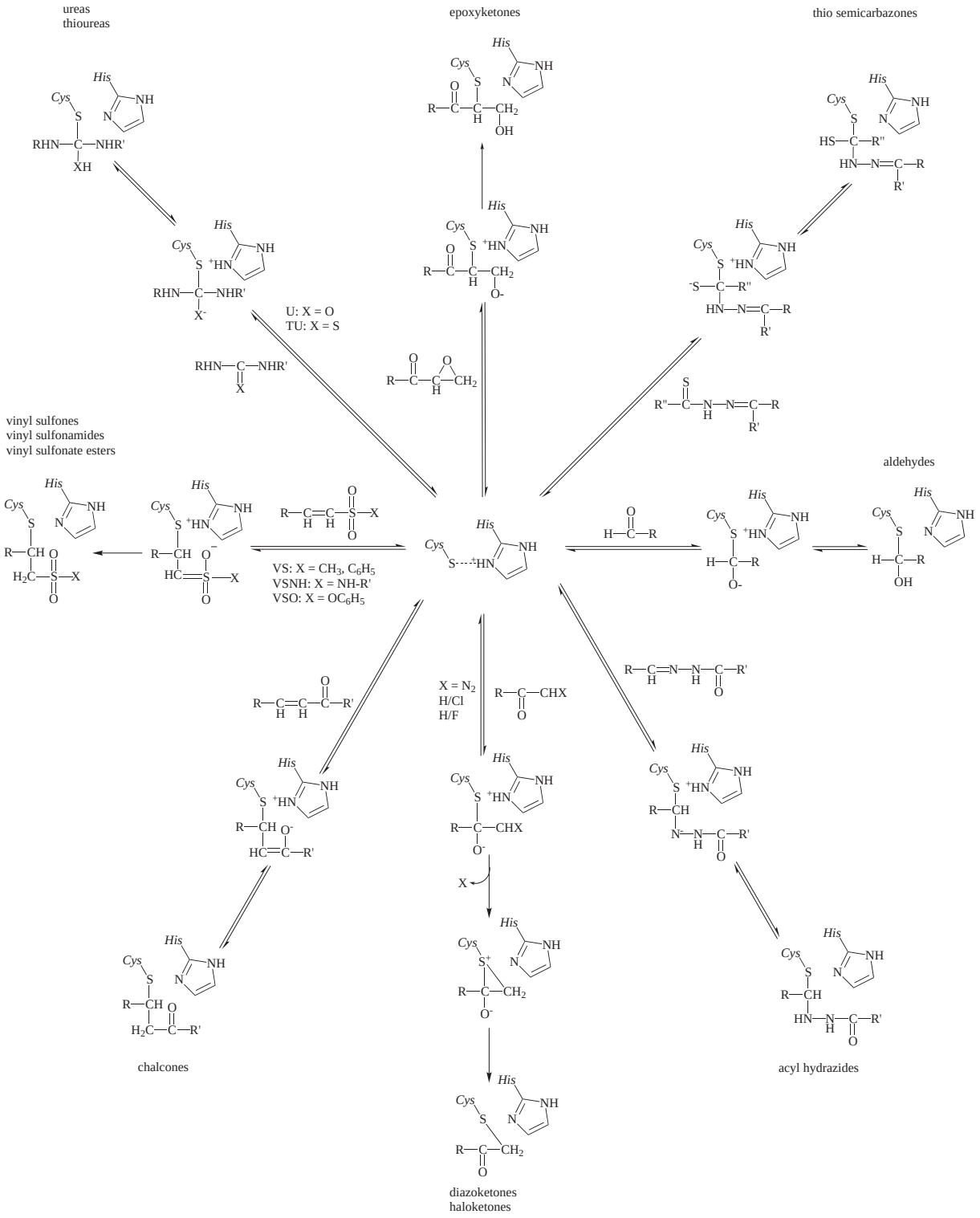


Fig. (2). Inactivation mechanisms of cysteine proteinase inhibitors.

using subcutaneous pumps (30 mg/kg/day for 14 days) [46]. These results with murine models clearly demonstrate the validity of targeting cysteine proteinases with dipeptidyl and peptidomimetic inhibitors for novel disease therapy.

NON-PEPTIDYL COMPOUNDS

In parallel with the above developments, new scaffolds of non-peptidyl reversible inhibitors of parasitic cysteine proteinases have been identified and synthesised [47, 49-58].

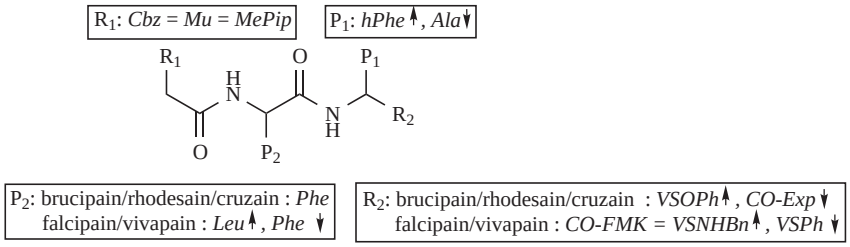
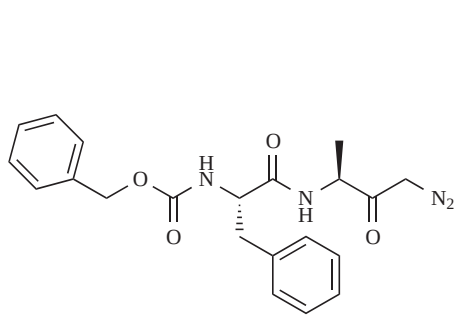


Fig. (3). Summary of structure-activity relationship of dipeptidyl compounds against parasite cysteine proteinases based on published data for second-order rate constants of enzyme inhibition [25, 29-38]. Cbz, carbobenzoxy; CO-Exp, epoxide ketone; CO-FMK, fluoromethyl ketone; MePip, *N*-methyl piperazine urea; Mu, morpholine urea; hPhe, homophenylalanine; VSOPh, phenyl vinyl sulfonate ester; VSPh, phenyl vinyl sulfone; VSNHBn, *N*-phenyl vinyl sulfonamide; _, favourable substituent; _, unfavourable substituent.

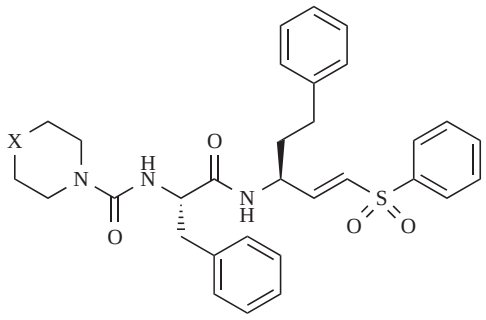
These include a wide range of acyl hydrazides, chalcones, ureas, thioureas and thiosemicarbazones. The reaction mechanism of these compounds with the cysteine residue of the active site is depicted in Fig. (**2**).

Structure-activity relationships of non-peptidyl inhibitors against parasite cysteine proteinases interestingly reveal recurring structures (Fig. **5**) [47, 49-58]. For **R1,** these include hydroxyl-substituted naphthyl groups, bromo- and chloro-substituted phenyl-2-furanyl groups, bromo-, chloro- and trifluoromethyl-substituted phenyl groups and the (1-methyl-3-trifluoromethyl-pyrazol-5-yl)-thiophenyl group. For **R2**, hydroxyl-substituted phenyl and naphthyl groups, and chloro- and iodo-substituted phenyl groups have been repeatedly identified. The same basic structures are also found for the structure-activity relationship of the compounds against live trypanosomes, *Leishmania* and malaria parasites [47, 49-58]. In addition, for *P. falciparum* at **R1**, quinolinyl and 2-chloroquinolinyl groups are favourable substituents [59].

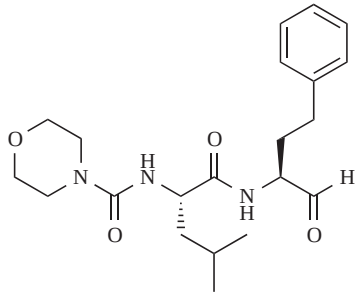
So far, few non-peptidyl compounds have been tested in animal models of parasitic diseases. The acyl hydrazide compound ZLIII43A (Fig. **6**) was shown to protect mice from a lethal *T. brucei* infection if given immediately after the infection (50 mg/kg) [52]. However, if the compound was administered 3 h after the infection, the treatment was ineffective. Another acyl hydrazide compound, ZLIII115A (Fig. **6**) delayed the development of footpad lesions in *L. major*-infected mice (100 mg/kg once daily i.p. for 28 days) [47]. However, at the end of the treatment period, lesion development was similar to that seen in control mice. Recently, a number of phenylurenyl chalcone derivatives (Fig. **6**) have been shown to significantly inhibit parasitemia on day 4 p.i. and increase the survival times of mice infected with *P. ber-*



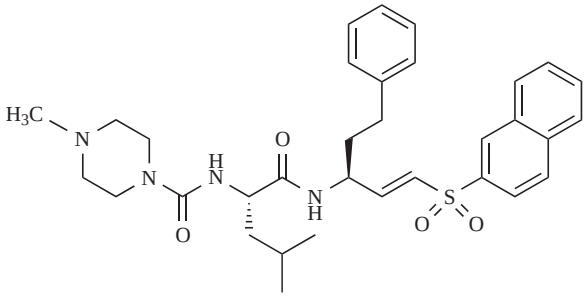
Cbz-Phe-Ala-CHN_2



Mu-Phe-hPhe-VSPh, X = O
MePip-Phe-hPhe-VSPh, X = NMe



Mu-Leu-hPhe-CHO



MePip-Leu-hPhe-VS2Np

Fig (4). Structures of peptidyl cysteine proteinase inhibitors tested in murine models of trypanosomiases, leishmaniasis and malaria.

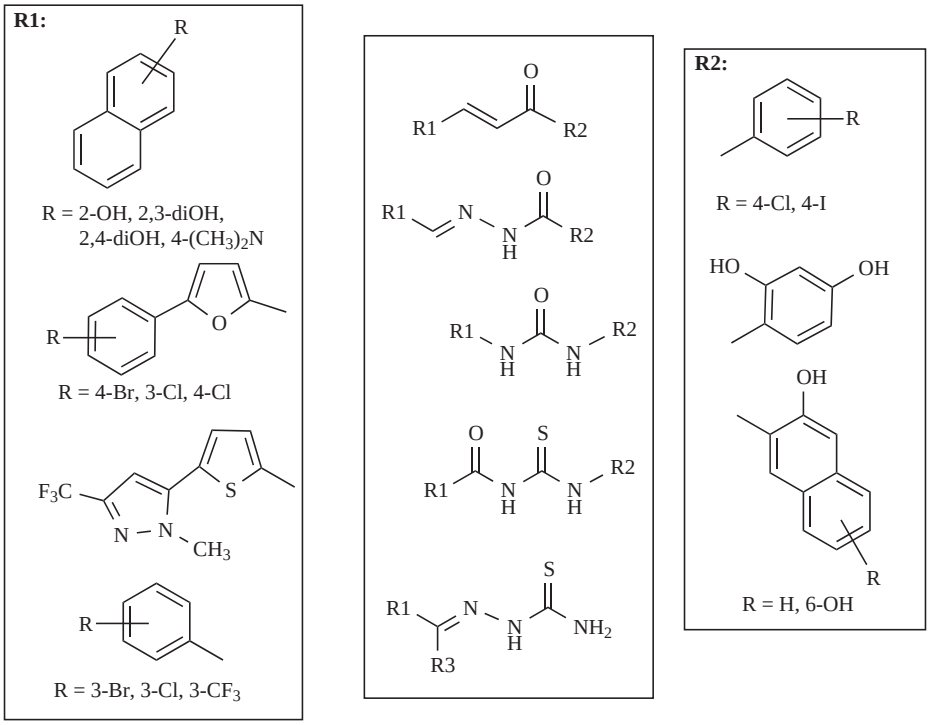


Fig. (5). Summary of structure-activity relationship of non-peptidyl compounds against brucipain, rhodesain, cruzain, *L. major* cpB, and falcipain based on published data for enzyme inhibition and DOCK analysis [47, 49-58]. Shown are recurring structures displaying potent inhibitory activity.

ghei (20 mg/kg once daily i.p. from days 0 to 3 p.i.) [57]. The foregoing data, therefore, demonstrate that non-peptidyl cysteine proteinase inhibitors can arrest parasitic diseases and that further development of these compounds is warranted.

NITRIC OXIDE-DONORS

Nitric oxide (NO) is an important cytotoxic and cytostatic agent that also possesses anti-parasitic activity. Different NO-donors have been shown to kill *P. falciparum* [60], *L. major* [61], and *T. cruzi* [61-63] in cell culture. There is increasing evidence that the parasiticidal effect of NO is through inhibition of cysteine proteinases. Recently, it has been demonstrated that the NO-donors sodium nitroprusside (SNP), *S*-nitroso-glutathione (GSNO), (±)-(*E*)-4-ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexenamide (NOR-3), 3-morpholinosydnonimine (SIN-1), 4-(phenylsulfonyl)-3-((2-(dimethylamino)ethyl)thio)-furoxan oxalate (SNO-102) and *S*-nitroso-acetyl-penicillamine (SNAP) inhibit the catalytic activity of falcipain, cruzain and leishmanial cysteine prote-

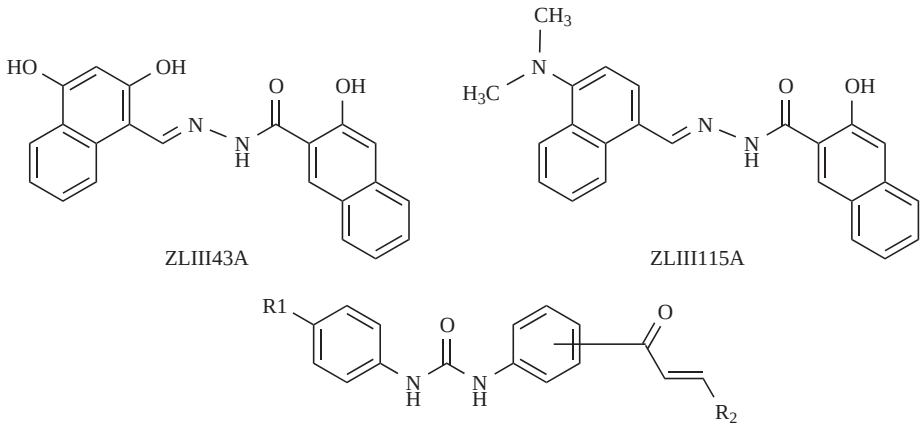


Fig. (6). Structures of non-peptidyl cysteine proteinase inhibitors tested in murine models of African trypanosomiasis, leishmaniasis and malaria.

inase [64-67]. It has been suggested that the inactivation of parasite cysteine proteinases by NO-donors occurs *via* NO-mediated S-nitrosylation of the catalytic cysteine residue [67, 68]. However, before considering NO-releasing drugs as potential chemotherapies of protozoal diseases, it has to be demonstrated that NO actually inactivates cysteine proteinases within parasitic cells.

AFFINITY LABELS

The covalent, activity-based modification of cysteine proteinases by peptidyl and peptidomimetic inhibitors together with their general non-reactivity toward free thiols in aqueous solutions [69], make them particularly suitable for the direct visualization of cysteine proteinases in cell lysates, living cells and whole tissues when 'tagged' with reporters such as biotin, fluorophores or radio-labels. Tagged dipeptidyl diazomethyl ketone inhibitors have been available for many years and used to resolve cysteine proteinases in cancer cells [70, 71] and parasite extracts [72]. However, only with the availability of quantitative mass spectrometry and searchable protein sequence datasets, combined with advances in synthetic organic chemistry that the necessary tools have become available to fully realize the biological potential of such 'activity-based probes' (ABPs). Progress in the design of ABPs has not been limited to targeting cysteine proteinases, but is now part of the expanding field of 'chemical genomics', *i.e.* the identification and profiling of a specific group or groups of enzymes based on their reactivity with small molecules in complex proteomes [73]. Activity-based profiling may be directed, whereby specific knowledge of an enzyme's mechanism directs design of ABPs, or non-directed, in which combinatorial libraries of small molecules are mixed with whole proteomes to identify and develop new ABPs that react with an expanded number of enzyme families [74, 75].

ABPs comprise a chemically reactive group (or 'warhead'), a linker, into which specificity for the given protease(s) is designed, and a 'tag' that allows subsequent visualization or purification of the modified protease target (Fig. **7**). Apart from diazomethyl ketones, the reactive groups incorporated into cysteine proteinases ABPs have included epoxides [76], vinyl sulfones [77] and acyloxy methyl ketones, the latter of which has proven particularly useful for whole-cell profiling of clan CD cysteine proteinases such as caspases and legumains [78]. Research by Matt Bogyo's group has demonstrated that exquisite selectivity can be built into ABPs to annotate and deconvolute the cysteine proteinase complement of eukaryotic cells during cellular differentiation [79] and progression to and through the cancerous state [80].

The identification of potential protease drug targets for treatment of tropical diseases has also benefited from the application of ABPs. Thus, Caffrey *et al.* [25], using a radiolabelled vinyl sulfone probe identified rhodesain/brucipain as the major cysteine proteinase in African trypanosomes. Inhibition of this activity was consistent with anti-parasitic effects both *in vitro* and animal models of disease [26]. For *P. falciparum*, biotinylated aziridine-2,3-dicarboxylates were directly parasiticidal at low micromolar concentrations [81], apparently by inhibiting falcipain-2 and -3, and prevented egress of the merozoite stage from infected red blood cells [82]. ABPs have facilitated the monitoring of the cysteine proteinase expression throughout the developmental cycle of

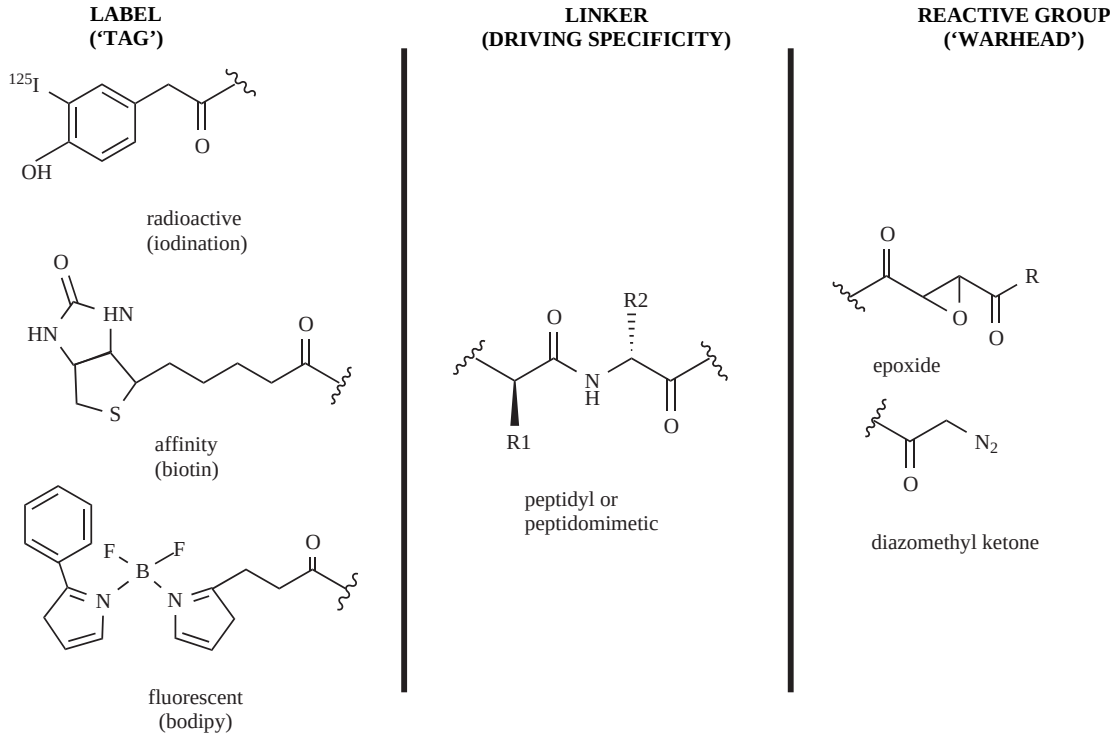


Fig (7). Structures of 'activity-based probes' (ABPs) to target cysteine proteinases.

P. falciparum, clearly indicating that certain cysteine proteinases (*e.g.* falcipain-2, and -3) are only expressed at discrete points in the life-cycle [83], whereas others (falcipain-1) are apparently constitutively expressed [79]. The same report also demonstrated the power of ABPs in combination with subsite positional scanning inhibitor libraries for identifying selective inhibitors of individual cysteine proteinases in cell lysates, thereby avoiding the need for prior preparation of pure native or recombinant enzyme. Accordingly, rapid progress can be made to identify *in situ* selective inhibitor scaffolds that can then be tested for anti-parasite activity.

CONCLUSION

Most of the current drugs for treatment of malaria, trypanosomiases and leishmaniases are unsatisfactory because of poor efficacy, toxic side effects, and emerging drug resistance. Therefore, new and affordable chemotherapies to treat these diseases are urgently required. Research over the last 15 years has demonstrated that cysteine proteinase inhibitors hold real promise.

Peptidyl and non-peptidyl compounds are generally small in size and relatively cheap to produce. The development of anti-protozoal drugs should also benefit from the current pharmaceutical interest in compounds that inhibit cysteine proteinases associated with diseases such as arthritis, osteoporosis, and cancer. In fact, 23 patents describing the synthesis and use of cysteine proteinase inhibitors against different parasitic diseases have been disclosed by various pharmaceutical companies in the last five years [84, 85]. One vinyl sulfone compound, MePip-Phe-hPhe-VSPh, which was developed at Khepri Pharmaceuticals (now Celera Genomics, South San Francisco, CA) and is now completing preclinical development for treatment of Chagas' Disease prior to further development under the auspices of the Drugs for Neglected Diseases Initiative (DNDi; www.dndi.org) [86].

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