Non-Peptidic Inhibitors of Cysteine Proteases

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Abstract: In comparison to the huge number of peptidic and peptidomimetic inhibitors of cysteine proteases which have been developed during the last twenty years the number of non-peptidic compounds with cysteine protease inhibiting properties is restricted to a few substance classes. In contrast to peptidic and peptidomimetic inhibitors the non-peptidic lead structures have mainly been discovered by computational or enzymatic industrial screenings and not by a rational approach. But, the growing number of resolved X-ray structures of the target enzymes as well as molecular modeling methods have supported the further development of potent inhibitors beginning from these lead structures. In this review we will focus on new non-peptidic cysteine protease inhibitors which have been developed during the last years. Discovery, structure-activity-relationship and inhibition mechanisms will be discussed.

INTRODUCTION

Until today more than 10 lysosomal papain-like cysteine proteases have been characterized including the well known cathepsins B [1], C [2], H [3], K [4], L [5] and S [6] as well as the recently discovered cathepsins F [7], O [8], P [9], V [10], W [11], X [12], Y [13], and Z [14]. These enzymes are supposed to be responsible for disregulated protein turnover in a variety of diseases. Examples are muscular dystrophy [15], chronic inflammatory diseases [16], tumour progression [17], osteoporosis [18], and asthma [19]. The number of the known calcium-dependent cytoplasmatic cysteine proteases, the calpains, has also increased since the publication of our review article in 1997 [20]. Today 14 calpain protease family members are known, calpains 1 to 3, and 5 to 13, as well as the small subunits 1 and 2 [21]. Overactivation of these enzymes is linked to a variety of neurodegenerative and neurological disorders [22], muscular dystrophy [23], cataract formation [24], myocardial infarct [25], and type 2 diabetes mellitus [26]. A third group of mammalian cysteine proteases comprises the aspartate-directed caspases. At the time of this writing, 14 mammalian caspases have been described [27]. Because of their roles in inflammation and apoptosis [28], caspases have received enormous research interest during the last years. These three families of mammalian cysteine proteases - cathepsins, calpains, and caspases - represent a huge pool of potential targets for the development of cysteine protease inhibitors as drugs. With respect to this research field we may not forget the various cysteine proteases of microbiological pathogens which are supposed to play key roles as virulence factors in infectious diseases caused by bacteria, viruses, and protozoa, respectively, and which therefore are excellent targets for new antiinfective drugs. Examples of important bacterial enzymes are streptopain [29], staphopain [30], clostripain [31], the gingipains [32] and sortase [33]. The picornains are examples for viral cysteine proteases [34]. AG-7088, an irreversible peptidomimetic inhibitor of human rhinovirus 3C protease, an enzyme which belongs to the picornain family, will probably be the first cysteine protease inhibitor managing the way to the drug market [35]. Cysteine proteases from pathogenic protozoa mainly belong to the papain family and are therefore related to mammalian cathepsins. Known members of these cathepsin-like protozoan enzymes are the cruzipains (cruzains) [36], trypanopains (brucipain, congopain, rhodesain) [37,38], falcipains [39] and the cathepsin B- and L-like proteases of several *Leishmania sp.* [40] as well as histolysain (histolysin) from *Entamoeba histolytica* [41]. As this listing of enzymes relevant for drug design does not correctly reflect strucural relationship between the enzymes we refer to a variety of excellent reviews [42-45] and the MEROPS database [46] (http://www.merops.co.uk/merops/index.htm), respectively, to achieve a deeper insight into the evolutionary lines and the terminology of cysteine protease families and clans. In this review we focus on new non-peptidic inhibitors of cysteine proteases which have been developed during the last years. Especially, we will discuss only those non-peptidic inhibitors which have not been developed from peptidic or peptidomimetic ones. In this respect this article is intended to be a continuation of our above mentioned review article [20]. What is new in cysteine protease inhibitor design? The overall build-up of most of the new low molecular weight cysteine protease inhibitors is essentially unchanged compared to previous structures: An electrophilic head group is linked to a substrate binding site directed chain. However, there are a variety of new electrophilic head groups and the peptidic recognition sequences have been altered to peptidomimetic ones in order to improve hydrolytic stability. The constantly growing number of known 3D-structures of enzyme inhibitor complexes has delivered insights into binding modes of inhibitors to the single enzymes and has facilitated the design of highly active and

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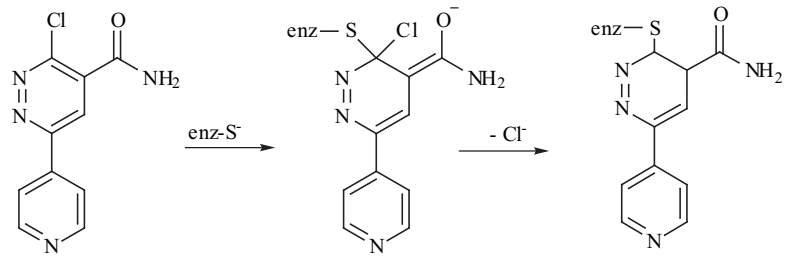


Fig. (1). Assumed mechanism of inactivation by 3-chloro-4-carboxamido-6-arylpyridazines.

selective inhibitors. To improve this selectivity among structurally related enzymes, inhibitors which span both the primed and non-primed substrate binding sites have been developed. Additionally, few new non-peptidic compounds have also been reported. In this review we will discuss these new cysteine protease inhibitors.

Irreversible Inhibitors

3-Chloro-4-Carboxamido-6-Arylpyridazines [47]

The lead compound 3-choro-4-carboxamido-6-(4-pyridyl)pyridazine (fig. **1**), found by a screening of a Sanofi Winthrop compound collection, was developed further. The halogen substituent of the pyridazine ring as well as both nitrogen atoms are necessary for irreversible ICE (caspase-1) inhibiting activitiy. The mechanism of inactivation is assumed to work by nucleophilic substitution of chlorine in position 3 by the thiolate anion of the catalytic center (fig. **1**).

According to this assumption the introduction of electron deficient aryl residues into the C-6 position of the pyridazine ring system resulted in more potent inhibitors (table 1).

Disulfiram [48]

The activity of disulfiram (fig. **2**) as alcohol deterrent is based on inhibition of aldehyde dehydrogenase by formation of mixed disulfides with essential SH-groups of the enzyme [49]. On the other hand it was known that disulfiram could inhibit lymphocyte apoptosis. This effect correlated with the proteolytic activation of pro-caspases-3 which can be mediated by the activity of caspases themselves. This led to the investigation of disulfiram as inhibitor of caspases-3 and –1. Actually, disulfiram acts as an irreversible inhibitor of these enzymes whereby thiol-disulfide exchange between

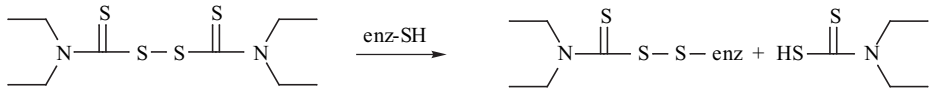
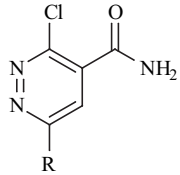
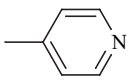
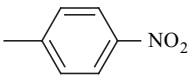
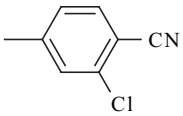
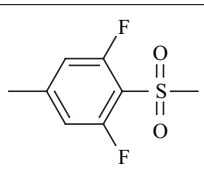


Fig. (2). Inhibition of caspases by disulfiram.

Table 1. 3-Chloro-4-Carboxamido-6-Arylpyridazines as ICE Inhibitors

	R	IC_{50} [μM]	k_{obs} / [I] [$M^{-1} \cdot s^{-1}$]
		3	70
		0,7	not determined
		0,5	225
		0,3	355

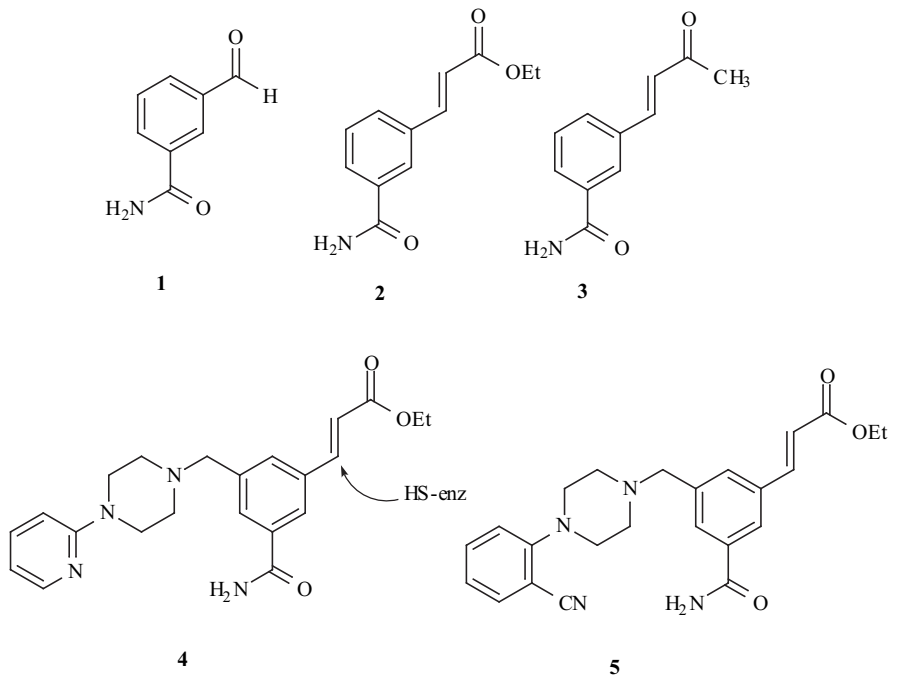


Fig. (3). Inhibition of HRV-3CP by benzamides.

inhibitor and enzyme is suggested (k_{app} = 450 M^{-1} s^{-1} caspase-3; k_{app} = 2200 M^{-1} s^{-1} caspase-1). The mixed disulfide formation between disulfiram and caspase-1 was proven using ^{35}S-labeled disulfiram (fig. **2**). Since caspases can also be regulated by protein thiol modification at sites other than the active site (e.g. inhibition of caspase-1 by thiomersal) [50] the target thiol for disulfiram is not clear yet.

Cinnamate Esters [51]

The structural relationship of human rhinovirus 3C protease (HRV-3CP) to the trypsin family of serine proteases led to the development of 3-carbamoyl benzaldehyde **1** (fig. **3**) [52] as reversible inhibitor of HRV-3CP (K_I = 104 μM). In this inhibitor the benzamide core mimicks the P1 recognition element of the natural cleaving site Gln-Gly. To improve the chemical stability of the compound the aldehyde moiety was replaced by an α,ß-unsaturated ester leading to the irreversible inhibitor **2** (k_{2nd} = 52 M^{-1} s^{-1}).[51] Substitution of the benzene ring at positions 4, 5, and 6 only led to a weak inhibition improvement in the case of compounds with a branched aminomethyl substituent in position 5. Cocrystal structures of HRV-3CP with benzamide inhibitors showed the inhibition to take place by covalent 1,4-addition of the nucleophilic catalytic cysteine. As predicted the benzamide moiety occupies the S1 pocket, H-bonds to His161 and Thr142 are formed. A cocrystal structure with inhibitor **4** (k_{2nd} = 269 M^{-1} s^{-1}) showed the substituent at position 5 to bind to the P4 subsite. Significant changes in protein conformation were observed. Analoguous α,ß-unsaturated keto benzamides are reversible inhibitors (e.g. **3**: K_I = 25 μM). In contrast to the esters these compounds are inactivated by free thiols (e.g. DTT), they are more toxic in the antiviral assays and have no measurable antiviral effect. Despite very weak inactivation constants (k_{2nd} = 139 M^{-1} s^{-1}) a submicromolar antiviral activity was observed with compound **5**.

Homophthalimides [53,54]

A blind screening program of Eli Lilly revealed the homophthalimide **1** (fig. **4**) amongst others as a time-dependent inhibitor of HRV-3CP (IC_{50} = 41.1 μM) [53]. Modification of the acetophenone moiety and substitution of the imide nitrogen led to compound **2** as most potent HRV-3CP inhibitor (IC_{50} = 22.1 μM) [53]. Molecular modeling studies suggest that the N-alkyl substituent of these compounds occupies the P1 pocket and mimics the Gln residue of the natural substrate. The acetophenone moiety is then placed into the P1′-P2′-pockets and the C3 carbonyl is the most likely to be attacked by the nucleophilic active site

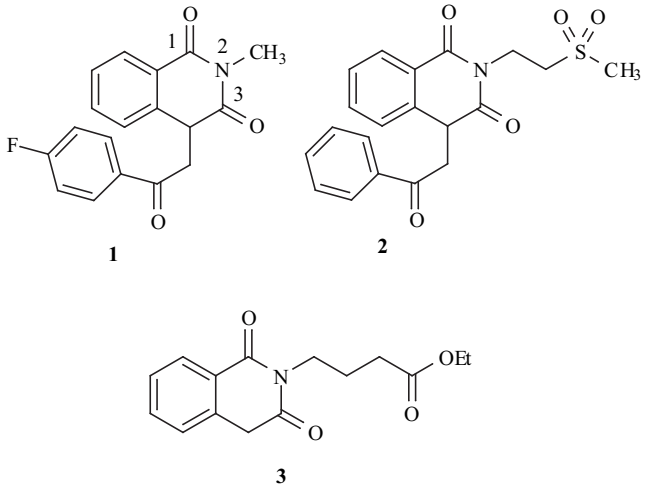


Fig. (4). Homophthalimides as HRV cysteine protease inhibitors.

cysteine. Mass spectrometry results propose that the inhibitors are covalently bound to the enzyme in a ratio of 1/1 (enzyme/inhibitor). According to tryptic peptide mapping homophthalimides inhibit HRV-3CP through an interaction with the active site cysteine residue. However, the antiviral activity of certain homophthalimides (e.g. compound **3**) did not always correlate with the level of HRV-3CP inhibition. This discrepancy can be explained by inhibition of another HRV cysteine protease [54], HRV14-2AP, an enzyme which, in contrast to HRV-3CP, has no specific requirement for the amino acid residue at P1 (inhibition by **3**: 2AP: IC_{50} = 18.2 µM; 3CP: IC_{50} > 200 µM). With this enzyme and also with the analogous protease of another serotype (HRV2-2AP) 1/1 and 1/2 adducts (enzyme/inhibitor) could be observed by mass spectrometry of enzyme inhibitor complexes [54].

Organotellurium (IV) Compounds [55]

Ammonium trichloro (dioxoethylene-O,O´)tellurate **(1)** (fig. **5**) is an organotellurium (IV) complex that exhibits immunemodulating activity. Te(IV) compounds like TeX_4 or $Te(OR)_4$ interact readily with nucleophiles, e.g. with thiols R´-SH, to yield $Te(SR')_4$. This complex may exchange ligands or hydrolyze to TeO_2. It may also undergo

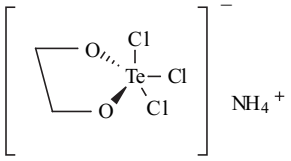


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Fig. (5). Organotellurium (IV) compounds as inhibitors of papain.

redox reactions to $Te(SR')_2$ + R´-S-S-R´. To investigate the protease inhibition potency of organotellurium compounds, **1** and related Te(IV) ($Te(ethylene\ glycol)_2$; $Te(citrate)_2$; TeO_2) and Te(VI) complexes ($Te(ethylene\ glycol)_3$; $Te(citrate)_3$; telluric acid $Te(OH)_6$) were tested against papain and cathepsin B as well as against several serine, metallo, and aspartic proteases. Only the Te(IV) compounds inhibited papain and cathepsin B selectively and irreversibly (inhibition by **1**: k_{2nd} = 310 M^{-1} s^{-1} papain; k_{2nd} = 37 M^{-1} s^{-1} cathepsin B). The enzymatic activity could be recovered by thiol treatment indicating that the inactivation involves oxidation of the active site cysteine to a disulfide *enzyme-S-S-R* with –S-R coming from the buffer cysteine or to an inactive *enzyme-S-TeL_3* species.

Nitric Oxide Donors [56]

Nitric oxide (NO) donors like S-nitrosoglutathione (GSNO), S-nitroso-N-acetyl-penicillamine (SNAP) as well as the non-thiol NO donors NOR-1 and NOR-3 (fig. **6**) can act as inhibitors of cathepsin K in a time- and concentration-dependent manner (table 2).

It was found evidence that the generally accepted S-nitrosylation as mechanism of inhibition of thiol-containing enzymes is not the only one. Inhibition of cathepsin K by GSNO and SNAP works most likely by formation of a mixed disulfide species with glutathione and N-acetyl-penicillamine, respectively, at the active site cysteine. This was supported by mass spectrometry. Additionally, the disulfide is reducible to the free thiol by DTT with recovery of enzyme activity.

Table 2. Inhibition of Cathepsin K by NO Donors

NO donor	IC_{50} [µM] cathepsin K, pH 7.5
GSNO	28
SNAP	105
NOR-1	0,4
NOR-3	10

In contrast, inhibition with NOR-1 is only partially (10 – 20 %) reversible by DTT and probably due to an oxidation of cysteine residues to sulfencic acid which results in formation of a mixture of sulfinic and sulfonic acids. The sulfenic acid was intercepted by reaction with dimedone to

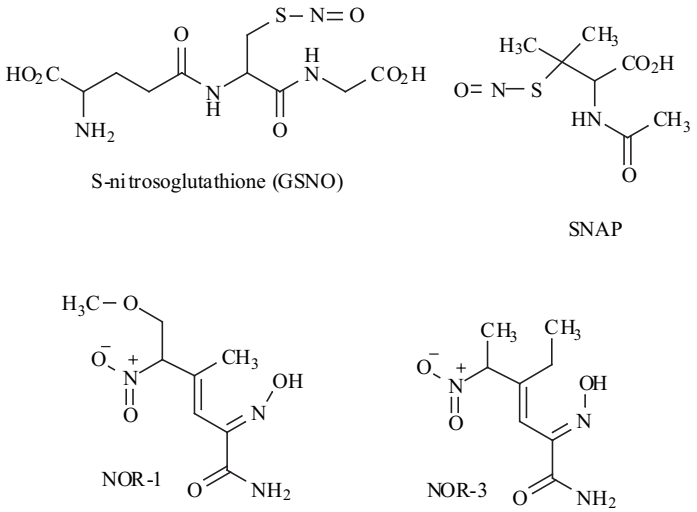


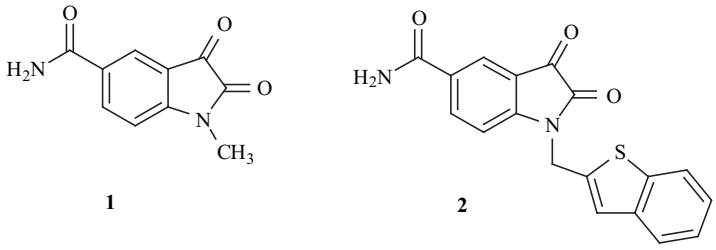
Fig. (6). NO donors.

form a thioether which was also proved by mass spectrometry.

Reversible Inhibitors

Isatins

Isatins as inhibitors of HRV-3C protease have also been found by the above mentioned Eli Lilly screening [53] and by a more rational approach by the Agouron group [57]. These compounds represent cyclic conformationally restricted α-keto amides which, as peptide derivatives, are well known reversible cysteine protease inhibitors. 1-Methylisatin-5-carboxamide **(1)** (fig. **7**) which contains the known benzamide moiety as P1 mimetic residue showed a nanomolar inhibition constant (K_I = 51 nM) [57]. A series of 1-methyl-substituted isatins revealed the carbonyl or a carbonyl isoster required at C-5. Molecular modeling studies led to the design of compounds with an aryl substituent within the N1-side chain with the benzothiophene derivative **2** as most potent inhibitor (K_I = 2 nM). X-ray studies confirmed the molecular modeling predictions. A covalent bond is formed between Cys147 and the C3 of the isatin, positioning the oxygen of the tetrahedral intermediate in the oxyanion hole. The carboxamide forms H-bonds to the S1-pocket and the benzothiophene is situated in the S2-pocket. 3C proteases of serotypes other than HRV14 (HRV89, -2, -16) are also inhibited by these compounds with K_I-values in the nanomolar range. Antiviral assays, however, showed that these isatins did not have any activity below their toxic concentrations [57].



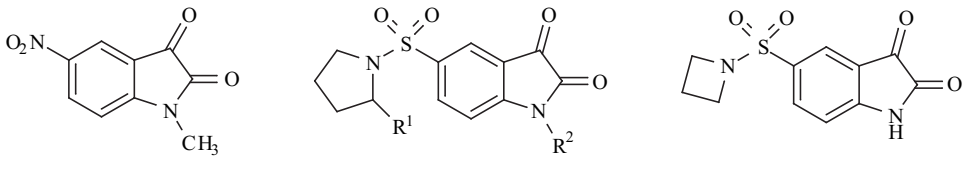
1 **2**

Fig. (7). Isatins as inhibitors of HRV-3C protease.

A high-throughput screening of the SmithKlineBeecham compound collection identified 5-nitroisatin **(1)** (fig. **8**) as a reversible inhibitor of caspase-3 [58]. Like HRV cysteine proteases caspases are inhibited reversibly, competitively and non-time dependently by isatins. The inhibition takes place in the same manner by nucleophilic attack of the catalytic cysteine at C3 carbonyl of the isatin and formation of a tetrahedral intermediate. A correlation between the electron-withdrawing ability of the 5-substituent and the potency of inhibition is observed [59]. To improve the metabolic stability of the nitroaromatic and to allow extension of the molecule at this position to gain a possible access to the S2-S4 pockets of caspases the nitro group was replaced by a sulfonamide function [58,59]. Although compound **2** (fig. **8**) showed a weaker inhibition the selectivity between caspases could be improved (table 3). Decreasing the ring size of this inhibitor from the pyrrolidine ring to the azetidine ring (cpd. **7**) resulted in a 10-fold increase in activity whereas ring expansions had no effects [59]. A series of isatin sulfonamides substituted at the pyrrolidine ring resulted in the identification of compounds **3** and **4** as highly potent and selective inhibitors of caspases-3 and –7 with the (*S*)-isomers being the eutomers (K_I = 18000 nM **3b**). No significant differences are found if the oxygen of the phenoxymethyl side chain is replaced by S or NH. The inhibition activity could be improved further by substitution of the isatin nitrogen with larger hydrophobic groups. Compound **6** is the most potent inhibitor of this series and exhibits a 1000-fold selectivity for caspases-3 and –7 vs. caspases-1, -2, -4, -6, and –8 and a 20-fold selectivity vs. caspase-9 [59]. Cathepsins B, K, L, and S and calpain I are not or only weakly inhibited. X-ray studies [58] with inhibitor **5** showed that the S1 pocket which normally has an absolute requirement for Asp is occupied only by a water molecule. The S2 pocket is involved in extensive hydrophobic contacts with the pyrrolidine ring which explains the selectivity for caspases-3 and –7. In contrast to other caspases the S2 pocket of these two enzymes contains three hydrophobic residues (Tyr204, Trp206, Phe256). The phenoxymethyl side chain occupies the S3 pocket.

Cyanamides [60]

A screening of the Merck sample collection identified the 1-cyanopyrrolidine **1** (fig. **9**) as a time dependent but fully reversible inhibitor of cathepsins K and L (IC_{50} = 0.37 and 0.45 μM). Removal of the quinoline moiety of **1** resulted in



1 **2-6** **7**

Fig. (8). Isatins as inhibitors of caspases-3 and –7.

Table 3. Inhibition of Caspase-3 by Isatins

compound	R^1	R^2	K_I / IC_{50} [nM][a]
1	-	-	500 / 1000
2	H	H	1400 / 2800
3a	(*S*)-CH_2-O-CH_3	H	60 / 120
3b	(*R*)-CH_2–O-CH_3	H	/ 18000
4	(*S*)-CH_2-X-Ph X = O, NH, S	H	/ 31-44
5	(*S*)-CH_2-O-Ph	CH_3	15 / 30
6	(*S*)-CH_2-O-Ph	CH_2-Ph	1.2 / 2.5
7	-	-	/ 170

[a] Values are given for caspase-3, values for caspase-7 differ only slightly.

a moderate decrease in inhibition while acyclic cyanamides were totally inactive. The most potent inhibitors of a series of 2- and 3-substituted 1-cyanopyrrolidines are benzenesulfonamide **2** (IC_{50} CK / CL = 0.05 / 0.08 μM) and benzylcarbamate **3** (IC_{50} CK / CL = 0.04 / 0.054 μM). Determination of association and dissociation rate constants showed that the increase of potency observed in this series of cyanamides is due to an increase of *k(on)* and that the inhibition fits an apparent single-step mechanism. Replacement of the 1-cyanopyrrolidine moiety by 1-cyanoazetidine led to a 10-fold increase in inhibition with the cyclohexylamide **4** (IC_{50} CK / CL = 0.005 / 0.006 μM) as the most potent inhibitor. This increase in inhibition potency is probably a result of a higher chemical reactivity towards the cysteinate of the enzyme´s active site. This was shown by the affection of the IC_{50} values by addition of glutathione at pH 7 which favors the formation of the glutathione thiolate anion. Cyanamides are structurally related to peptidyl nitriles which are known to form thioimidate ester adducts with cysteine proteases. As could be expected ^{13}C-NMR experiments with papain showed the inhibition by cyanamides being due to reversible formation of a covalent isothiourea ester adduct (fig. **9**).

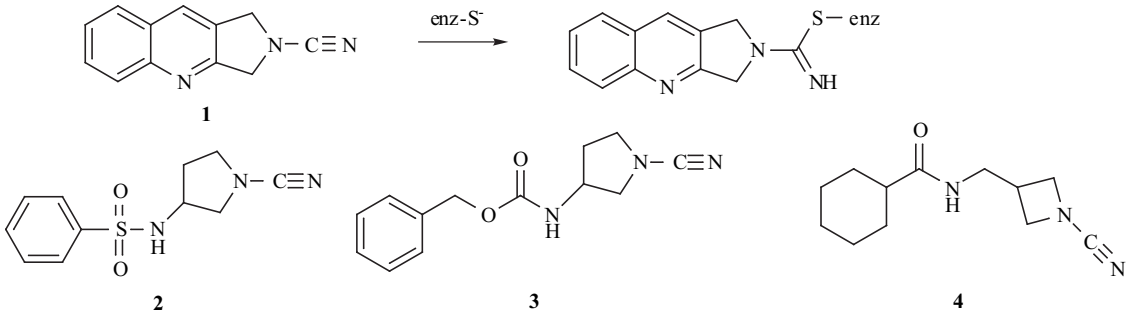


Fig. (9). Cyanamides as inhibitors of cathepsins K and L.

α-Mercaptoacrylic Acids [61,62]

A Parke-Davis random screening of more than 150.000 compounds revealed several mercaptoacrylic acid derivatives as selective and reversible inhibitors of human μ-calpain. Two representative compounds are shown in figure **10** (inhibition of μ-calpain by **1** and **2**: K_I = 0.21 / 0.26 μM; inhibition of m-calpain by **1** and **2**: K_I = 0.37 / 5.33 μM). In these compounds the free thiol and carboxylate group as well as the double bond are necessary for inhibition. These compounds operate by binding to both calcium binding sites of calpain leading to uncompetitive inhibition. They do not interact with the catalytic centre. This was shown by the reduction of TNS-caused fluorescence enhancement and by the non-protection of mercaptoacrylic acid inhibited calpain to inactivation by the irreversible and active-site directed inhibitor E-64c. The necessity of unmodified thiol and carboxylate groups suggests these groups to act as calcium chelators.

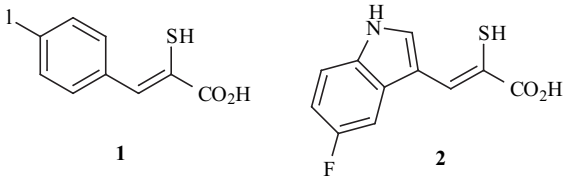


Fig. (10). Mercaptoacrylic acids as calpain inhibitors.

1-Carboxymethylnicotinic Acid [63]

1-Carboxymethylnicotinic acid (fig. **11**), a compound which was synthesized first in 1991, was isolated from a

marine sponge. This compound inhibits papain with an IC_{50} value of 80 mg/mL.

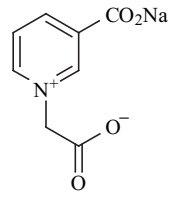


Fig. (11). 1-Carboxymethylnicotinic acid as a papain inhibitor from marine sponge.

Acyl Hydrazones, Chalcones and α,β-Unsaturated Amides

A model structure of the trophozoite cysteine protease of *Plasmodium falciparum* falcipain, designed on the basis of the X-ray structures of papain and actinidin, was used as a receptor for ligand docking using DOCK 3.0 to search the Fine Chemicals Directory (Molecular Design Ltd., San Leandro, CA) of more than 55.000 commercially available small molecules. 31 compounds were chosen for testing against falcipain [64]. The most active compound, oxalic bis[(2-hydroxy-1-naphthylmethylene)hydrazide] **(1)** (fig. **12**)

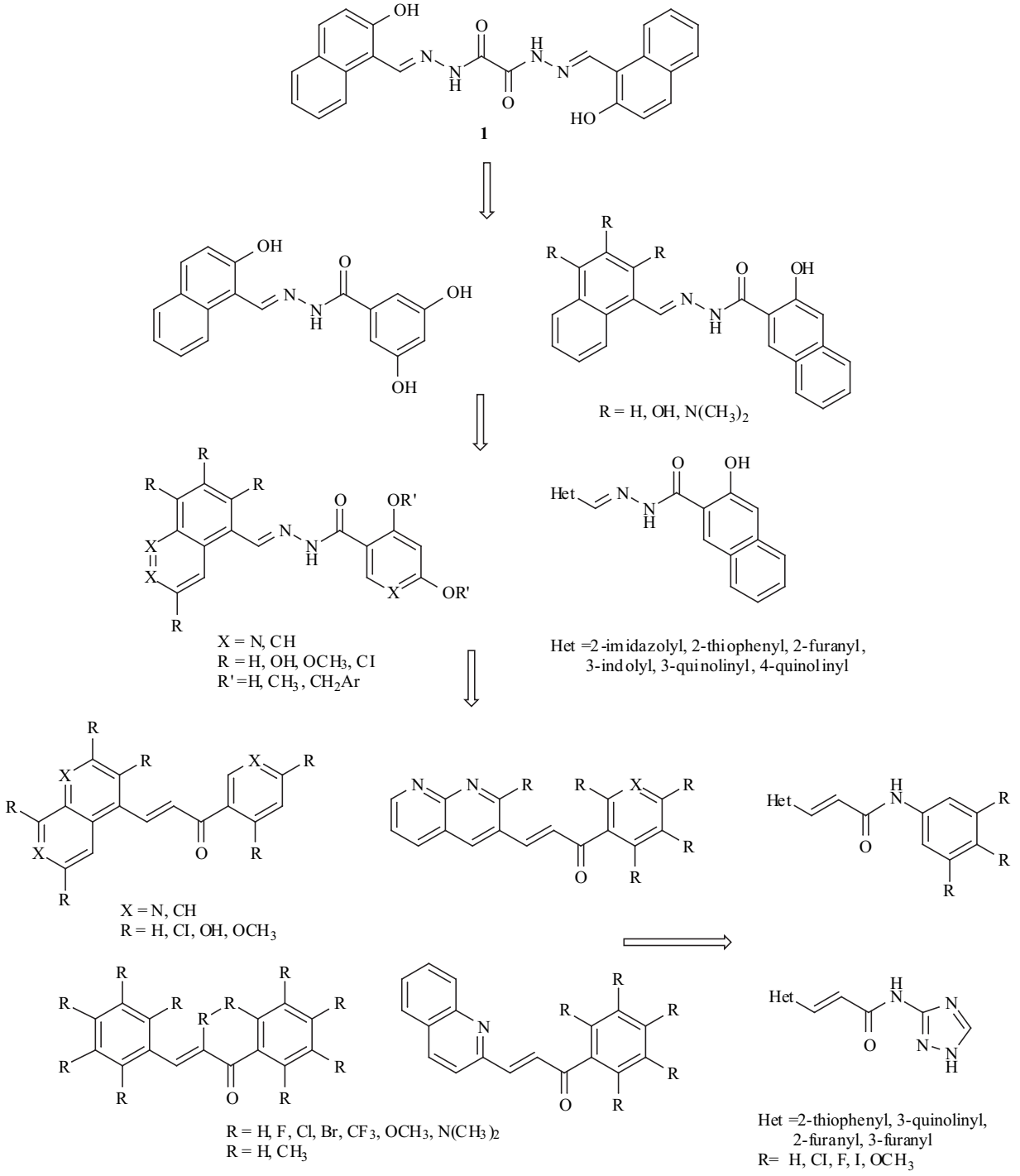


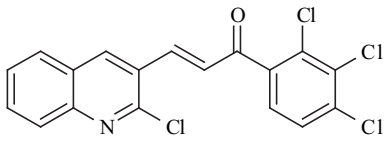
Fig. (12). Evolution of acyl hydrazones and chalcones as inhibitors of protozoan cysteine proteases.

inhibited falcipain with an IC_{50} value of 6 µM. The DOCK generated enzyme inhibitor complex showed that one naphthol group fills the hydrophobic S2 site and that the other naphthol group interacts with Trp177 of the S1´ site. Beginning with **1** as lead compound a variety of chemical modifications were made in order to identify more active antimalarial agents (fig. **12**). The length of the backbone was shortened leading to asymmetric acyl hydrazones with less conformational heterogeneity than the symmetric ones. Additionally the aromatic rings were replaced by nitrogen containing heteroaromatics in order to improve water solubility and to enhance electrostatic interactions with His67 of the S2 site. To increase chemical and metabolic stability the hydrazide linker was replaced by an α,ß-unsaturated ketone backbone leading to chalcones. Another modification comprises the exchange of the α,ß-unsaturated ketone against an α,ß-unsaturated amide.

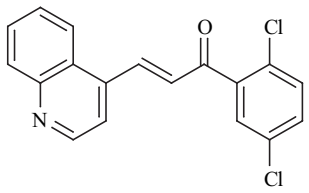
These modifications have led to a variety of highly active reversible inhibitors of the cysteine proteases not only of *Plasmodium falciparum* but also of *Trypanosoma brucei brucei* (trypanopain-Tb, brucipain), and *Trypanosoma cruzi* (cruzain) (fig. **13**) [65-71]. Even if the molecules contain electron withdrawing groups on the aromatic rings they do not react as Michael acceptors.

During the time the chalcones were identified as possible antiparasitic agents by the rational design described in (figure **11**) licochalcone A (fig. **14**), isolated from Chinese licorice roots, was found to inhibit *in vitro* growth of *Plasmodium falciparum* (IC_{50} 5.6 µM) as well as *Leishmania major* (IC_{50} = 7.2 – 13 µM) and *L. donovani* (IC_{50} = 0.9 µM) promastigotes and amastigotes. Together with the synthetic acyl hydrazones and chalcones described above a large number of other naturally occurring and semisynthetic chalcones have now been identified in cell culture and *in vivo* assays as potent antimalarial, antitrypanosomal and antileishmanial agents [66,72-75]. However, there are doubts if cysteine proteases are the only targets for these compounds within the parasite cells. Thus, no direct correlation between trypanocidal activity and inhibition of purified trypanopain-Tb could be observed (fig. **13**) and *Leishmania* mutants lacking cysteine proteases are as sensitive to some of the chalcones as wild type parasites. The activity against *Leishmania* might be the result of interference with the function of the parasite mitochondria. Possible other targets may be glycosomal glyceraldehyde-3-phosphate dehydrogenase [76] or fumarate reductase [77].

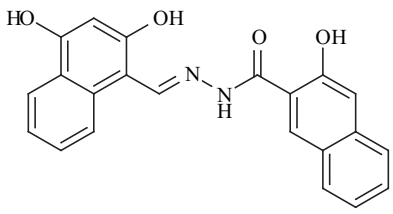
Although the cysteine proteases of *Plasmodium*, *Leishmania*, and *Trypanosoma* have not been unequivocally identified as intracellular targets of acyl hydrazones and



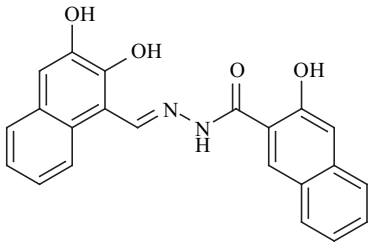
trypanopain-TB; K_1 = 0.37 µM
cathepsin L : K_1 = 7.16 µM
Trypanosoma b. brucei: IC_{50} = 13.8 µM



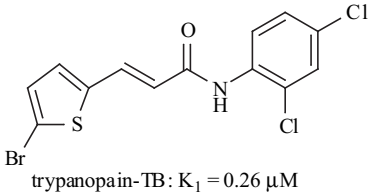
trypanopain-TB: K_1 = 17. 14 µM
cathepsin L : K_1 = 6.95 µM
Trypanosoma b. brucei: IC_{50} = 1.88 µM
Plasmodium falciparum: IC_{50} = 0.19-0.23 µM



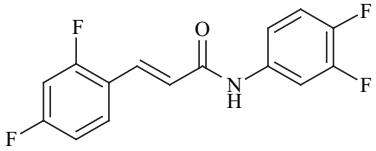
trypanopain-TB: K_1 = 0.027 µM
cathepsin L : K_1 = 1.49 µM
Trypanosoma b. brucei: IC_{50} = 1.53 µM



trypanopain-TB: K_1 = 0.64 µM
cathepsin L: K_1 = 1.07 µM
Trypanosoma b. brucei: IC_{50} = 0.42 µM



trypanopain-TB: K_1 = 0.26 µM
cathepsin L : K_1 = 1.42 µM
Trypanosoma b. brucei: IC_{50} = 10.0 µM



trypanopain-TB: K_1 = 0.50 µM
cathepsin L: K_1 = 52.17 µM
Trypanosoma b. brucei: IC_{50} = 1.82 µM

Fig. (13). Chalcones, acyl hydrazones and α,ß-unsaturated amides as inhibitors of protozoan cysteine proteases.

chalcones, these compounds do inhibit the purified enzymes and are effective antiprotozoan agents [78,79]. A discrimination between parasite enzymes and related host cathepsins is possible to a certain degree (fig. **13**).

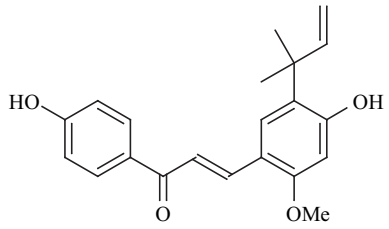


Fig. (14). Licochalcon A, a powerful antimalarial and antileishmanial agent.

Aryl Ureas and Aroyl Thioureas [80]

The optimization of the bisaryl acyl hydrazones described above to produce inhibitors that were more potent than 300 nM for cruzain and 420 nM for rhodesain, the major cysteine protease of *Trypanosoma brucei rhodesiense*, failed. To develop more potent inhibitors for these enzymes a computational screen of ACD (Available Chemicals Directory) database using DOCK 4.0.1 targeted against the solved crystal structure of cruzain was carried out. 21 compounds were selected for testing against cruzain and rhodesain, nine of them exhibiting IC_{50} values < 10 μM. These nine compounds provide a wide variety of new scaffolds for potential antiparasitic drugs (fig. **15**). All the hits are calculated to make substantial hydrophobic interactions with cruzain indicating that cruzain and also rhodesain accept a wide variety of inhibitors with two aryl groups connected with an appropriate linker. In this respect the molecular overall structure of these inhibitors resembles the structures identified in the acyl hydrazone and chalcone series. A pharmacophore based miniscreen using GenX identified additional 38 compounds, 16 of them showing IC_{50} values below 10 μM against the two enzymes. Thus, the aroyl thiourea (5 cpds), aroyl urea (3 cpds), urea (5 cpds), sulfonamide (2 cpds), and benzamide (1 cpd) scaffolds were filtered off as new leads for cruzain inhibition. Due to synthetic and pharmakokinetic reasons the aroyl thiourea and urea compounds were selected for cell culture assays of *T. cruzi*. With one exception the results of the thioureas were disappointing. As a common feature of the active compounds a six-membered aryl ring connected with a five-membered heteroaromatic at one side of the urea or thiourea linker and a six-membered ring alone or a benzyl substituted six-membered ring at the other side were identified (fig. **16**). The six-five combination possibly interacts with the S2 pocket whereas the benzyl-substituted six-six combination might result in a better interaction with the S1´site. A representative set of inhibitors from the aroyl thiourea and urea series were studied for selectivity between cruzain and papain, cathepsin B and trypsin showing that the

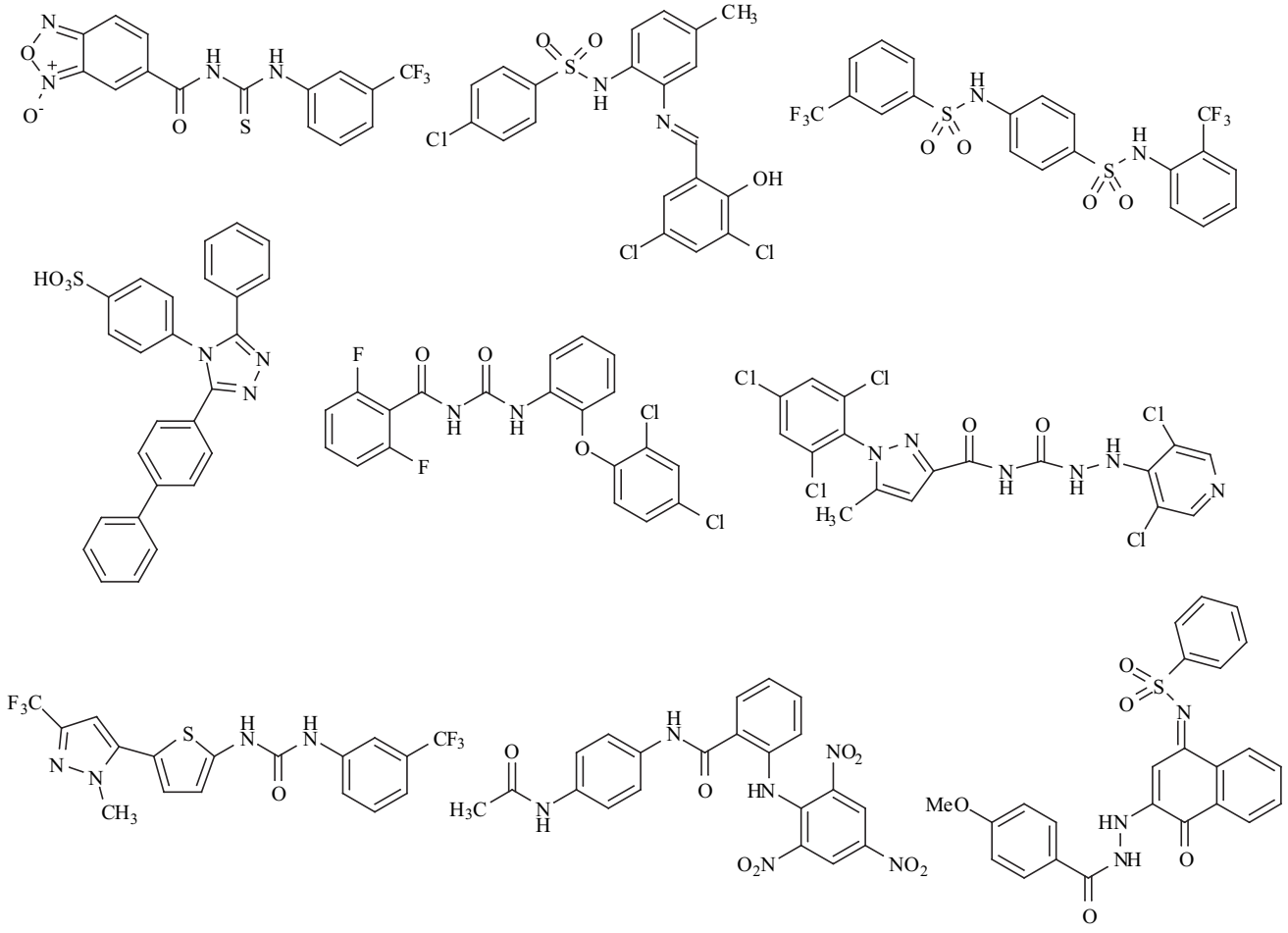


Fig. (15). Cruzain and rhodesain inhibitors found by computational screening.

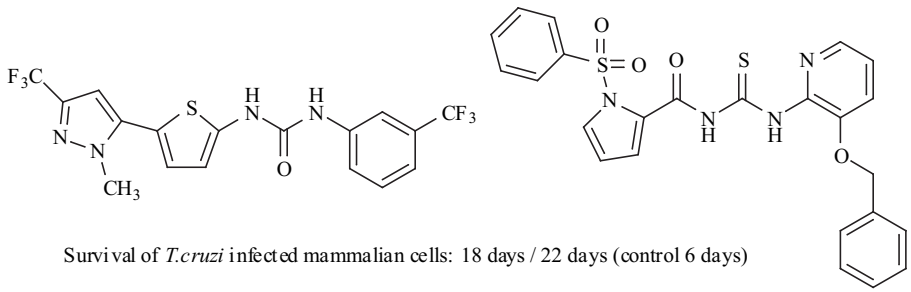


Fig. (16). Cruzain inhibitors active *in vivo* [80].

compounds are selective for the protozoan enzymes over other cysteine and serine proteases (fig. **17**, table 4). Kinetic studies showed all these inhibitors to be competitive and reversible.

Phenothiazines [81]

A series of phenothiazin-4-ones and phenothiazin-4-one-5,5-dioxides have been evaluated as falcipain inhibitors. Their development is based on several acridinediones (e.g. inhibitors these two exhibited a lower activity against cultured *Plasmodium falciparum* parasites. Thus, two independent antimalarial mechanisms of action are proposed. The first is probably the same unknown mechanism that is the basis of the antimalarial activity of the previously reported acridinediones. The second mechanism is the inhibition of falcipain and as a consequence the inhibition of hemoglobin degradation. This can be assumed since the same morphological abnormalities can be found as seen with the potent cysteine protease inhibitor E-64.

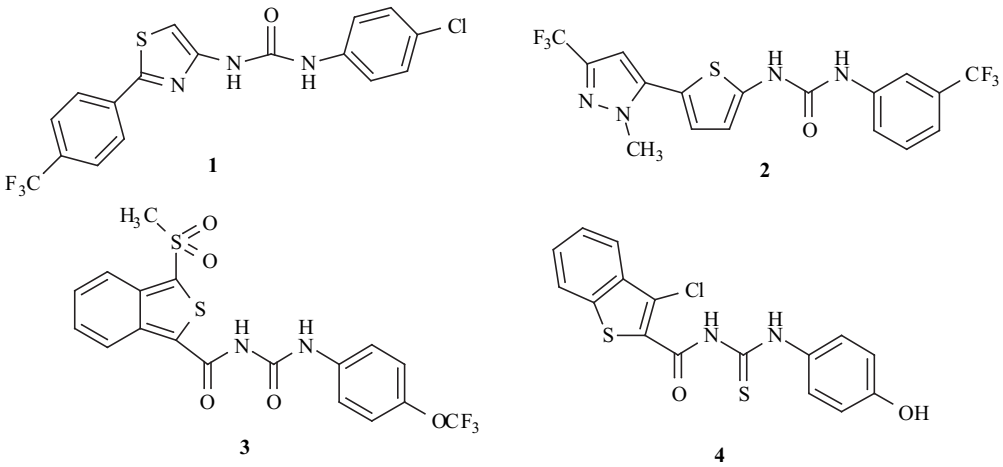


Fig. (17). Cruzain and rhodesain inhibitors with urea or acyl thiourea scaffold.

floxacrine, fig. **18**) which have been described as potent antimalarial agents [82-87]. The phenothiazines have been developed as sulfur isosters. The most potent phenothiazines show IC_{50} values against falcipain in the lower μM range. They bear a Cl- or F-substituent at position 7 and a 4-chlorophenyl-, 2,3-dimethoxyphenyl-, 3,4-dimethoxyphenyl- or 3-methoxyphenyl-substituent at position 2. No differences can be found between the phenothiazine-4-ones and the corresponding 5,5-dioxides (fig. **19**). Additionally, two compounds with two methyl groups instead of the phenyl group at C-2 are just as potent. But, in contrast to the other

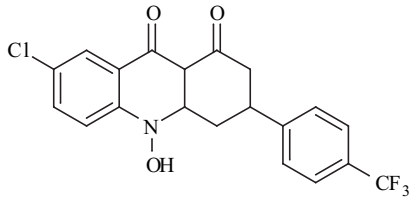
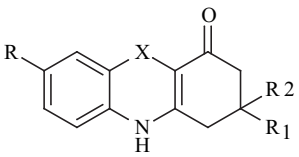


Fig. (18). Floxacrine, a potent antimalarial acridinedione.

Table 4. Selectivity of Cruzain and Rhodesain Inhibitors with Acyl Thiourea and Urea Scaffold

IC_{50} [μM] / compound	cruzain	rhodesain	papain	cathepsin B	trypsin
1	0.8 – 2.9	2.5	> 50	10.8	> 50
2	2.0 – 3.1	3.0	> 50	11	> 50
3	2.1 – 6.9	4.2	> 50	11	> 50
4	1.5 – 2.7	4.0	> 50	10.7	> 50



R = Cl, F
R1 = H, CH_3
R2 = CH_3; 4-Cl-Ph; 2,3-$(OCH_3)_2$-Ph; 3,4-$(OCH_3)_2$-Ph; 3-(OCH_3)-Ph
X = S, SO_2

Fig. (19). Phenothiazin-4-ones and –5,5-dioxides as falcipain inhibitors with IC_{50} = 4-5 μM.

Indomethacin [88]

Since cyclooxygenase pathways generate free radicals in CNS diseases and trauma causing cell death via calpain stimulation, the inhibitory effect of indomethacin, a non-steroidal anti-inflammatory drug, has been studied upon calpain degradation of casein. A dose-dependent inhibition was observed (IC_{50} = 1.1 mM, complete inhibition with 3.3 mM concentration) [88]. Compared to calpeptin, a physiological inhibitor of calpain, indomethacin is not a very strong inhibitor but it acts better than methylprednisolone or hydrocortisone. The mechanism of inhibition (reversible or irreversible and site of action) is unknown and still to reveal.

ABBREVIATIONS

TNS	=	2-(p-Toluidino)naphthalene-6-sulfonate
E-64c	=	*Trans*-(epoxysuccinyl)-L-leucyl-amido-3-methylbutane
NOR-1	=	(*E*)-Methyl-2-((*E*)-hydroxyimino)-5-nitro-6-methoxy-3-hexenamide
NOR-3	=	(*E*)-Ethyl-2-((*E*)-hydroxyimino)-5-nitro-3-hexenamide
DTT	=	Dithiothreitole
ICE	=	Interleukin converting enzyme

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