Inhibitors of Cathepsin B

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Abstract: Cathepsin B is an abundant and ubiquitously expressed cysteine peptidase of the papain family. It is involved in many physiological processes, such as remodeling of the extracellular matrix (wound healing), apoptosis, and activation of thyroxine and renin. In addition to its physiological roles, cathepsin B is important in many pathological processes, such as inflammation, parasite infection and cancer, where it is highly up-regulated. In cancer patients, elevated cathepsin B activity correlates to poor therapy outcome. Therefore, it is not surprising that the use of cathepsin B inhibitors reduces both tumor cell motility and invasiveness *in vitro*. This review summarizes recent developments in cathepsin B inhibition. To date, numerous protein inhibitors of cathepsin B have been described, some of which are of endogenous origin and function as regulators of cathepsin B activity in the cell, such as the cystatins. In addition, some exogenous protein inhibitors of cathepsin B have been isolated from various natural sources, and the use of X-ray crystal structures of cathepsin B complexed with such protein inhibitors has resulted in the design and synthesis of many new small-molecular-weight compounds as inhibitors of cathepsin B. These synthetic compounds generally contain an electrophilic functionality that reacts with cathepsin B. In the present review, these inhibitors are divided according to their mechanisms of action, as reversible and irreversible, and then further subdivided into groups for their full descriptions.

Keywords: Cathepsin B, inhibitors, cancer, anticancer drugs, cysteine proteases, cathepsins.

1. INTRODUCTION

The cathepsins are abundant and ubiquitously expressed peptidases that belong to the papain family of enzymes. They are present in all mammalian cells and have traditionally been viewed as lysosomal mediators of protein turnover [1]. However, recent findings have extended their roles as key enzymes in other physiological processes, including remodeling of the extracellular matrix (ECM; wound healing) [2], bone remodeling [3], apoptosis [4,5] and cancer (for reviews see [6-9]). This is particulary valid for one of the best characterized mammalian cysteine peptidases - cathepsin B (catB, E.C.3.4.22.1). In higher organisms, catB is present and active intracellularly and extracellularly in almost all tissue types [10]. Intracellularly, catB is localized in the lysosomes, where it is partly responsible for the terminal degradation of intracellular proteins, whereas extracellularly, catB can be found both in a soluble form and bound to the plasma membrane or ECM proteins, where it has many important physiological functions [11]. For example, catB is involved in thyroxine synthesis [12], in site-selective cleavage of human prorenin [13], in processing of antigens to preferentially activate Th2 cells [14], and in self-protection of cytotoxic T lymphocytes during degranulation [15,16].

However, in addition to its well established physiological roles, catB has been implicated in a variety of diseases involving tissue-remodeling states, such as inflammation [17,18], parasite [19] and virus infections [20], Alzheimer's disease [21], ischemic neuronal death [22] and tumor metastasis [23-25]. In malignant tumors and premalignant lesions, the expression of catB is highly up-regulated. This has been reported for a variety of cancers, including prostate [26], colorectal [27], breast [28], colon [29], esophageal [30], oral [31], gastric [32], lung [33], ovarian [34] and thyroid [35] carcinomas and gliomas, as well as melanomas. Furthermore, elevated serum levels of catB are associated with advanced tumor stages and the progression of disease, where the survival rate has been inversely correlated to the catB activity ratio [36-38]. In addition, elevated levels of catB expression have been seen in premalignant lesions [39] and in tissues of patients with post-operative tumor recurrence [40]. Increases in catB expression are known to be mediated on many levels, ranging from gene amplification, elevated transcription, alternative promoters and in the use of alternative splicing to increase the levels and stability of its mRNA and of catB itself [41,42]. The activity of catB is controlled by specific endogenous inhibitors (e.g. the stefins and the cystatins) [43] and cellular receptors. Under physiological conditions, a balance exists between proteases and their inhibitors [44], the loss of which is believed to promote tumor progression and to be an index of invasive properties [45-49].

Elevated catB activity is directly related to degradation of ECM proteins, including fibronectin, laminin and type IV collagen, which are all part of an important structural barrier that cells must cross to reach the vasculature [50,51]. In addition to extracellular catB activity, intracellular catB contributes to tumor invasion, as these cells can take up the ECM by endocytosis and degrade it intracellularly, enabling tumor cells to invade basement membranes [52]. Indirectly, catB is also involved in the activation of other enzymes that form a proteolytic cascade that mediates ECM degradation. These include both soluble and receptor-bound urokinase plasminogen activator [54] and the matrix metalloproteinases (e.g. collagenase I) [56]. Recently, a close relationship between the intensity of the angiogenesis response and the

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overexpression of catB in cancer cells has also been reported [57]. It appears that catB acts on tumor cells and/or on the ECM to promote angiogenesis, although it may also have an important role in normal physiology as a suppressor of angiogenesis by acting at the level of the endothelium [58]. A dual role for catB has been established in apoptosis as well, where it has been demonstrated to promote both caspase-dependent and caspase-independent apoptosis [59]. On the other hand, catB also down-regulates p53 (a well known transcription factor that negatively regulates cell proliferation) activity in mammalian cells, and may thus have anti-apoptotic features as well [60].

2. RECENT REVIEWS

The pertinent reviews, monographs and trends on the cathepsins, on the cysteine proteases, and on their inhibitors that have been published recently are given below. However, to our knowledge, no reviews that deal exclusively with catB inhibitors have been published to date.

1. General reviews on inhibitors of cathepsins or cysteine proteases: [61].
2. Reviews including pathophysiological roles of catB: [44,65].

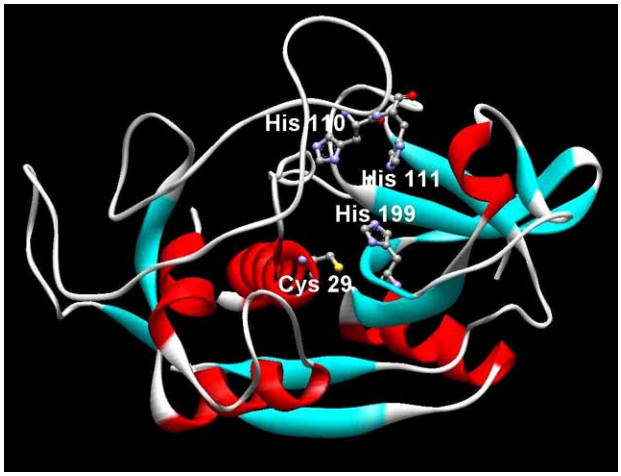


Fig. (1). X-ray structure of human catB. The important amino-acid residues of Cys 29, His 199, His 110 and His 111 are presented as balls and sticks.

3. THE STRUCTURE OF CATHEPSIN B

CatB is a 30-kDa, bilobal protein that consists of a left (L-) and a right (R-) domain, in accordance with the orientation used from the standard viewpoint [66]. The active site and substrate-binding cleft are located at the interface between the two domains. Peptide bond cleavage by catB is catalysed by a cysteine residue (Cys 29) that is located on the left domain, and which interacts with a histidine residue (His 199) that is located on the right domain (Fig. (**1**)) [67]. In the specialized enviroment of the active site, the thiol and imidazole side chains of Cys 29 and His 199, respectively, form an ion-pair over the pH range of 4.0-8.5. Cleavage of the substrate peptide bond is mediated by nucleophilic attack of the thiolate group from Cys 29 of catB on the carbonyl carbon atom, followed by proton donation from His 199 [68]. The polypeptide substrate binds along the active-site cleft in an extended conformation, and its backbone amides form a hydrogen-bonded network with the conserved residues of the underlying enzyme surface, while its side chains bind into relatively shallow binding sites to either side of the active site [66]. On the left domain, the loops form the S3, S1 and S2' binding areas, while on the right domain, they form the S1' and S2 binding sites (Fig. (**2**)).

Access of the substrate into the active site of catB is controlled by an 18-residue-long insertion (Pro 107-Asp 124) that is known as the occluding loop [67]; this provides two His residues (His 110 and His 111) for binding to the C-terminal carboxylic groups of the substrate [66]. This explains the preferred carboxypeptidase activity of the enzyme. However, catB can also act as an endopeptidase, since the occluding loop is flexible and can move away from the active-site cleft when an endopeptidase substrate binds to catB [69]. It appears that the stability of this occluding loop is pH dependent, with an endopeptidase activity at neutral pH (typical of the enviroment of the membrane-bound or extracellular catB) and an exopeptidase activity at acidic pH (typical of the environment of the lysosomes) [71]. This suggests that the catB substrates differ depending on the location of the enzyme [72].

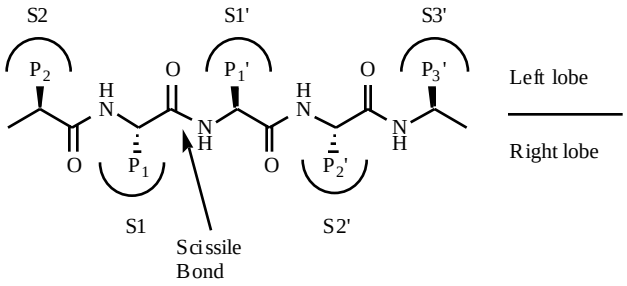


Fig. (2). Schematic representation of the binding of a substrate to the active site of cathepsin B. The substrate side chains (P) bind to specific subsites (S) in the left and right lobes of catB. The peptide bond that is cleaved in this catB catalysed reaction is marked as the scissile bond.

4. INHIBITORS OF CATHEPSIN B

Since an elevated catB activity has a well known role in the development of cancer, it is not suprising that the use of catB inhibitors leads to reduced levels of both cancer metastasis and invasiveness, as has been demonstrated in various *in vitro* studies. Wickramasinge and colleagues, for example, recently demonstrated that stable transfection of 1386Tu oral carcinoma cells with two different ribozymes targeted to catB mRNA resulted in a significant inhibition of both cell motility and invasiveness [73]. In addition, human glioblastoma cells that had been stably transfected with a plasmid containing antisense catB cDNA showed decreased MMP-9 activity, decreased cell-cell interactions with human microvascular endothelial cells, and reduced microvasculature development [74]. Furthermore, prolonged inhibition of the lysosomal proteolytic pathway has been shown to be incompatible with cell survival, leading to apoptosis [75]. Therefore, catB inhibitors could significantly reduce the invasiveness of tumor cells, and thus have a potential in cancer chemotherapy [76]. However, despite the

well established role of catB in cancer and more than 20 years of pharmaceutical reasearch there are no *in vivo* studies which would serve as a proof of concept for pharmacological inhibition of catB and establish the role of catB inhibitors as anticancer drugs. However, catB inhibitors might prove useful in diseases other than cancer. Using animal testing it was demonstrated that selective catB inhibition reduced liver damage [77], prevented trypsinogen activation, reduced pancreatitis severity [78] and acted cerebroprotective in rats undergoing transitient middle cerebral artery ischemia [79].

Numerous endogenous protein inhibitors of the cysteine proteases have been described. The most abundant and characterized are the endogenous inhibitors from the cystatin superfamily, which are further subdivided into four families. While the first three families, the stefins, cystatins and kininogens, are truly inhibitory, the fourth family consists of non-inhibitory homologs of the cystatins. All of the cystatins are competitive, reversible, tight-binding inhibitors of the cysteine proteases, and they inhibit their targets in the micromolar to sub-picomolar range [80]. In addition to the cystatins, several other endogenous inhibitors have been described, including Pro-Phe-Pro-Gly-Ile and Gly-Pro-Phe-Pro-Ile that were isolated from bovine β-caseine [81], and Cabin-1, -2, -3, and -4 [82] that were isolated from human plasma.

Although these endogenous protein inhibitors can inhibit catB with great efficiency and sometimes even with extreme selectivity, they have not been used as lead compounds for new anticancer drug discovery. Instead, information from X-ray crystal structures of complexes between macromolecular inhibitors and catB have been used in the design of small-molecule compounds [80]. A good approach to the design of potent catB inhibitors was a consequence of the isolation of various catB inhibitors from different microorganisms (*Streptomyces* [84], *Aspergillus* [84], *Theonella* [85]) and from higher organisms (*Rana catesbiana*- Saxiphilin [86]), which have indeed been used as lead compounds. Another approach was described by Fox and colleagues, who used the structural information of the procathepsin B proregion, and who were the first to show that a synthetic peptide that mimics the proregion of procathepsin B is a strong inhibitor of catB (K_i = 0.4 nM), but not of papain [87].

Synthetic catB inhibitors generally contain an electrophilic functionality that reacts with the catB Cys29 –SH functional group. Examples include aldehydes, disulfides, vinylsulfones and halomethyl ketones, which have all been used as synthetic 'warheads' directed towards various cathepsins [88]. An interesting feature of the catB exopeptidase activity is that access to the substrate-binding site is *via* the occluding loop, which distinguishes catB from other members of the papain family [90]. Hence, specific inhibitors of catB can be designed to take advantage of this feature. Usually, the peptidic sequence Leu-Pro-OH is used to interact with the His 110 and His 111 residues of the occluding loop [66,91].

4.1. Cathepsin B Inhibitors of Natural Origin

Three types of natural cathepsin inhibitors have been isolated from terrestrial microorganisms to date: peptidyl aldehydes, aziridinyl peptides and epoxysuccinyl peptides. These inhibitors are generally non-selective since members of the papain family possess similar tertiary structures and have similar specifities towards their substrates. However, certain selectivities can result from variations in the amino acids in the P1 and P2 subsites (see Fig. (**2**)) of the prefered substrates of the individual enzymes [92].

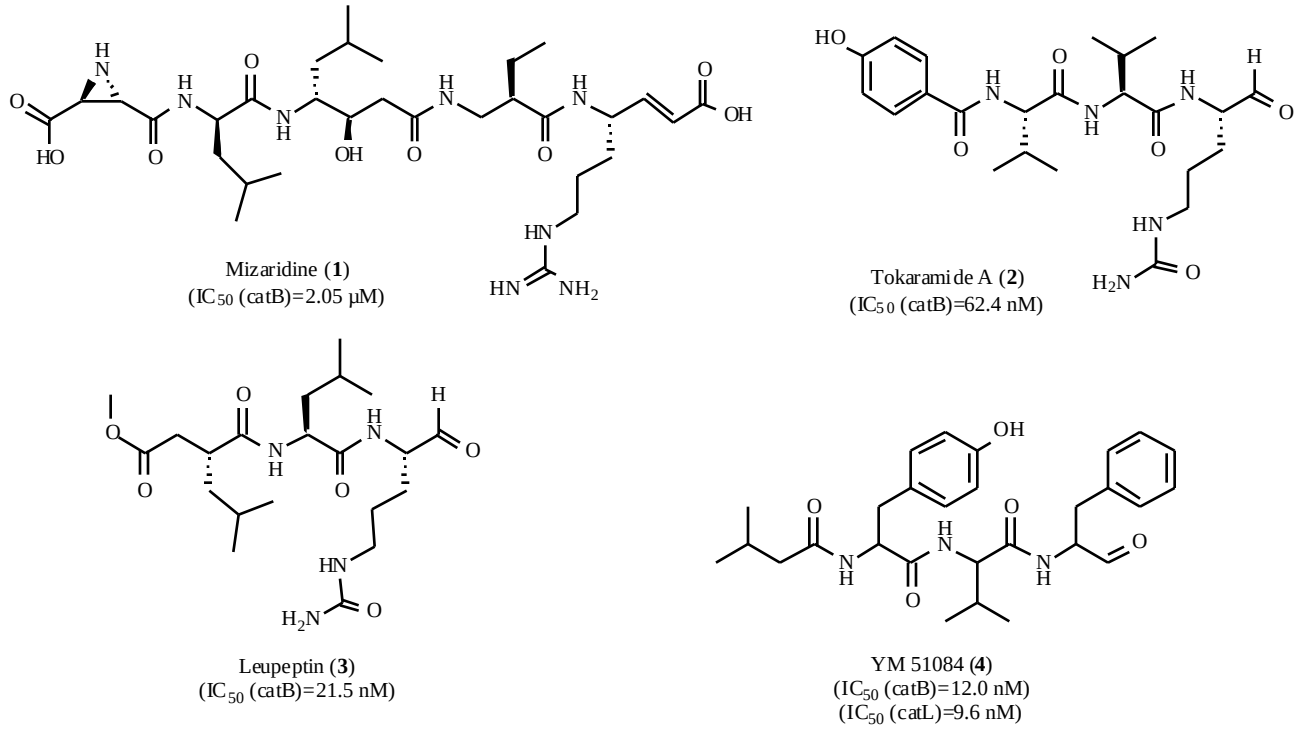
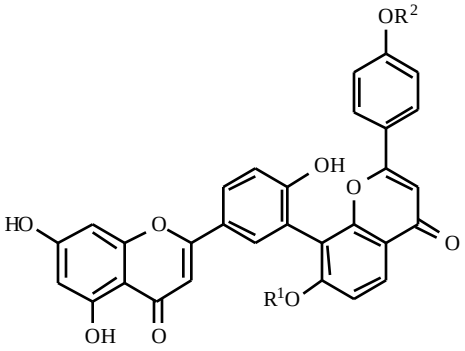


Fig. (3). Aziridines and aldehydes - natural peptidic inhibitors of catB.

The aziridine inhibitor miraziridine (**1**) [93] and an aldehyde inhibitor known as tokaramide A (**2**) [85] were isolated from extracts of the marine sponge *Theonella mirabilis* by Schirmeister and collegueas [85]. As illustrated in Fig. (**3**), miraziridine (**1**) and tokaramide A (**2**) showed inhibition of catB with IC_{50} values of 1.4 μg/mL (2.05 μM) and 29 ng/mL (62.4 nM), respectively. Many potent aldehyde inhibitors have also been isolated from different culture broths of *streptomyces* strains, including leupeptine (**3**) as one of the most potent catB inhibitors, with an IC_{50} of 9.2 ng/mL (21.5 nM) [94]. Another potent inhibitor of catB and also cathepsin L (catL) is YM-51084 (**4**) from *streptomyces* sp. Q21705, with IC_{50} values for catB and catL of 12.0 nM and 9.6 nM, respectively [95]. In addition, the most systematically investigated and the most well known member of epoxysuccinyl peptides (described in chapter 4.2.1), E-64 ((**8**), Fig. (**5**)), was isolated from *Aspergillus japonicus* by Hanada *et al.* with IC_{50} values for catB, catL and papain of 55 nM, 68 nM and 580 nM, respectively [96].

Recently, other non-peptidic natural compounds, such as the flavonoids, were indentified as strong catB inhibitors. The flavonoids amentoflavone (**5**), 4'''-methylamentoflavone (**6**) and 7'',4'''-dimethylamentoflavone (**7**) have been isolated from numerous plants, including *Ginkgo biloba* L. and *Hypericum perforatum* L, and they inhibit catB with IC_{50} values in the low micromolar range (see Fig. (**4**) [97].



R_1=R_2=H, amentoflavone (**5**) (IC_{50}=1.75 μM)
R_1=H, R_2=CH_3, 4'''-methylamentoflavone (**6**) (IC_{50}=1.68 μM)
R_1=R_2=CH_3, 7'',4'''-dimethylamentoflavone (**7**) (IC_{50}=0.55 μM)

Fig. (4). Structures of the natural flavonoid catB inhibitors amentoflavone (**5**), 4'''-methylamentoflavone (**6**) and 7'',4'''-dimethylamentoflavone (**7**), with their inhibitory activities.

4.2. Irreversible Cathepsin B Inhibitors

4.2.1. The Epoxysuccinyl Cathepsin B Inhibitors

The epoxysuccinyl peptides represent one of the most extensively studied classes of catB inhibitors [96,98,100]. In 1978, Hanada *et al*. succeeded in isolating E-64 ((**8**), Fig. (**5**)) from *Aspergillus japonicus*, which became the most systematically investigated and the most well known member of this class of natural compounds. Derivatives of E-64 soon emerged, but unfortunately they only displayed marginal selectivity between the different cysteine proteases. The X-ray structure analysis of different epoxysuccinyl inhibitors complexed to papain revealed the nature of binding of these inhibitors to the active site [92,101]. The standard nomenclature [103] used to designate the inhibitor residues (*i.e.* P1, P2, *etc.*) that bind to the corresponding subsites in papain (*i.e.* S1, S2, *etc.*) are shown in Fig. (**5**). Interestingly, the peptide chain direction of E-64 and its analogs bound to the active site is the reverse of that of the substrate (Fig. (**5**)). In addition, the epoxysuccinic residue interacts irreversibly with Cys 29 in the S1 subsite, while the S2 subsite is occupied by either a hydrophobic Leu or Ile, or by the aromatic rings of Phe or Tyr residues [84]. Generally, the substituents at the P2 position are very important for the selectivity towards catB, and substrates with Leu or Ile in this position show a preference for catB inhibition over catL inhibition of up to 8-fold (inhibitors **8**, **10**, **35** and **37** in Table (**1**)). The opposite is true for substrates with Phe or Tyr, where catL inhibition is preferred to catB inhibition by some 10- to 70-fold (inhibitors **15-23**, **26** in Table (**1**)) [105-111]. The P3 position is usually occupied with C3, C4 or linear C5 CH_2 spacers that terminate in basic functionality [112]. The potency of the inhibitors is influenced by the configuration of the epoxide ring, where SS conformers (*trans*) are preferred over RR (*cys*) conformers (Table (**2**), see **8**, **10** versus **8a**, **10a**). Furthermore, the free carboxylate substituent on the epoxide ring has a key role in the selectivity of these compounds for the cysteine proteases over other biological sulphur nucleophiles [113]. In addition, if the free carboxylate is substituted, the activity of the compounds decreases. The following sequence of inhibitory activity was seen: COOH > $CONH_2$ > $COCH_3$ > COOEt ≈ $COOCH_3$ > CH_2OH ≈ H [112]. Natural *trans*-epoxysuccinyl inhibitors and their derivatives are shown in Table (**1**).

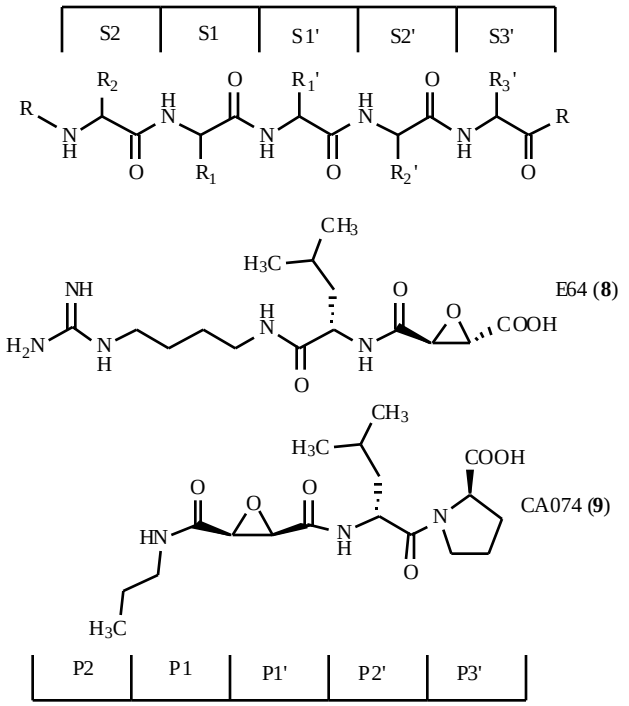
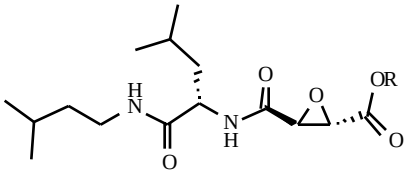


Fig. (5). Standard nomenclature for the inhibitors and enzyme subsites. Epoxysuccinyl inhibitors that bind to the S (8) and S' (9) subsites are shown.

In some cases, cell permeability of these compounds was improved by esterification (e.g. loxistatin (E-64d, **11**), Fig (**6**)). Even if the esters are 100- to 1000-fold less active *in vitro* as compared to the free acids, they are used as prodrugs



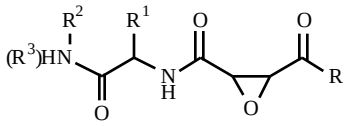
R=H, E-64c, (**10**)
R=Et, E-64d, Loxistatin, (**11**)

Fig. (6). Structures of E-64c (**10**) and its prodrug Loxistatin (**11**).

that are easily absorbed and subsequently rapidly hydrolysed to their active forms [114]. Loxistatin (**11**), which is a prodrug of E-64c (**10**), has undergone tested in Japan for the treatment of muscular dystrophy, but its development was stopped in 1992 in phase III [115] due to a low efficacy [116] and hepatic injury in rats [117].

Other epoxysuccinyl inhibitors that show better selectivity for catB than seen for E-64 and its analogs have also emerged [118]. A representative member of these is seen with CA074 (**9**), which is shown in Fig. (**5**). Analysis of X-ray crystal structures has revealed that the largest part of the CA074 molecule is bound to the S' subsites in a substrate-like direction (Fig. (**5**)) [119,120]. The enzyme recognition sequence for inhibition by CA074 and its analogs is Leu(Ile)-Pro-OH, a dipeptide that is complementary to the

Table 1. The Natural Peptidic *Trans*-Epoxysuccinyl Peptidic Inhibitors and some of their Analogues (11-13). Their Structures, IC_{50} (nM) Values and Selectivities for catB over catL (CB/CL) and Papain (CB/PP) are also Given.



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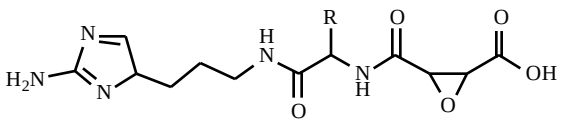

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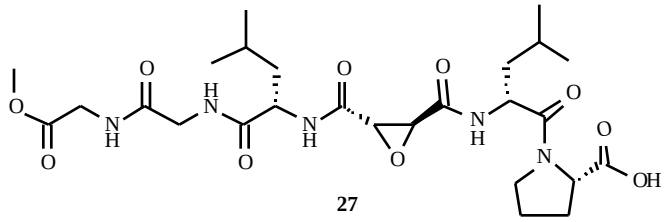
No.	Compound	R^1	R^2	R	Papain[a]	CatB[a]	CatL[a]	CB/CL[c]	CB/PP[c]	Ref.
8	E-64	Ile	Agm	H	580	55	68	0.81	0.65	[105]
9	CA074		Pro-OH	Pr		1.94	233000	0.00		[99]
10	E-64c	Leu	Iam	H		8.70	3.5	2.49		[99]
11	CA030		Pro-OH	Et		4.38	40000	0.00		[99]
12	CA028	Ile	Pro-OH			140	625	0.22		[99]
13	AM4299A		$-(CH_2)_5OH$		88	73	390	0.19	0.83	[107]
14	AM4299B		$-(CH_2)_5NH_2COOH$		280	130	1000	0.13	0.46	[107]
15	Estatin A[b]	Phe	$-(CH_2)_4NHC(=NH)NH_2$		130	270	4	68	2.08	[108,109]
16	Estatin B[d]	Tyr	$-(CH_2)_4NH(=NH)NH_2$		180	320	6	53	1.78	[108,109]
17	Cathestatin A[d]	Phe	$-(CH_2)_4NH_2$	H	360	260	7	37	0.72	[109]
18	Cathestatin B[d]		$-(CH_2)_4NH_2$		230	280	9	31	1.22	[109]
19	Cathestatin C	Tyr	$-(CH_2)_5NH_2$		20	114	11	10	5.70	[110]
20	TMC-52A[h]		$-(CH_2)_3NH(CH_2)_4NH_2$		44	320	13		7.27	[111]
21	TMC-52B[h]		$-(CH_2)_4NH(CH_2)_3NH_2$		7	200	10	20	28.27	[111]
22	TMC-52C[h]	Phe	$-(CH_2)_3NH(CH_2)_4NH_2$		88	460	10	46	5.23	[111]
23	TMC-52D[h]		$-(CH_2)_4NH(CH_2)_3NH_2$		49	280	6	47	5.71	[111]
24	WF14865A[f]			Ile	650	8.4	66	0.13	0.01	[105]
25	WF14865B[g]			Leu	390	13	72	0.18	0.03	[105]
26	WF14861[e]	Tyr	$-(CH_2)_3NH(CH_2)_4NH_2$	H	850	16	1.1	15	0.02	[105,106]

[a] IC_{50} (nM)
[b] IC_{50} in ng/mL
[c] CB/CL – ration between IC_{50} of catB and catL
[d] CB/PP – ratio between IC_{50} of catB and papain
[e] $R^3=(CH_2)_4NH_2$, isolated from *Aphanoascus fulvescens*
[f] isolated from *Colletotrichum* sp
[g] isolated from *Aphanoascus fulvescens*
[h] isolated from *Gliocladium* sp.



27

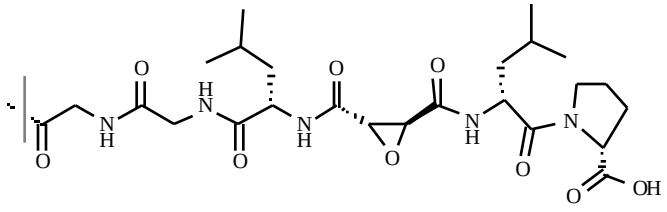
$k_{2nd\ rate}$=1,520,000 $M^{-1}s^{-1}$

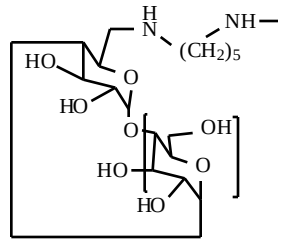
Fig. (7). The very potent and selective epoxysuccinyl inhibitor that spans both the S' and S subsites, with a $k_{2nd\ ord}$ of 1,520,000 $M^{-1}s^{-1}$.

S1'-S2' binding subsites [92,103]. Interestingly, in contrast to E-64 and its analogs where the SS configuration is preferred over the RR configuration, the opposite is true for CA074 and its analogs (Table (**2**), **27**, **43**, **44, 47, 48** versus **27a**, **43a**, **44a, 47a, 48a**) [121]. In structure-activity-relation (SAR) studies, it has been reported that these compounds are selective and highly potent inhibitors of catB if they contain an ester or amide substituent on their epoxide ring, and simultaneously, a free carboxylic acid substituent at their C-terminus (Table (**1**), **9**, **11**, **12** and Table (**2**), **43**, **44**, **46**-**48**), which will interact with the positively charged His 110 and 111 residues [98,119,120]. The preferences for catB over catL (CB/CL) and over papain (CB/CL) are about 200-22,000 and 30-10,000, respectively.

This approach was further extended by Moroder and colleagues in the design of inhibitor **27**, which occupies both the S' and S subsites. The tripeptide moiety Leu-Gly-Gly-OMe (the sequence portion that corresponds to the amino-acid sequence 46-48 of the propeptide) was used to bind to the S subsites, and the Leu-Pro-OH fragment to occupy the S' subsites, resulting in one of the most potent inhibitors of catB ($k_{2nd\ ord}$=1,520,000 $M^{-1}s^{-1}$) [121]. Similar to what was seen for the other epoxysuccinyl inhibitors, the stereochemistry of the ring is very important, with the RR diastereomer (**27**) being 13-fold more potent as compared to the SS diastereomer (**27a**). Compound **27** is also one of the most selective inhibitors, and its preferences for catB over catL (CB/CL) and over papain (CB/PP) are 1,262 and 103, respectively (Table (**2**)) [121].

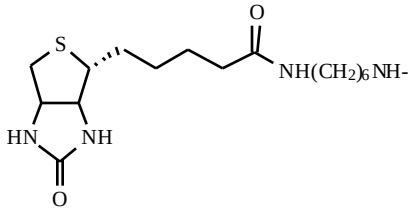
The parent compound **27** was conjugated with mono-(6-deoxy-6-amino)-β-cyclodextrin using a -$NH(CH_2)_5NH$- spacer, which was attached to the free carboxylate group, to give compound **28**. Using this approach, 70% of the activity of the parent compound was still retained; however, its selectivity for catB over catL and over papain was increased by 3- and 40-fold, respectively. Both parent compound **27** and its cyclodextrin conjugate **28** are not cell permeant (MCF-7 breast cancer cells, HaCaT-cells and fibroblasts) at the concentrations needed for full inhibition. Hence, it has been shown that the conjugated inhibitor is fully water soluble and that it is targeted only to the extracellular and membrane-bound catB. Furthermore, the cyclodextrin





=R **28**

RhodamineB-NH-$(CH_2)_6$-NH- =R **29**



=R **30**

Fig. (8). Structures of the most potent and most selective epoxysuccinyl catB inhibitors synthesized to date (cyclodextrin derivative 28, rhodamine B derivative 29 and biotinylated derivative 30).

moiety can form inclusion complexes with other cytotoxic drugs, such as methotrexate, which can offer many advantages; e.g. such conjugates could be used in certain cancers, for example, as site-directed drug carrier systems, for delivery to where the active catB is known to be present [100].

Two similar conjugates with rhodamine B (**29**) and biotin (**30**) have also been prepared using a -$NH(CH_2)_6NH$- spacer. Both of these compounds still retain the inhibitory activity of the parent compound; however, their selectivity for catB over catL is increased 4-5-fold. In addition, affinity blot analysis has demonstrated that these conjugates can be

Table 2. Second-Order Rate Constants of Inactivation of Cysteine Proteases by the *Trans*-Epoxysuccinyl Peptides

No.	Conf.	Inhibitor	Papain[c]	CatB[c]	CatL[c]	CB/CL[a]	CB/PP[b]	Ref.
E-64 analogs								
8	SS	E64 (HO-EpsLeu-Agm)	869000	81400	43 800	1.9	0.09	[121,123]
8a	RR	E64 (HO-EpsLeu-Agm)	86000	1170	4930	0.2	0.01	[121]
10	SS	E64c (HO-EpsLeu-iAm)	357000	298000	206000	1.5	0.83	[96]
10a	RR	E64c (HO-Eps-Leu- iAm)	38000	6900			0.18	[96]
11	SS	E-64d (EtO-EpsLeu-iAm)		750				[113]
31	RR	EtO-EpsLeuArg-OH	92	291000	81	3590	3163	[121,123]
32	RR	HO-EpsLeuArg-OH	3890	520	680	0.8	0.13	[121,123]
33	RR	EtO-EpsLeu-Agm	1030	73	93	0.8	0.07	[121,123]
34	RR	HO-EpsLeu-Agm	86000	1170	4930	0.2	0.01	[121,123]
35	SS	(E-64a) HO-EpsLeu-$NH(CH_2)_4NH_2$		69500	27500	2.5		[96]
36	SS	HO-EpsLeu-$NH(CH_2)_4$NH-Z	471000	175000	231000	0.8	0.37	[96]
37	SS	HO-EpsLeu-$NH(CH_2)_7NH_2$	874000	339000	142600	2.4	0.39	[96]
38	SS	(E-64b) (HO-EpsLeu Leu-OH)	122000	64500			0.38	[96]
39	SS	HO-EpsLeu-NBzl	90000	37600			0.42	[124]
40	SS	Bzl-DL-EpsIleTyr-OMe	11340	49400	12660	3.9	4.37	[124]
CA074 analogs								
27	RR	MeO-GlyGlyLeuEpsLeuPro-OH	14800	1520000	1204	1262	103	[121]
27a	SS	MeO-GlyGlyLeuEpsLeuPro-OH	870	21460	269	798	25	[121]
28	RR	Cyclodextrin derivative of **27**	1047	1050000	393	2672	1003	[100]
29	RR	Rodamine derivative of **27**		1530000	323	4736		[122]
30	RR	Biotin derivative of **27**		1726000	256	6742		[122]
41	RR	HO-EpsLeuPro-OBzl	176000	8700			0.05	[98]
41a	SS	HO-EpsLeuPro-OBzl		388				[96]
42	RR	EtO-EpsLeuPro-Obzl	110	30			0.27	[98]
43	RR	HO-EpsLeuPro-OH	30000	2200			0.07	[98]
43a	SS	HO-EpsLeuPro-OH	3270	270	74	3.6	0.08	[121]
44	RR	EtO-EpsLeuPro-OH	56 760	567000 13800	26	21800	10125 18	[98,121,123]
44a	SS	EtO-EpsLeuPro-OH	6130	44400	170	260	7	[121,123]
45	RR	*i*Bu-EpsLeuPro-OBzl	2070	206			0.1	[98]
46	RR	*i*Bu-EpsLeuPro-OH	558	52000			**93**	[98]
47	RR	nPrNH-EpsLeuPro-OH	103	153000	22	6960	149	[121,123]
47a	SS	nPrNH-EpsLeuPro-OH	5	29400	46	640	5880	[121,123]
48	RR	Agm-OrntEpsLeuPro-OH	6220	197000	250	790	32	[121,123]
48a	SS	Agm-OrntEpsLeuPro-OH	225	63300	26	790	281	[121,123]

[a]CB/CL - ratio between second-order rate constants of catB and catL.

[b]CB/PP - ratio between second-order rate constants of catB and papain.

[c] Second-order rate constants ($k_{2nd\ rate}(M^{-1}min^{-1})$) expressed as k_{inact}/K_i ($M^{-1}s^{-1}$) with k_{inact} as reaction rate and K_i as pre-association equilibrium constant.

used for highly sensitive, selective and non-radioactive detection of catB. These conjugates could therefore be used for the development of 'tool kits' for the detection of catB activities in diseases where extracellular catB is elevated. As they are membrane impermeant, all of these derivatives offer another advantage: they target only the extracellular and/or membrane-bound catB, leaving the intracellular catB intact to perform its housekeeping functions [122].

4.2.2. The Aziridine Cathepsin B Inhibitors

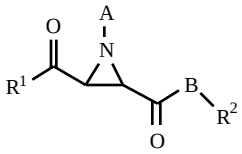
The synthesis of epoxysuccinyl analogs containing aziridine-2,3-dicarboxylate (Azi) as the electrophilic building block has also been reported. As with the epoxysuccinyl derivates, these Azi-containing inhibitors react irreversibly with Cys 29 in the active site of catB. In contrast to the epoxides, the aziridine ring can also be derivatized on its heteroatom, which offers greater variability for SAR studies [125-126]. Many different types of pseudopeptides containing Azi have been obtained, among which remarkable differences have been seen concerning their stereospecificity, pH dependency of inhibition, selectivity and the importance of a free carboxylic function on the aziridine ring. In general, *N*-unsubstituted analogs have a higher selectivity towards catL as compared to catB or to papain (Table (**3**), **51**, **52**) [127]. The opposite holds true for *N*-substituted inhibitors, where the aziridine ring is located in the middle of the peptide chain, and where a much higher selectivity towards catB and papain was obtained (Table (**3**), **53**-**56**). A carboxylic acid attached to the aziridine ring enchanced the activities of a series of *N*-unsubstituted aziridines (Table (**3**), **52, 58** versus **51** and **59**) [125,126], which was not seen within the series of *N*-acylated inhibitors (**56** versus **55**) [127]. The most potent aziridine inhibitors of catB are shown in the Table (**3**).

The aziridine ring can be also protonated on its nitrogen. Consequently, the inhibitory activity of aziridine inhibitors has been shown to be strongly pH dependent, with their maximum activity observed at pH 4. The interactions of an aziridine ring with Cys 25 and Gln 19, which form the oxyanion hole, is not as strong as with E-64 and its analogs as a result of the different stereochemistry and because of the protonation of the aziridine ring [126]. In addition, it has also been shown that a free carboxylic acid group on the aziridine ring enhances the ring opening to a much greater extent than in the epoxides [112].

4.2.3. The 1,2,4-Thiadiazole Cathepsin B Inhibitors

Leung-Toung and collegues described the synthesis of the 1,2,4-thiadiazoles, which can act as thiol trapping agents as they undergo selective ring opening with thiols, but not with amines and/or alcohols [128]. The design of a wide variety of catB inhibitors involved the use of substituent X (see Fig. (**9**)) at the C5 position as a recognition arm for binding to catB, and substituent Y (the C3 position), which

Table 3. The catB, catL and Papain Inhibitory Activities of the Aziridines



49-59

No.	Conf.	R^1	A	B	R^2	Papain[a]	CatB[a]	CatL[a]	CB/PP	CB/CL	Ref.
49	RR+SS	HO	BocPhe		OEt	505	1230	188	2,44	6,54	
50	RR+SS	HO	BocPhe		OH	3544	807	38081	0,23	0,02	
51	RR	EtO	H	Leu-Pro	OBzl	1533	1607	16261	1,05	0,1	
52	RR+SS	HO	H	Leu-Pro	OBzl	7660	3870	16930	0,51	0,2	
53	RR	EtO	BocPhe	Leu-Pro	OH	176	6538	655	37,15	10	
54	SS	EtO	BocPhe	Leu-Pro	OH	704	6859	212	9,74	32	
55	RR	BzlO	BocPheAla		OEt	1232	433	281	0,35		
56	RR	H	BocPheAla		OEt	1456	676	843	0,46		
57	RR	EtO	H	Leu	OBzl	1533	1607	16261	1,05	0,1	
57a	SS	EtO	H	Leu	OBzl	214	41	3130	0,19	0,01	
58	RR	HO	H	Leu	iAm		14400				
58a	SS	HO	H	Leu	iAm		1800				
59	RR	EtO	H	Leu	iAm			55			
59a	SS	EtO	H	Leu	iAm			3			

[a] $k_{2nd\ rate}$ ($M^{-1}min^{-1}$)

can adjust the reactivity of the ring opening (Fig. (**9**)). Thus electron withdrawing substituents at the C3 position lower the electron density and consequently increase the rate of ring opening. The opposite is true for electron donating substituents [91,131]. A C5 substituent can be tailored with the dipeptide sequence to achieve recognition by the catB active site and not by other cysteinyl peptidases. The most potent inhibitor here is the 3-MeO analog **60** (Y=MeO, X=Leu-Pro-OH), with a second-order rate constant of k_i/K_i=5630 $M^{-1}s^{-1}$. In addition, the 1,2,4-thiadiazoles are presumed not to be able to inhibit catB inside the cell because they rapidly react with reduced glutathione. This feature offers several advantages for these 1,2,4-thiadiazoles over other classes of catB inhibitors because the housekeeping function of intracellular catB is again left intact, with only extracellular catB being inhibited [132].

The mechanism of inhibition by the 1,2,4-thiadiazoles is shown in the Fig. (**9**), where the N-S bond of the 1,2,4-thiadiazole moiety is cleaved by the thiolate group of catB. As a result, the disulfide adduct was suggested to occur in numerous studies [133-136], and the X-ray crystal structure of papain complexed with the 1,2,4-thiadiazole inhibitor Apo1073 later proved this to be so [137].

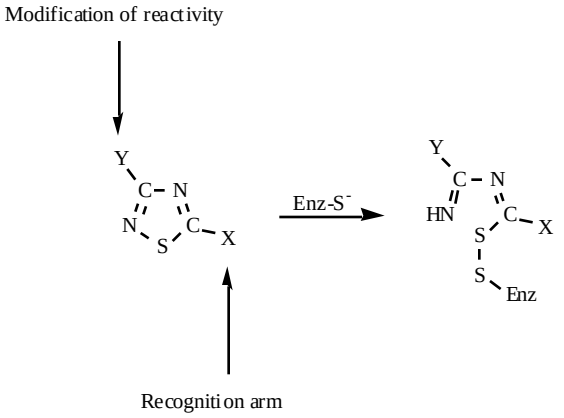


Fig. (9). Mechanism of catB inhibition by the 1,2,4-thiadiazoles.

4.2.4. The Acyloxymethylketone Cathepsin B Inhibitors

Fig. (**10**) and Table (**5**) show the structures of the acyloxymethylketones, which are irreversible catB inhibitors that are composed of a peptide recognition sequence and a space-filling leaving group, which reacts with Cys 29 of catB [138]. A wide variation of both of these parts of the acyloxymethylketones is possible, thus enabling regulation of their selectivity and potency [139]. A number of acyloxymethylketones have been synthesized, and compound **68** is representative of this group of catB inhibitors. The recognition sequence for **68** is *N*-carbobenzyloxy-L-phenylalanyl-L-alanyl (Z-Phe-Ala), which binds to the S2-S1 subsites [141]. Compounds **69** and **70** are representative of the newer acyloxymethylketones. Interestingly, the second-order rate constant of compound **70** is 3,000-fold greater than for reference compound **69**, which is probably due to the interaction of an additional peptidyl sequence with the S' subsite of catB [142]. The other acyloxymethylketones inhibitors that have been investigated to date are structurally related, and the most potent of them are given in Table (**5**).

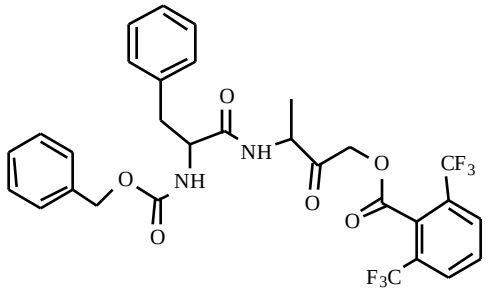
4.2.5. The β-Lactam Cathepsin B Inhibitors

The β-lactam core (azetidin-2-one) was used by Zhou and colleagues for the design of potent irreversible protease inhibitors. The chemical reactivity, inhibition potency and selectivity of the β-lactam inhibitors were adjusted by substituents at the C4 position (Fig. (**11**)). Some very potent inhibitors of catL, catK and catS that are selective towards serine proteases and human elastase have been obtained, with compounds **76** and **77** being the most potent of these. Unfortunately, inhibition of catB by these β-lactams was much weaker when compared to the other cathepsins tested [144]. However, because the β-lactam ring can be substituted at the C3 and C4 positions, a proper combination of modifications to the C3 and C4 substituents to provide a better fit into the active site of catB could provide further selective β-lactam-based catB inhibitors in the future.

Table 4. The 1,2,4-Thiadiazole Based Inhibitors and their Second-Order Rate Constants for catB, Cathepsin S (catS), Cathepsin H (catH) and Papain

Comp.	Y	X	catB		$k_{2nd\ rate}$ ($M^{-1}s^{-1}$)		
			K_i[a] (μM)	$k_{2nd\ rate}$ ($M^{-1}s^{-1}$)	catS	catH	Papain
60	MeO	-NH-Leu-Pro-OH	2.6	5630	NI	200 μM[b]	49
61	Ph	-NH-Leu-Pro-OH	74	175	NI	NI	120 μM[a]
62	Me	-NH-Leu-Pro-OH	447	55	Nd	Nd	Nd
63	COOH	-NH-Leu-Pro-OH	300	293	Nd	Nd	Nd
64	MeO	-NHCONH-Leu-Pro-OH	390	36	NI	NI	Ni
65	MeO	-NHCONH-Leu-Pro-Isoamyl	367	84	nd	Nd	Nd
66	MeO	CBz-Phe-NH-	21	658	50 μM[a]	45 μM[a]	NI
67	MeO	CBz-Phe-Ala-NH-	37	864	50 μM[a]	50 μM[a]	145 μM[a]

[a]K_i is dissociation constant for non-covalent enzyme inactivator complex
[b]IC_{50} for reversible inhibition determined at 10 μM substrate concentration



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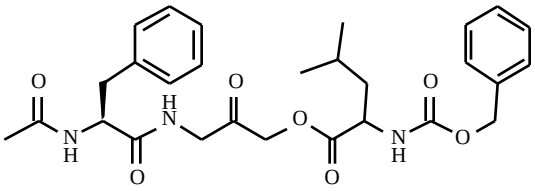

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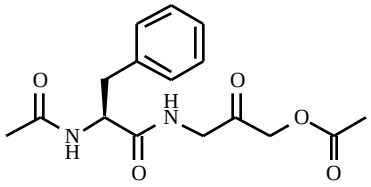


70

Fig. (10). Representative α-acyloxymethylketones.

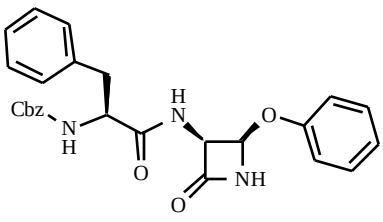
Table 5. Some Potent α-Acyloxymethylketones

No.	Compounds	$K_{2nd\ rate}$ ($M^{-1}s^{-1}$)		Ref.
		catB	catL	[139]
71	Z-Phe-Ala-CH_2-O-CO-(2,6-$(CF_3)_2$)-Ph	1,600,000	332,000	[143]
72	Z-Phe-Lys-CH_2-O-CO-(2,4,6-Me_3)-Ph	2,000,000		[139]
73	Z-Phe-Ala-CH_2-O-CO-(2,4,6-Me_3)-Ph	14,000	4,200	[139]
74	Z-Phe-Cys(SBzl)-CH_2-O-CO-(2,6-$(CF_3)_2$)-Ph	2,900,000	10,700,000	[139]
75	Z-Phe-Ala-CH_2-O-CO-(2,5-$(CF_3)_2$)-Ph	38,000	2,400	[139]

Using the β-lactam scaffold, Zhou and collegues prepared four different groups of inhibitors: the penam oxides (e.g. **78**) [145], the sulfoxides (e.g. **79**), the sulfides (e.g. **80**) and the sulfones (e.g. **81**) [146]. These were shown to be similar to the β-lactam series above, where their inhibitory activity for catB is much lower when compared to other cathepsins. Among these inhibitors, the sulfoxides were the most potent (Fig. (**12**)).

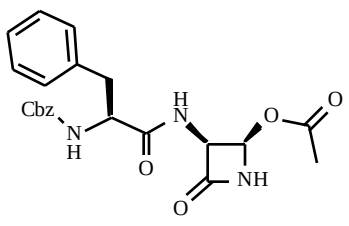
4.2.6. The Organotellurium (IV) Inhibitors of Cathepsin B

Cunha and colleagues recently described the inhibitory activity of organotellurium (IV) compounds, which are shown in Fig. (**13**). The high reactivities of these inhibitors could be associated with the Lewis acid character of the tellurium atom. A model reaction with cysteine has also suggested that substitution of the chloride ion takes preference over the opening of the telluroxetane ring (Fig. (**14**)). However, these organotellurium (IV) compounds are only effective against the extracellular form of catB, since the cytoplasmic environment of the cell contains high amounts of reducing agents, such as glutathione, cysteine and NADPH. Compound **82** (Fig. (**13**)) had the highest inhibitory activity in the series, showing a second-order rate constant of 36,000 $M^{-1}s^{-1}$ [147].



IC_{50} (catB)=0.43 μM
IC_{50} (catL)=2.6 nM
IC_{50} (catK)=16 nM

76



IC_{50} (catB)=0.47 μM
IC_{50} (catL)=42 nM
IC_{50} (catK)=0.100 μM
IC_{50} (catS)=0.207 μM

77

Fig. (11). Two representatives of the β-lactam cathepsin inhibitors, 76 and 77, and their inhibitory activities towards catB, catL, catK and catS.

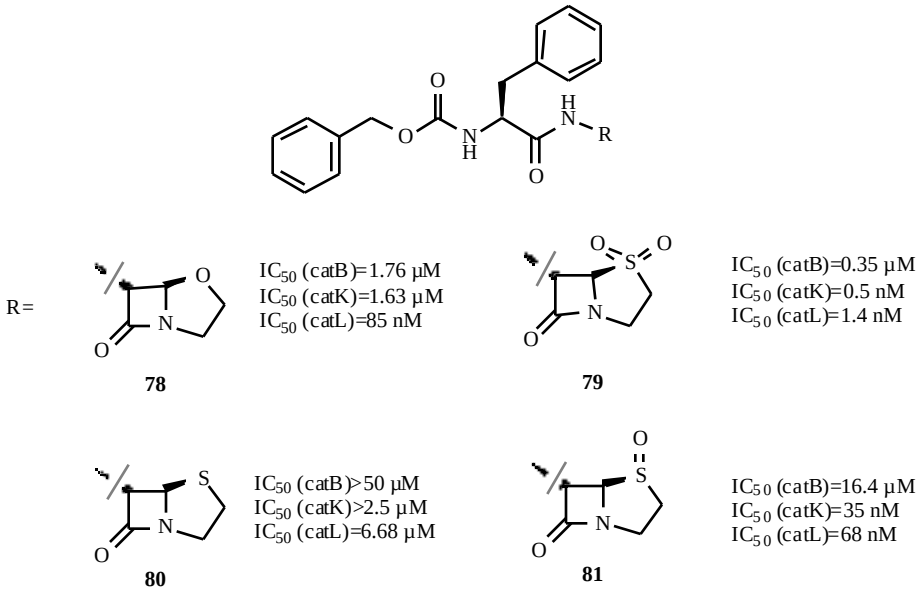


Fig. (12). The structures of the penames **78**, **79, 80** and **81**, with their inhibitory activities towards catB, catK and catL.

4.2.7. Other Irreversible Inhibitors of Cathepsin B

On the basis of computational methods, Lim and colleagues [148] have reported the synthesis of new irreversible inhibitors, where the structural diversity was provided by the synthesis of a focused library of compounds. Compound **85** (Fig. (**15**)) is the prototype of these inhibitors, with a K_i of 4.4 ±0.6 µM ($k_{2nd\ ord}$ = 150,000 $M^{-1}s^{-1}$), and with an interesting proposed mechanism of action (Fig. (**15**)). This compound can undergo nucleophilic attack by Cys 29 of catB, to give **86**, as shown in the Fig. (**15**). The intermediate **86** may fragment to give species **87**, which would be expected to form hydrogen bonds with the unprotonated His 199 of catB and attenuate its basicity. By doing so, not only will this histidine be a poorer base for the promotion of water molecules for the second step of catalysis, but also the hydroxyl moiety is positioned such as to hinder the incoming hydrolytic water molecule.

4.3. Reversible Cathepsin B Inhibitors

Since reversible inhibitors usually have reduced toxicities, they are very important from a therapeutic point of view. A lot of effort has been put into the synthesis of reversible and tight-binding catB inhibitors. However, attempts to design such inhibitors of catB that form interactions with the S and S' subsites have had limited success because the electrophilic 'warheads' used allow the modification of these molecules in only one direction.

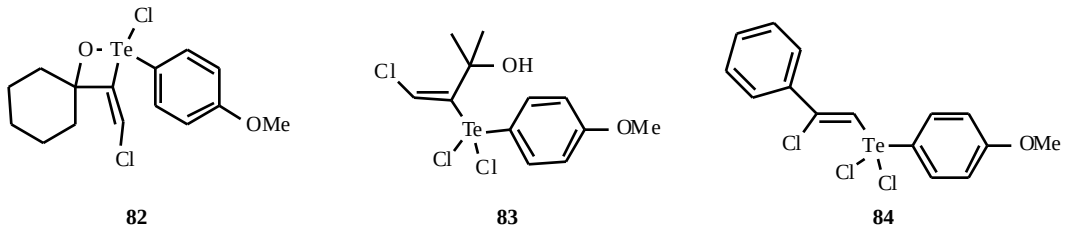


Fig. (13). The organotellurium (IV) inhibitors.

4.3.1. The Aldehyde Cathepsin B Inhibitors

The aldehyde inhibitors are believed to inhibit serine and cysteine proteases by forming a tetrahedral hemi(thio)acetal between the aldehyde and the hydroxylate (thiolate) in the active site of the enzyme (Fig. (**16**)). This reaction has been confirmed by NMR studies [149]. Despite the covalent binding to the enzyme, peptidyl aldehydes are reversible

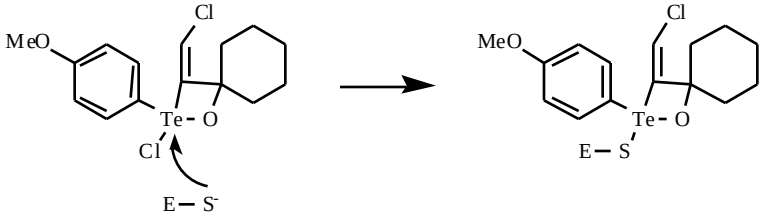


Fig. (14). Proposed interaction of the catB thiolate with compound 82.

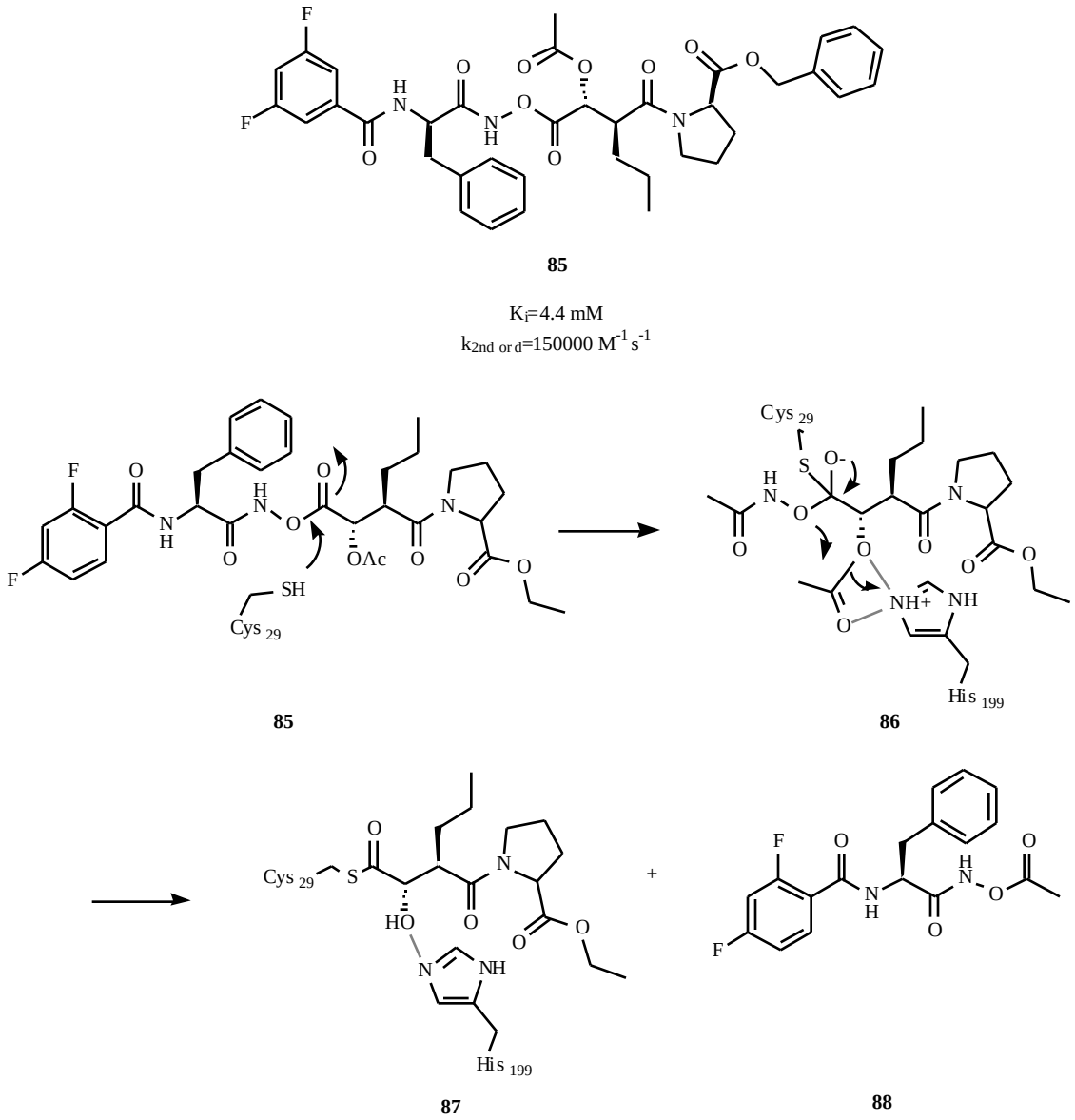


Fig. (15). The new irreversible inhibitor **85**, and its proposed mechanism of action.

inhibitors [92]. A large number of potent aldehyde inhibitors have been synthesized over the last 15 years, among which only a few have proven to be catB selective.

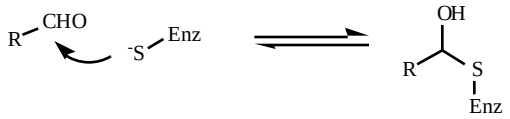


Fig. (16). Formation of the tetrahedral hemithioacetal, as demonstrated by NMR studies.

McConell and colleagues prepared a series of leupeptin (**3**) analogs, which are given in Table (**6**). Some of these have proven to be selective and very potent inhibitors of catB against some of the serine proteases [151].

Scheidt reported the synthesis of some cruzain inhibitors, among which some were also active against catB. Two of the most potent of these, **95** and **96**, are shown in the Fig. (**17**) [152].

4.3.2. The Ketone Cathepsin B Inhibitors

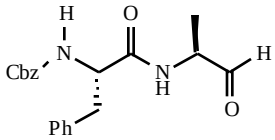
Ketones are potent cysteine protease inhibitors that are well known to interact with the cathepsins [153]. As with the aldehyde catB inhibitors, the ketones also react with the nucleophilic thiolate (Cys 29 of catB), to give a reversible hemithioketal adduct that also mimics the tetrahedral intermediate [154].

Abato and collegues have described the synthesis and screening of a 400-membered combinatorial library of inhibitors that were based on a cyclohexanone nucleus (Fig. (**18**)). The X_{aa} position in compound **97** was designed to fit into the S2 specificity pocket, the carbonyl moiety of the cyclohexanone ring would react with Cys 29 and the Y_{aa} position was designed to interact with the S2' site [154]. They also observed the known preference of the S2 subsite for hydrophobic amino acids, such as Ile and Leu. On the other hand, the S2' subsite did not appear to have a strong preference for any particular amino acid, and binding in this position led to only incremental increases in affinity [155].

Table 6. Some of the Most Potent Lysinal and Arginal Leupeptin Analogs and their Inhibitory Activity Towards Different Proteases

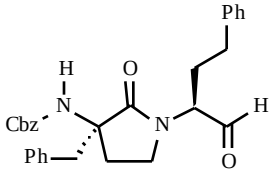
No	Compound	catB[a]	Trypsin[a]	Kallikrein[a]	Thrombin[a]	Plasmin[a]
3	Leupeptin	0.31	8.1	40	12	3.7
89	Acetyl-Leu-Ile-arginal	0.15	9.1	35	18	11
90	Acetyl-Leu-Phe-arginal	1.1	NA	41	8.2	3.6
91	Acetyl-Phe-Val-arginal	0.039	12	28	1.8	2.2
92	Acetyl-Leu-Leu-lysinal	0.13	NA	NA	NA	18
93	Acetyl-Leu-Val-lysinal	0.004	45	NA	140	13
94	Acetyl-Leu-Phe-lysinal	0.1	NA	NA	NA	NA

NA, data not available
[a] IC_{50} (μM)
NA, data not available



95

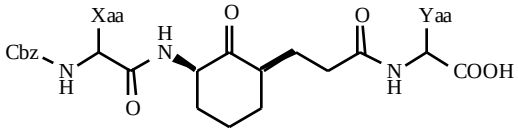
IC_{50}(catB)=0.05 μM
IC_{50}(cruzain)=0.01 μM



96

IC_{50}(catB)=1.0 μM
IC_{50}(cruzain)>10 μM

Fig. (17). The aldehyde cruzain inhibitors **95** and **96**.



97

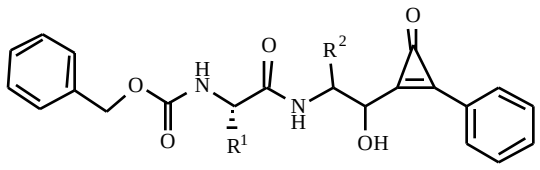
Fig. (18). General structure of the cyclohexanone inhibitors gained from combinatorial library screening of pools of compounds.

4.3.3. The Cyclopropenone Cathepsin B Inhibitors

The cyclopropenone ring has amphiphilic properties - it can act as a good electrophile for 1,2 or 1,4 additions that receive various nucleophiles, or as a precursor for the formation of the stable 2π-aromatic hydroxycyclopropenium cation that can be generated readily by protonation of the carbonyl group [156]. The former property may be amenable to the design of reversible catB inhibitors by taking advantage of the reaction with a cysteine or serine residue in the active site of the enzyme. Cyclopropenone may react with the thiol residue of Cys 29 of catB, or could even be protonated by this acidic Cys 29 if these two residues exist within a suitable distance on the surface of the enzyme.

Ando and collegues have described the synthesis of active-site-directed inhibitors by using the cyclopropenone moiety, to which a peptide chain was attached. These inhibitors showed strong inhibitory activities towards the cysteine proteinases, such as calpain, papain, catB and catL, but not to the serine or aspartic proteinases. The general structure of a cyclopropenone inhibitor is given in Table (**7**). In general, these inhibitors show some geometrical

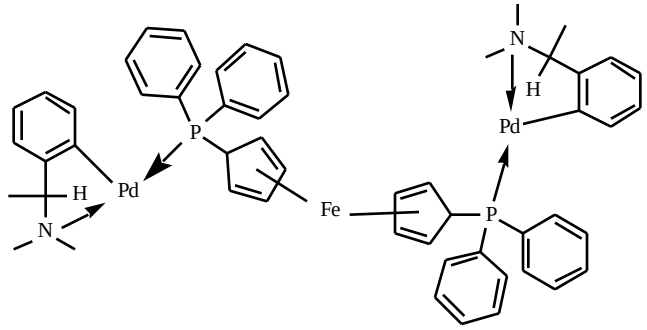
Table 7. The Cyclopropenone Inhibitors of catB



98-103

No.	Compound	R^1	R^2	IC_{50} (μM)
98	1S	$-CH(CH_3)_2$	$-CH(CH_3)_2$	0.71
99	1R	$-CH(CH_3)_2$	$-CH(CH_3)_2$	0.044
100	1R	$-CH(CH_3)_2$	$-CH_3$	22.9
101	1R	$-CH(CH_3)_2$	$-CH(CH_3)_2$	9.45
102	1R	$-CH(CH_3)_2$	$-CH_2CH(CH_3)_2$	29.0
103	1R	$-CH(CH_3)_2$	$-CH_2Ph$	>100

resemblance to the epoxysuccinyl derivatives. The results of catB inhibition by these cyclopropenone inhibitors are also given in Table (**7**), from which it can be easily seen that $1R^1$ enantiomers are the preferred isomers for better inhibitory potencies. Furthermore, SAR studies have demonstrated that the *i*-Pr group is the best substitution at position R^2 [157].



104

Fig. (19). The cyclopalladated complexes with inhibitory activity towards catB (Kh = 12 ±1 μM, αKh = 2.4 ±0.3 μM).

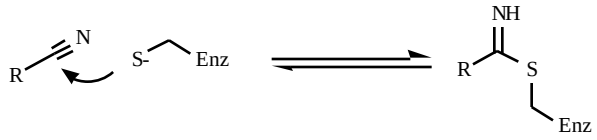


Fig. (20). Mechanism of catB inhibition by nitriles.

4.3.4. The Cyclometallated Complexes and Cathepsin B Inhibition

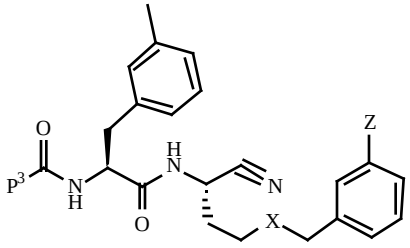
Caires and colleagues have reported the synthesis of chiral cyclopalladated complexes (Fig. (**19**)) that were derived from *N*, *N*-dimethyl-1-phenethylamine and the coordinating ligand 1,1'-bis(diphenylphosphine)ferrocene. These complexes were able to inhibit catB activity in a reversible fashion. The palladacycle compound **104** binds to free catB as well as to the enzyme-substrate complex, with dissociation constants Kh and αKh of 12 ±1 μM and 2.4 ±0.3 μM, respectively. The use of this complex with Walker tumor-bearing rats (single doses of 2.0 mg/kg) resulted in a 90% inhibition of tumor growth and a reduction in the tumor mass. On the other hand, the same complex did not offer any protection to mice bearing the non-metastatic Ehrlich-ascites tumor, which shows that these compounds are more selective towards malignant tumors, such as the Walker carcinoma. Toxicological studies using mice treated with a single high dose of complex (**104**) (100 mg/kg) did not show any alterations in red and white blood cell morphology, and in hepatic, kidney and spleen tissues, 14 days after administration of **104**. These results presented cyclopalladated complexes as promising antitumoral drugs with reduced toxicity in experimental studies [158].

4.3.5. The Nitrile Cathepsin B Inhibitors

Nitriles have long been known to inhibit the cysteine proteases [92]. NMR studies have indicated that this inhibition occurs through the reversible formation of a thioimidate intermediate (Fig. (**20**)) [159]. Mutational studies have suggested that this intermediate is stabilized by a neighboring Gln 19 residue [160], which, along with the backbone NH of the catalytic Cys 29 residue of catB, forms the putative oxyanion hole of the enzyme [161].

Greenspan and colleagues have reported the successful application of structure-based design on a series of peptidyl nitrile inhibitors of catB. Through systematic and iterative optimisation of a series of substituents at the P3 and P2

Table 8. The Dipeptidic Nitriles with Different P^3 and Z Substituents

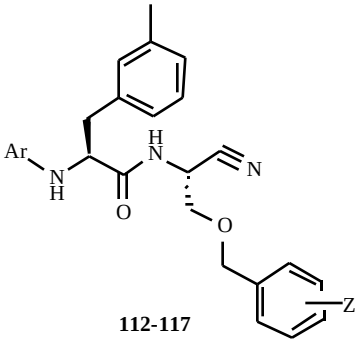


105-111

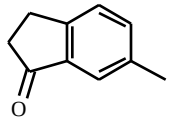
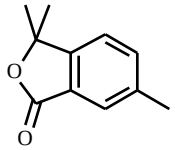
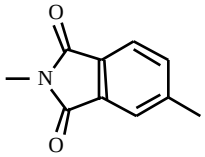
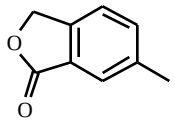
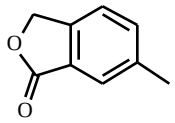
No.	P^3	X	Z	IC_{50} (nM)
105	2,4-difluorophenyl	O	COOH	6.8 ±0.6[a]
106	Ph_2CH	O	H	10.2 ±0.6
107	Ph_2CH	O	3-COOH	1.8 ±0.75
108	Ph_2CH	CH_2	3-COOH	18
109	Ph_2CH	O	5-COOH	5.0 ±1.5
110	2-fluoro-4-chlorophenyl	O	3-COOH	2.0 ±0.1
111	Ph	O	3-COOH	9.4 ±1.6

[a]IC_{50}(catL), 554 nM; IC_{50}(catS), 937 nM

Table 9. The Different *N*-Arylaminonitriles, their IC_{50} Towards catB, catL and catS, and their Plasma Concentrations



112-117

Compd	Ar	Z	IC_{50} (nM)			Plasma conc. (μM)[a]
			Cat B	Cat L	Cat S	
112		3-COOH	5.3	ND	ND	0.07
113		3-COOH	5.3	560	780	0.11
114		3-COOH	4.9	>1,000	>1,000	0.015
115		3-Tetrazole	5.0	2,400	1,000	0.037
116		3-COOH, 4-F	4.1	1,100	1,000	0.056
117	Ph	3-COOH, 4-F	12.2	1,400	1,600	5.27

[a]Plasma concentration is measured 4 h after a 30 mg/kg po dose. ND, not determined

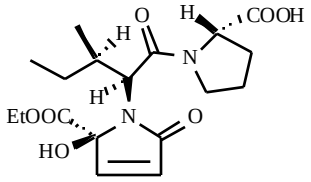
positions, they were able to achieve a 1,000-fold improvement in potency, and thus to produce catB inhibitors with IC_{50} values in the nanomolar region. Their inhibitory potencies and selectivities were further enchanced by tethering the carboxylate functionality and carbon α to the nitrile, to interact with His 110 and His 111 in the S2' subsite of catB. The most promising has proven to be compound **105**, which has an excellent selectivity over other cathepsins. Some of the most potent inhibitors are given in Table (**8**) [162].

Later on, the same group reported the synthesis of the *N*-arylaminonitriles, a structural modification of the template presented above, where the P2-P3 amide group was replaced by an arylamine. As expected, the activities of the different P3 substituents were identical to those of the P3 acyl series. Selectivity over other cathepsins was further improved by using different Z and Ar substituents (see Table (**9**)), to gain substances with high selectivity, *in vitro* potency (**112-117**) and oral availability (**117**). Some of these are given in the Table (**9**) [163].

4.4. Other Cathepsin B Inhibitors

Nagao and colleagues have reported the synthesis of 3-pyrrolin-2-one-L-Ile-L-Pro-OH derivatives as a new type of inhibitor of the cysteine proteases (Fig. (**21**)). As with the

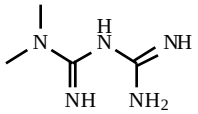
other inhibitors discussed, the Ile-Pro-OH moiety provides the recognition sequence in **118** [164].



118

Fig. (21). The most potent catB inhibitor of the 3-pyrrolin-2-one-L-Ile-L-Pro-OH series, with 90.7% inhibition at 0.1 μM.

Some older drugs have also been recognized as being catB inhibitors. The biguanide derivatives (Fig. (**22**)) are widely used against malaria and type II diabetes; however, their mechanisms of action were not proposed until recently. Thus various biguanide derivatives were hypothesized to be metal-interactive inhibitors of catB (metformin (**119**) caused 80% antilysosomal activity at 100 μM), which is also involved in insulin and hemoglobin degradation [165].



119

Fig. (22). Metformin.

5. CONCLUSIONS

The catB cysteine protease is no longer considered to be a 'garbage-disposal' enzyme as it has very important roles in many physiological and pathological processes. Under physiological conditions, a balance exists between catB and its inhibitors; this balance can, however, be destroyed under many pathological conditions, such as with inflammation, infection, Alzheimer's disease, ischemic neuronal death and cancer. The proteolytic activity of both intracellular and extracellular catB promotes tumor invasion and metastasis by degrading the ECM. Therefore, inhibition of catB results in a significant reduction in both motility and invasiveness of cancer cells, and can also lead to apoptosis. Thus new information relating to the role of catB in cancerogenesis in the mid 1990s and the availability of crystal structures increased interest in the search for new inhibitors of catB. However, two decades of research and development gave almost no preclinical data which would confirm the enormous potential of catB inhibitors in cancer chemotherapy, which was demonstrated in *in vitro* tests.

Despite the similarity in tertiary structures of the cysteine proteases belonging to the papain family, amino acids His 110 and His 111 on the occluding loop of catB have allowed the design of potent and selective inhibitors. The majority of these selective inhibitors bear carboxylic functionality substituted to the recognition sequence Leu-Pro-OH, which is complementary to the S1'-S2' substrate-binding subsites and which forms ionic interactions with His 110 and His 111. Furthermore, these inhibitors usualy contain synthetic 'warheads', which react reversibly or irreversibly with Cys 29 in the active site of catB.

Due to the great number of possible therapeutic applications, catB inhibitors still have a potential in drug design and development. Because the latest reports show that catB also has a major role in pathological processes other than cancer metastasis and invasiveness, the design and synthesis of selective and potent catB inhibitors could prove to be of even greater interest in the future.

ACKNOWLEDGEMENT

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ABBREVATIONS

Agm	=	agmatine
Azi	=	aziridine-2,3-dicarboxylate
catB	=	cathepsin B
catH	=	cathepsin H
catK	=	cathepsin K
catL	=	cathepsin L
cats	=	cathepsin S
CB/CL	=	ratio between second-order rate constants of catB and of catL
CB/PP	=	ratio between second-order rate constants of catB and of papain
ECM	=	extracellular matrix
Eps	=	epoxysuccinyl-2,3-dicarboxylate
iAm	=	isoamyl
OBzl	=	benzyl protective group
SAR studies	=	structure-activity-relation studies.

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