Protease Inhibitors in the Clinic

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Abstract: This review describes the clinical status (based on available information) of experimental drugs that inhibit enzymes called proteases, or more precisely a sub-class of proteases called peptidases that catalyse the hydrolysis of polypeptide main chain amide bonds. These peptidases are classified by the key catalytic residue in the active site of the enzyme that effects hydrolysis, namely aspartic, serine, cysteine, metallo or threonine proteases. In this review we show structures for 108 inhibitors of these enzymes and update the clinical disposition of over 100 inhibitors that have been considered worthy enough by pharmaceutical, biotechnology or academic researchers and their financial backers to be trialed in humans as prospective medicines. We outline some of their chemical and pharmacological characteristics and compare the current status of protease inhibitors in the clinic with what was observed about 5 years ago (Leung et al, J. Med. Chem. **2000**, 43, 305-341). We assess the progress of protease inhibitors into man, predict their futures, and outline some of the hurdles that have been overcome and that still remain for this promising class of new therapeutic agents.

Key Words: Protease, proteinase, peptidase, inhibitor, review, clinic.

1. INTRODUCTION

Proteolytic enzymes, known as peptidases or proteases (see www.protease.net.au and www.protease.net), are very important enzymes, accounting for ~2% of the genes in humans, infectious organisms, and other forms of life. Significantly, proteases regulate most physiological processes by controlling the activation, synthesis and turnover of all proteins. They are consequently pivotal regulators of conception, birth, growth, maturation, ageing, diseases and death. Genetic and environmental factors can disturb the balance of protease-catalysed human physiology leading to abnormal development, poor health, disease and death. Proteases are also essential for replication/transmission of viruses, parasites and bacteria that cause infectious disease in mammals; for the proliferation of insects and agricultural pests that transmit disease, damage plant crops, and spread infection through animal stocks; and for growth and yields of all marine and terrestrial food sources. Based on their importance in health and disease, protease inhibitors have already been developed into blockbuster drugs and diagnostics, many others are in clinical trials, and some proteases are themselves being trialed as vaccines or diagnostics. Proteases are also important in domestic industry, including the action of washing powders, cheese manufacture, meat tenderisation and processing, baking and as targets in plants, insects, and animals for new herbicides, pesticides, and growth regulators.

There are five classes of peptidase enzymes categorized by the catalytic residue that effects enzymatic hydrolysis, namely aspartic, serine, cysteine, metallo and threonine enzymes. All proteases bind their substrates in a groove or cleft, where amide bond hydrolysis occurs. Amino acid side chains of substrates occupy enzyme sub-sites in the groove, designated [1] as S3, S2, S1, S1', S2', S3', that bind to corresponding substrate/inhibitor residues P3, P2, P1, P1', P2', P3' with respect to the cleavable amide bond, as shown below (Figure **1**).

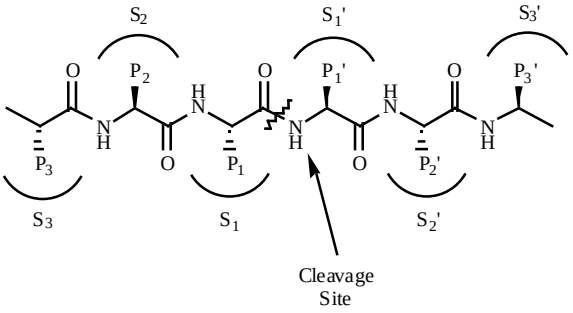

Fig. (1). Cartoon showing substrate/inhibitor residues (P) and protease binding sites (S). Prime and non-prime designations distinguish C-versus N-sides respectively of cleavage site.

Over 1500 crystal structures are available for peptidases in the pdb alone. These proteases are known to *universally* recognize their inhibitors/substrates in a shape equivalent to the peptide β-strand backbone conformation, [2-4] a property that should inspire the development of new generic approaches to β-strand mimics as protease inhibitors [5, 6]. Inhibitors of such proteases could potentially be useful in the treatment of diseases as diverse as cancer,[7-9] parasitic, fungal and viral infections (e.g. schistosomiasis, [10, 11] malaria, [12, 13] *C. Albicans*, [14, 15] HIV, [16-18] Hepatitis, [19, 20] Herpes [21, 22]); inflammatory, immunological, respiratory, [23-26] cardiovascular [27] and neurodegenerative disorders including Alzheimer's disease [28, 29].

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Many protease inhibitors have displayed promising therapeutic activities in preclinical trials in animals and in early clinical trials in man for viral and parasitic infections; cancer; inflammatory, immunological, and respiratory conditions; [23-26] cardiovascular [27] and degenerative disorders including Alzheimer's disease [28-30]. However despite a great deal of research over the last two decades, there are relatively few protease inhibitors that have successfully progressed through clinical trials and are currently available as relatively safe and effective medicines for humans. They include ACE inhibitors for treating high blood pressure, HIV-1 protease inhibitors for treating HIV/AIDS, thrombin inhibitors for treating stroke, and an elastase inhibitor for treating systemic inflammatory response syndome (SIRS). This relatively low success rate raises questions as to whether protease inhibitors are truly realising their earlier promise, and prompts us to review now the recent clinical status and future prospects of protease inhibitors. Is the development of protease inhibitors stagnating or is it still maturing, and is it likely to realise its promise of delivering efficacious therapeutics for improving the human/mammalian condition?

This review now describes some of the better studied small molecule inhibitors of aspartic, serine, cysteine, metallo and threonine proteases, illustrating briefly how effectively they inhibit enzymes, exert pharmacological properties (where reported), and their stage of clinical development as potential new medicines. Much of the information had to be obtained through literature clues from, or access to, the Clinical Trials Database: Pharmaprojects (Sept. 2004), Annual Reports in Medicinal Chemistry 1998-2003, recent conference abstracts, or company websites and press releases because pharmaceutical companies often do not report their data in peer-reviewed publications, or at least in a timely manner. We analyse herein some of the progress made in recent years to advance experimental protease inhibitors through clinical trials and into man for the treatment of a wide variety of diseases that require 21st Century medical solutions. We also outline some of the hurdles that have been overcome and that still remain for this class of new therapeutic agents, and make some predictions for the future of protease inhibitors as therapeutics.

2. SERINE PROTEASE INHIBITORS

A well accepted classification system for serine proteases is based on the nature of the P1 residue in their peptide substrates. [31-34] Three major classes are designated as trypsin-like (positively charged residues Lys/Arg preferred at P1), elastase-like (small hydrophobic residues Ala, Val at P1) or chymotrypsin-like (large hydrophobic residues Phe/Tyr/Leu at P1) [35]. A catalytic triad of residues (Ser195, His57 and Asp102, chymotrypsin numbering system) is responsible for amide bond hydrolysis.

2.1. Thrombin

Thrombin is a serine protease that plays a central role in blood coagulation and fibrinolysis [36]. This enzyme is also a potent inducer of platelet aggregation due to its activity on the protease activated receptor-1 (PAR-1) [36-38]. It is a member of the trypsin family of serine proteases and the final enzyme in the blood coagulation cascade. Thrombin, through its actions on factors V, VIII, XIII and fibrinogen, leads to the generation of fibrin which subsequently polymerises to form the core of a blood clot. The X-ray crystal structures of thrombin complexed with the irreversible and reversible inhibitors PPACK and NAPAP [39, 40] have revealed an aspartic acid residue Asp189 which sits at the bottom of the S1 pocket, explaining its preference for Arg/Lys at P1 in its substrates. An occluding loop composed of amino acids Y-P-P-W restricts access to S2 subsites, therefore thrombin prefers small hydrophobic residues, especially proline at P2. The S3 pocket can bind larger residues (e.g. D-Phe).

Only a few anticoagulant strategies are in clinical use to control hyper-coagulation by excessive thrombin activity or generation. Vitamin K agonists (eg. Warfarin) have been used since the 1950's, but administration needs careful monitoring due to a narrow therapeutic range and various food and drug interactions. Heparin and Low Molecular Weight Heparin (LMWH), which act to increase the activity of antithrombin, also need to be monitored clinically due to variability in dose efficacy, need antithrombin as a cofactor, and are unable to inhibit clot-bound thrombin. These factors result in limited efficacy in these anticoagulant drugs, and therefore an enormous effort over the last twenty years has been invested in the design of low molecular weight, selective and orally bioavailable direct inhibitors of thrombin. [37, 38, 41] The compounds described below have either been approved or are in clinical trials in 2004.

Melagatran (**1**) and its ethyl ester-amidoxime prodrug, [42] *Ximelagatran* (**2**) are the result of a 20 year research program directed towards the inhibition of thrombin by AstraZeneca. An excellent review describing the history of the development of these compounds has been published recently [43]. Melagatran is a non-covalent thrombin inhibitor that is currently in pre-registration for the treatment of general thrombosis and as an adjunct to surgery. It is a potent inhibitor *in vitro* (Ki, thrombin 2nM) but not very selective over the similar enzyme trypsin (Ki, trypsin 3.6 nM). Melagatran is not metabolised in the body, 80% being cleared by the kidneys and excreted in the urine. It has shown promise as an antithrombotic in animals models of thrombosis [44] and in clinical trials involving orthopedic surgery and deep vein thrombosis [45]. However, **1** has very low oral bioavailability (~5%, man). Plasma levels after oral administration are variable and unpredictable and there is pronounced food interaction, thus making **1** unsuitable for oral administration.

Ximelagatran (Exanta) (**2**) was developed in order to increase the bioavailability of melagatran without resorting to developing a new thrombin inhibitor [46]. Introducing ethyl ester and hydroxyamidine functions into the molecule increased lipophilicity by 170 times with a Ki 360nM for thrombin. Consequently, this prodrug is rapidly absorbed and metabolised to melagatran (bioavailablity ~20%) [47]. Ximelagatran is the first orally bioavailable inhibitor to reach the market (launched in 2004). It is available in the UK for treating venous thrombosis and is undergoing trials for cerebral ischaemia and myocardial infarction.

Table 1. Serine Protease Inhibitors In Clinical Development.

Target	Indication	Drug Name	Company Name	Clinical Status
Thrombin	Venous Thrombosis	Ximelagatran	Astra Zeneca	Launched
	Thrombosis, general	Melagatran	Astra Zeneca	Pre-registration
	Arterial Thrombosis	Argatroban	Mitsubishi Pharma	Launched
	Venous Thrombosis	BIBR-1048	Boehringer Ingelheim	Phase III
	Thrombosis, general	MCC-977	Mitsubishi Pharma	Phase II
	Thrombosis, general	TGN-167, TGN-255	Trigen	Phase I
	Thrombosis, general	SSR-182289	Sanofi-Synthlabo	Phase I
	Thrombosis, general	AZD-0837	Astra Zeneca	Phase I
	Thrombosis, general	E-5555	Eisai	Phase I
	Venous Thrombosis	LB-30870	LG Life Sciences	Preclinical
Factor Xa	Thrombosis, Angina	DX-9065a	Daiichi	Phase II
	Venous thrombosis	DPC-906	BMS	Phase II
	Thrombosis	CI-1031	Berlex Biosciences	Phase II
	Venous thrombosis	JTV-803	Japan Tobacco	Phase II
NS3-protease	Hepatitis C Virus Infection	BILN-2061, Ciluprevir	Boehringer-Ingleheim	Phase II
	Hepatitis C Virus Infection	VX-950	Vertex	Phase I
Elastase	SIRS, Inflammation,	Sivelestat, Elaspol	Ono	Launched (Japan)
	COPD	Midesteine	Medea Research	Pre-registration (Italy)
	COPD	AE-3763	Dainippon	Pre-clinical
	COPD	R-448	Roche	Phase I
Broad-Spectrum	Pancreatitis, Inflammation	Nafamostat, FUT-175	Japan Tobacco	Launched
	Pancreatits	Camostat mesilate	Ono	Launched
Urokinase	Cancer, Gastrointestinal	WX-UK1	Wilex	Phase II
Chymase	Restenosis	NK-3201	Nippon Kayaku	Preclinical
DPP IV	Diabetes Type II	LAF-237	Novartis	Phase III
	Diabetes	MK-0431	Merck	PhaseII
	Diabetes	P32/98 (P3/01)	ProBiodrug	Phase I
	Diabetes	T-6666	Tanabe Seiyaku	Phase I
	Diabetes	NN-7201	Novo-Nordisk	Phase I

Argatroban (**3**), a potent non-covalent reversible thrombin inhibitor (K_i 39 nM), has been launched for treating arterial thrombosis, peripheral vascular disease, thrombocytopenia, cerebral ischemia and stroke. Originally developed by researchers at University of Kobe (Japan), Mitsubishi Pharma, and Daiichi Pharmaceutical, **3** has been used in Japan since the early 1980's. It is a 64:36 mixture of 21-(*R*) and 21-(*S*) diastereomers, with the latter being twice as potent as the former in an *in vitro* coagulation assay, but less soluble in aqueous buffer [48]. **3** lacks oral bioavailability, has a short duration of action ($t_{1/2}$ 40 min, man), and is therefore used intravenously [49, 50].

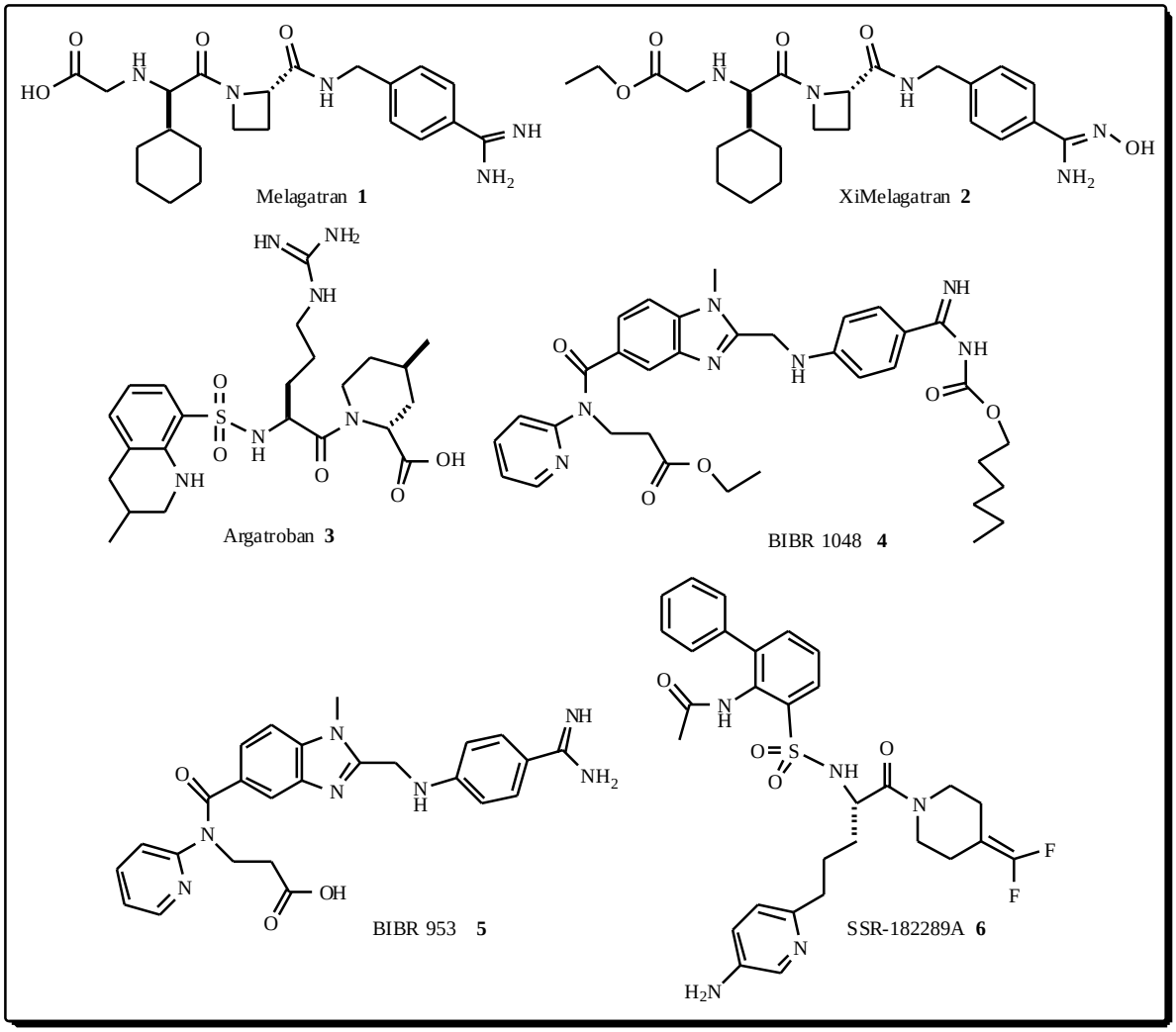
BIBR 1048 (**4**) is an orally active, non-covalent thrombin inhibitor developed at Boehringer Ingelheim. The compound is in phase III trials for venous thrombosis. Compound **4** was developed through structure-based drug design from the crystal structure of NAPAP (IC_{50} 0.2 μM) [40]. Modification of this lead by replacing the central glycine residue with a tri-substituted benzimidazole led to *BIBR 953* (**5**) (IC_{50}

thrombin 9.3 nM; trypsin 50.3 nM). This compound lacked oral bioavailability but was an active anti-coagulant *in vivo*. It was orally active (~5%, $t_{1/2}$ ~12h) after conversion to the double ester pro-drug **4** [51,52].

SSR-182289A (**6**) is an orally active, non-covalent thrombin inhibitor developed by Sanofi-Synthelabo through rational drug design from argatroban. Unlike prodrugs **2** and **4**, **6** does not require metabolism to an active form to achieve oral activity. Compound **6** displays potent activity against thrombin (Ki 31 nM) and is very selective over other enzymes of the clotting cascade (Ki > 50 μM), or trypsin (Ki 54 μM) and chymotrypsin (Ki 580 nM). Its anticipated binding in the active site of thrombin has been published [53]. Compound **6** inhibited thrombin-induced aggregation of human platelets (IC_{50} 32 nM). In dogs, 1-5 mg/kg produced dose-related increases in clotting times, with a maximum anti-coagulant effect after 2 h and lasting for 3-5 h [54]. SSR-182289A displays antithrombotic activity in a number of animal models, including the rat [55], rabbit [56] and macaque. It is in phase I clinical trials for general thrombosis.

Trigen, a UK-based pharmaceutical company, has two compounds *TGN-255* (parenteral formulation) and *TGN-167* (oral formulation) [57] in phase I clinical trials for the treatment of thrombosis. They are salts/esters of *TRI50c* (**7**, free boronic acid) which is the active principle. TRI50c is a highly potent (Ki 22nM) and selective competitive inhibitor of thrombin that has high efficacy in animal models of venous and arterial thrombosis with minimal effect on bleeding [58]. It is an electrophilic inhibitor that forms a reversible covalent bond between boron and the active site serine of thrombin.

LB-30870 (**8**) is a more recent compound designed and developed by the Korean company, LG Life Sciences Ltd. Clinical trials of a former developmental candidate *LB-30057* (**9**) (Ki 0.4 nM) were suspended in 2003, due to its short duration of action and low efficacy in Phase I. The design of LB-30870 is based on non-covalent inhibitor D-Phe-Pro-agmatine and incorporates a thienylamidine to replace the more basic agmatine residue. Although this compound is a potent inhibitor of thrombin (Ki 15 pM), it still has good potency for trypsin (Ki 300 pM). It does



however display good oral pharmacokinetic profiles in rats, (10mg/kg, $t_{1/2}$ 47min, bioavailability 43%) dogs (5mg/kg, C_{max} 3.8 μg/mL, $t_{1/2}$ 68 min, bioavailability 42%) and monkeys (20 mg/kg, $t_{1/2}$ 58 min, bioavailability 15%) [59].

Researchers at Johnson and Johnson have developed non-covalent oxyguanidine inhibitors of thrombin, examples including **10** and **11** which are orally bioavailable. The oxyguanidine functional group is much less basic (pKa 7.2) than guanidine and contributes to reasonable pharmacokinetics for these compounds. A 10 mg/kg p.o. dose of **11** (Ki 9nM, thrombin) in beagle dogs gave Cmax 4.5μM after 1h, and F 62% [60]. Compound **10** (Ki 12nM) was more orally bioavailable (95%, dog) [61].

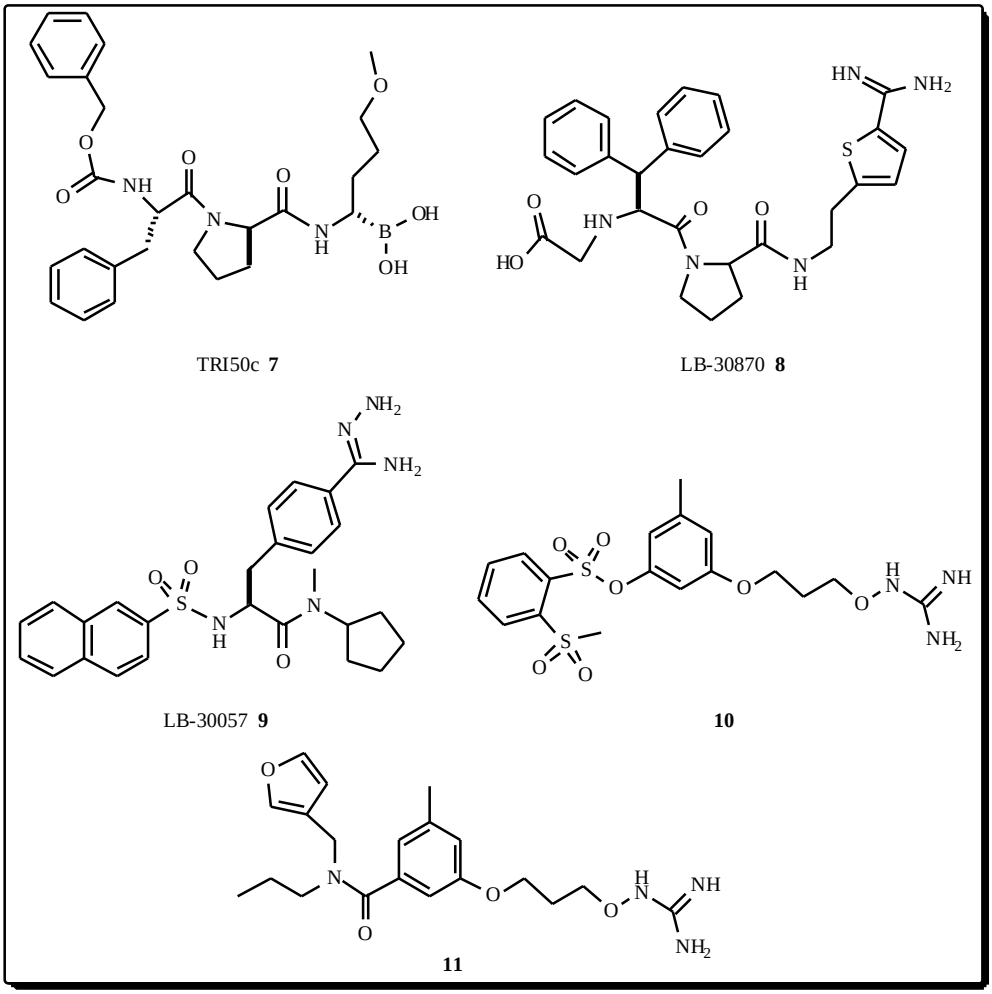
Other thrombin inhibitors reported to be in clinical trials, but for which no structure or pharmacokinetic data is reported, include *MCC-977* (Mitsubishi Pharma) in phase II trials for general thrombosis, *E-5555* (Eisai, Japan) in phase I trials, and *AZD-0837* (AstraZeneca), structure probably based on modification of Ximelagatran, which is in phase I trials.

Since (Leung *et al*, *J. Med. Chem*., **2000**, *43*, 305) when Argatroban, Napsagatran, RO46-6240, Inogatran, Efegatran, CVS-1123, DuP714, UK156406 and Melagatran were discussed, only Argatroban and Melagatran have progressed while clinical trials for the others appear to have been discontinued or halted.

2.2. Factor Xa

Factor Xa is trypsin-like serine protease that is at the convergence of the intrinsic and extrinsic coagulation pathways of the blood coagulation cascade. It is responsible for cleaving pro-thrombin to thrombin [62]. To date no factor Xa inhibitors have been realised as therapeutic agents, but they could conceivably become valuable anticoagulants in the future since they demonstrate good anti-thrombotic activity without the bleeding complications associated with thrombin inhibitors. Research in this area has been very fruitful over the last five years with 4 inhibitors in phase II clinical trials.

DX-9065a (**12**, K_i = 41 nM) is an inhibitor of factor Xa developed by Daiichi pharmaceutical. Currently it is in Phase II clinical trials in Japan and the USA for the treatment of general thrombosis and unstable angina via subcutaneous administration. It is an amidinonaphthalene



derivative with poor oral activity but highly selective over other serine proteases (e.g. K_i(thrombin) > 2000 μM; (chymotrypsin) > 2000 μM; (trypsin) 0.62 μM) [63,64]. It does not affect platelet aggregation, has prolonged anti-factor Xa activity and inhibition of thrombin generation, causes a reduction in tissue factor-induced mortality in mice (ED_{50} 56 mg/kg), and inhibits stasis-induced thrombosis in rabbits (ED_{50}(i.v.) 0.03 mg/kg; (s.c.) 0.3 mg/kg; (p.o.) 50.5 mg/kg) [65]. A crystal structure of **12** bound to the enzyme has been reported [66]. The naphthamidine group binds in the S1 pocket via a salt bridge to Asp189. Hydrophobic interactions around the pocket also contribute to the high binding affinity, while the pyrrolidine ring binds to the other major interaction site at S4. In a phase I clinical study with ^{14}C labelled **12**, a 10 mg intravenous dose was found to give a plasma concentration of 380 ng/mL after 1 hour with a $t_{1/2}$ 7 h. DX-9065a was excreted in the urine (77%) and faeces (5%) unchanged, with no evidence of other radioactive metabolites after 14 days [67].

Razaxaban (**13**,DPC-906, BMS-561389) is a factor Xa inhibitor in development by Bristol-Myers Sqibb for venous thrombosis and is in phase II trials. It is a potent, selective non-covalent inhibitor of Factor Xa (Ki; factor Xa 0.19nM, thrombin 540nM), with IC_{50} values >2 μM against all other enzymes in the coagulation cascade. In dogs at 0.2 mg/kg p.o. it was 84% bioavailable. A crystal structure of the inhibitor bound to factor Xa reveals the aminobenzisoxazole group of the inhibitor binding to the S1 subsite and phenylimidazole group in the S4 sub-pocket. Razaxaban inhibited thrombus formation in a dose-dependant manner with an ID_{50} 1.6 μmol/kg/h in a rabbit arterio-venous shunt thrombosis model [68].

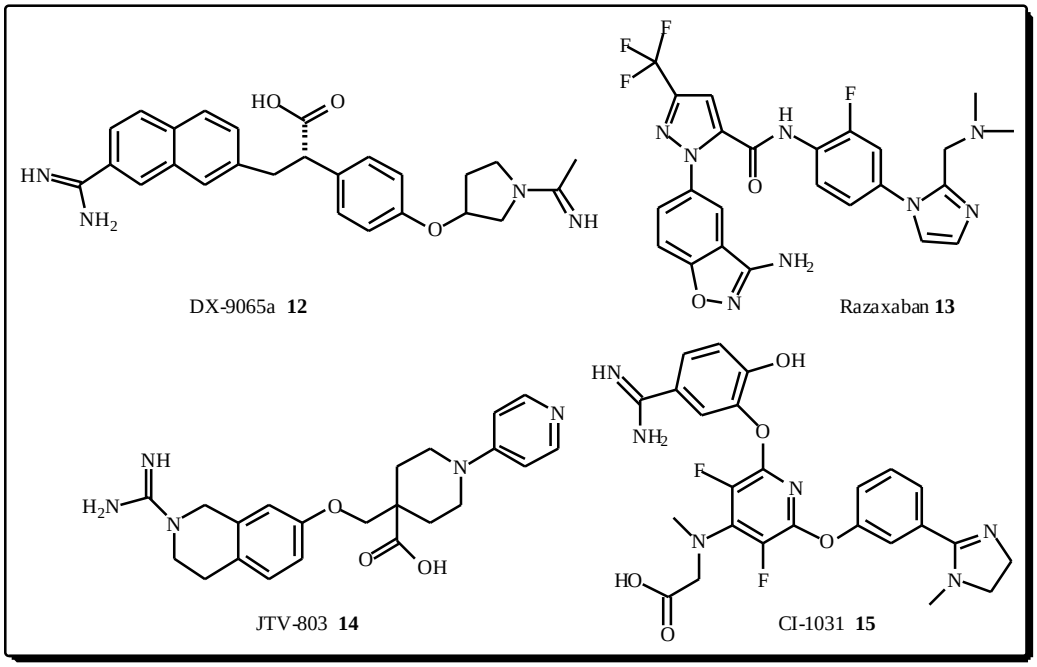
JTV-803 (**14**) was discovered via a structure-based drug design effort by Japan Tabacco. This compound is in phase II trials for treating general thrombosis via intravenous administration. JTV-803 is a potent (Ki 19nM) and selective inhibitor of factor Xa and 100 times less active against for thrombin, plasmin and trypsin. In addition, **14** displayed > three times better selectivity for human factor Xa as compared to activated factor Xa of monkey, rat, dog and hamster. In cynomologous monkeys a 10mg/kg dose gave C_{max} 0.39 μg/mL and $t_{1/2}$ 3.6h [69]. An oral formulation of **14** (JTC-803) was discontinued after phase I trials.

CI-1083 (ZK-807834,**15**) is an aminopyridine based Factor Xa inhibitor (Ki 110pM) in development by Berlex Biosciences. It is in phase II trials for venous thrombosis and unstable angina by intravenous administration. A crystal structure of **15** bound to the active site of factor Xa shows the benzamidine binding in S1 and the imidazoline ring in S4 [70]. Interestingly, this compound displays > 2500 times selectivity for factor Xa over trypsin (Ki 280nM) or thrombin (Ki 2μM). It is however not suitable for oral administration due to its negative log D value (-0.40) and poor cell permeability [71].

2.3. Hepatitis C NS3 Protease

Chronic infection by the flavivirus hepatitis C can lead to progressive liver injury, cirrhosis and liver cancer. A serine protease known as NS3 protease is thought to be essential for viral replication and has become a target for anti-HCV drugs [72]. A crystal structure shows that the NS3 protease is monomeric with two domains, a trypsin-like fold, and a structural zinc-binding site [20,73,74]. Its substrate specificity is however different from cellular serine proteases, with a cysteine residue in the P1 position of peptide substrates [75,76]. NS3 protease is becoming the most studied target for the development of anti-HCV therapeutics.

Ciluprevir (BILN-2061,**16**) is a non-covalent inhibitor of the HCV NS3 protease and has been developed for treating hepatitis C by Boehringer Ingelheim. It has passed phase I



trials. Compound **16** was developed from hexapeptide H-DDIVPC-OH (IC_{50} 71 μM), which is an N-terminal cleavage product of a peptide substrate [77, 78]. BILN-2061 displays good affinity and potency *in vitro* (IC_{50} 3 nM, ED_{50} 1 nM) and has a good pharmacokinetic profile in rats (20mg/kg p.o., C_{max} 2.5μM, bioavailability 42%) [78]. In a phase I trial in humans, **16** was well tolerated at 5-2000 mg p.o. with no adverse effects and $t_{1/2}$ 4h. In HCV infected patients, a 200 mg twice daily treatment for 2 days resulted in a rapid decline in viral load, reaching undetectable levels in 24 hours after administration [79]. Although these results are encouraging, long term toxic effects still need to be determined. One animal study using very high doses of **16** revealed some side-effects which may impede its progress [80].

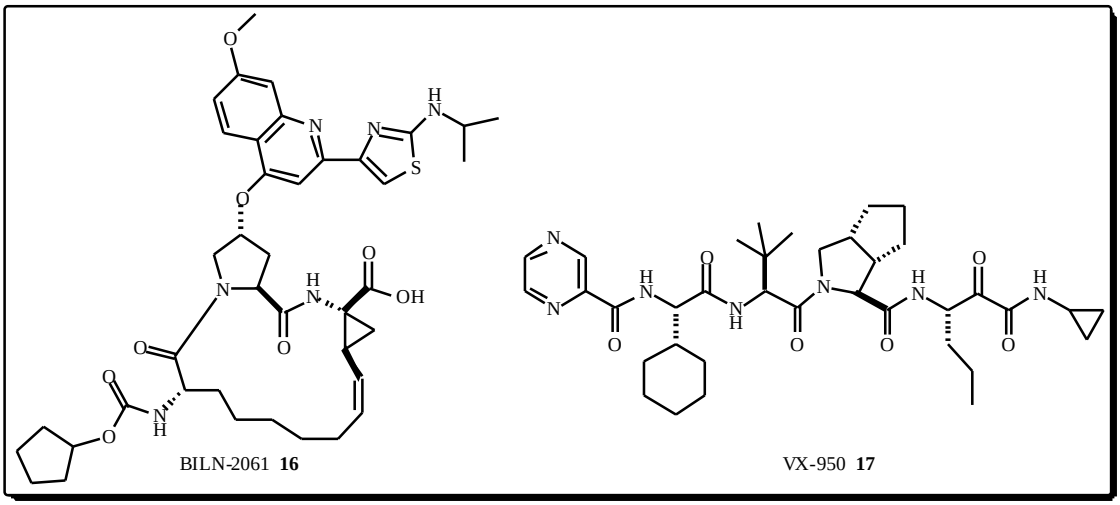
VX-950 (**17**) is an HCV NS3 protease inhibitor recently disclosed by Vertex pharmaceuticals, and currently in phase I clinical trials. It displays apparent non-competitive inhibition and tight binding [81]. The active site serine of the enzyme forms a reversible covalent bond to the α-keto amide of **17**, which was derived through structure-based drug design methodology, based on the natural NS5A-NS5B substrate cleavage site. VX-950 (Ki 44 nM, HCV NS3 protease) is orally bioavailable in man and several other species with a favourable pharmacokinetic profile. The drug is concentrated in the liver due to first pass hepatic extraction, which should be beneficial for treatment of HCV infection [82]. Interestingly, an *in vitro* resistance study has recently been published [83] comparing the actions of VX-950 and BILN-2061 on HCV. Drug resistant NS3 protease mutants were identified for both inhibitors. The major BILN-2061 resistant mutations occurring at Asp-168 were fully susceptible to VX-950. In addition, the dominant resistant mutation at Ala-156, induced by VX-950 remained susceptible to BILN-2061. This result suggests that much like HIV protease, combination therapy will be the most likely strategy for future treatment of HCV.

2.4. Elastase

Elastase (EC 3.4.21.37) is a serine protease implicated in adult respiratory distress syndrome (ARDS), rheumatoid arthritis, pulmonary emphysema, cystic fibrosis and chronic bronchitis. Human neutrophil elastase is released from human polymorphonuclear leukocytes (PMN) in response to inflammatory stimuli, and is responsible for the degradation of connective tissue proteins such as collagen, elastin, laminin, fibronectin and proteoglycan. Normally elastase activity is tightly regulated by endogenous inhibitors such as secretory leukocyte protease inhibitors (SLPI) and α1-protease inhibitors, but an imbalance between proteases and anti-proteases can lead to degradation of healthy tissue and disease development. Many inhibitors of elastase have been reported during the past 20 years [84], but not much clinical progress has been made.

Sivelestat (ONO-5046,**18**) is a competitive inhibitor (K_i 0.2 μM, IC_{50} 0.044 μM) which suppresses lung damage induced in hamsters by elastase administered intratracheally (ID_{50} 82 μg/kg) [85]. Interestingly, when this drug was injected (100 mg/kg/day/i.p.) to arthritic rats, it protected cartilage from degradation and reduced the incidence and severity of collagen-induced arthritis [86,87]. This compound has also been investigated for lung cancer [88] and idiopathic interstitial pneumonia [89]. Unfortunately, the bio-availability for **18** is low (1.5%, rat) which is mainly due to intestinal first pass metabolism [90]. Sivelestat has been launched in Japan (2002) as an injectable formulation (Elaspol) for the treatment of acute lung injury associated with systemic inflammatory response syndrome (SIRS).

Midesteine (MR-889,**19**) is a reversible, slow binding, fully competitive serine protease inhibitor. It has some selectivity for neutrophil elastase (Ki 1.4 μM), but also inhibits chymotrypsin. This compound is currently in pre-registration (Italy, Medea Research) for the treatment of chronic obstructive pulmonary disease (COPD). The active site serine of elastase is thought to attack the molecule at its thio-ester forming a thiophencarbonyl acyl-enzyme complex, which resists further hydrolysis and effectively inhibits the enzyme. A crystal structure of the thiophencarbonyl adduct supports this hypothesis [91]. A double-blind, randomised, placebo-controlled clinical trial in COPD patients showed that the compound was well tolerated at doses of 500 mg b.i.d for 4 weeks [92]. Further studies have not been published.



BILN-2061 **16** VX-950 **17**

AE-3763 (**20**) is in preclinical development by Dainippon (Japan). It is a reversible, covalent human neutrophil elastase inhibitor (IC_{50} 29nM) which utilises a trifluoromethyl ketone moiety for interaction with the active site serine. The compound is not effective orally, however i.v. bolus (ED_{50} 1.3 mg/kg) or infusion (ED_{50} 0.42 mg/kg/h) was found to reverse HNE-induced haemorrhage in hamsters [93]. Another compound that is reportedly in phase I trials is *R-448* (Hoffman-La Roche) an elastase inhibitor for treating COPD, but no structural or pharmacokinetic data is available.

2.5. Urokinase-Type Plasminogen Activator

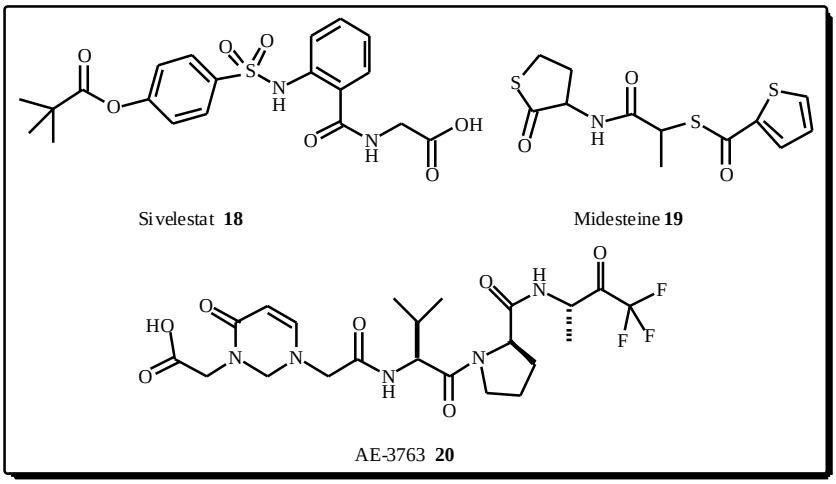
The urokinase-type plasminogen activator is a serine protease that converts plasminogen to plasmin. Plasmin is a protease of broad specificity that digests various components of the extracellular matrix, including fibronectin, laminin, collagen type I,V and also activates pro-enzyme forms of matrix metalloproteases. In tumour cells uPA is directed to the cell surface via a specific receptor (uPAR). Urokinase-type plasminogen activator, in complex with uPAR, retains its proteolytic activity and the conversion of plasminogen to plasmin increases several fold at the surface of cancer cells. Increased expression of uPA in tumour tissues is highly correlated with tumor cell migration, invasion, proliferation, progression and metastasis. Urokinase inhibitors are considered to be attractive targets for preventing metastasis of tumours [94-96].

Human urokinase (uPA) is a trypsin-like, arginine-specific serine protease. Therefore most attention has been directed to the development of arginine mimetics that can interact with the Asp-189 residue, located at the bottom of the S1 binding pocket. So far, *WX-UK1* (**21**) is the most advanced of these compounds being in phase II i.v. clinical trials for cancer (Wilex). WX-UK1 is a low molecular weight 3-amidinophenylalanine with moderate activity (Ki uPA 410 nM), but is not very specific (Ki : plasmin 390 nM, thrombin 490 nM, trypsin 37 nM) [97]. Nevertheless, *in vitro* invasion models using fibrocarcinoma and breast cancer cells showed that **21** effectively inhibited cellular migration through fibrin matrices [97]. In addition, a 50% decrease in tumour cell invasion capacity has been reported in two cancer cell lines when treated with **21** [98]. Various crystal structures have been published with inhibitors bound in the active site of uPA [99, 100]. A recent series of 2-naphthamidine inhibitors of uPA has also been described, the most selective being **22** (Ki uPA 40 nM, trypsin 2 μM, plasmin 16 μM) [101].

2.6. Dipeptidyl Peptidase IV

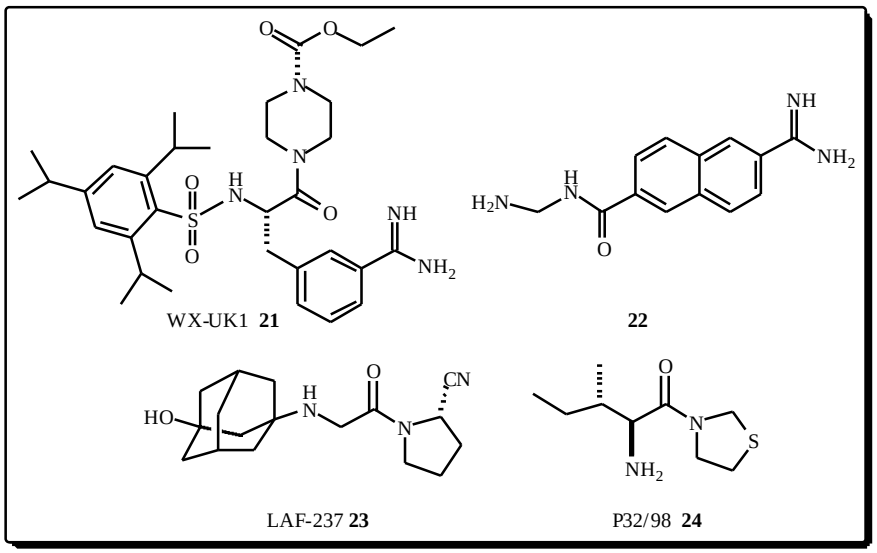
The incretin, human glucagon like peptide 1 (GLP-1), stimulates insulin release and biosynthesis and inhibits glucagon release. GLP-1 is rapidly degraded *in vivo* by dipeptidyl peptidase IV (DPP-IV), which cleaves the two N-terminal amino acids to give an inactive form. In recent years there has been increased effort to develop inhibitors of DPP IV for the treatment of Type II diabetes. DPP-IV is a membrane bound serine protease predominantly found in the kidneys, liver, pancreas, thymus and spleen. A soluble form is also shed into the circulation. DPP-IV has a strong preference for a proline residue at the P1 position in its substrates and accepts just about any residue at P2 and in prime side positions. Most inhibitors to date have incorporated a proline or proline mimetic at P1, with either a reversible or irreversible electrophilic isostere to interact with the active site serine of the enzyme [102]. So far, two of these inhibitors (**23**, **24**) have progressed in clinical trials.

LAF-237 (**23**) is a DPP-IV inhibitor under development by Novartis and is in Phase III clinical trials. It is a potent inhibitor of DPP-IV and is very selective with respect to the homologous peptidases, post-proline-cleaving enzyme (PPCE) and DPP-II (IC_{50}: DPP-IV 3.5 nM, DPP-II 500 μM, PPCE 210 μM). The nitrile of **23** forms a reversible covalent imidate with the serine hydroxyl of DPP-IV. Oral administration of **23** (10 μmol/kg, p.o.) in obese Zucker rats inhibited plasma DPP-IV activity by 90% and increased plasma GLP-1 levels by 60%. A glucose challenge after drug administration showed that **23** was able to significantly decrease glucose excursions and stimulate insulin secretion. In cynomolgus monkeys oral bioavailability was >90% following 1 μmol/kg, C_{max} 293 nM, T_{max} 72 min, $t_{1/2}$ 90 min. Maximum inhibition of DPP-IV (95%) occurred after 2 h and persisted for 10 h. The compound has commenced phase III trials as a once-a-day treatment for diabetes [103].



Sivelestat **18** Midesteine **19**

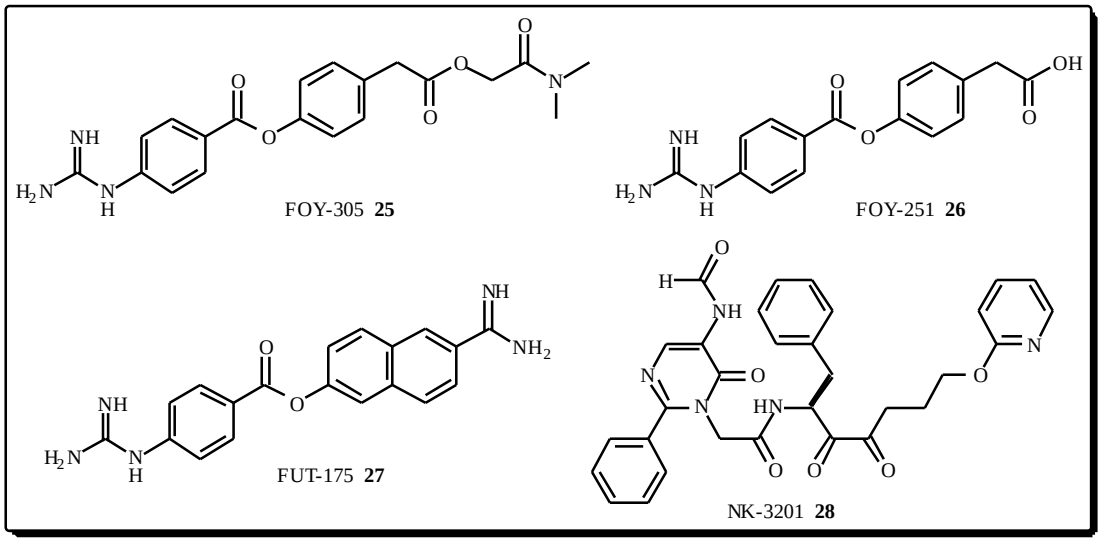
AE-3763 **20**



P32/98 (P93/01, **24**) is a non-covalent inhibitor of DPP-IV (Ki 126 nM), [104] being developed by Probiodrug in phase II trials as an oral antidiabetic. The molecule lacks an electrophile and thus does not interact covalently with the enzyme. Administration of **24** to VDF Zucker rats resulted in a progressive decrease in both fasting and peak blood glucose levels with respect to control [105]. A recent study in Wistar rats showed that **24** displayed a cytoprotective effect upon streptazotocin induced destruction of pancreatic β-cells [106]. In clinical studies, **24** was reported to be safe and well tolerated up to 240mg doses in healthy volunteers, [107] with a therapeutic effect observed for 60mg given to diabetic patients [108]. Other compounds reportedly in clinical trials, but for which no structure or data have been reported, include *T-6666* (Tanabe Seiyaku), a fluoropyrrolidine inhibitor of DPP- IV in phase 1; *MK-0431* (Merck) in Phase II; and *NN-7201* (Novo Nordisk), a xanthine based inhibitor in Phase I.

2.7. Multiple Serine Proteases

Camostat Mesylate (Gabexate Mesylate, FOY-305,**25**) and its active metabolite *FOY-251* (**26**) are potent small molecule broad spectrum inhibitors of mutliple serine proteases. They not only inhibit the hydrolytic activities of thrombin, but also inhibit trypsin, kallikrein, plasmin, C1 esterase, phospholipase A2 and prostaglandin synthesis. Camostat (**25**) has been used for several years in clinical therapy of acute pancreatitis and disseminated intravascular coagulation [109]. Camostat has also been shown to modulate the immune response in an experimental model of lung injury by acute pancreatitis [110]. It significantly improved mortality in severe pancreatitis and achieved significantly earlier recovery of abdominal pain, hyperamylasmia and leukocytosis in mild to moderate pancreatitis [111]. In a pilot clinical trial, where the effect of **25** on blood loss in cardia valve replacement surgery was



assessed, it was concluded that **25** may be a promising alternative to aprotinin [112]. *Nafamostat* (FUT-175,**27**) is a closely related naphthamidine derivative of camostat, and is also widely used for the treatment of acute pancreatitis and disseminated intravascular coagulation in Japan. Compound **27** can also exert beneficial effects on glomerulonephritis with hypocomplementemia by inhibiting complement activation [113]. This compound is even more potent than **25** in its inhibitory effect on other proteases (IC_{50}; thrombin 1.9 μM, plasmin 2.9 μM, collagenase 0.42 mM, cathepsin D > 1 mM, factor Xa 2.1 μM). It inhibits tryptase 1000x more potently (Ki_{app} 95 pM) than camostat (Ki_{app} 95 nM) and it has been suggested that beneficial effects *in vivo* might be due to tryptase inhibition[114]. However, it has been reported to cause hyperkalemia because of reduced urinary excretion of potassium [115]. FUT-175 also caused 50% inhibition of the production of C3a and C5a generated by C3/C5 convertase activity at 4 μM [116].

2.8. Chymase

Chymase is a chymotrypsin like serine protease that is present in the secretory granules of mast cells. In general it cleaves substrates with Phe, Tyr or Trp at the P1 position. It has been recently shown that human and dog chymases are able to cleave the Phe^8-His^9 amide bond of Angiotensin I to give Angiotensin II, while the rat chymase cleaves Angiotensin I at the Tyr^4-Ile^5 yielding inactive fragments [117]. A recent paper has shown that chymase-dependent angiotensin II formation plays an important role in the development of vascular proliferation after injury [118]. Inhibitors of this enzyme may therefore be useful for preventing vascular proliferation after surgery.

NK2301 (**28**) is an orally active chymase inhibitor currently under development by Nippon Kayaku for the treatment of various chronic diseases including restenosis (the re-narrowing of a heart valve after corrective surgery). The compound presumably interacts reversibly with the active site serine by forming a hemi-acetal with the β-diketone portion of the molecule. It inhibits human, dog and hamster chymases (IC_{50} 2.5, 1.2, 28 nM respectively) and does not inhibit ACE at 100μM. In dogs, the concentration of **28** in plasma, heart and aorta was 470, 195, and 78nM respectively 8 h after oral administration of a 1 mg/kg dose [119]. Furthermore, oral administration in dogs (1mg/kg per day p.o.) was found to significantly reduce (50%) the development of intimal hyperplasia in balloon injured arteries [118].

3. THREONINE PROTEASE INHIBITORS

The only enzymes that utilise a threonine residue as a catalytic nucleophile for amide bond hydrolysis are the proteasomes. High levels of proteasomes are found in both the nucleus and cytoplasm of all eukaryotes. They are barrel-shaped proteins whose primary function is the degradation of ubiquitin-labelled proteins, aiding the removal of damaged proteins and the regulation and turnover of many critical proteins that control cellular function. Proteasomes are multifunctional, possessing three distinct cleavage preferences; trypsin-like (Arg/Lys at P1), chymotrypsin-like (Phe/Tyr/Leu at P1), and caspase-like (Asp/Glu at P1) [120]. Proteasome inhibitors can stabilise many cell cycle inhibitory proteins and can cause cell cycle arrest and apoptosis. As it has been found that cancer cells are more susceptible to the proapoptotic effects of proteasome inhibitors than normal cells, there has been great interest in the development of proteasome inhibitors in recent years [121-123].

Bortezomib (Velcade, MLN-341, **29**) was developed by Millenium Pharmaceuticals as an anti-cancer agent and was launched in the USA in 2003. It is also registered in the EU for treating relapsed and refractory multiple myeloma via i.v. administration. Its use in other forms of cancer is under investigation. Bortezomib is a potent and very selective inhibitor of the chymotrypsin activity of the proteasome (Ki : 20S proteasome 0.62 nM, HLE 2.3 mM, cathepsin G 630 nM, chymotrypsin 320 nM, thrombin 13 μM). It is a dipeptide boronic acid that interacts covalently and reversibly with the active-site threonine hydroxyl of the proteasome. The compound was discovered through substrate-based design from tripeptide aldehyde inhibitor Cbz-LLL-H (Ki 4 nM). Replacement of the aldehyde with electrophilic isosteres revealed that boronic acid derivatives gave optimal activity [124]. The compound is not devoid of side-effects. A steep dose-toxicity effect was observed at doses ≥ 0.9mg/m^2 in rat and ≥ 3mg/m^2 in monkeys, resulting in cardiovascular collapse and death. In humans, a phase I trial on 43 patients showed that **29** could be administered at 0.13 - 1.56 mg/m^2/dose with minimal side-effects, including diarrhoea and sensory neurotoxicity, fatigue, fever, anorexia, nausea, vomiting, rash, pruritus, and headache [125]. The compound has a $t_{1/2}$ 9-15 hours at 1.45-2.00 mg/m^2. In a phase II trial in patients with relapsed or multiple myeloma, a 35% response rate was observed including 4% with a complete response. [126] Accelerated approval of **29** was granted by the FDA for treating multiple myeloma in patients who have received at least two prior therapies, but

Table 2. Threonine Protease Inhibitors In Clinical Development.

Target	Indication	Drug Name	Company Name	Clinical Status
Proteasome	Myeloma cancer	Bortezomib	Millenium	Launched
	Cerebral Ischaemia	MLN-519	Millenium	Phase I
	Anticancer	ER-807446	Eisai (Japan)	Pre-clinical
	Anticancer	TMC-95A		Pre-clinical

show no improvement. Recommended dose of **29** is 1.3 mg/m^2 administered twice weekly for two weeks followed by a 10 day rest period [127].

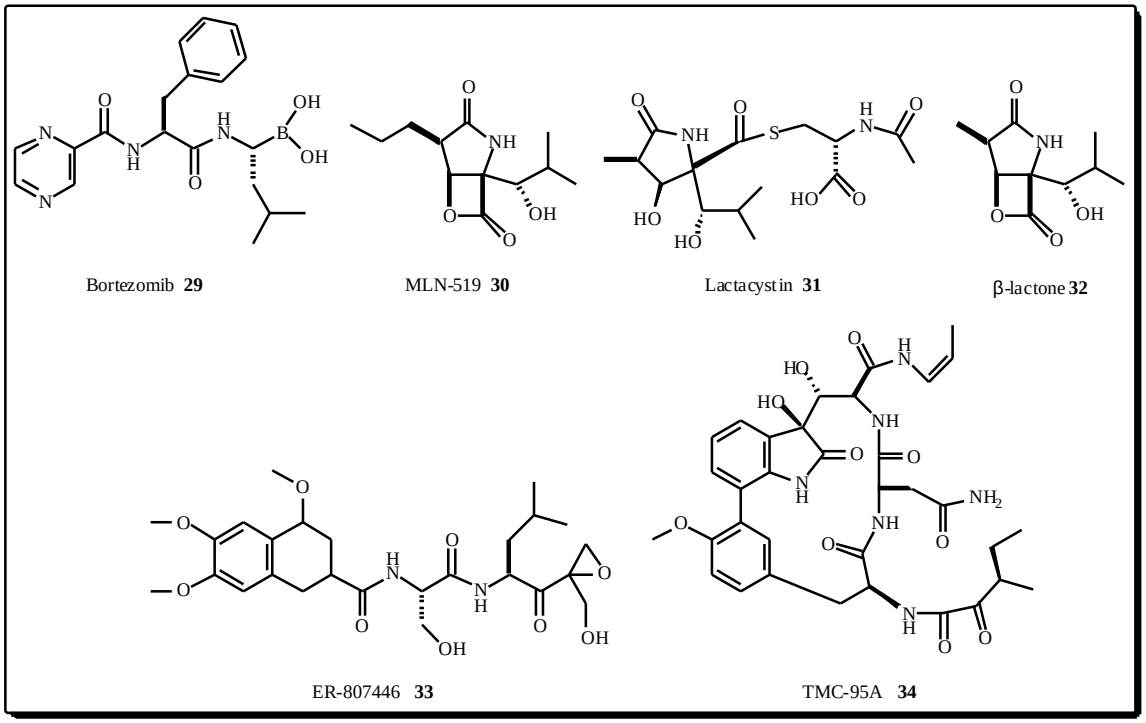
MLN-519 (**30**) is a derivative of the natural product Lactacystin (**31**) which rearranges at neutral pH to give the highly reactive β-lactone **32**. The latter inhibits the proteasome by acylation of active site threonine (Kobs/[I] 20,000 $M^{-1}s^{-1}$). The inhibition is irreversible, although the resultant acyl-enzyme complex is slowly hydrolysed ($t_{1/2}$ 20 h) to regenerate active proteasome. **30** is more active than **32** (Kobs/[I] 46,500 $M^{-1}s^{-1}$) and is in phase I trials for stroke via parenteral and topical administration. Dose-response studies in rats established that a 70-80% reduction in proteasome activity *in vivo* correlates with maximum neuroprotection [128]. Early results from the phase I study showed that doses of 0.5mg/m^{-2} – 1.6mg m^{-2} for three consecutive days were well tolerated in 28 patients, with no side-effects. Proteasome inhibition in blood samples (measured by a 20S proteasome assay) achieved the intended maximum target level of 70-80% with the 1.6mg m^{-2} dose [129].

ER-807446 (**33**) is representative of a series of epoxyketone inhibitors of the proteasome in preclinical development by Eisai (Japan) and based on the natural product eponemycin [130]. The epoxyketone inhibitors bind to the active site threonine in a novel and specific manner. A hemi-acetal is first formed between the inhibitor ketone and threonine hydroxyl, followed by epoxide ring opening by the free amine of the N-terminal threonine to give a morpholino adduct [131]. Compound **31** is potent (IC_{50} low nM) and exhibits antitumor efficacy (Akasaka et. al., 227th ACS National meeting, Anaheim CA, Mar 28th, 2004).

Although covalent irreversible/reversible inhibitors of the proteasome are the most advanced clinically, at least two series of non-covalent inhibitors have been described. *TMC-95A* **34** is a natural product with potent (Ki : chymotrypsin 1.1nM, trypsin 43 nM, caspase 650 nM) affinity for the proteasome. A crystal structure of **34** bound to the proteasome shows the biphenyl ring constraining the peptide in an extended β-strand conformation, with the Z-propenyl moeity at S1 and the central asparagine residue in the S3 subsite [132]. In addition, a series of 2-aminobenzylstatine non-covalent inhibitors have been described [133].

4. CYSTEINE PROTEASE INHIBITORS

There are three main classes of cysteine (thiol) proteases, papain-like (e.g. cathepsins), ICE-like (caspases), or picorna-viral (similar to serine proteases with cysteine replacing serine) [134]. Cysteine proteases hydrolyse amide bonds in a similar way to serine proteases. A non-covalent Michaelis complex is formed upon substrate binding. The thiolate anion then attacks the carbonyl carbon of the scissile amide bond. A tetrahedral intermediate is produced which is stabilised by the oxyanion hole. This is followed by the acylation of the enzyme and the liberation of the first product amine. Hydrolysis of the acyl enzyme leads to the formation of the second tetrahedral intermediate. Following the collapse of the second intermediate, the product acid is released and the free enzyme regenerated. Most inhibitors developed to date tend to be 2-4 amino acids or their equivalent in length, interacting with the non-prime subsites of the enzymes and terminating with various electrophilic isosteres. Because of their similar mechanism of amide bond hydrolysis, most design strategies incorporate an electrophilic isostere (aldehyde, α-ketoamide, ketone, vinyl sulfone etc.) in inhibitors, although non-covalent inhibitors are also being reported for a few cysteine proteases (eg. Cathepsin L [135] & Cathepsin K [136]).



4.1. Caspase-1 (ICE)

Interleukin-1 converting enzyme ICE (EC 3.4.22.36), now renamed caspase-1, hydrolyses the 31 kDa pro-IL-1 in monocytes to the active 17 kDa cytokine IL-1β. Among its many functions, this cytokine is a key inflammatory mediator and ICE inhibitors are promising therapeutic candidates for the treatment of inflammatory diseases.

Pralnacasan (**35**,VX-740) is a caspase-1 inhibitor in Phase III clinical trials for osteoarthritis and psoriasis (Vertex Pharmaceuticals). Pralnacasan has an oral bioavailablity of 50%. It is an ethyl acetal pro-drug of *VRT-18858* (**36**), which has limited (4%) oral bioavailability. **36** is a potent inhibitor of ICE (IC_{50} 1.3 nM) and inhibits (IC_{50} 0.85 μM) IL-1 release from LPS- challenged human peripheral blood mononuclear cells [137]. In an animal model of collagen-induced arthritis, **35** was effective at 50 mg/kg b.i.d. In a phase IIa study involving 285 patients, it was shown that the compound was safe at 100mg or 400mg thrice daily, well tolerated, and had positive anti-inflammatory effects for rheumatoid arthritis. In November 2003 Vertex (Press Release, Nov. 2003) however, announced a discontinuation of Phase IIb clinical trials for rheumatoid arthritis due to liver abnormalities detected in animal toxicology studies when administered for prolonged periods (9 months) in high doses. A phase II trial for osteoarthritis (OA) involving 522 patients with knee OA treated with one of three dosages of pralnacasan was completed in 2003. Preliminary results (Vertex Press Release, Jan. 2004) showed that, although pralnacasan was well tolerated at the three different doses, there were no statistically significant differences in the change of signs and symptoms of OA between placebo and drug groups over a twelve week period. Although a phase II trial for psoriasis was planned for 2003, no data has as yet been reported.

VX-765 (**37**) is another caspase-1 inhibitor in the vertex pipeline currently in phase 1 trials for inflammation. VX-765 is a pro-drug of VRT-43198 and is slightly more potent than pralnacasan *in vitro* (IC_{50} = 0.8 nM). The structure of **37** was recently reported (Randle, MEDI 200, 228th ACS National Meeting, Aug 2004). The compound possesses the masked aldehyde function of VX-740, but incorporates a t-butyl glycine–proline dipeptide at the P3-P2 positions. The 4-amino-3-chlorophenyl portion of the molecule presumably interacts with the S4 subsite of the enzyme. VX-765 inhibits IL-1 release from LPS-challenged human PBMCs (IC_{50} 0.47 μM). In an oxazolone-induced mouse ear inflammation model, VX-765 dose-dependently (10-100 mg/kg) inhibited ear inflammation, and had comparable efficacy to prednisolone (Randle J.C.R., Advances in Anti-Arthritic Agents - SMi's Third Annual Conference 23-24 April 2002, London, UK) [137]. Vertex has completed Phase I trials for this compound and based on promising results has initiated a phase II study (Vertex Press Release, Jan. 12, 2004).

4.2. Multiple Caspases

IDN-6556 (**38**) is a broad-spectrum irreversible inhibitor of multiple caspases and is being investigated for treating diseases were liver damage can occur due to increased apoptosis. The drug is in phase II trials for hepatic dysfunction and Hepaptitis C infection, phase I for Hepaptitis B, and preclinical for heart failure. It has been recently granted orphan drug status for liver and solid organ transplantation (diseases that affect <200,000 USA patients). Although no data has yet been published, this drug selectively and irreversibly inhibits caspases 1, 3, 6-9, at low to sub nM concentrations, presumably via displacement of the tetrafluorophenoxy substituent by the active site thiol of these enzymes. The structure has only been recently disclosed [138]. The compound has been shown to be efficacious in a phase II study of HCV patients at doses of 5-200 mg p.o. over 2 weeks [139]. In preclinical animal studies, **38** displayed potent *in vivo* activity (ED_{50} < 0.01mg/kg) although it displayed low oral bioavailability (~4%). Further investigation revealed significant first pass effect with a high proportion of the drug concentrating in the liver.

MX1013 (**39**) is an irreversible caspase inhibitor currently in preclinical development for the treatment of hepatitis,

Table 3. Cysteine Protease Inhibitors In Clinical Development.

Target	Indication	Drug Name	Company Name	Clinical Status
Caspase-1 (ICE)	Osteoarthritis, Psoriasis	VX-740-Pralnacasan	Vertex	Phase II
	Inflammatory disease	VX-765	Vertex	Phase II
Caspases-General	Hepatic dysfunction	IDN-6556	Idun	Phase IIb
	Myocardial Infarction, Cerebral Ischaemia	MX-1013	Maxim	Preclinical
Cathepsin K	Osteoporosis	AAE-581	Novartis	Phase II
	Osteoporosis	SB-462795	Glaxo Smith Kline	Phase I
Rhinovirus 3C	SARS	Ruprintrivir	Pfizer	Preclinical
	Common Cold	Rupintrivir		Phase II/III (Disc)

stroke and myocardial infarction. **39** inhibits caspases 1,3,6-9 with IC_{50} 5-20 nM and good selectivity with respect to other cysteine proteases. Interestingly, **39** is more water soluble than the well known longer tripeptide z-VAD-fmk and is more active in cell-based assays [140]. It can block apoptosis in various cell models at 0.5μM. In rats a single 5mg/kg dose gave Cmax 13 μg/mL, $t_{1/2}$ 1h, 10% bioavailability. The compound prevented liver damage in an anti-Fas liver apoptosis model at a dose of 1mg kg^{-1}. Compound **39** was efficacious in models of ischemia/reperfusion injury and myocardial infarction at 20mg/kg(i.v. bolus).

4.3. Cathepsin K

A large number of cathepsins are intracellular cysteine proteases of the papain superfamily, usually, but not exclusively, found in lysosomes, and are active at acidic to neutral pH. Cathepsin K is selectively expressed in osteoclasts where it represents 98% of the total cysteine protease activity. Studies involving Cathepsin K antisense nucleotides [141] and cathepsin K-deficient mice [142, 143] have established that this enzyme is primarily involved in osteoclast-mediated bone resorption. Cathepsin K inhibitors therefore are currently thought to be promising therapeutics for the treatment of diseases characterized by excessive bone loss such as osteoporosis. One important consideration in the design of cathepsin K inhibitors is selectivity with respect to the highly homologous lysosomal cysteine proteases cathepsin L, V and S [144]. This has been shown to be possible in many series of inhibitors that have been described over the last decade.

One example is **40,** a biphenyl that incorporates a nitrile function that interacts reversibly with the active site thiol. The compound is potent and selective (IC_{50}: CatK 3 nM, CatL 3.8 mM, CatS 2.0 mM, CatB 4.0 mM) [145]. Compound **40** also displays good bioavailability in monkeys (F 32%, $t_{1/2}$ 9.6h) and is efficacious with respect to a lowering of bone resorption markers [146]. *BS-357114* (**41**) is an azepanone inhibitor of cathepsin K with potent activity (Ki: CatK 0.16nM, CatL 2.2nM, CatS 4.0nM, CatB 500nM) but modest selectivity. Although bioavailability of **41** varies between species (rat, F 42%; monkey, F 4.8%), it was used to establish the efficacy of cathepsin K inhibitors in primates [147]. Most designed inhibitors of cathepsin K make use of an electrophilic isostere to reversibly or irreversibly covalently interact with the active site thiol, however in recent years a series of non-covalent inhibitors have also been described by Novartis [136, 148]. The arylaminoethyl amide **42** is one example which shows excellent selectivity and potency (IC_{50}; CatK 6 nM, CatL 2.3 μM, CatS 1.9 μM).

Two compounds have been reported in clinical trials for treating osteoporosis. *AAE-581* (Novartis) is in phase IIb clinical trials. The structure has not been disclosed but is reported to have a potent and rapid action on bone resorption as gauged from monitoring the bone resorption marker sCTX (C-terminal telopeptides of bone resorption) in a phase IIa clinical trial at 10-50mg/day p.o. Preliminary results suggest that bone resorption is prevented without affecting bone growth. *SB-462795* (GlaxoSmithKline) is currently in phase I clinical trials but no structure or pharmacological data has been published.

4.4. Rhinovirus 3C Protease

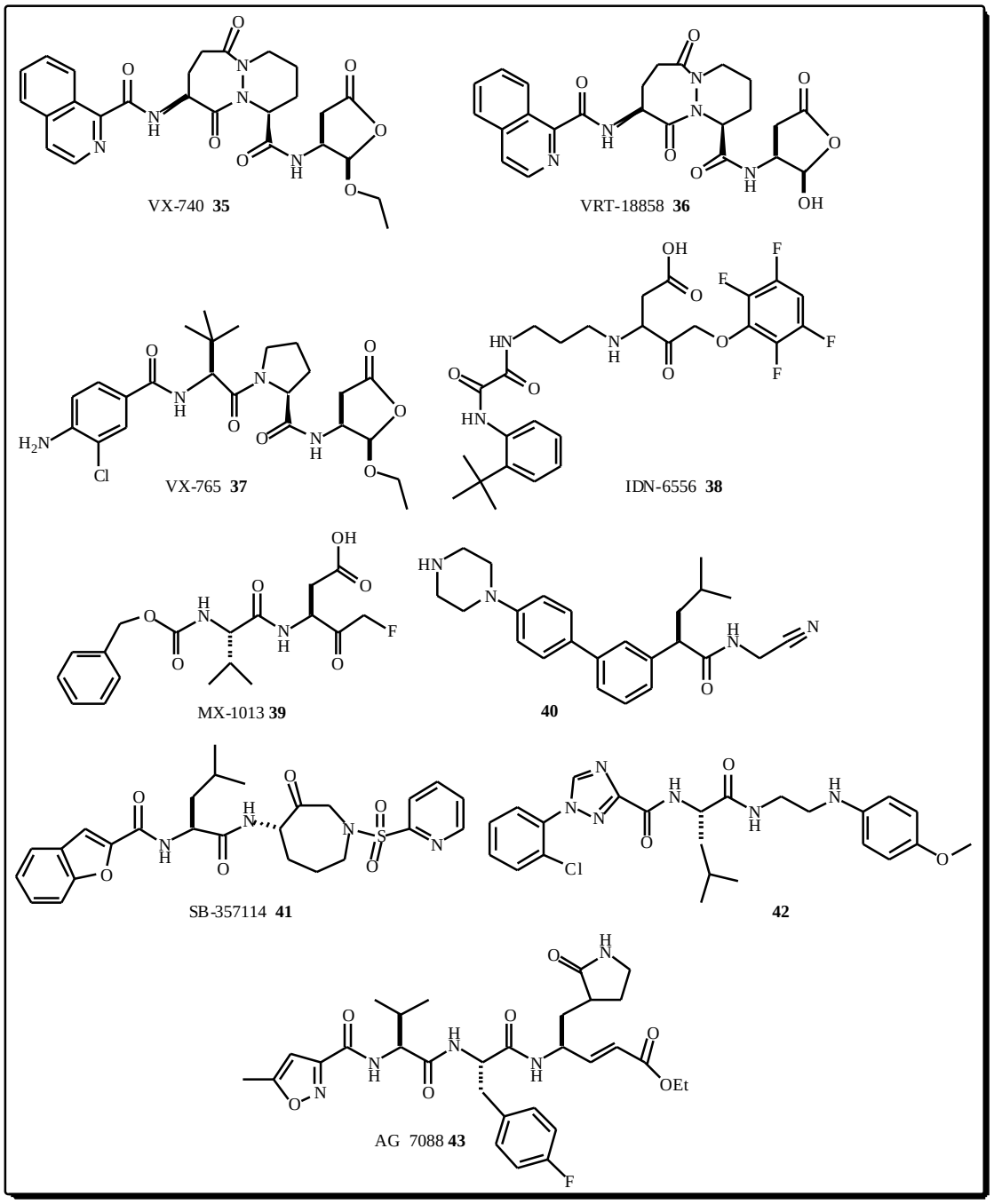
Rupintrivir (**43**) is a human rhinovirus 3C protease inhibitor that had reached phase III clinical trials for the treatment of common cold via intranasal delivery in 2003 by Pfizer. The inhibitor forms an irreversible covalent bond with the active site cysteine of the enzyme kobs/[I] 1,470,000 $M^{-1}s^{1}$ [149]. It was however realised that oral delivery was necessary in order to target viral replication sites throughout the body, and not just those available through intranasal delivery [150]. Unfortunately, the compound displays poor bioavailability and pharmacokinetics in dogs, partly due to the α,β unsaturated ester function and the peptidic nature of the molecule. A 20-30mg/kg p.o. dose in dogs gives C_{max} 0.50 μg/mL, T_{max} 12min, $T_{1/2}$ 27min, and oral bioavailability of 8%. Development has since ceased, and more bioavailable derivatives are presently being investigated [151-153]. The compound has also been suggested from *in silico* studies as a possible lead for developing inhibitors of the SARS coronavirus main protease [154].

5. ASPARTIC PROTEASE INHIBITORS

Aspartic proteases [155, 156] tend to use two catalytic aspartic acid residues to catalyse the hydrolysis of polypeptide substrates, hexa-deca peptide segments of which normally bind within the active site of the protease. Most inhibitors have been derived from such substrate segments by first replacing the cleavable peptide bond with a transition state isostere. Most aspartic proteases have one or more flaps that close down on top of the inhibitor, forming the active site with pockets or indentations on both sides of the catalytic residues. There are many hundreds of crystal structures now deposited in the pdb database [157] for inhibitor-bound and uncomplexed aspartic proteases including viral proteases (HIV-1, HIV-2, SIV, FIV), cathepsin D, renin, rennin/chymosin, penicillopepsin, secreted aspartic protease, pepsin, mucoropepsin, retropepsin, saccharopepsin, rhizopuspepsin, and plasmepsin II.

5.1. HIV Protease

Saquinavir (**44**), ritonavir (**45**), lopinavir (**46**), indinavir (**47**), nelfinavir (**48**), amprenavir (**49**), atazanavir (**50**) and fosamprenavir (**51**) have all been launched for the treatment of HIV/AIDS in humans, with tripanavir (**52**) expected to launched in late 2004 or early 2005. Some of these HIV protease inhibitors are key components of many highly active antiretroviral therapy regimens, and the early examples were among the first successfully validated examples of structure-based drug design [158-160]. They are all potent, low molecular weight peptidomimetic inhibitors of the protease of the human immunodeficiency virus (HIV-1 protease) that is known to be essential in the latter stage of viral replication. They all feature a transition state analogue replacement for the cleavable bond of a polyprotein substrate, but have then been truncated and extensively derivatised to make them less peptidic and more druggable. All are very potent inhibitors of viral replication *in vitro* in both acute and chronically infected cells, preventing maturation of the virion.



However most have fairly poor pharmacokinetic or pharmacodynamic profiles, necessitating oral administration of multiple high daily doses at ~1g each time. All suffer from drug-induced phenotypic/genotypic resistance when used as monotherapies, trough concentrations of free drug being too low to inhibit viral mutants that are selected for under drug pressure. Consequently these drugs tend to be most useful *in vivo* either in combination with one another or in combination with drugs that target a different HIV protein required for replication. Combination therapy usually results in greater than additive increases in CD4 counts, synergistic reductions in viral loads, and longer delays in viral resistance to drugs. As a class most HIV-1protease inhibitors cause alterations in lipid metabolism (lipodystrophy) [161] with long term use and each compound has its own unique toxicity profile. Nevertheless the use of HIV-1 protease inhibitors has been largely responsible for prolonging the lives of HIV/AIDSpatients for as long as 5-10 years, without many serious side effects.

Saquinavir (**44**, Invirase®, Hoffmann-La Roche) was the first example in the clinic of an HIV-1 protease inhibitor (IC_{50} 0.4nM) to be obtained through structure-based design. It was designed on the basis of Phe-Pro substrates not being

Table 4. Aspartic Protease Inhibitors In Clinical Development.

Target	Indication	Drug Name	Company Name	Clinical Status
HIV-1 Protease	HIV/AIDS Infection	Ritonavir, Ritonavir soft gel	Abbott	Launched
	HIV/AIDS Infection	Lopinavir	Abbott	Launched
	HIV/AIDS Infection	Nelfinavir Mesylate	Pfizer	Launched
	HIV/AIDS Infection	Atazanavir Sulfate	Bristol-Myers Squibb	Launched
	HIV/AIDS Infection	Amprenavir	Vertex Pharmaceuticals	Launched
	HIV/AIDS Infection	Saquinavir, Saquinavir soft gel	Hoffmann-La Roche	Launched
	HIV/AIDS Infection	Crixivan	Merck & Co.	Launched
	HIV/AIDS Infection	Fosamprenavir Calcium	GlaxoSmith Kline	Launched
	HIV/AIDS Infection	Tipranavir	Pfizer	Phase III
	HIV/AIDS Infection	KNI-272	Japan Energy	Phase II (No Dev)
	HIV/AIDS Infection	TMC-114	Johnson & Johnson	Phase II
	HIV/AIDS Infection	NV-RX	Novartis	Phase II
	HIV/AIDS Infection	NAR-DG-35		Phase II (No Dev)
	HIV/AIDS Infection	VX-385	Vertex Pharmaceuticals	Phase I
	HIV/AIDS Infection	DPC-681		Phase I
	HIV/AIDS Infection	Ro-03-34649	Hoffmann-La Roche	Phase I
	HIV/AIDS Infection	PL-100	Procyon BioPharma	Preclinical
	HIV/AIDS Infection	C Sixty	C Sixty	Preclinical
	HIV/AIDS Infection	SM-309515	Sumitomo	Preclinical
	HIV/AIDS Infection	GS-9005	Gilead Sciences	Preclinical
	HIV/AIDS Infection	protease inibitor	Zapaq	Preclinical
Renin	Hypertension	Aliskiren	Novartis	Phase II (No Dev)
BACE	Alzheimer's Disease	LY-450139	Eli Lilly	Phase II
	Alzheimer's Disease	TGCN-001	The Genetics Company	Preclinical
	Alzheimer's Disease	β-secretase inhib	Acetilon	Preclinical
	Alzheimer's Disease	β-secretase inhib	Astex Technology	Preclinical
	Alzheimer's Disease	β-secretase inhib	Cellzome	Preclinical
	Alzheimer's Disease	β-secretase inhib	De Novo	Preclinical
	Alzheimer's Disease	β-secretase inhib	Elan	Preclinical
	Alzheimer's Disease	β-secretase inhib	Glaxo Smith Kline	Preclinical
	Alzheimer's Disease	β-secretase inhib	Locus	Preclinical
	Alzheimer's Disease	β-secretase inhib	NeoGenesis Pharm	Preclinical
	Alzheimer's Disease	β−secretase inhib	Sunesis	Preclinical
	Alzheimer's Disease	β−secretase inhib	Zapaq	Preclinical
	Alzheimer's Disease	TGC-2	The Genetics company	Preclinical

(Table 4. Contd….)

Target	Indication	Drug Name	Company Name	Clinical Status
Plasmempsins	Malaria infection	Plasmepsin-1 inhibitor	Medivir	Preclinical
	Malaria infection	Plasmepsin-2 inhibitor	Fulcrum Pharmaceuticals	Preclinical
Aspartic protease	Unspecified indication		Zapaq	Preclinical

known (at that time) to be cleaved by mammalian proteases but it was cleaved by HIV-1 protease, and thus perhaps a basis for selectivity. Compound **44** is a pentapeptide analogue featuring an hydroxylethylamine transition state analogue in place of the cleavable peptide bond, a bulky decahydroquinoline in place of proline at P1' and a quinoline at P3. It inhibits one of the last stages of viral replication, and it was active in cell culture against both HIV-1 and HIV-2 viruses (EC_{50} = 1-30 nM) though typically with 10 fold lower potency in cells than against enzyme. A 10mg/kg/po dose in the rat (C_{max} 150nM, T_{max} 66min, F 3%) did not suggest ideal kinetics but, since drug concentrations remained >15nM over more than 6h and as this was the first HIV protease inhibitor, clinical trials proceeded. Administration of 600mg tid to humans gave C_{max} 70nM. A Phase I trial in HIV-infected patients at 1200mg tid produced an AUC 7.3 μg/hr/mL and C_{max} 2.5 μg/mL, while a series of Phase III trials in 800-900 patients for 12-48 weeks showed reduced viral loads to 60-80% after 1200mg tid or 1600 mg bid plus nelfinavir or 2 nucleoside analogues. Saquinavir (Invirase) was approved for human use in 1996, [72, 162] but suffered from very low oral bioavailability (1-4 %) because of poor absorption and extensive first pass metabolism by cytochrome P450 (CYP 3A4) [163]. A soft gel formulation (Fortovose, launched 1997) reportedly gives 8x the bioavailability of Invirase and is approved for co-administration with other antiretroviral drugs for treating HIV diseases.

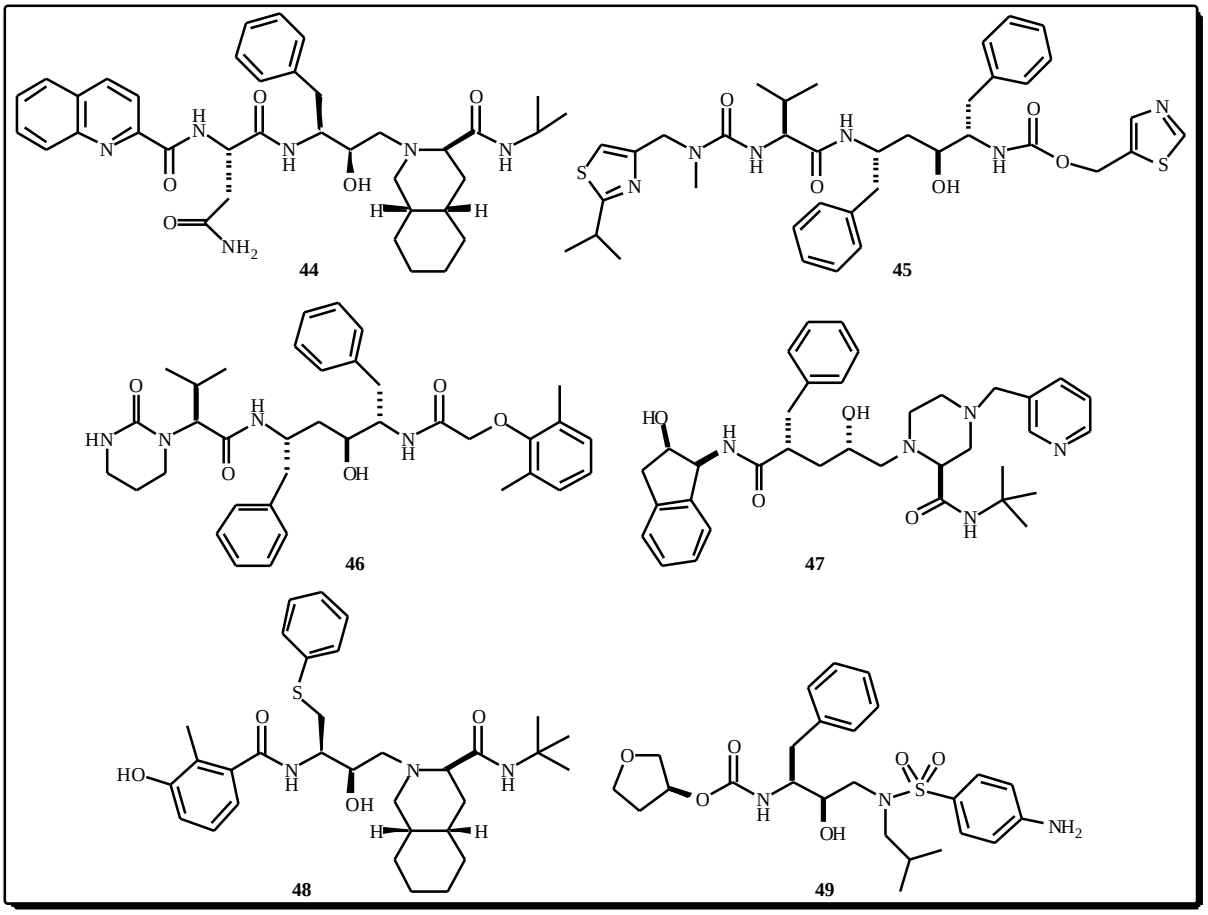
Ritonavir (**45**, Norvir®, Abbott/Gilead) is a potent inhibitor of HIV-1 protease (Ki 15 pM) and HIV in MT4 cells (EC_{50} 0.03μM, EC_{90} 0.13μM) without killing normal cells (IC_{50} > 50μM). Its hydroxyethyl transition state isostere, two thiazole termini, and hydrophobic Phe and Val substituents conferred higher oral bioavailability than **44**. In rats a single 10mg/kg oral dose resulted in 78% oral bioavailability, $t_{1/2}$ 3-5h, C_{max} 2.6 μM, T_{max} 2 h compared with $t_{1/2}$ 1.2 h after a 5 mg/kg i.v. dose [164]. However it also bound strongly to plasma proteins (>98%). In humans a 600mg dose bid po gave $t_{1/2}$ 4h,T_{max} 4h,C_{max} 7.6μg/kg, Cl 5.6L, AUC 129μghr/mL, Vd 20L (600mg po) and a single 10mg po dose gave plasma concentrations that were higher than the EC_{50} *in vitro* for over 12h. Ritonavir also inhibits cytochrome P450-3A and has consequently been used to inhibit rapid metabolism of other HIV protease inhibitors, including **44**-**48**, boosting the plasma concentrations of such drugs and delaying development of resistance [165].

Lopinavir (**46**, Abbott) also known as ABT-378 is an analogue of **45** with about 10 fold higher potency as an inhibitor (Ki 1.3 pM) of HIV-1 protease, and is effective against ritonavir-resistant HIV strains [73]. Its oral bioavailability is substantially boosted (After 8h, C_{max} < 0.1 μg/mL to 5.5 μg/mL) when administered with ritonavir and the compound is in early phase II clinical trials. It is consequently formulated with **45** to reduce its metabolism by CYP450 [166]. In rats a single 10mg/kg dose of **45** + **46** gave plasma concentrations of **3** that were 50x EC_{50} for 8h, due to P450 inhibition by **45**, and high antiviral activity in MT2 cells (EC_{50} 100nM). Phase I trials typically involved 400mg bid **46** plus 100mg bid **45** giving plasma levels of **46** (10μg/mL for 24h, EC_{50} 100ng/mL), and over 3-4 weeks showed T_{max} 4h, C_{max} 9.6 μg/mL. Phase II trials over 3 weeks showed decreased viral loads by 2log.

Indinavir (**47**, *Crixivan*®, L735524, Merck) is another potent inhibitor of HIV-1 (K_i 0.52 nM) and HIV-2 (K_i 3.3 nM) proteases and features the novel 2-aminoindanol substitutent at P2 and the unusual transition state isostere [167]. It stops the spread of HIV infection in MT4 lymphoid cells at 25-50 nM. In preclinical studies, a 10mg/kg/po dose was orally bioavailable in the rat, dog and monkey (22%, 70-90%, 13-20% respectively) with $t_{1/2}$ (28, 34, 73min), T_{max} (30, 30, 65 min) and C_{max} (2.8 (25mg/kg dose), 11.4, 0.7 μM) [158]. In humans, **47** is rapidly absorbed, there is significant binding to plasma proteins (60%), and 800 mg p.o. every 8 h shows $t_{1/2}$ 1.8h, T_{max} 0.8 h, C_{max} 12.6 μM, AUC 30.7 μM/hr, F 65%. The plasma concentration after 8h was 257 nM. The main degradation pathway is via cytochrome P450 [168]. A Phase III trial in Brazil (996 patients) of 4 ± zidovudine reduced disease progression or death by 61 and 70% respectively. Press releases from Merck have claimed that 1200 mg + 200mg **45** given orally once daily with two non-nucleoside RT inhibitors reduce viral loads in > 20/30 patients to 400-500 copies/mL after 48 or 100 weeks treatment.

Nelfinavir (**48**, AG1343, Viracept®, Agouron) couples the same bulky hydrophobic P1' substituent and hydroxyethylamine transition state isostere as **44** with an extended P1 substituent to produce a lipophilic (LogP = 4.1) protease inhibitor (K_i 2 nM, EC_{50} 20-60nM), approved as the mesylate salt for human use in 1997. Preclinical studies [169] showed good oral bioavailability in rats (52-80%), dogs (20%) and monkeys (20-40%), and Phase I trials with 1250 mg po bid for 28 days gave C_{max} 4mg/L, $t_{1/2}$ 3.5-5h,Vd 2-7L/kg. Three Phase III trials suggested that **48** was perhaps the best tolerated of the first generation HIV protease inhibitors and displayed acute viral reductions > 90%.

Amprenavir (**49**, Agenerase®, VX478, Vertex/GSK/ Kissei) is a smaller tetrapeptide analogue, featuring an unusual tetrahydrofuran substituent at P2 and an hydroxyethylamine isostere that has been converted to a sulfonamide. It is another potent inhibitor of HIV-1protease (K_i = 0.6 nM) and HIV-1 replication (IC_{90} 80nM). It is water



soluble and orally bioavailable (> 70% humans) with plasma concentrations 20x EC_{90} after 900-1200 mg doses and good brain penetration. Its long duration enables fewer doses compared with other first generation inhibitors like **44**, **45**, **47**, **48** [170, 171]. In Phase I trials it showed $t_{1/2}$ of 7-10 h, T_{max} 1.9-2.1h, Vd 430L, and in HIV-infected patients 1200mg bid for 3weeks showed C_{max} 7.7 μg/mL, T_{max} 1h, AUC 17.7 μg/hr/mL and enhanced activity when in combination with **44** and **47**. The same dose in Phase III trials, usually with two non-nucleoside inhibitors, reduced viral loads to <400 copies/mL in >70% patients versus in <20% patients without **49**.

The above six compounds were the first generation HIV protease inhibitors approved for use in humans. All had substantial efficacy in the clinic in short term studies, significantly reducing viral load in HIV-infected patients. However, as long ago as 1996 it was realised that the fairly rapid onset (days-weeks) of resistance to these drugs, due to selection of pre-existing virus mutants in which the mutated protease no longer binds tightly to these active site directed inhibitors, rendered monotherapy ineffective in the clinic. Triple drug cocktails of different HIV protease inhibitors sometimes lasted longer before development of resistance, but the most highly active antiretroviral therapies (HAART) were those that included drugs that target different proteins required for HIV replication, particularly nucleoside and non-nucleoside inhibitors of reverse transcriptase. Unfortunately, around one third of even those HAART regimens have still failed over longer term (> 1year) studies. The quest for more effective HAARTs has led to second generation compounds with improvements in HIV protease inhibitor potency, reduced binding to plasma proteins, better pharmacokinetic profiles (including higher oral bioavailability, longer plasma lifetimes, reduced clearance), more potent antiviral activity and especially greater clinical efficacy against virus variants that are resistant to first generation protease inhibitors.

Atazanavir (**50**, BMS-232632, CPG73547) [172] is a second generation tetrapeptide-mimicking protease inhibitor featuring a 2-hydroxy-1,3-diaminopropane transition state isostere, an aza-dipeptide core, and an extended P1' substituent. It inhibits WT HIV-1 protease (Ki 10 pM) and HIV infected PBMCs (IC_{50} 8-12 nM), as well as indinavir-resistant (IC_{50} 0.03-0.1 μM) and saquinavir-resistant (IC_{50} 0.004-0.1 μM) strains of HIV-1. It is orally bioavailable (20% in rats; C_{max} 90 min) [173]. In humans a 400mg po dose results in $t_{1/2}$ 6.5-7.9h, T_{max} 2-2.5h, C_{max} 3-5 μg/mL, AUC 22-30 μghr/m, F 60%. For doses above 300mg, plasma concentrations were > EC_{50} for over 24h. In a 14 day Phase II trial, 400mg qd gave steady state concentrations of **50**, with $t_{1/2}$ 5.3h, C_{max} 7.6 μM, T_{max} 2.2h, AUC 42 μM/h, C_{trough} 225 nM. Monotherapy produces similar efficacy results to nelfinavir, but its long half life permits single daily dosing and it produces a unique resistance profile in HIV patients

with the I50L and A71V double mutation [174]. Interestingly Phase III clinical data suggests that 400-600mg/day doses of **50** do not alter LDL cholesterol or triglyceride concentrations after 48 weeks [175].

Fosamprenavir (**51,** GW433908, GSK/Vertex) is a phosphate prodrug analogue of amprenavir that is a similarly potent inhibitor of HIV-1 protease [176]. In dogs it displayed C_{max} 6.1 μg/mL,AUC 16.7μghr/mL. In Phase I trials in humans ($t_{1/2}$ 7-10h) it showed bioequivalence and dose proportionality to amprenavir, but has much higher oral bioavailability than **49** and its greater water solubility enables much lower doses to be administered.

Tipranavir (**52**, Pfizer/Boehringer Ingelheim) [172, 177] or PNU-140690 features a 5,6-dihydropyran-2-one sulfonamide scaffold and, while claimed to have been derived from a non-peptidic screening program, looks to combine some features of emprenavir and early coumarin/γ-pyrone inhibitors of HIV-1protease. Interestingly it has no classic transition state unit. It is a potent second generation HIV-1 protease inhibitor (Ki 4 pM) that similarly inhibits HIV-2 protease and many clinically resistant isolates (to **44**, **45**, **47**, **48**) in cell culture. It has good antiviral potency (IC_{90} 100 nM), high protein binding (75% human serum), and has been reported to inhibit around 100 multidrug resistant clinical HIV isolates with <5x decrease in IC_{50}. Its broad spectrum of activity may be due to its flexibility allowing binding to a range of mutated proteases, although the fairly unusual V82T and L90M mutations in HIV protease do lead to drug resistance. It is well tolerated in rats at 500mg/kg/day with doses of 5-10 mg/kg po giving plasma drug levels of $>IC_{90}$ for HIV in cells. In male beagle dogs, 5mg/kg i.v. resulted in AUC 32.6μMhr and $t_{1/2}$ 0.93h, while a 10mg/kg po dose showed 20% oral bioavailability, $t_{1/2}$ 1.6h,T_{max} 2h, C_{max} 3.9h, AUC 13.7 μMhr. In Phase I at 900mg tid it gave trough concentrations >1μM. In Phase II at 1200 mg bid alone, or at 300 mg + 200mg **45**, or 1200 mg plus 200mg **45**, concentrations of **52** at trough were 0.8, 21 and 67μM respectively. In Phase III trials in humans tipranavir (500 mg bid) has typically been used in combination with **45** (e.g. 200 mg bid) due to its extensive metabolism by CYP450-3A, and has been shown to significantly reduce viral load (1.4-1.6 log HIV RNA reduction after 2 weeks treatment) in people with up to 20 resistance mutations that render other HIV protease inhibitors ineffective, and may therefore be helpful for salvage therapy. It appears poised to be approved for human use in late 2004 or in 2005.

KNI-272 (**53**, Japan Energy) is a substrate-derived inhibitor with an allophenylnorstatine transition state isostere, a hydrophobic quinoline at P3, and a bulky thiazoline at P1'. The latter helps pre-organize the molecule to adopt a β-strand, the solution structure being almost identical to its crystal structure in complex with HIV-1protease [178, 179]. Compound **53** is 33% orally bioavailable, $t_{1/2}$ 3.5h (50mg/kg/po, rat) or 1h (25mg/kg/po, monkey) [158]. It appears to have been halted in Phase II trials, although has not been listed as discontinued.

TMC-114 (**54**, J&J) or UIC-96017 is a second generation analogue of amprenavir with a bis-THF terminus. It has good antiviral activity in cell culture (IC_{50} 3 nM, IC_{90} 9nM) and against multiple HIV strains that are resistant to **44**, **45**, **47**, **48** (IC_{50} 3-30 nM) but not **49** (IC_{50} 220 nM) [180, 181]. Its crystal structures with HIV-1 protease and V82A and I84V mutants show crucial hydrogen bonds with conserved mainchain atoms of Asp29and Asp30 of the protease, these possibly being responsible for retention of activity in the face of resistant proteases [182]. In dogs 20mg/kg/po gave C_{max} 7.5 μg/mL, while Phase I trials in humans with 400mg bid – 1200 mg tid via oral delivery in PEG for 14 days gave $t_{1/2}$ 10h, T_{max} 3h, C_{max} 2.2 μg/mL. Coadministration with **45** reduced diarrhoea, nausea, headache and skin disorders. The compound is in Phase II trials for treating primary HIV-1 infections and those resistant to multiple protease inhibitors.

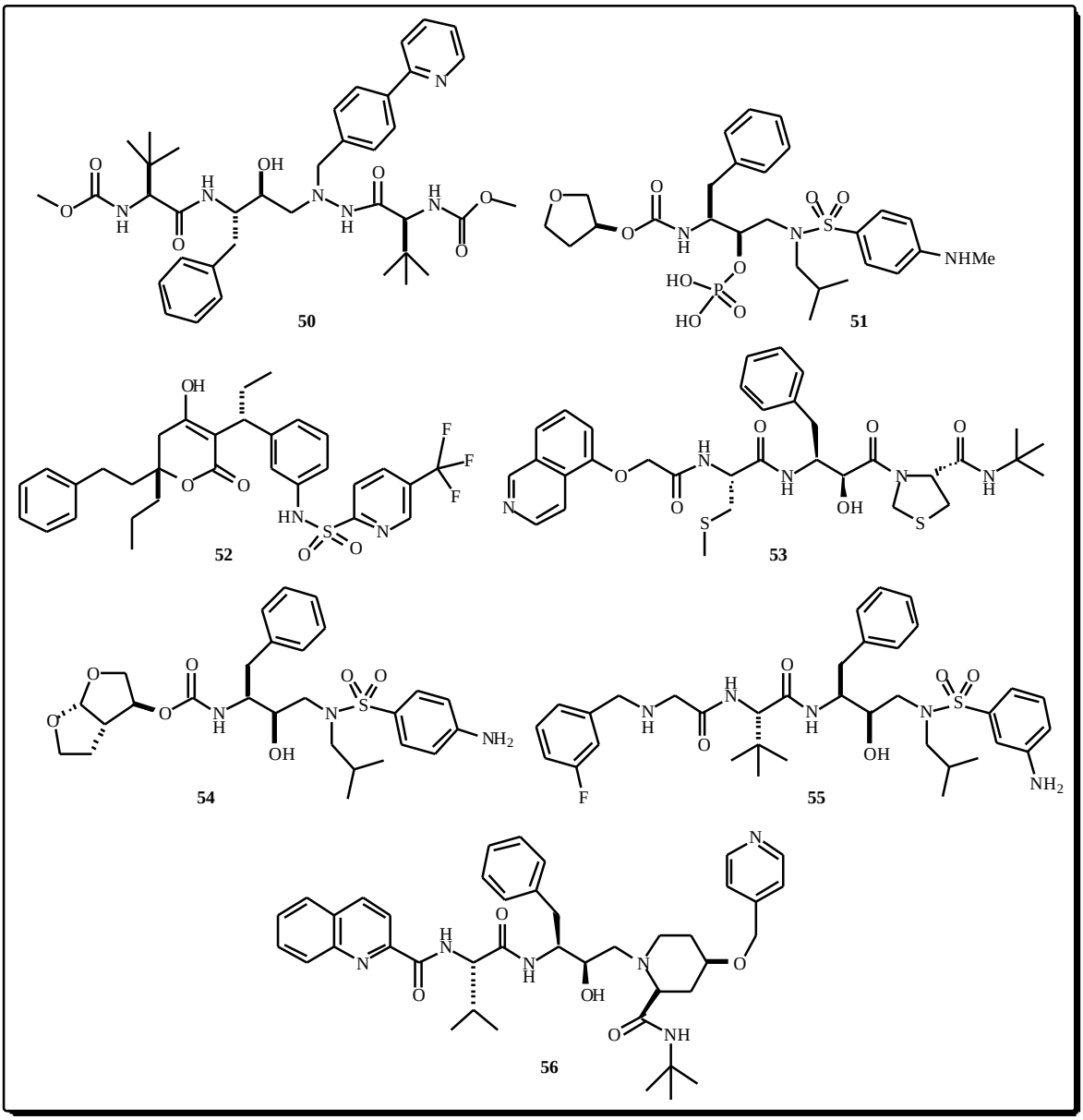
DPC-681 (**55**) is representative of a series of newer aminoacyl sulfonamides based on amprenavir with a 2-hydroxy-1,3-diaminopropane isostere and an extension at P3. This compound (Ki 12pM for **55** vs 270nM for **44**, 370nM for **47** under same conditions) and an analogue DFC-684 have been reported [183] to be potent inhibitors of HIV protease, with high enzyme selectivity (IC_{50} > 13μM renin, pepsin, cathepsins D and G). They have potent antiviral activity with IC_{90} 4-40 nM against laboratory and clinical isolates of HIV in MT-2, MT-4 and PMBC cells. They also showed some impressive inhibitory potencies against clinically relevant mutant variants, viruses that were not inhibited by other protease inhibitors such as amprenavir and nelfinavir, and data suggest that blood levels of **55** need to be only 1-10% of those of **47**-**49** to suppress viral replication, although **55** is 98% serum bound. Preclinical studies indicated that 10mg/kg/po **55** in dogs produced C_{max} 0.9μM (C_{6h} 0.012μM), $t_{1/2}$ 1h, F 18% while 30mg/kg/po gave Cmax 8.5μM (C_{6h} 0.2μM), $t_{1/2}$ 1.7h, F 78%. A 1mg/kg/iv dose gave $t_{1/2}$ 1h and CL 1.8L/h/kg. The plasma levels of drug 3-6h after the 30mg/kg/po dose were sufficient to inhibit 90% wild type and mutant viruses but this dog PK data probably do not predict human trough levels, as found for the structurally related drug **49**.

Palinavir (**56**) combines some of the features of **44** and **48**, namely a quinoline/Val/Phe nonprime side reminescent of P3-P1 segment of **1** and its analogues with a piperidine carboxyl t-butyl amide much like the prime side of **47**. It is a very potent orally active inhibitor of HIV-1 (K_i 31pM) and HIV-2 (K_i 134 pM) proteases with high antiviral activity (EC_{50}(HIV-1) 0.5-28 nM; (HIV-2) 4-30 nM), low cytotoxicity (CC_{50} 30-45 μM; TI > 1000). But like many such promising candidates 5 years ago, this has not progressed. In fact its pharmacokinetics were not optimal (rats: $t_{1/2}$ 0.7 h, C_{max}(5mg/kg) 1.6 μmol/L, F 37%) [184] and Val32Ile and Ile84Ala were among resistance mutations that may have halted development of this series.

Other HIV-1 protease inhibitors listed to be in clinical development but either have not yet progressed or appear to have halted are *NV-RX* (Novartis), *PL-100* (ProcyonBioPharma), *SM-309515* (Sumitomo), *NAR-DG-35*, *VX-385* (Vertex), *Ro-03-34649* (Hoffmann-LaRoche) and *GS-9005* (Gilead Sciences).

5.2. Renin

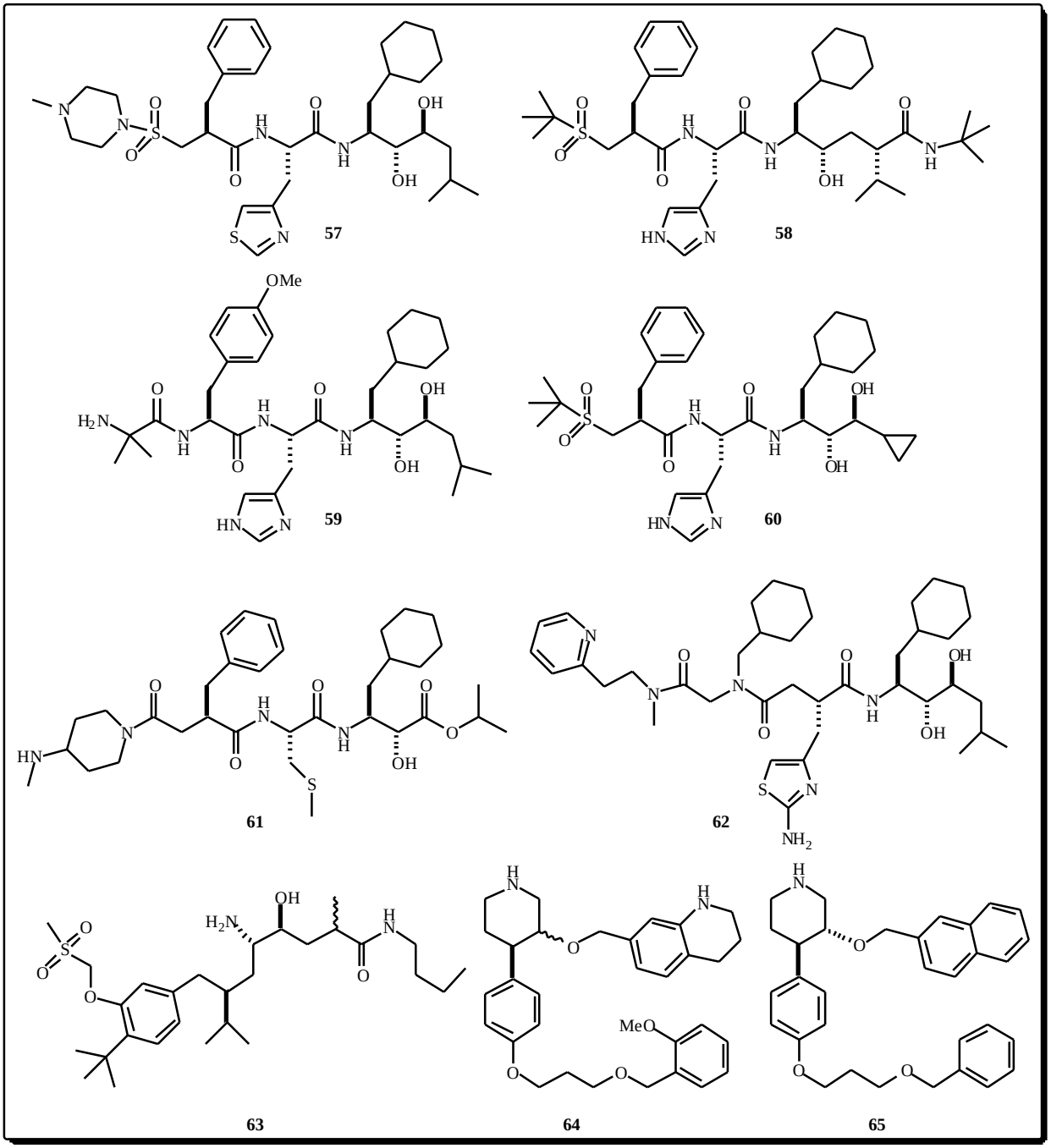
Renin catalyses the rate-limiting first hydrolysis step of the renin-angiotensin system, releasing the decapeptide



angiotensin I from α_2-globulin angiotensinogen. Renin inhibitors might therefore be potential antihypertensive agents with an action similar to, but mechanistically different from, ACE inhibitors. Unfortunately, renin inhibitors to date have mainly been substrate analogues with high peptidic character [185]. They therefore have tended to be proteolytically degraded and exhibit poor oral bioavailability, properties that have compromised their clinical progress [186]. Among the more notable renin inhibitors are zankiren (**57**), CGP 38 560 (**58**), enalkiren (**59**), remikiren (**60**), CP-108,671 (**61**), BILA 2157 BS (**62**), and **63**-**65**. Only Aliskiren (Novartis) is listed in Phase II trials, but it should be noted that most research activity on renin involved peptide ligands and preceded the development of HIV-protease inhibitors. The success of the latter drugs may spark renewed enthusiasm for developing inhibitors of renin.

Zankiren (A-72517) (**57**) is a potent inhibitor (IC_{50} 1.1 nM, pH 7.4) of human plasma renin and reduces dry cough associated with ACE treatment [187]. It is absorbed orally (8% monkey, 53% dog, 24% rat, 32% ferret) [186]. An intraduodenal dose (10 mg/kg) in sodium-depleted monkeys reduced mean arterial blood pressure by 32 mmHg (37%) for 2 hours. Doses of 125 mg and 259 mg to humans produced C_{max} 0.43 and 1.15 μL/mL respectively [188], reducing blood pressure by 16 (systolic) and 8 (disystolic) mmHg after a 125 mg dose. Some other potent renin inhibitors that are largely peptidic include *CGP 38 560* (**58**), [189] < 1%, K_i 0.7nM; *enalkiren* (**59**), [185] 0.5%, K_i 14 nM; and *remikiren* (**60**), [190] 0.2%, K_i 0.7 nM.

The less peptidic *CP-108,671* (**61**), with a cyclohexyl norstatine transition state isostere and a (R)-benzylsuccinate at P3 for chymotrypsin stability, potently inhibits human



plasma renin (IC_{50} 4 nM), is orally bioavailable (60% dog, marmosets; > 27% cynomolgus monkeys) with a half-life of 2.4 h (dog) after i.v. administration, and lowers mean arterial blood pressure in sodium-depleted marmosets by 25 mmHg for >5h after 3 mg/kg po. It is selective over most aspartic proteases (IC_{50}(porcine pepsin) 2100 nM) but inhibits cathepsin D (IC_{50} 550 nM) [191].

BILA 2157 BS (**62**) also inhibits renin (IC_{50} 1.4 nM, pH 6.0) with some selectivity (IC_{50}(catD) 550 nM) and oral activity (40% cynomolgus monkeys, 3 mg/kg) [192]. The P2-P3 amide bond has been replaced in this compound by a 2-substituted butanediamide moiety, the (2-amino-4-thiazolyl)methyl group at P2 and N-methyl N-2-(2-pyridinyl)ethyl moeity at P4 are needed for oral activity, and there is only one stereogenic centre and only one amino acid residue (glycinamide) left in the molecule.

Inhibitor **63** (IC_{50} 13 nM) [193] extends the P1 residue into the S3 subsite. Despite lacking the P2-P4 amide backbone of CGP38560 it retains good activity and specificity. Compound **64** (IC_{50} 37 nM) is representative of a series of non-peptidic piperidine-based inhibitors of human plasma renin [194] with low MW (517Da), membrane

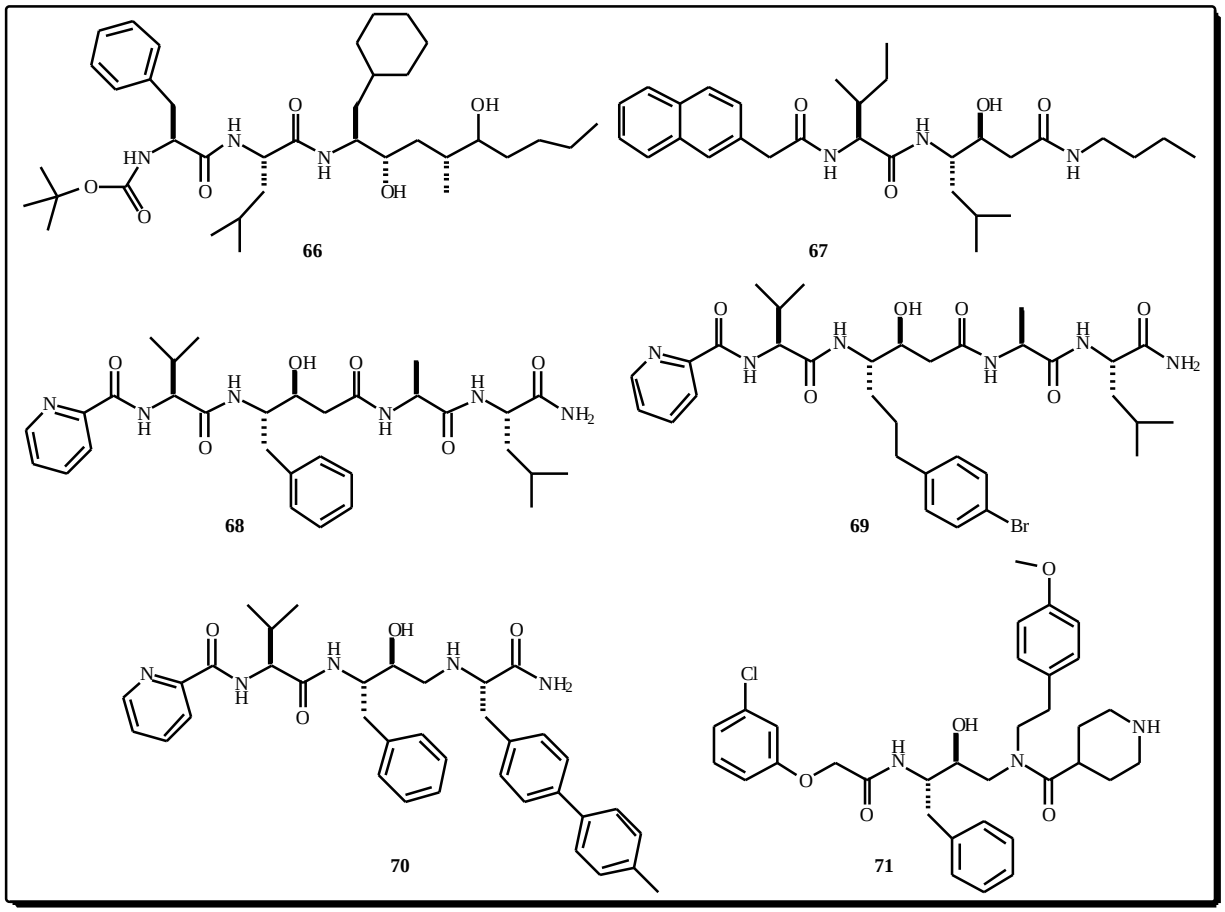
permeability (LogD 1.9, pH 7.4), and oral activity in sodium-depleted marmosets with a maximal decrease in mean arterial blood pressure of 19 mmHg at 8.5 h after a 3 mg/kg dose. Unlike most renin inhibitors, the protonated nitrogen of the piperidine ring hydrogen bonds to each of the two active site aspartates like transition-state isosteres do for other inhibitors [195]. The naphthyl substituent fills the large hydrophobic S1/S3 subsite of renin, the 4-phenyl and the hydrophobic tail disrupting a hydrogen bond between Tyr75 and Trp39, lifting the from Thr72 to Ser81. This creates a new hydrophobic pocket in the enzyme to accommodate the hydrophobic extension and terminal phenyl group. Non-peptidic compounds like **64** and **65** have been studied by many companies in the last few years but clinical progress is still elusive.

5.3. Plasmepsins

Aspartyl proteases found in the malarial parasite *Plasmodium falciparum* include Plasmepsins I and II, which have high sequence homology with human cathepsin D (73% and 37% respectively). These enzymes degrade human hemoglobin, the major source of nutrients for the parasite, cleaving Phe33-Leu34 of the alpha chain of haemoglobin before degrading the polypeptides to smaller fragments that are processed by other enzymes such as falcipain [196]. The classic aspartic protease inhibitor pepstatin A inhibits both plasmepsins as well as human cathepsin D, but a crystal structure of its complex with plasmepsin II has been useful in developing inhibitors of plasmempsins.

SC-5003 (**66**) was the first inhibitor reported to selectively inhibit plasmepsin I (IC_{50} 500 nM) over plasmepsin II (22% at 10 μM) and it prevented hemoglobin degradation *in vitro* with IC_{50} 2-5 μM (parasites) [197]. Libraries of peptidic inhibitors of plasmemsin II have since been created, including the leucine-statin **67** (IC_{50}(plm II) 50 nM; IC_{50}(catD) 320 nM) [198] and the phenylalanine-statin analogues **68** and **69**. Compound **68** is of comparable potency to **67** as an inhibitor of plasmepsins (IC_{50} 0.54 nM) and starves the live parasite *in vitro* (54% at 20 μM) but also inhibits human cathepsin D (IC_{50} 21 nM) [12]. Such compounds do not penetrate cell membranes readily and thus tend to have low potencies against *P.falciparum*. However Medivar is reportedly investigating statin-like inhibitors (e.g. **69**) of Plasmepsins I and II for the treatment of malaria, their best inhibitors having Ki values 0.5-74 nM for both enzymes and up to 1000 fold selectivity over cathepsin D.

Compounds **70** and **71** were also developed using combinatorial libraries and feature the hydroxyethylamine transitions state isostere. Compound **70** is a reasonably potent inhibitor of plasmepsin II (Ki 68nM) with better potency against the parasite in infected erythrocytes *in vitro* (ED_{50} 1.6 μM) [199]. Compound **71** (K_i 4.3 nM plm II) retains only two amide bonds, has a low molecular weight



(594Da) and a Log P (3.71) suggesting better cell permeability, and 10 fold selectivity over human cathepsin D (K_i 63 nM) [200]. It seems likely that **67-71** will similarly inhibit plasmepsin I, and better selectivity is needed for the parasite enzymes over human aspartic proteases.

6. METALLOPROTEASE INHIBITORS

All of the metalloproteases are zinc enzymes that catalyse the hydrolysis of a peptide bond. Metalloproteases for which inhibitor-protease crystal structures are available on the pdb [157] include ADAM17 endopeptidase (TNF

Table 5. Metalloprotease Inhibitors In Clinical Development.

Target	Indication	Drug Name	Company Name	Clinical Status
ACE	Hypertension	Trandolapril, Enalapril, Captopril + 8 more		Launched
General Metalloprotease	Acne	Dermostat		Phase II
	Cancer, lung, non-small cell	Neovastat	Aeterna Zentaris	Phase III
	Cancer, Kaposi's sar	CMT-3	CollaGenex	Phase II
	Cancer, general	S-3304	Shionogi	Phase II
	Osteoarthritis	CPA-926	Kuhera Chemical	Phase II
	Cancer, general	XL-784	Exelixis	Phase I
	COPD	Ilomastat	Arriva	Preclincial
	Unspec. Indication	BMS-2 MMP inhibitors	Bristol-Myers Squibb	Preclincial
	Inflammation general	Metalloenzyme inhibitor	Serono	Preclincial
	Cancer, general	SC-77964	Pfizer	Preclincial
	Osteoarthritis	AZD-8955	Astra Zeneca	Preclincial
	Cancer, general	AMEP ligands	Bio Alliance Pharma	Preclincial
MMP-1	Peridontitis	Doxycycline	CollaGenex	Launched
	Inflammation, MS, Pancreatic Cancer	Marimastat	Vernalis	Phase III
	Cancer	Marimistat	British Biotech	Discontinued
MMP-2	Peridontal Disease	Periostat		Launched
	Cancer	Rebimistat		Phase III
	Prostate Cancer	Rebimastat	Celltech	Phase I
	Osteoarthritis	S-3536	Shionogi	Phase I
	Cancer, geneal	Ro-28-2653	Hoffmann-La Roche	Preclinical
MMP-3	Unspec. Indication	Neurolysin inhibitors	Aventis	Preclinical
MMP-8	Osteoarthritis	Glucosamine Sulfate	Rottapharm	Launched
MMP-9	Inflammation general	REGA-3G12	Biophage Pharma	Preclinical
	Prostate Cancer	MMP-9 inhibitor	AVI Biopharma	Preclinical
	Cancer, general	BDI-7800	Biopharmacopae	Preclinical
MMP-12	Multiple sclerosis	MMP-12 inhibitor	Serono	Preclinical
MMP-13	Osteoarthritis	MMP-13 inhibitor	Novartis	Preclinical
TACE	Arthritis, MS	BMS-561392	Bristol-Myers Squibb	Phase II
	Inflammation general	TMI-005	Wyeth	Phase I
	Asthma, COPD	PKF-241-266, 242-484	Novartis	Preclinical
NEP	Hypertension	Candroxatril	Pfizer	Phase III (disc)

alpha converting enzyme), Adamalysin, Aeruginolysin, Angiotensin Converting Enzyme-1 (ACE), Anthrax L.F., Astacin, Atrolysin C and E, Bontoxilysin, Collagenase-1 (interstitial, MMP-1), Collagenase-2 (Neutrophil, MMP-8), Collagenase-3 (MMP-13), Gelatinase A (MMP-2), Gelatinase B (MMP-9), Matrilysin (MMP-7), Membrane-type matrix metallo-proteinase 1 (MMP-14), Nepralysin, Serralysin, Stromelysin-1 (MMP-3), Stromelysin-3 (MMP-11), and Thermolysin.

Most inhibitors are small molecules that bind only with the prime side pockets of metalloproteases, and this has led to difficulties in obtaining enzyme selectivity especially for matrix metalloproteases. All metalloprotease inhibitors possess a zinc-binding ligand like carboxylate, sulfhydryl, phosphinate, hydroxamate or formylhydroxylamine. ACE inhibitors were the first protease inhibitors to reach the market place in the early 1980's and there are now quite a few available in man.

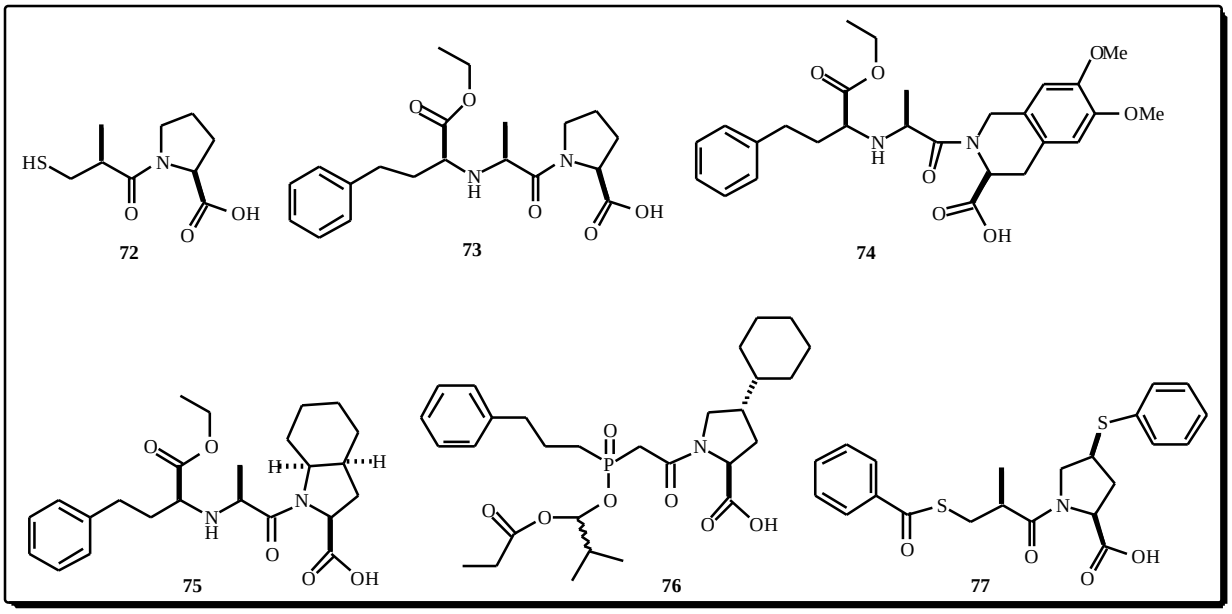
6.1. Angiotensin-Converting Enzyme (ACE)

Angiotensin converting enzyme (ACE) is an important regulator of the renin-angiotensin sytem but is a relatively non-specific enzyme. While it catalyses the hydrolysis of decapeptide angiotensin I, cleaving two amino acids from the C-terminus, to produce octapeptide angiotensin II, it also cleaves bradykinin, substance P, and other peptide hormones. The inhibition of ACE is a recognized target for antihypertensive drugs that can reduce blood pressure, angiotensin II causing potent vasoconstriction through stimulating angiotensin II (type I) GPCRs [201, 202].

A range of ACE inhibitors have been approved for use in man for treating hypertension, and also exhibit efficacy for treating endothelial dysfunction, myocardial infarction, congestive heart failure, and provide renal protection [203]. Such ACE inhibitors include *captopril* (**72**) (IC_{50} 23 nM), *enalapril* (**73**) and *enalaprilat* (diacid metabolite of enalapril) (IC_{50} 4.5 nM), *lisinopril* (IC_{50} 4.7 nM), *benazepril* (IC_{50} 1.7 nM), *moexipril* (**74**) [204] (IC_{50} 2.6 nM), *trandolapril* (**75**) [205] (IC_{50} 0.93 nM), *fosinopril* (**76**) (IC_{50} 1 nM), *ramipril* (IC_{50} 4 nM), *quinapril* (IC_{50} 8.3 nM), *zofenopril* (**77**) (IC_{50} 0.4 nM). The IC_{50} values shown are for de-esterified active metabolites. As a result of the non-specific hydrolytic action of ACE, the above inhibitors exhibit side-effects such as cough, skin rash and angio-neurotic oedema usually attributed to bradykinin.

The inhibitors characteristically are low molecular weight compounds that target only S1, S1' and S2' subsites of the protease. They all use a chelating group to bind to the active site zinc, resulting in high inhibitor potency and selectivity. Captopril (**72**) and zofenopril (**77**) [206] use a thiol ligand for zinc, fosinopril (**76**) uses a phosphinate, while most others use a carboxylate to bind zinc. Except for captopril and lisinopril, the ACE inhibitors are orally administered as ester pro-drugs for good oral bioavailability, the ester being cleaved *in vivo*. Enalaprilat is available for iv administration. Oral bioavailability in humans for all compounds except enalprilat is 50-80%. Based on enalapril, other homophenylalanine derivatives with a long duration of action (> 24 h) have been developed, including moexipril (**74**) and trandolapril (**75**). Except for captopril (duration of action is 6-10 h) all ACE inhibitors can be dosed once per day. ACE inhibitors are only effective in 40-50% of people suffering from hypertension, usually those with high renin activity, and is enhanced by coadministration of diuretics which can also lead to side-effects such as potassium depletion.

In recent years, angiotensin II receptor antagonists such as losartan, [207] irbesartan [208], candersartan cilexitil [209], and olmesartan [210] have been approved for the treatment of hypertension and are as effective as ACE inhibitors but without side-effects like cough, angioneurotic oedema that are associated with ACE inhibitors [211].



6.2. Neutral Endopeptidase

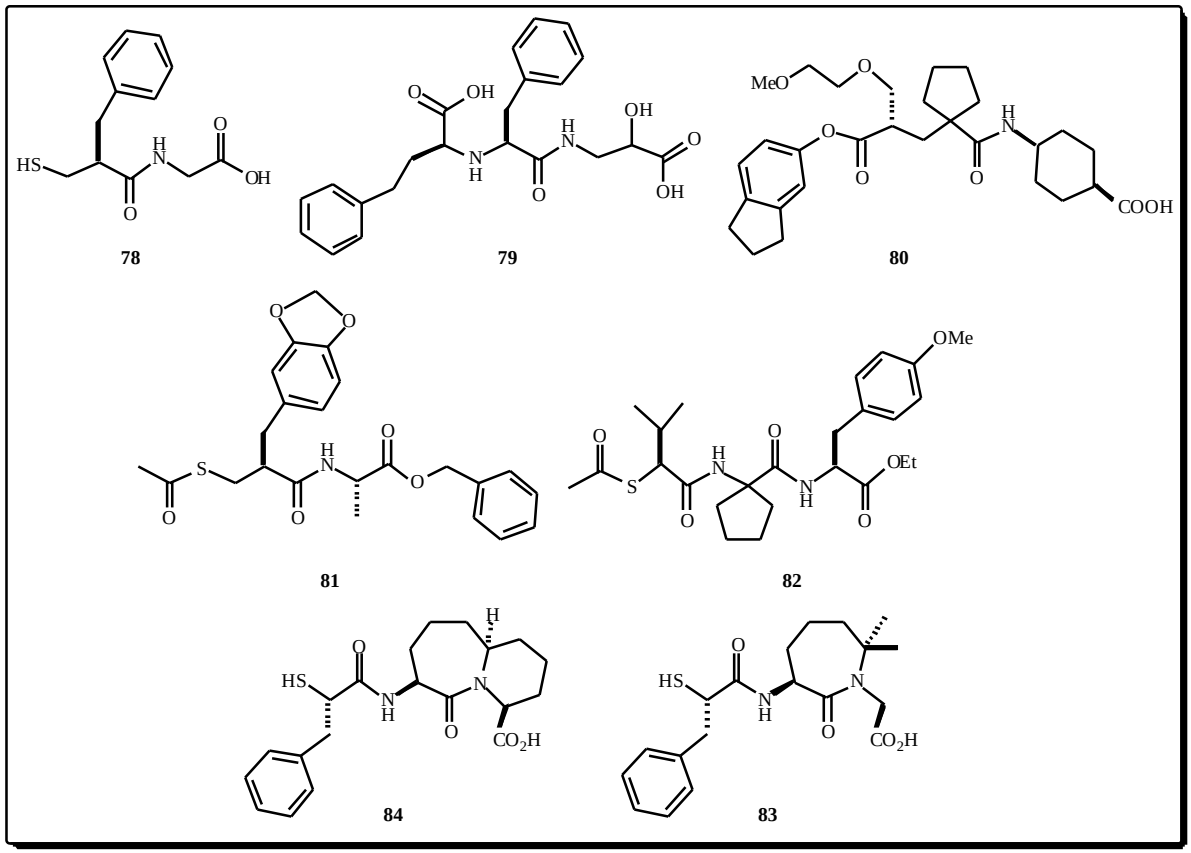
Neutral endopeptidase (NEP) is quite similar to ACE but catalyses the hydrolysis of atrial natriuretic peptide (ANP), which is secreted by the heart into the circulation to decrease blood pressure, raise the urinary excretion of water and sodium, and lower plasma renin and aldosterone levels. Like ACE it is non-specific and also cleaves endogenous enkephalins in the brain. Inhibitors have been designed to target S1, S1'and S2' subsites of NEP as prospective treatments for hypertension and analgesia [212].

Thiorphan (**78**, IC_{50} 4 nM) was among the first NEP inhibitors, but its enantiomers also inhibit ACE (IC_{50}(*R*) 860 nM, (*S*) 140 nM). By exploiting differences in the size of the S1 and S1' subsites between NEP and ACE, selectivity has been obrtained for NEP. Thus, *SCH39370* (**79**) projects its homotyrosine substituent into S1 which, together with the β-hydroxy GABA residue at the C-terminal end of the molecule, confers highly specific for NEP (IC_{50} 11 nM vs IC_{50} > 10000 nM for ACE). No NEP inhibitor has yet progressed through clinical trials, though *candoxatril* (**80**, Pfizer), [213] an ester pro-drug of *UK-69578* (candoxatrilat) (IC_{50} 28nM), was recently discontinued after late-stage Phase III clinical trials for the treatment of conjestive heart failure.

Since NEP inhibitors show significant diuresis and naturesis in humans without potassium loss, attempts have been made to create compounds that inhibit both NEP and ACE. Such dual inhibitors can possess synergistic actions that are superior to individual ACE or NEP inhibitors alone. [214] *Fasidotril* (**81**), [215] is such a dual inhibitor derived through modification of thiorphan (**78**). The methylenedioxy substituted phenyl ring of **78** was proposed to bind to the S1 subsite of ACE and the S1' subsite of NEP. The metabolite *fasidotrilat* (with free acid and thiol) is a potent inhibitor of ACE (K_i 9.8 nM) and NEP (K_i 5.6 nM), while **81** has high oral activity against both enzymes *in vivo* (ED_{50} 0.2-0.5 mg/kg mouse). Fasidotril (Phase II clinical trials) and other NEP/ACE inhibitors are useful in disorders associated with sodium retention such as congestive heart failure [216]. Other dual NEP/ACE inhibitors in clinical trials include *CGS30440* (**82**), *sampatrilat* [217], *omapatrilat* (**83**) (IC_{50} (ACE) 5 nM, (NEP) 8 nM) [218-220] and derivative **84** (IC_{50} (ACE) 12 nM, (NEP) 63 nM) [221]. Omaprilat (**83**) advanced to phase III clinical trials, effectively inhibiting both enzymes *in vivo* at 0.1 μmol/kg, and is long-acting. A single dose of 12 mg/kg in rats decreased mean arterial pressure by 40 mmHg for 24 h and is as effective as fosinopril (**76**) as an ACE inhibitor.

6.3. Matrix Metalloprotease Inhibitors

Matrix metalloproteases (MMPs) are zinc metallo-proteases that degrade and remodel structural proteins (e.g. membrane collagens, aggrecan, fibronectin, laminin) in the extracellular matrix. Natural endogenous tissue inhibitors of metalloproteases (TIMPs) tightly regulate their activities *in vivo*, but over-expression and activation of MMPs leads to tissue degradation. MMPs are associated with tissue



remodeling in many stages of human development, physiology and ageing, as well as in immunity, wound healing, growth and spread of malignant tumours, and chronic diseases like multiple sclerosis, arthritis, fibrosis and other inflammatory conditions [218]. There are almost two dozen human MMP enzymes now known, all sharing significant sequence and structural homology that has made obtaining selective inhibitors of individual MMPs a very challenging objective. Fibroblast collagenase-1 (MMP-1), gelatinase A (MMP-2), stromelysin-1 (MMP-3), matrilysin (MMP-7), neutrophil collagenase-2 (MMP-8), gelatinase B (MMP-9), stromelysin-2 (MMP-10), stromelysin-3 (MMP-11), collagenase-3 (MMP-13), membrane-type matrix metalloproteinase 1 (MMP-14), and aggrecanase have probably been the main focuses of therapeutic strategies [222,223]. Three-dimensional crystal and/or solution structures of inhibitors bound to MMP-1, 2, 3, 7, 8, 9, 11,13, 14 and MT1-MMP have all been determined in the last 10 years [157] and this structural data is greatly enhancing drug design research. There are numerous reviews on metalloprotease inhibitor development now available [7, 224-227] and at least 20 MMP inhibitors are undergoing clinical trials for diseases like cancer, arthritis and multiple sclerosis [225]. Like other metalloproteases, high potency can be obtained with just two of S1, S1', S2' subsites of MMP enzymes occupied by inhibitor substituents. The S1' subsite (in particular) differs most among MMPs and specificity can often be obtained by varying P1' of inhibitors. Larger P1' substituents often lead to greater specificity for MMP-2, 3 and 9 over MMP-1 and 7. More recently, increased selectivity between MMPs has been achieved by designing molecules to make non-covalent interactions at the S2 and S1 subsites as well as S1', S2' and S3' subsites. Oral bioavailability has been a major hurdle for these substrate-based inhibitors.

6.3.1. MMP Inhibitors - Hydroxamates

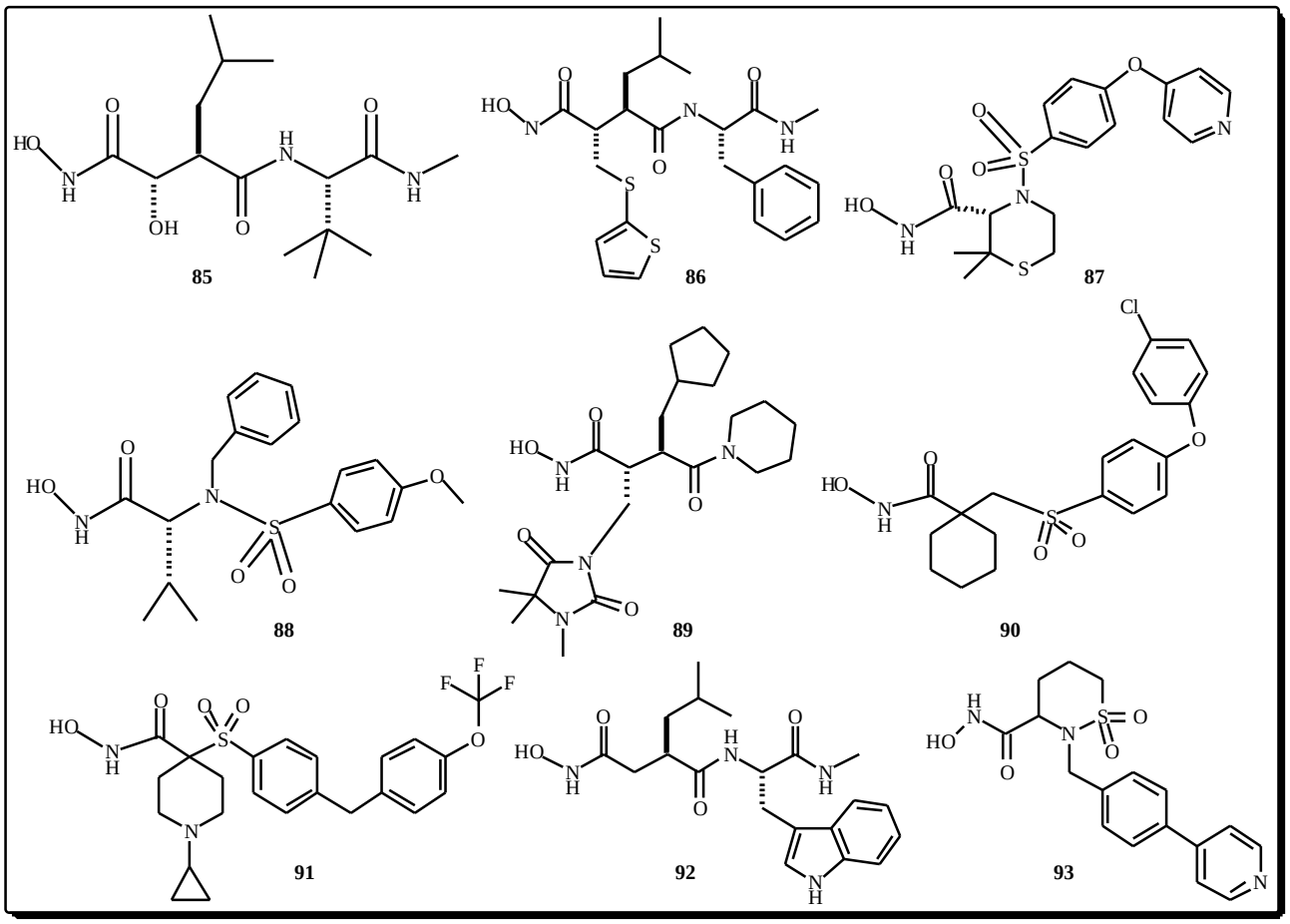
Marimastat (**85**) and *batimistat* (**86**) are two hydroxamates developed by British Biotech (Vernalis). Although both have been discontinued as anticancer agents following a series of unsuccessful Phase III clinical trials for invasive cancers and metastasis [226], they have provided a great deal of information about MMP inhibition. Compound **85** was designed based on the Gly-Ile and Gly-Leu collagenase (MMP-1) cleavage sites and has features characteristic of other MMP inhibitors, especially a zinc-binding terminal hydroxamate ligand which has been shown to form tighter binding complexes with fibroblast collagenase (MMP-1) than with formylhydroxylamine > sulphydryl > phosphinate > aminocarboxylate > carboxylate analogues [7]. Marimistat (**85**) is a broad spectrum MMP inhibitor of MMPs 1, 2, 3, 7, 8 and 9 (IC_{50} 5, 6, 200, 20, 2, 3 nM respectively). An α-cyclopentyl derivative had IC_{50}s 4, 3, 30, 20 and 900nM against MMP-1, 2, 3, 7 and TACE. The combination of α-hydroxy and P2' tert-butyl groups in **85** gave high oral bioavailability (20-50%), and a 50 mg bid oral dose to healthy volunteers produced C_{max} 200 ng/mL, T_{max} 1-3 h, $t_{1/2}$ of 7-10 h, AUC 1.5 μghr/mL.

Receptor structure-based drug design has been instrumental in recent years for the design specific inhibitors for individual MMPs. X-ray crystal structures have been determined for the orally active compounds **87** (phase III), [228] **88** (phase I), [229] **89** (phase I), [230] all developed by exploiting structural differences in the active site of MMPs. Such compounds are usually more potent and specific for particular MMPs, though lack of selectivity is still an critical stumbling block for most MMP inhibitors. Thus AG3340 (**87**, *prinomastat*) has Ki 8.2, 0.083, 0.27, 0.038 nM for MMPs 1, 2, 3, 13 respectively; *CGS-27023A* (**88**) has Ki 33, 20, 43, 8nM for MMPs 1,2, 3, 9 respectively) [231]; while *Ro-3203555* (**89**) has Ki 3, 154, 527, 4, 59 and 3nM for MMPs 1, 2, 3, 8, 9, 13 respectively.

RS-130830 (**90**) and *SC-77964* (**91**, Pfizer) are structurally related sulfonamides with hydroxamate at one end. The former displays Ki values 590, 0.22, 9.3, 1200, 0.58 and 0.52 nM against MMPs 1,2, 3, 7, 9, 13, with notable poor activity against MMP1 and 7. The latter is in early preclinical development and shows 28% oral bioavailaibility in rats and efficacy in a mouse model of angiogenesis and tumour growth. *Ilomastat* (**92**, CS-610, Arriva) is listed as a broad spectrum MMP inhibitor and is a simple hydroxamate-dipeptide. It is currently in preclinical studies for the treatment of chronic obstructive pulmonary disease (COPD) and emphysema. Bronchoalveolar lavage data suggested that inhaled ilomastat inhibited a smoke-induced increase in proteases in mice, and when administered once daily for 6 months **92** reportedly reduces neutrophil and macrophage accumulation in lung tissue, dose-proportionately reducing lung damage by 96% of controls. Previous Phase 1 and II/III trials by Glycomed led to granting of orphan drug status in the USA for treating corneal ulcers.

BMS-2 (**93**) is one of a series of sultam hydroxamate inhibitors of MMPs in preclinical trials by BMS for potential in the treatment of arthritis, angiogenesis, periodontal disease, multiple sclerosis and restenosis. It is said to display excellent selectivity over MMP-1. In preclinical trials in mice at 10mg/kg iv, **93** gave Cl, Vss, $t_{1/2}$, AUC values of 5.6 L/h/kg, 7.3ug/mL, 2.5h, 3.77μM/h respectively. At 30mg/kg po the AUC was 4.9μM/h. *UK-383367* (**94**, Pfizer) is a non-peptidic inhibitor of procollagen C-proteinase in clinical trials for application to skin following surgery and is anticipated for treating and preventing dermal scarring and possibly other forms of fibrotic diseases. Preclinical studies in pigs suggested that topical administration for 7 days gave a flux of 30μM at 24h, and in a human fibroblast model **94** was highly selective against MMPs involved in wound healing and inhibited collagen deposition.

Procter and Gamble researchers have reported bis-sulfonamides as potent MMP inhibitors, including **95** (Ki 24, 18, 30,2.7 nM against MMPs 1, 3, 7, 9) for which a crystal structure with stromelysin showed that one para-methoxyphenyl substituent extended into the S1' subsite while the other binds in the S1/S2 pocket [232]. Du Pont researchers reported that macrocyclic MMP inhibitors like **96** behave like derivatives of marimastat, with the macrocyclic tether directed into solvent and low nanomolar potency for MMPs 1, 3 and 3 (Ki <1, 3, <1nM respectively [233]. Rhone-Poulenc reported that **97** inhibits MMP-1 (K_i 2 μM), MMP-2 (K_i 10 nM), MMP-3 (K_i 500 nM) as well as PDE4 (K_i 30nM) [234]. PDE4 inhibitors have also been



shown to be effective for treating inflammatory diseases (arthritis, multiple sclerosis, atopic dermatitis, psoriasis), [235] so this dual action could be therapeutically advantageous.

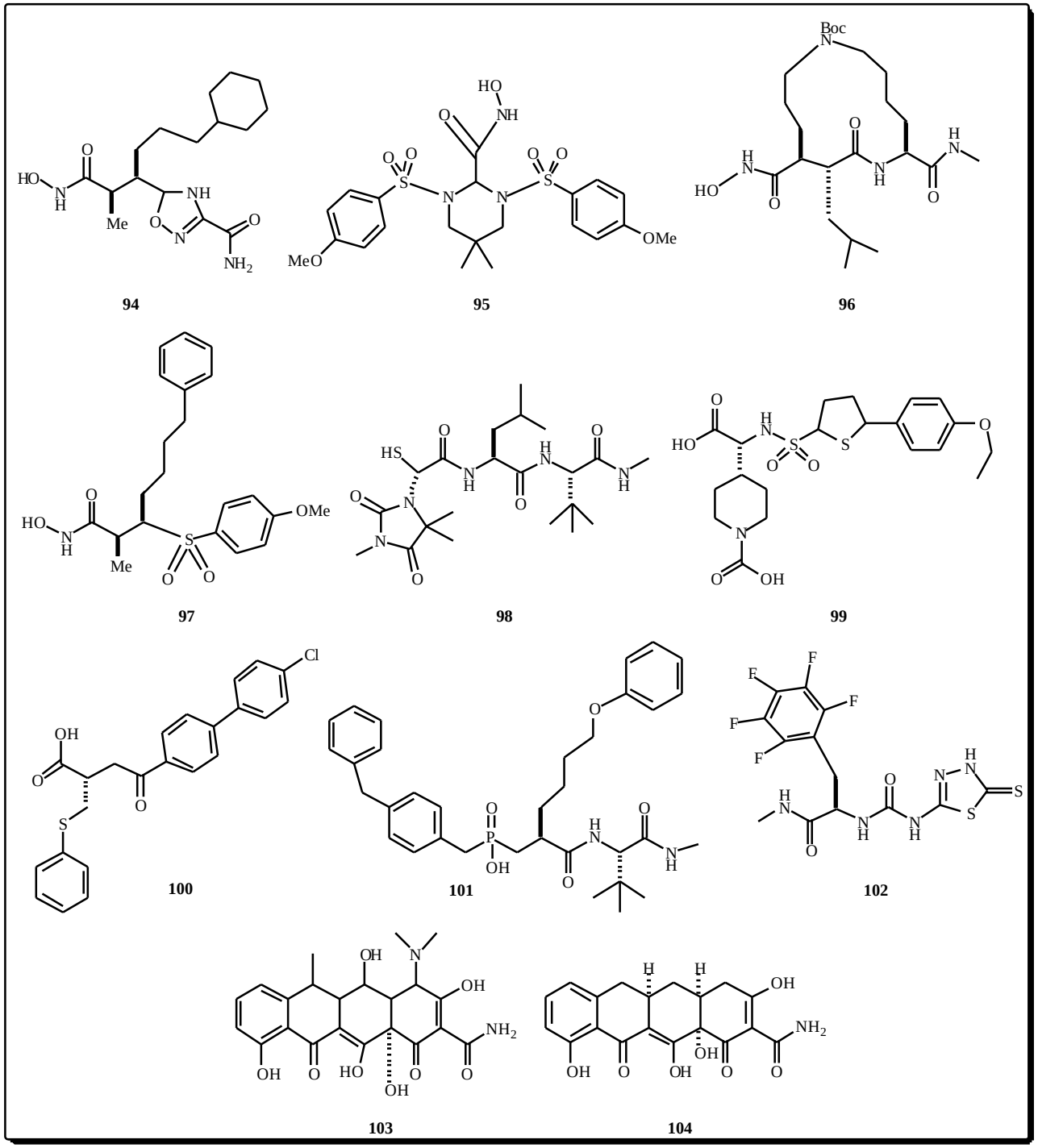
6.3.2. MMP Inhibitors - Non-Hydroxamates

There are some indications that researchers are moving away from hydroxamate-containing protease inhibitors, no doubt because their high affinities for metals is accompanied by less discriminate target receptor binding and unwanted toxicities. *Rebimastat* (**98**, BMS/Celltech, BMS-275291) is a non-hydroxamate inhibitor of MMP 2 and 9 in development for cancer, especially prostate cancer, though it also inhibits angiogenesis. Preclinical studies suggested that it is orally bioavailable and selective for MMP enzymes without affecting TNFα or IL-1β release. A Phase I study revealed that **98** was well tolerated at 1200mg/day and $t_{1/2}$ > 50h suggested possible use once daily. A Phase II study in 75patients with Stage IIb/IV nsclc with 1200mg po bid **98** plus paclitaxel + carboplatin gave low response rates (20%) and the trial was halted when it was judged unlikely to achieve an efficacious endpoint. Phase I studies for prostate cancer and Kaposi's sarcoma have been reported. The similarly small non-hydroxamate inhibitor **99** (Novartis) is in preclinical development as a MMP-13 inhibitor for osteoarthritis. It is said to have no MMP-1 activity and oral bioavailability in the rat (47%), though few other details are presently available.

Due to the difficulty of developing orally bioavailable inhibitors via the substrate-based approach, synthetic and natural product libraries have also been screened in order to obtain leads for the design of non-peptidic MMP inhibitors with better bioavailability. Like **88**, *Bay-12-9566* (**100**) [236] was identified in this way. It is selective for MMP-2 (Ki 5μM, 11nM, 143nM, 301nM, 1.5μM against MMPs-1, 2, 3, 9, 13), orally bioavailable, and in phase III clinical trials.

While not in clinical trials, as far as we know, **101** is a phosphinate inhibitor that binds to S2, S1' S2', S3' of MMPs 1 and 13. Pfizer reports that **101** is >1000 fold more selective for MMP-3 (Ki 30nM) than MMP-1 (Ki > 30μM), [237] Pharmacia and Upjohn reported that **102** is typical of a series of thiadiazole urea inhibitors that are selective for MMP-3 (Ki 18nM) over MMP-2 (K_i 3 μM) and other collagenases. A crystal structure showed that **100** bound to MMP-3 at S1, S2, S3 with the exocylic sulfur interacting with zinc [238].

Tetracycline analogues *periostat* (**103**, docycline, Collagenex) and *metastat* (**104**, CMT-3, Collagenex) are clinically advanced. Compound **103** is an orally administered collagenase and MMP inhibitor that has been launched for the treatment of periodontal disease, although Phase III trials for periodontitis are still in progress, and Phase II trials have



94 **95** **96**

97 **98** **99**

100 **101** **102**

103 **104**

proceeded for acne, occular inflammation and acute coronary syndrome. A non-antibiotic chemically modified tetracycline analogue [239], **104** is an MMP inhibitor that is claimed to down-regulate inflammatory cytokines and is in development for treating metastatic cancer and acute lung injury. It has anti-tumour and anti-metastatic activity at low μg/mL concentrations. In preclinical studies it had anticancer activity at 2-12μg/mL and a protective effect in models of acute lung injury. Phase I and II trials for HIV-associated Kaposi's sarcoma patients, suggested that **104** caused tumour regression at 50 or 100 mg/day (36% and 28% response rates) over 6 months–2 years and median levels of circulating MMP-2 levels were reduced.

Among other inhibitors that are either not well reported, structurally uncharacterised, in early stage clinical trials, not especially novel, or not efficacious are *glucosamine* sulfate (inhibits collagenase and stromelysin), *S-3304* (Shionogi),

XL-784 (Exelixis), *CPA-926* (Kureha), and the shark cartilage-derived neovastat (*Aeterna Zentaris*).

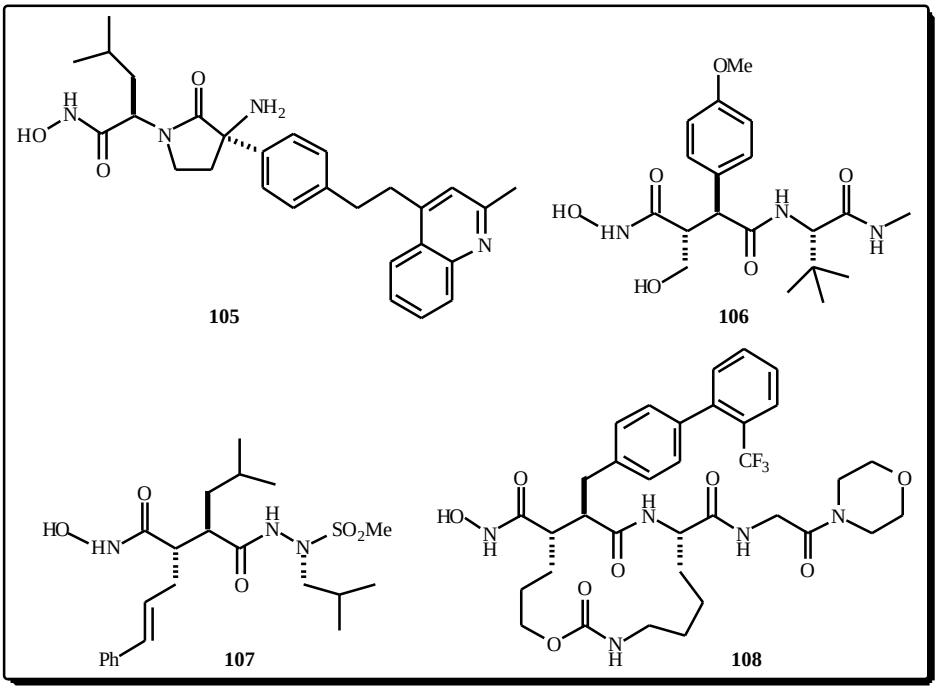
6.4. TACE

Tumor Necrosis Factor-Alpha Converting Enzyme (TNF-α convertase, TACE) is a zinc metalloenzyme that catalyses the hydrolysis of Ala-Val bond of a membrane-bound 233 residue 26kDa pro-TNF-α, releasing a soluble 17 kDa immunoregulating cytokine (tumour necrosis factor-alpha, TNF-α) into the circulation [240]. TNF-α is a very important proinflammatory mediator that is overproduced in inflammatory diseases such as rheumatoid arthritis (RA), multiple sclerosis, diabetes, ulcerative colitis and Crohn's disease, and congestive heart failure [241]. It plays central roles in the recruitment and retention of inflammatory cells and the production and regulation of inflammatory mediators such as IL-1β, IL-6, prostaglandins, leukotrienes and it also stimulates release of MMPs [242]. TNF-α and its receptors are over-expressed in the synovium and cartilage-pannus junction of RA joints [243] and monoclonal antibodies against TNF-α (e.g. infliximab or Remicade®) as well as soluble TNF-α receptor-Fc dimer (etanercept or Enbrel®) have become impressive, if expensive, anti-inflammatory agents in patients with RA [243,244]. Therapeutic effects of anti TNF-α proteins only last a short time due to their inappropriate pharmacokinetic profiles, and small orally bioavailable TACE inhibitors could have enormous potential as new therapeutics.

The enzyme TACE was purified and cloned in 1997, a crystal structure was published in 1998 and features large connected S1'-S3' subsites, [245] and there are quite good TACE inhibitors being pursued [246, 247]. The most advanced compound **105** (*BMS-561392*, DPC-333) entered Phase II clinical trials in 2001 for rheumatoid arthritis and is also under investigation for potential treatment of inflammatory bowel disease [248]. In preclinical studies **105** had oral bioavailability (53% dog, 25% rat), in an LPS-mouse model it was orally active and dose-dependently inhibited TNFα production (ED_{50} 6mg/kg), and 50mg/kg bid/tid in collagen-induced arthritic mice reduced paw and ear inflammation to histologically similar levels to non-arthritic control mice. We are not aware of any other TACE inhibitors that have advanced to Phase II, though there are many compounds in preclinical studies, [246] including the broad spectrum MMP/TACE inhibitors **106** (*PKF242-484*, Novartis) and **107** (*Ro 32-7315*), and a macrocyclic inhibitor **108** that is more selective for TACE over a panel of MMPs.

7. SUMMARY AND FUTURE

Proteolyic enzymes are essential for most physiological processes, but their overexpression or unregulated actions can lead to many debilitating diseases associated with, for example, the CNS and cardiovascular systems, inflammatory and neurodegenerative conditions, and viral and parasitic infections. In addition specific mutations in protease genes are now known to be responsible for over 50 human diseases [249]. It also seems likely that the mutation/regulation of protease precursors, protease folding, endogenous protease inhibitors, cofactors, receptors and transporters can all indirectly influence protease activity. In the face of such very strong evidence for proteolytic enzymes being crucial mediators of mammalian physiology and disease, it is not surprising that there has been extensive research activity and resources targeted to the development of potent and selective protease inhibitors, particularly over the last 15 years. However what is perhaps surprising is that so few



compounds have actually arrived in the marketplace to date.

We have listed the protease inhibitors currently available as human medicines in Tables **1**-**5**. Although many compounds have entered clinical trials over the past 5 years since our previous survey, most have not progressed as expected. Even those that have survived the rigours of clinical evaluation and penetrated the marketplace, like inhibitors of HIV protease, thrombin, ACE and elastase are not without side effects that warrant a new generation of improved candidates. It is clear that potent inhibitors can now readily be obtained for most proteases given sufficient time and resources, but target *selectivity* is still a major problem. Some of the larger pharmaceutical companies have been building libraries of all known human proteases specifically to screen *in vitro* for non-selective actions of putative protease inhibitors. This may assist in improved selection of clinical candidates, although there is no real way of accurately predicting *in vivo* selectivity for proteases. Similarly selectivity is difficult to predict over other targets, the *in vivo* human side effects of ACE, HIV protease and thrombin inhibitors were not realised during their early stage development. It seems likely that more chemical foliage will be needed in future to enhance selectivity and minimise side effects.

Other key problems concern *delivery* and although promising new predictive methods for *ADMET* properties for example are being developed, it remains practically very difficult to predict oral bioavailability and other pharmacokinetic and pharmacodynamic parameters in humans. This review is littered with testimony to the difficulties associated with predicting and optimising human *pharmacokinetics*, *metabolism* and *pharmacodynamics*. Patient compliance with alternative methods of delivery has always been a significant problem in the clinic even for terminal illnesses.

There is the further issue of the development of *drug resistance* to protease inhibitors in the face of a build up of substrate pressure, and selection of catalytically active mutant or other salvage proteases that do not have complementarity for carefully designed inhibitors of wild type proteases. On this issue, it may not be desirable to have too much selectivity for a particular protease if a variant can escape the inhibitor and execute the same function.

In summary, despite and perhaps because of these drawbacks, a great deal has been learned about drug design and development. The future appears to still hold considerable promise for protease inhibitors. We can anticipate new, overexpressed proteases from genomic/biochemical comparisons made between normal/diseased cells, host/pathogen, healthy/unhealthy subjects leading to more effective and efficient validation of proteases as drug targets. We can also expect that new advances in protein chemistry will lead to faster production and greater quantities of pure recombinant proteases. New advances in structural biology (crystallography, NMR spectroscopy) will produce faster and more accurate inhibitor-protease structures, and new robot/chip assays are already providing faster evaluation of protease inhibitors *in vitro* as potent and selective inhibitors. Advances in solid phase organic chemistry are producing structurally more diverse chemical libraries for inhibitor screening, and faster and more reliable predictive *in silico* methods are enabling better inhibitor design with desirable ADMET properties. These advances, together with more careful attention to inhibitor conformation, mechanism of action, and drug-like composition are expected to result in more potent, more selective, more bioavailable inhibitors with a higher probability of success in the clinic.

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