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Inhibitors of Proteinases as Potential Anti-Cancer Agents

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1. Introduction

Cancer is a collection of over 100 devastating diseases that share a number of characteristics, a primary hallmark of which is out-of-control growth. However, in reality there are significant differences among these diseases, a fact that underlies the difficulties in the past few decades in their chemotherapeutic intervention. It is becoming evident that there are multiple routes to development of cancer, in part because so many distinct metabolic and biochemical steps can be altered to give rise to uncontrolled cell growth.

There is a positive correlation between the aggressiveness of a tumor and the secretion of various proteinases. Using bioinformatic analysis approximately 600 proteinases have been determined in human and mouse genomes (2-4% of the genome), many of which are orthologous (Puente et al., 2003). Only some of them are involved in tumor progression and growth, both at the primary and metastatic sites. As tumor progresses towards increased malignancy, it passes through several important stages that require the action of proteinases. First, the induction of angiogenesis requires degradation of the vascular basement membrane and the release of matrix-bound proangiogenic growth factors. Second, invasion of cancer cells into the surrounding tissue involves the dissolution of cell-cell junctions, degradation of the epithelial basement membrane and remodeling of extracellular matrix to allow cancer cells to be released from the primary tumor mass. Third, at least two key steps in metastasis require proteolysis: intravasation of cancer cells into the blood or lymphatic circulation at the primary site and then extravasation at the secondary site, where proteinases can play a part in promoting the colonization and growth of cancer. Proteinases may co-operatively mediate these steps with individual ones having distinct roles. Therefore, inhibition of their activity might be one of the means to combat the development of cancer. Despite of the described facts, recent findings have revealed that the functions of proteinases in tumors are significantly more complex and varied. For example, they are now seen as extremely important signaling molecules that are involved in numerous vital processes. Proteinase signaling pathways are strictly regulated, and the deregulation of their activities can lead to various pathologies, including cancer. Thus, construction of the inhibitor, which should have an impact on tumor progression and metastasis, cannot be done without placing certain proteinase in the proper metabolic context. Inhibitor therapy design is further complicated because different types of cancers utilize diverse proteinases at varying stages of cancer development.

Through the evolution, proteinases have adapted to the wide range of conditions found in human organism (variations in pH, reductive environment and so on) and they use several catalytic mechanisms for substrate hydrolysis. Basing on the chemical mechanism of their action human proteinases may be classified as: cysteine, serine, threonine, aspartic acid and metallo proteinases. In most cases specific inhibitors for each class of these enzymes are being designed.

A number of reviews on various aspects of the use of proteinase inhibitors as a mean to combat cancer have been published recently (Castro-Guillen et el., 2010; Lee et al., 2004; Magdolen et al., 2002; Pandey et al., 2007; Puxbaum & Mach, 2009; Turk, 2006). Therefore, in this review the current trends in designing of such inhibitors will be presented. Special emphasis will be put on rational design using the techniques which are based either on the knowledge of detailed mechanism of enzymatic catalysis or on three-dimensional structure of active sites of chosen enzymes. Indeed, several small-molecule drugs targeting proteinases obtained in that manner are already on the market and many more are in development.

2. Cysteine proteinases

Despite mounting evidence in the last 30 years showing that expression, localization and activation of lysosomal cysteine proteinases are aberrant in tumor cells, when compared to normal cells, this class of proteases has received little attention. Studies on increased expression, elevated activity and mislocalization of certain enzymes have indicated that members of the cysteine proteinases have been implicated in cancer progression. In mammalian cells, cysteine proteinases are localized mainly in the cytoplasm (calpain and caspase families) and lysosomal compartments (cathepsin and legumain families). Cathepsins are the most directly involved in tumor progression. There are 11 human cathepsins: B, C, F, H, K, L, O, S, W, V and X. These enzymes alongside with aspartic proteinases - cathepsins D and E are mainly involved in intracellular proteolysis within lysosomes. Their increased expression correlates with more aggressive tumors and poorer prognoses for patients (Berdowska, 2004). Cathepsins B and L expression is increased in many human cancers and these enzymes have been investigated most intensively (Bell-McGuinn, et al., 2007; Koblinski et al, 2000). In addition, the predominant expression of cathepsin K in osteoclasts has rendered this enzyme as a major target for the development of novel drugs against bone tumors (Lindeman et al., 2004).

The common belief is that cathepsin-mediated degradation of he extracellular matrix is primarily extracellular at the invasive front of tumor cells. This proteolytic process is associated both with early tumor development, affecting tumor cell proliferation and angiogenesis, and with dissemination of malignant cells from primary tumors (Turk et al 2004). Therefore inhibitors of cathepsins are most intensively studied.

Recent evidence reveals that tumor-promoting proteinases act as part of an extensive multidirectional network of proteolytic interactions. These networks involve various constituents of the tumor microenvironment, with cathepsin B being one of the best examples. An aspartic enzyme - cathepsin D converts pro-cathepsin B into cathepsin B. Cathepsin B can be also activated by a series of other proteinases with cathepsins C and G, urokinase-type plasminogen activator and tissue-type plasminogen activator being the most active ones. Finally, cathepsin B may undergo auto-activation under certain conditions. Activated cathepsin B cleaves a wide variety of targets depending on its subcellular

localization in the tumor microenvironment. Some of its best-known substrates are proteins of extracellular matrix, as well as several important proteinases and their inhibitors (Skrzydlewska et al., 2005; Mason & Joyce, 2011). This complicated pattern of activity emphasizes the central role of cathepsin B in tumor progression simultaneously showing that design of its inhibitors as anticancer agents is a difficult task.

2.1 Cystatins

Cystatins are a superfamily of endogenous inhibitors of proteinases of papain family. So far, 25 representatives of these proteins have been determined. Their main function is to ensure protection of cells and tissue against the proteolytic activity of lysosomal peptidases that are released during normal cell death, or intentionally by proliferating cancer cells or by invading organisms, such as parasites. They exhibit low specificity towards their target proteases, meaning that one cystatin can inhibit several cathepsins. This is because they have apparently similar three-dimensional structure. In some types of cancers, the changes in cysteine cathepsin epression or activity have diagnostic or prognostic value with imbalance between cathepsins and cystatins being associated with tumor phenotype. Since the latter ones are able to inhibit cathepsins tumor-associated activity many studies have indicated their potential use in therapeutic approaches (Keppler, 2006; Kopitz et al, 2005; Palermo & Joyce, 2007). Indeed, one of these inhibitors, cystatin C (mostly the one isolated from egg white) has been used in preclinical research studies for more than 20 years, however, it has been introduced into clinical practice quite scarcely. Despite some isolated promising results (Saleh at al., 2006) this approach is also highly criticized (Keppler, 2006; Mussap & Plebani, 2004) with the greatest problems being high cost of the inhibitor (140 $ USA per milligram), its low bioavailability and short circulation time, and general skepticism amongst clinicians.

Despite the fact that cystatins of different families posses different biochemical properties their inhibitory properties are rather common. They are tight and reversible inhibitors of cathepsins and interact with the active sites of these proteinases via their inhibitory reactive site, made up of the juxtaposition of three regions of the molecule, which form a wedge-shaped edge that is highly complementary to the active site of papain family of proteinases (Fig. 1).
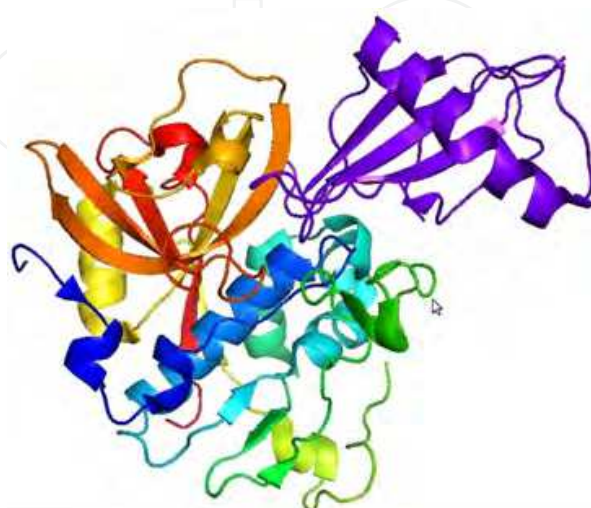

Fig. 1. Stefin A (violet) complexed with cathepsinB.

Thus, mimicking the segment of cystatin interacting with the cathepsin active site (Fig. 1) seems to be the method of choice. This approach is well represented by highly active inhibitor (N-1845, Fig. 2) of cathepsin B (K_i value of 0.088nM) containing an azaglycine residue in place of evolutionary conserved glycine residue in the N terminal part of cystatin (Wieczerzak et al., 2002). Further modification of this molecule, enforced by the use of molecular dynamic and NMR, afforded next potent and selective inhibitor of cathepsin B (K_i of 0.48 nM, Fig. 2) (Wieczerzak et al.; 2007).
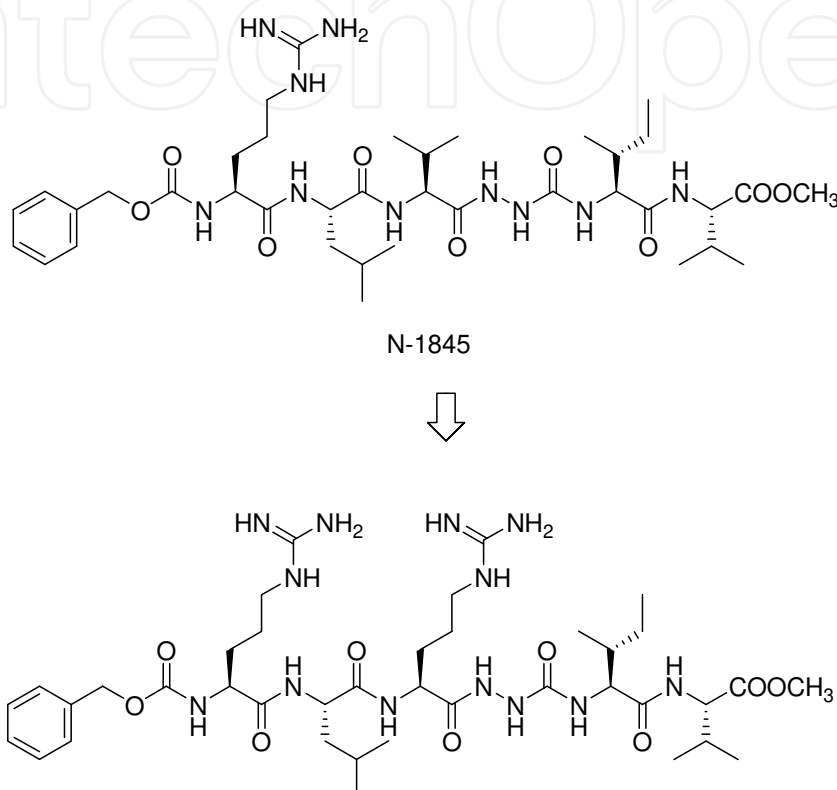

N-1845

Fig. 2. Two potent azapeptide inhibitors of cathepsin B.

2.2 Inhibitors from natural sources

General strategy employed for discovery of a new drug relays on random screening of libraries of newly available compounds and selection of these, which exhibit desired activity at micromolar range. The leads are then being modified in order to obtain significantly more potent and selective inhibitors, which might be further introduced as drugs. Nature is strongly exploited as a source of lead substances. Isolated in 1978 from *Aspergillus japonicus*, non-specific, irreversible inhibitor of cysteine proteinases, E-64 can serve as a good example (Hanada et al, 1978). The epoxysucciante fragment of this molecule reacts with active-site cysteine and binds covalently to the enzyme. By using this inhibitor as a frame and applying X-ray crystal structures of cathepsins B and L, specific inhibitors of these enzymes were designed (Fig. 3), prepared and shown to have promising anticancer activity in animal studies (Katunuma, 2011).

Traditionally, secondary metabolites from streptomyces show a wide range of diversity with respect to their biological activity and chemical nature. Therefore it is not surprising that their secondary metabolites appear to be interesting lead compounds. A mixture of two

peptide metabolites from *Streptomyces* NCIM 2081 (Fig. 4) exhibited potent inhibitory action against papain and significantly inhibited tumor cell migration at subcytotoxic concentrations, indicating its remarkable potential to be developed as antimetastatic drug (Singh et al, 2010).
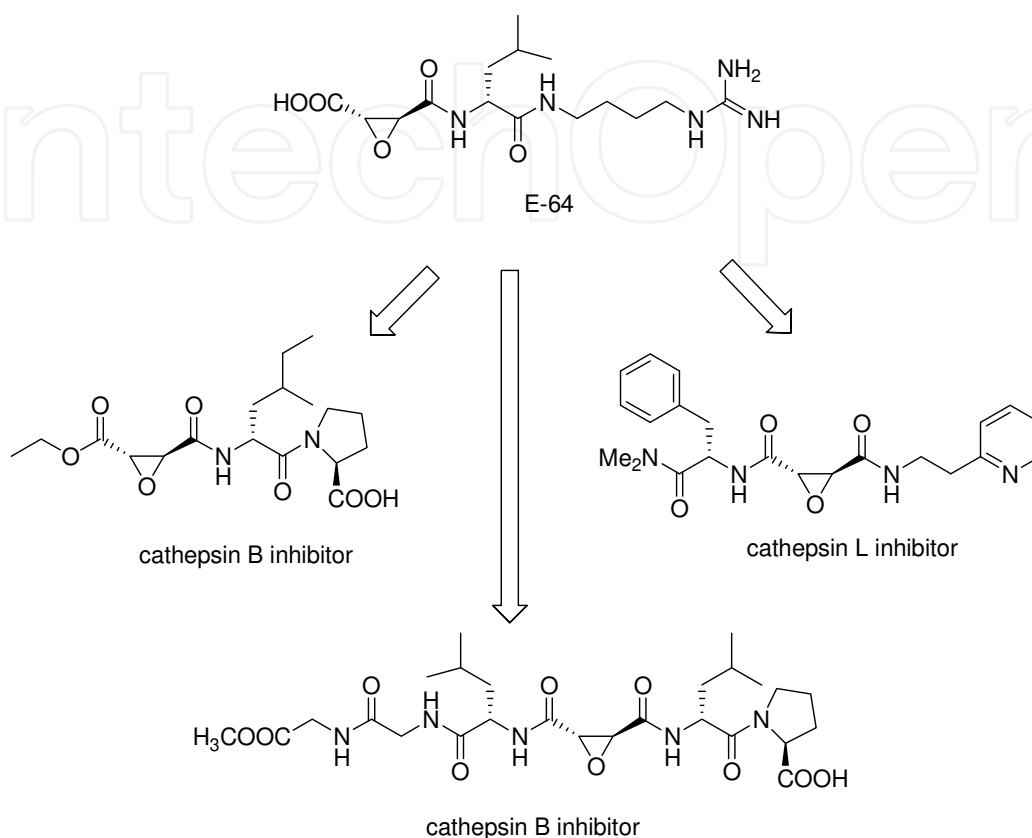

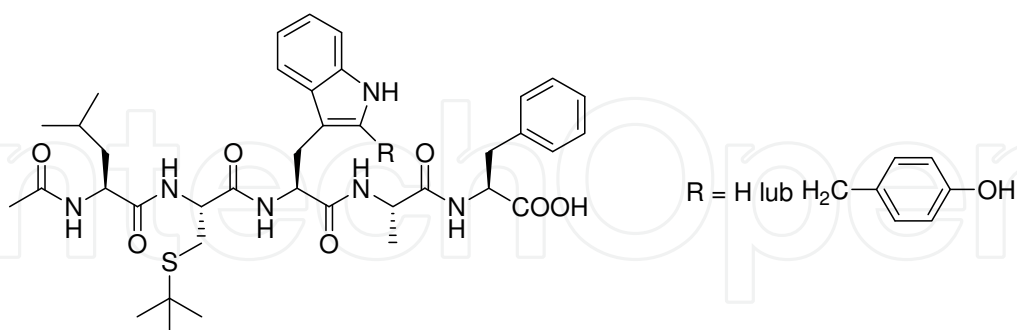Fig. 3. Specific inhibitors of cathepsins B and L built up on the frame of E-64.

Fig. 4. Anticancer peptides produced by *Streptomyces* NCIM 2081.

2.3 Irreversible inhibitors

The majority of synthetic cysteine proteinase inhibitors contain a peptide segment for recognition by the chosen enzyme and an electrophilic functionality that is able to react with the thiolate moiety of active site cysteine. In most cases this results in covalent modification of the enzyme and irreversible inhibition. A wide variety of such reactive groups have been employed, including: azomethyl- or halomethyl ketone, acyloxymethyl ketone,

acylhydroxamate, vinyl sulfone and chloromethyl sufoxide functions. It is also worth to mention that epoxysuccinates, described earlier, also fall within this class of inhibitors. Representative examples of structurally variable inhibitors are shown in Figure 5.
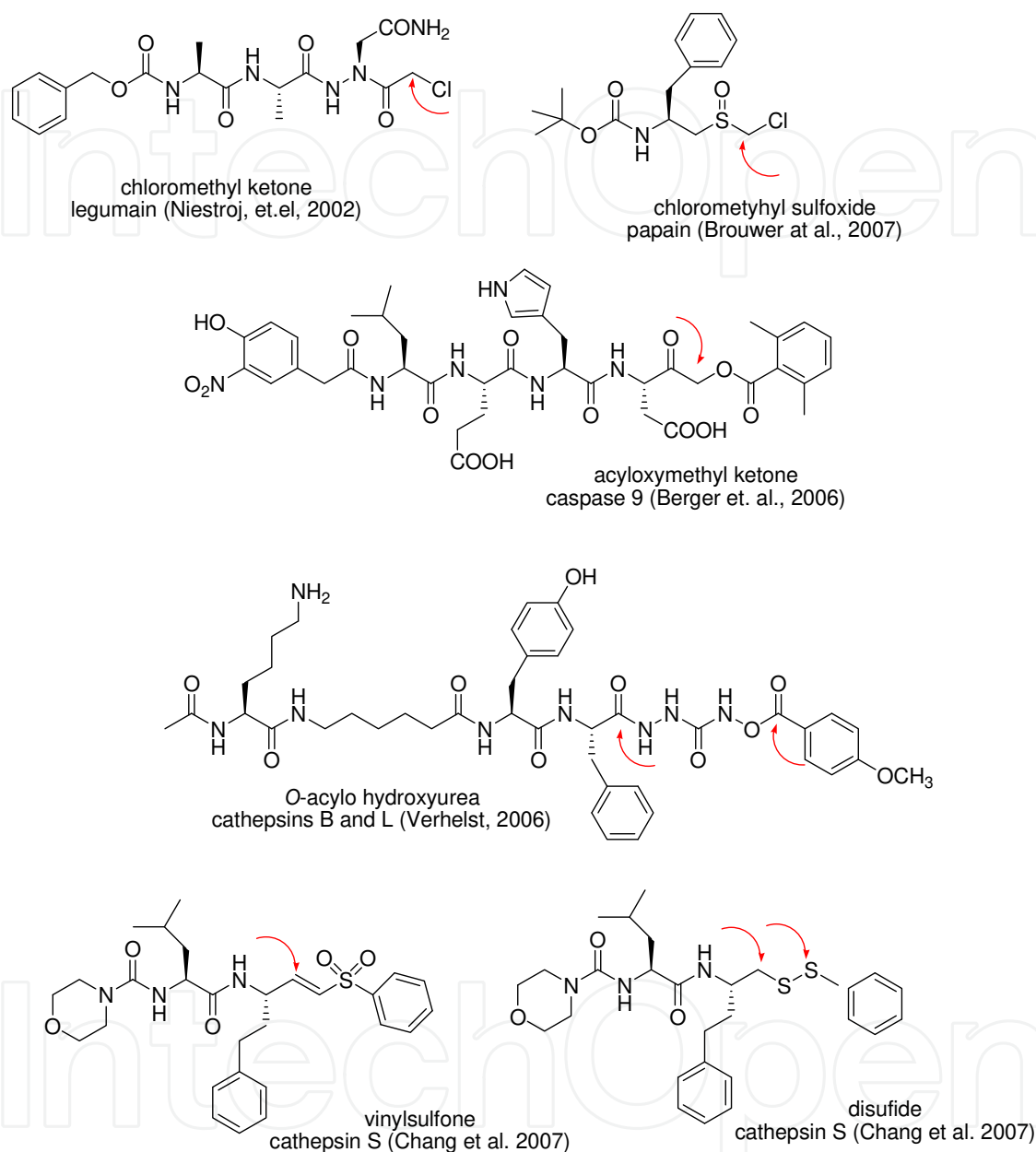

Fig. 5. Representative examples of irreversible inhibitors of cysteine proteinases. The curved arrows indicate possible sites of nucleophilic attack by the active site –SH of active site cysteine.

The reactivity of the electrophilic group greatly determines the selectivity and reaction rate of the formation of the covalent enzyme-inhibitor complex. With this respect halomethylketones are known to react not only with cysteine but also with serine proteinases, thus being non-selective. Although irreversible inhibitors possess high potency and selectivity, they are not considered to be viable drug candidates for treating diseases like cancer, osteoporosis or arthritis. This is because such inhibitors often react over time

with other cysteine proteinases, thus causing toxic side effects or generating immunogenic adducts (Joyce et al., 2004).

Rational design of the peptidyl or peptidomimetic part of inhibitor requires X-ray determination of either cysteine proteinase alone or complexed with already known inhibitors. This provides the detailed insight into the active site and binding pockets of certain enzyme and makes the design process viable. The knowledge of the architecture of the active site of cathepsin B and molecular docking studies were used to design the mechanism-based inhibitor of this enzyme with dual action (Lim et el., 2004)). First, active site Cys-29 is acylated by the inhibitor, which is followed by transfer of acetyloxy moiety of the inhibitor catalyzed by His-199. Thus, two vital active site amino acids are blocked irreversibly (Fig. 6).
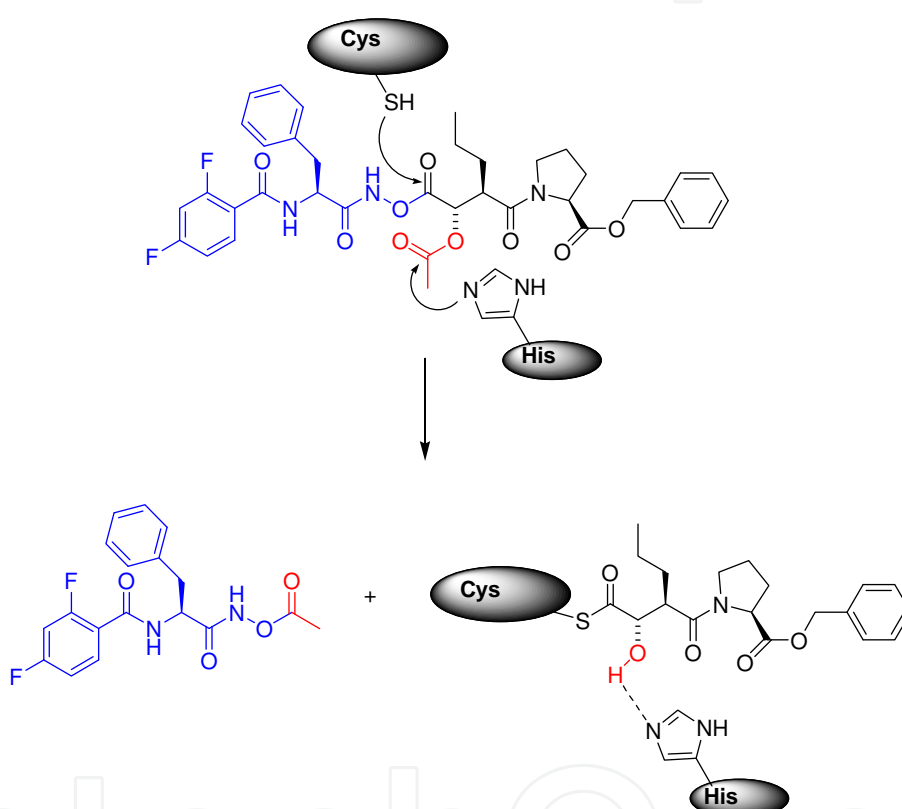

Fig. 6. Inhibition of cathepsin B by mechanism-base dual inhibitor.

2.4 Reversible inhibitors

The strategy in design of reversible inhibitors of cysteine proteinases is commonly the same as in the case of irreversible ones with the exception that the reaction between electrophilic warhead of the inhibitor and the enzyme is reversible. An aldehyde, a metyl ketone, a α-ketoamide or a nitrile groups usually act as the reactive electrophiles. Representative examples of such inhibitors are shown in Figure 7. Some of them are currently being profiled in animal models to further delineate the role of these enzymes in cancer disease processes.

A wide variety of these inhibitors were obtained using computer-aided design. For example, high-resolution X-ray crystallographic data and molecular modeling studies were used to find out one of the most potent inhibitors of cathepsin B (K_i=7nM) - dipeptide nitrile shown

in Figure 8 (Greenspan et al., 2001). In the Figure 8 also the mechanism of reversible binding of this inhibitors was outlined.
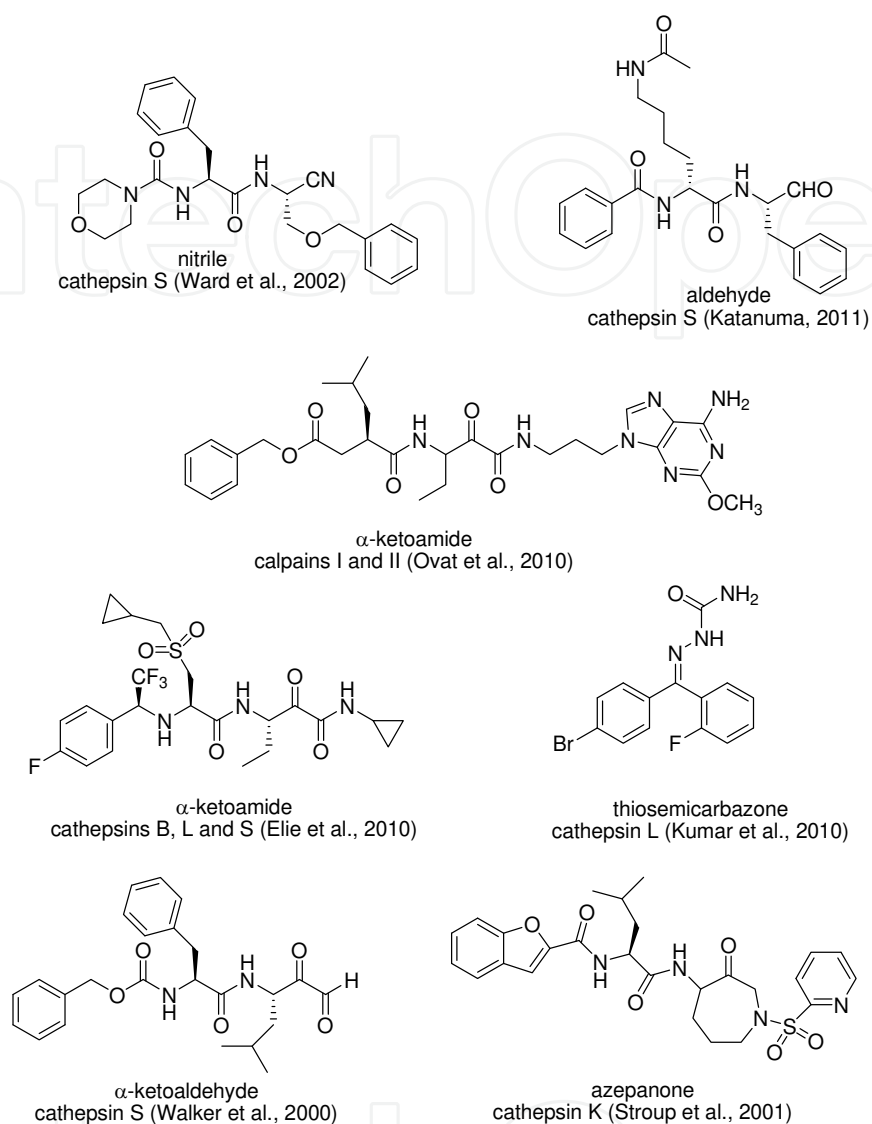

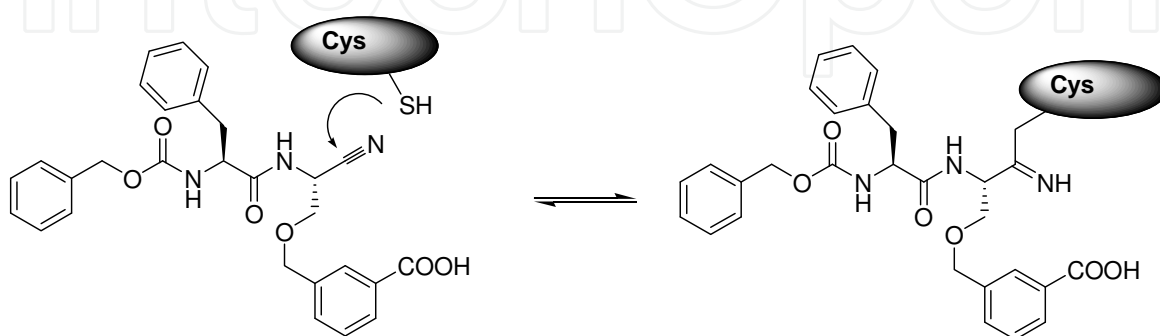Fig. 7. Representative examples of reversible inhibitors of cysteine proteinases.

Fig. 8. Mechanism of inhibition of cathepsin B by dipeptidyl nitrile.

2.5 Metalloinhibitors

The field of metallodrugs is dominated by compounds, which interact with DNA and cause its direct damage. In recent years, however, it was well established that some of them exert cytotoxic activity affecting certain enzymes. Rhutenium (II)–arene derivatives exhibit remarkable selectivity towards solid tumors, most likely by inhibiting two vital enzymes for cancer development – thioredoxin reductase and cathepsin B. The most active inhibitor of cathepsin B is reversibly bound to the active site of the enzyme (Casini et al., 2008). Docking studies revealed that the most important interactions responsible for its activity are those with the residues flanking the active site (Fig. 9).
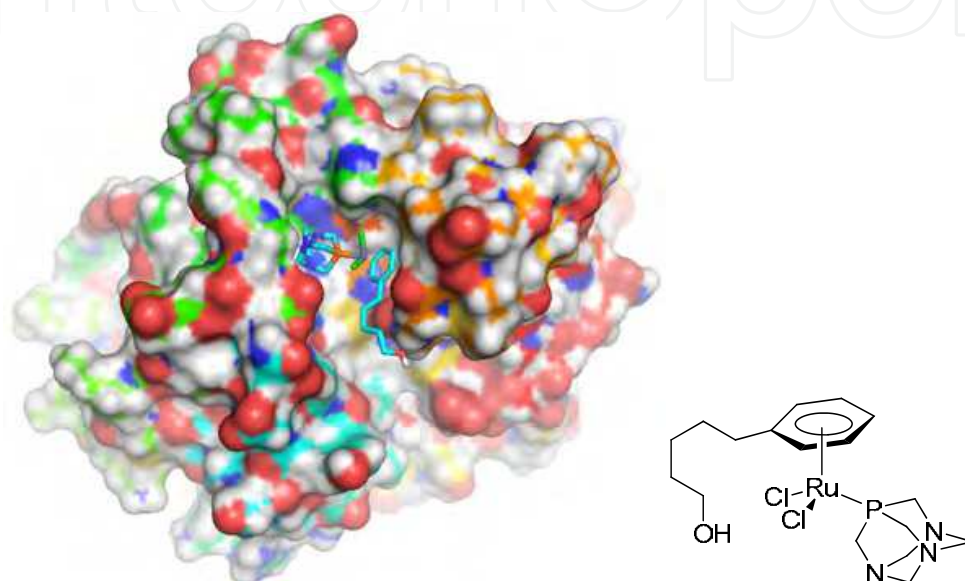

Fig. 9. The most active organorhutenium inhibitor of cathepsin B and its mode of binding in the active site of the enzyme as modeled by docking approach.

Quite contrary, newly synthesized series of organotelluranes appeared to be potent, irreversible inhibitors of cathepsins V and S (Piovan et al., 2011). Tellurium atom is an electrophilic center, which undergoes nucleophilic attack of cysteine thiol at the active site of the enzyme. In this reaction tellurium-halogen bond is broken and new tellurium sulfur bond is formed (Fig. 10). Considering the electrophilicity of the chalcogen, it is known that tellurium is less electronegative than selenium and, due to its greater capacity to stabilize the negative charge, bromide is a better leaving group than the chloride, we can explain the highest reactivity of the dibromo-organotelluranes toward cysteine cathepsins.
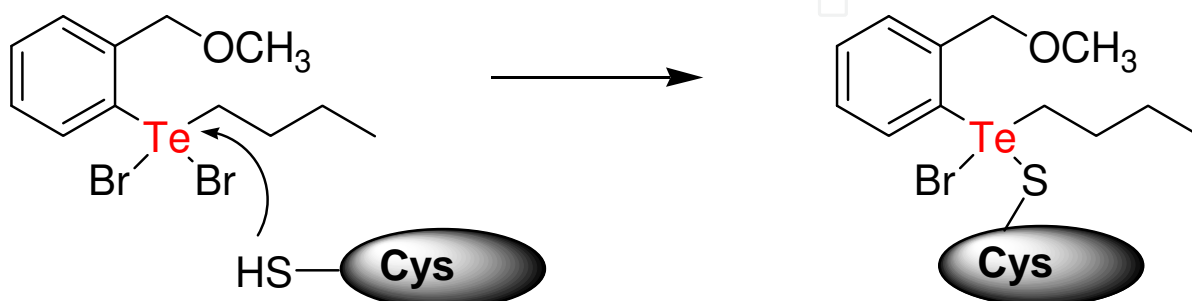

Fig. 10. Mechanism of irreversible inhibition of cathepsins by organotellurane.

3. Serine proteinases

Serine proteinases emerged during evolution as the most abundant and functionally diverse group of proteolytic enzymes - over one third of them belong to this class. They typically contain a catalytic triad of serine, histidine and aspartic acid residues in their catalytic active sites, which are commonly referred to as the charge relay system. This implies common mechanism of peptide bond hydrolysis. It goes through two-step hydrolytic process, which allows acylation of the serine residue by peptide substrate followed by hydrolysis of this adduct and regeneration of the enzyme.

Several serine proteinases have been implicated as important regulators of cancer development. This family includes enzymes involved in mediating of plasminogen (urokinase-type and tissue-type plasminogen activators), as well as serine proteinases stored in secretory lysosomes of various leukocytes, namely mast cell chymase, mast cell tryptase, and neutrophil elastase. Although most secreted serine proteinases emanate from host stromal cells, recent studies implicate a superfamily of cell-surface associated serine proteases, also known as Type II Transmembrane Serine Proteinases (TTSP), such as matriptase and hepsin, as important regulators of cancer development.

Plasmin proteolytic cascade is functionally contributing to neoplastic progression, including acquisition of a migratory and invasive phenotype by tumor cells, as well as remodeling of extracellular matrix components via activation of matrix metalloproteinases. Urokinase-type and tissue-type plasminogen activators (uPA and tPA respectively) regulate enzymatic activity of plasmin. uPA plays a crucial role in tissue remodeling, while tPA is important in vascular fibrinolysis (Naffara et al., 2009).

Mast cell-derived chymases and tryptases are stored in secretory granules. Their release into the extracellular milieu triggers a proinflammatory response as well as induces a cascade of protease activations, culminating in activation of matrix metalloproteinase 9. As a result neoplastic progression is observed (Fiorucci & Ascoli, 2004; Takai et el., 2004).

Neutrophil elastase, a serine protease abundantly present in neutrophil azurophilic (primary) granules, is transcriptionally activated during early myeloid development. Little is known about the role of this proteinase in cancer progression, however, it has the ability to cleave almost every protein contained within the extracellular matrix including, but not limited to: elastin, collagen, fibronectin, laminin, and proteoglycans. Interest in neutrophil elastase during neoplastic processes stems from recent clinical reports that correlate elevated expression of this enzyme with poor survival rates in patients with primary breast cancer and non-small cell lung cancer. It also has recently been found to initiate development of acute promyelocytic leukemia (Naffra et al., 2009; Sato et al., 2006)

Most serine proteinases are expressed by supporting tumor stromal cells, whereas membrane-anchored serine protease appear to be largely expressed by tumor cells at the cell surface and are thus ideally located to regulate cell–cell and cell–matrix interactions. Increasing evidence demonstrates that aberrant expression of enzymes such as matriptase and hepsin is a hallmark of several cancers and recent studies have defined molecular mechanisms underlying TTSP-promoted tumorigenesis, a processes causing carcinomas of skin, breast, and prostate (Choi et al., 2009). Similar association with cancer has led to great interest in kallikreins (Di Cera, 2009), a large family better known for its role in regulation of blood pressure through the kinin system. Prostate-specific antigen (PSA), a serine protease also belonging to the human kallikrein family, is best known as a prostate cancer biomarker since its expression is highly restricted to normal and malignant prostate epithelial cells.

3.1 Proteinous inhibitors

Typically serine proteinases have active site clefts that are relatively exposed to solvent. This permits the access to polypeptide loops of substrates and endogenous inhibitors. By forming strong proteinase-inhibitor complexes the latter ones regulate the activity of proteolytic enzymes and play important physiological roles in all organisms. Therefore, it is not surprising that they are considered as potential anticancer drugs and are already being tested in clinics.

Proteinous serine proteinase inhibitors were the first used against cancer and are so far the most intensively studied (Castro-Guillén et al., 2010; Otlewski et al., 2005). A small metalloprotein, Birk-Bowman inhibitor, isolated from soybeans as far as in 1946, is 8kDa polypeptide of the documented activity in a variety of tumors. Other members of this family have also proved their anti cancer activity, with field bean protease inhibitor being strongly active against skin and lung tumors, and tepary bean inhibitor affecting proliferation and metastasis of fibroblast (Castro-Guillén et al., 2010; Joanitti et al., 2010; Sakuhari et al., 2008). Another classes of similar inhibitors of serine proteinases also exhibit promising anti-cancer properties, to mention only: Kunitz-type inhibitors (Sierko et el., 2007; Wang et al., 2010), serpins (Catanzaro et al., 2011; Li et al., 2006), antileukoprotease (Xuan et el., 2008), nexin (Candia et el., 2006) and lunasin (Dia & de Meija, 2010; Hsieh et al., 2010).

Paradoxically, the action of proteinase inhibitors in some cases results in poorer prognosis and promotion of the cancer development (Fayard et al, 2009; Ozaki et al., 2009). This is contrary to what would be expected from proteinase inhibitor and shows that more detailed studies are required in order to understand their action. These observations also indicate the need for development of inhibitors of different types. Examination of crystal structures of inhibitors bound by various proteinases is a useful tool to study architecture and requirements of serine proteinase binding sites. This is because 3-5 amino acid residues of proteinaceus inhibitor, properly spatially located with respect to each other, interact with small binding region of the enzyme. The binding modes of Bowman-Birk inhibitor from *Vigna unguinocula* with β-chymotrypsin (Barbosa et. al., 2007), and structure of textilinin-1 from the venom of Australian *Pseudonaja textilis* snake complexed with trypsin (Millers et al., 2009) are shown in Figure 11 as representative examples.
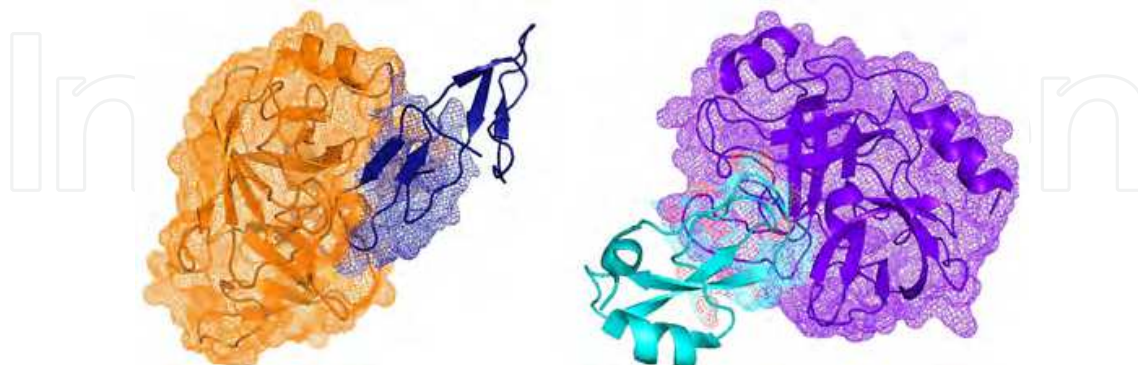

Fig. 11. The binding modes of Bowman-Birk inhibitor with β-chymotrypsin (left-hand side) and textilinin with trypsin

Mutation of the already known protein inhibitors is one of the means to construct highly specific inhibitors of chosen proteinase. Such strategy was applied to obtain specific and potent inhibitors of human kallikrein 14. A human serpin, named α-1-antichymotrypsin,

was used to change its specificity by modifying five amino acids of its reactive center loop, a region involved in inhibitor–protease interaction. This region was replaced by two pentapeptides, previously selected by kallikrein 14 using phage-display technology. In this manner inhibitors with high reactivity towards the enzyme were generated (Fleber et al., 2006).

Sensing the binding site of chosen proteinase by studying structure of bound regions of its inhibitors and substrates is a classical tool for the design of new inhibitors of these enzymes. This concept is well illustrated by the discovery of cyclic peptides mimicking binding fragment of plasminogen activator (uPA) to its cell surface associated receptor (uPAR). The minimal portion of uPa able to bind effectively to uPAR was selected by systematic deletions of peptidyl fragments from the N- and C-terminus of the starting protein inhibitor. Cyclization of the minimal effective structure results in introduction of constrains that limit the conformational freedom of the molecule and ensure proper spatial arrangement of the amino acid residues interacting with the receptor. In that manner cyclic peptides, mimetics of uPA, (the most effective one is shown in Figure 12) were synthesized and found to effectively compete with uPA binding (Schmiedeberg et al., 2002).
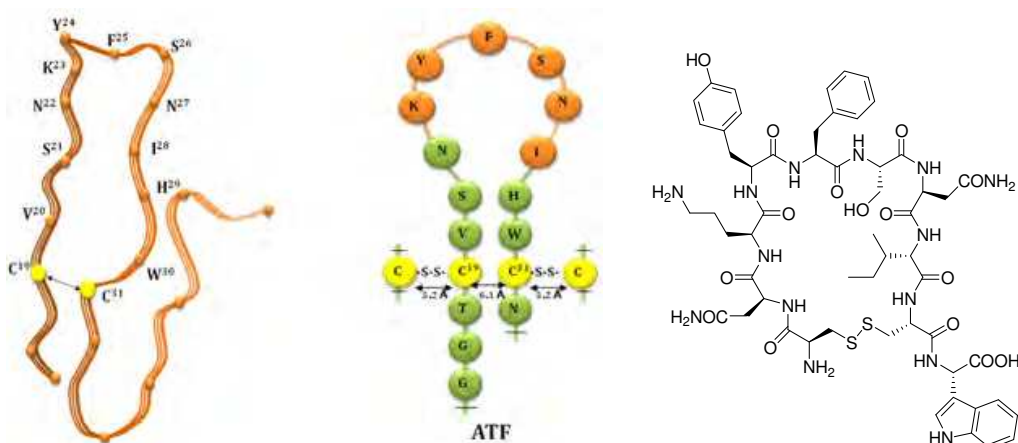

Fig. 12. Fragment of uPA selected as a scaffold for the preparation of cyclic peptidomimetics and the structure of the most effective of them
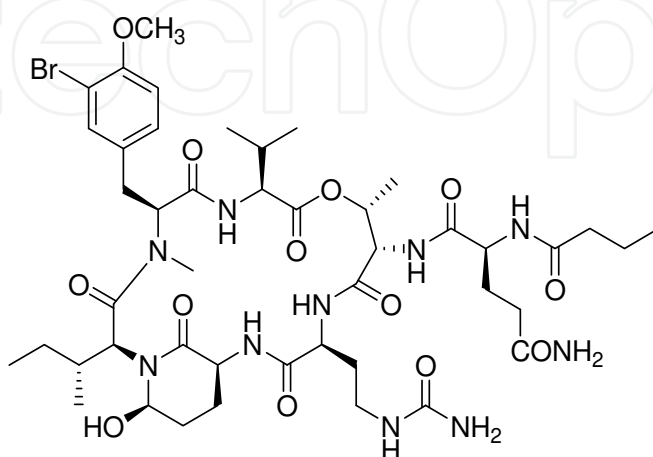

Fig. 13. Structure of Symplocamine A

Similar cyclic peptides, inhibitors of various proteinases, were also isolated from natural sources. For example, out of more than 100 compounds of this class isolated from cyanobacteria about half have been reported to inhibit trypsin or chymotrypsin. Recently isolated Symplocamine A (Figure 13), molecule of strong serine protease inhibitory activity, appeared to exert high level of cytotoxicity against variety of cancer cells *in vitro* thus being a potential agent against cancers (Linington et el., 2008).

3.2 Irreversible inhibitors

Similarly as in the case of cysteine proteinases irreversible inhibitors of serine proteinases are prominent class of their inactivators. They usually bind covalently to one of the nucleophilic moieties of amino acids present in an active site of the enzyme (most likely hydroxylic group of serine) using an electrophilic warhead. Although there are many classes of irreversible inhibitors of serine proteinases available today (Powers, et al., 2002) only limited examples entered clinical studies as anticancer agents. Therefore, new low-molecular inhibitors of enzymes involved in cancer development and metastasis are still strongly desirable. Recent studies are concentrated on the synthesis of inhibitors containing non-typical warheads (representative examples are shown in Figure 14).
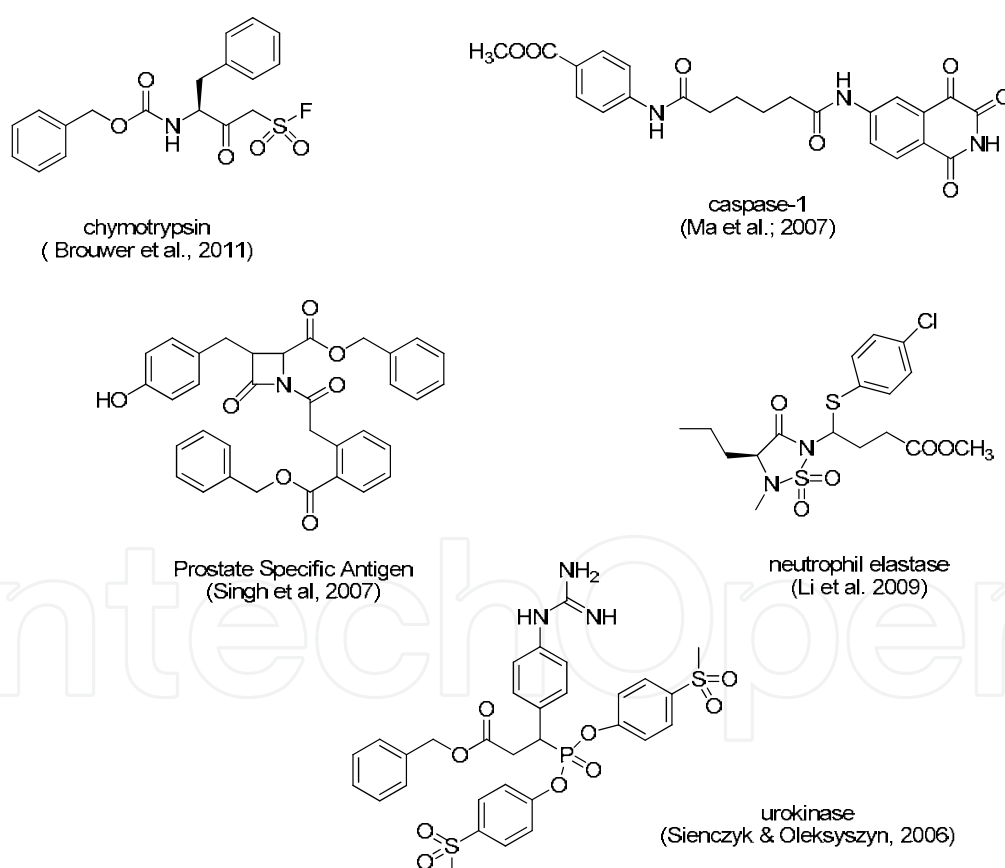

Fig. 14. Representative examples of irreversible inhibitors of serine proteinases.

Diphenyl phosphonates seem to be the most promising and general group of these inhibitors. They may be also classified as competitive transition-state analogues. On a molecular level they phosphonylate specifically active-site serine residue thus blocking the catalytic triad of serine, histidine and aspartic acids responsible for the formation of enzyme-substrate acyl

intermediate and its further hydrolysis (Fig. 15). Anyway, the mode of action of phosphonates towards serine proteinases is not yet fully elucidated and minor variations were observed, depending on the targeted enzyme and conditions (Grzywa et al. 2007; Joossens et al., 2006; Sieńczyk et al., 2011; Sieńczyk & Oleksyszyn 2006; Sieńczyk & Oleksyszyn, 2009).
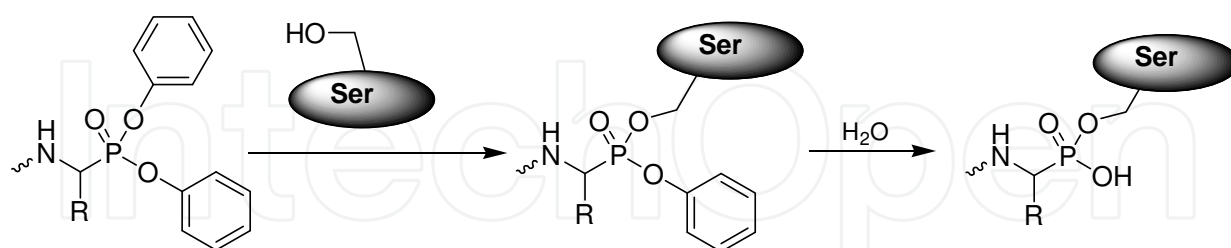

Fig. 15. Schematic illustration of the mechanism of action of the diphenyl α-aminophosphonate inhibitors of serine proteinases

3.3 Reversible inhibitors

Inhibitors of urokinase (also called urokinase-type plasminogen activator, uPA) are the biggest family of reversible serine protease inhibitors. Development of small molecule uPA inhibitors has began with aryl guanidines, aryl amidines, and acyl guanidines, molecules that contain positively charged guanidine, amidine, or simple amines as anchors able to interact with the negatively charged site chain of Asp189 (Lee et el., 2004). Although they exhibited moderate potency and poor selectivity they constituted a good starting point for the development of new effective generations of uPA inhibitors. Intensive studies using various approaches resulted in many inhibitors, which quite frequently revealed *in vitro* anticancer properties. Determination of three-dimensional structure of this enzyme either in native state or complexed with various inhibitors is vital for the design of new effectors of urokinase (Huai et al. 2008; Klinghofer et al. 2001; Sperl et. al. 2000).

For, example, an extremely simple inhibitor UK 122 (Fig. 16) was designed in a stepwise process. The first step was a selection of moderate inhibitors of uPA by screening a library of 16,000 synthetic compounds. This resulted in four promising inhibitors of the enzyme sharing very similar chemical structures. They were further optimized by using crystal structure of the enzyme-Amiloride complex and by applying molecular modeling methods. As a result UK 122 was found (Zhu et al., 2007). This compound significantly inhibited the migration and invasion of pancreatic cancer cell line.

Another example may be the use of three-dimensional quantitative structure-activity relationship (3D QSAR) studies to elucidate structural features required for uPA inhibition and to obtain predictive three-dimensional template for the design of new inhibitors. 3D QSAR was performed on five reported classes of the urokinase inhibitors by employing widely used CoMFA (Comparative Molecular Field Analysis) and CoMSIA (Comparative Molecular Shape Indices Analysis) methods (Bhongade & Gadad 2006). As a result the significance of various structural elements bound at different urokinase subsites was identified. These subsites may be combined to improve overall activity of newly designed inhibitors.

Inhibitors of other serine proteinases were studied as anticancer agents quite scarcely. Most of the obtained inhibitors were designed to affect with prostate specific antigen (PSA) and matriptase by adopting the procedures used for designing of other serine proteinase inhibitors. Some of them exhibit promising anticancer properties in cell culture systems. Representative examples of these inhibitors are shown in Figure 16.
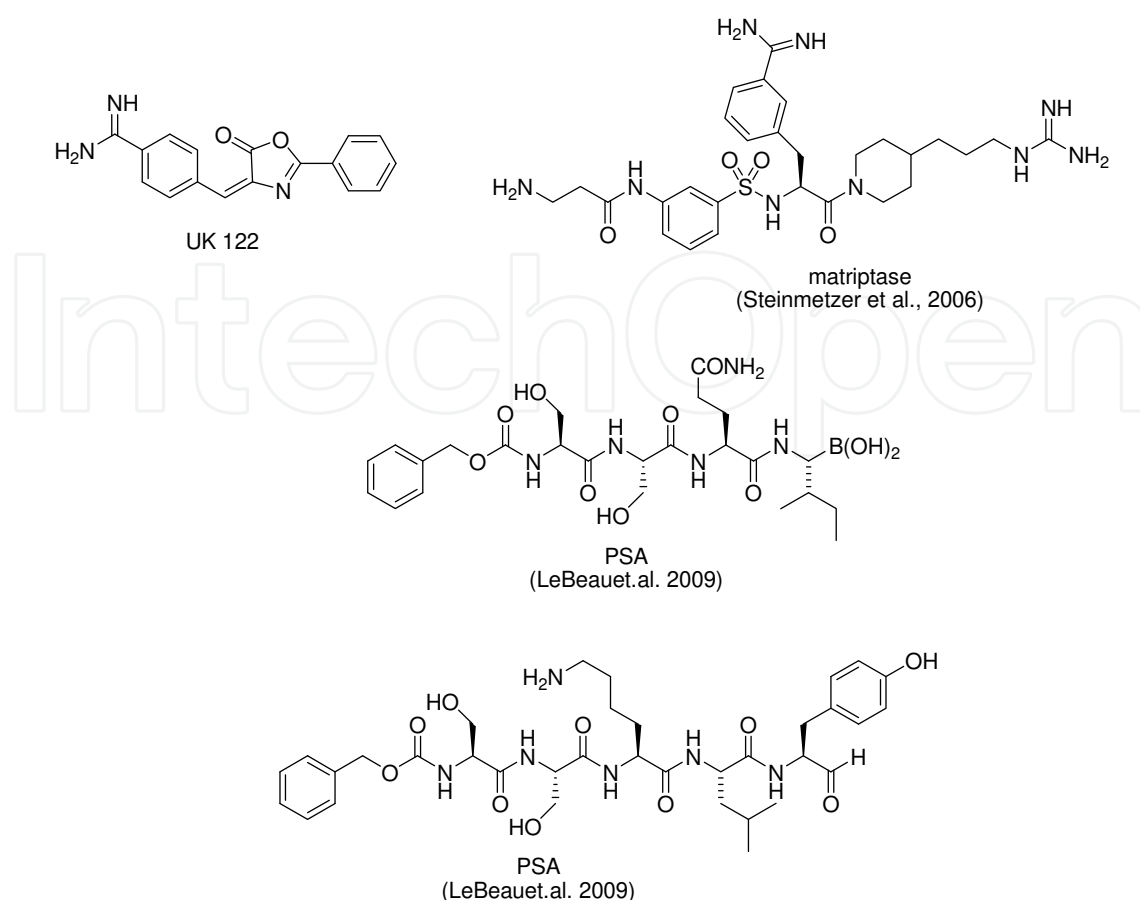
Fig. 16. Representative examples of reversible inhibitors of serine proteinases.

4. Threonine proteinases

The sequencing of human genome revealed that threonine proteinases account only for about 5% of the whole pool of proteinases. From these proteinases, only proteasome is considered as a target for potential anticancer agents. Since tightly ordered proteasomal degradation of proteins plays crucial role in the cell cycle control potential of proteasome inhibitors is currently under intensive investigations.

The proteasome is a highly conserved intracellular nonlysosomal multicatalytic protease complex, degrading proteins usually tagged with a polyubiquitin chain. The 26S proteasome is a 2,000 kDa multisubunit cylindrical protein comprised of a 20S core catalytic component (the 20S proteasome) capped at one or both ends by 19S regulatory components (Figure 17). Proteasome 20S has three major sites of different activities designed as "chymotrypsin-like", "trypsin-like" and "caspase-like". These three activities are responsible for the cleavage of protein after hydrophobic, basic, and acidic amino acid residues, respectively. Analysis of the proteasome catalytic mechanism has revealed the importance of the N-terminal threonine as catalytic nucleophile. Thus, proteolytic machinery of the proteasome is an important target for the design of anticancer drugs (Abbenante & Fairlie, 2005; Delcros et al., 2003; Goldberg, 2007). A wide variety of inhibitors of proteasome were developed and evaluated (Delcros et al., 2003). This process culminated in discovery of bortezomib (*Velcade*, Figure 17), which decreases proliferation, induces apoptosis and enhances sensitivity of tumor cells to radiation or chemotherapy (Adams, 2002; Goldberg, 2007).

The most significant step in development of proteasome inhibitors was the decision by A. L. Goldberg and colleagues to create in 1993 the company *MyoGenics*. The goal was to synthesize proteasome inhibitors that could prevent muscle atrophy that occur in various disease states, such as cancer cachexia. This led to the production of a series of inhibitors that were freely distributed to academic laboratories and contributed to the enormous leap forward in understanding the multiple roles of the proteasome in cells.
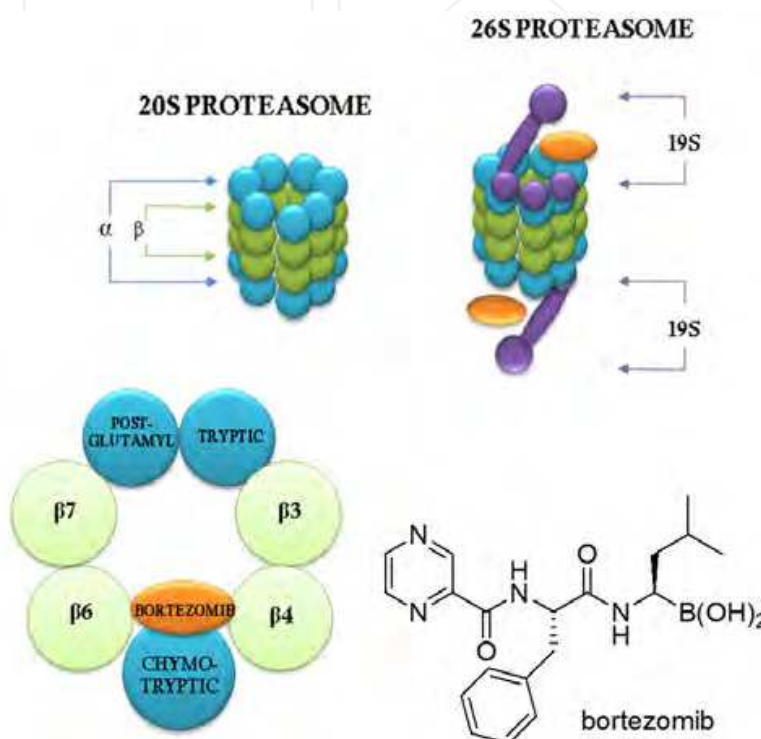

Fig. 17. Schematic structure of proteasome with indication of the binding site of bortezomib

4.1 Inhibitors from natural sources

The 20S proteasome is a tubular molecule with the proteolytic active sites on the inner surface. Thus, substrate molecules have to be translocated through the internal cavity to the catalytic sites. The X-ray crystallographic analysis has shown that the translocation channel is too narrow to allow passage of folded proteins. Protein substrates should be firstly unfolded and then degraded. Quite surprisingly, classical protein inhibitor of serine proteinases, bovine pancreatic trypsin inhibitor (BPTI) appeared to exert similar activity against proteaseome *in vitro* and *ex vivo* (Yabe & Koide, 2009). The molar ratio of BPTI to the proteasome 20S in the complex was estimated as approximately six to one, suggesting that two out of three proteinase activities of this complex were inhibited. This interesting finding has opened a new front in proteasome inhibition studies.

The majority of proteasome inhibitors have a structure of small cyclic and linear peptides built on scaffolds provided by natural substances. Lactacystin (Figure 18), produced by *Strepromyces* (Omura et. al., 1991), rearranges in neutral pH to highly reactive lactone-Omuralide, which irreversibly acylates proteasome active site threonine. Minute modification of the latter one led to the more potent inhibitor MNL-519 (Abbenante & Fairlie, 2005). Isolation of *Actinomycete* products – epoxomycin and eponemycin, and evaluation of their inhibitory activity (Hanada, et. al, 1992; Sugawara et al, 1990) has

stimulated studies on their analogues (representative structure is shown in Fig. 18). This resulted in several potent inhibitors, which display non-typical mechanism of action (Elofsson e., 1999; Zhou et el., 2009). A hemiacetal is first formed between the ketone portion of the inhibitor and threonine hydroxyl, followed by epoxide ring opening by the free amine of the N-terminal threonine to give a stable morpholino adduct.
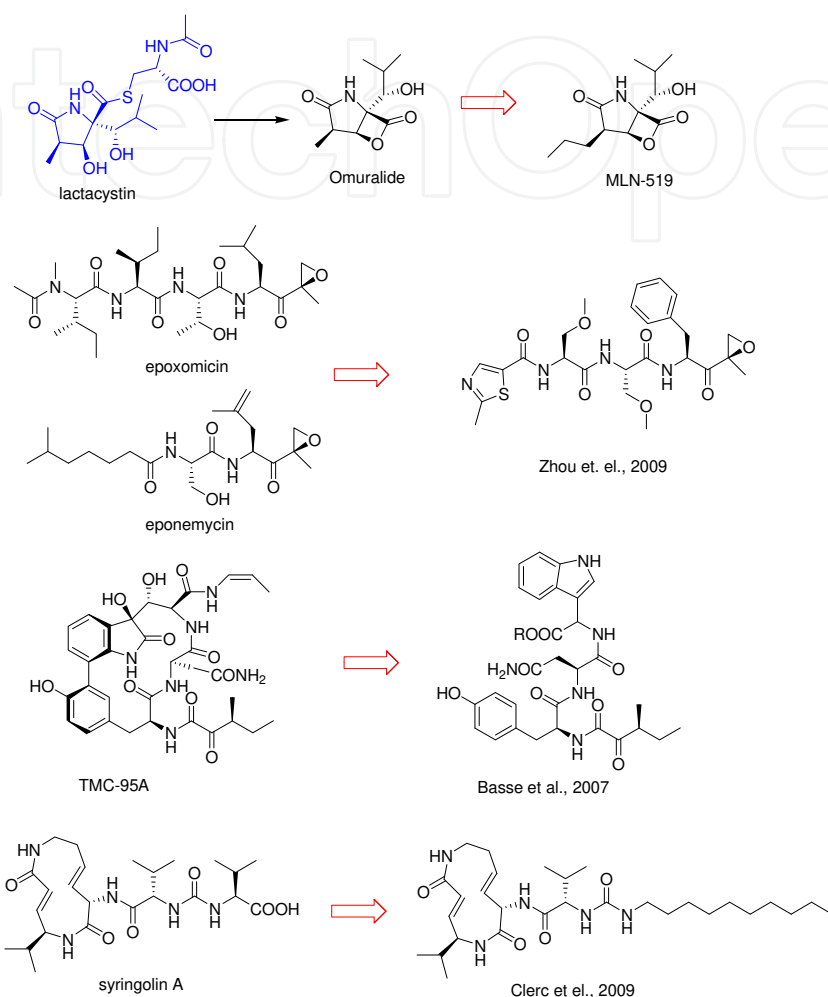

Fig. 18. Natural inhibitors of proteasome activity as scaffolds for synthesis improved ones.

Next example considers syringolines, reversible inhibitors of proteasome produced by *Pseudomonas siringae* (Coleman, et al. 2006). Elucidation of the crystal structure of syringolin B complexed with proteasome gave an insight into the structural requirements of good inhibitor. These findings were used successfully in the rational design and synthesis of a syringolin A-based lipophilic derivative, which proved to be one of the most potent proteasome inhibitors described so far (Clerc et el., 2009).

A limiting factor in the efficiency of peptidic inhibitors is that they are unstable in living organism because they are easily degraded by endogenous proteinases. This explains growing interest in non-peptidic inhibitors. Nature is an acknowledged source of such compounds and many inhibitors of proteasome were isolated and identified. These include such structurally diverse compounds as: ajoene isolated from garlic (Hassan, 2004), gliotoxin produced by *Aspergillus fumigatus* (Pahl et al., 1996), or triterpene-celastrol isolated from the root bark of medicinal plant *Tripterigium wolfordii* (Yang et al., 2006).

4.2 Synthetic inhibitors

The first inhibitors of proteasome were identified among the commercially available reversible tripeptide inhibitors of serine and cysteine proteinases. The easy access to the peptide aldehydes had lead to the development of a wide variety of inhibitors with an improved potency and selectivity. MG-132 (for its chemical structure see Figure 20) was one of the first synthetic inhibitors to be described and used in cell culture system (Adams & Stein, 1996). It exerts both, direct antiproliferative and cytotoxic effects towards tumor cells, and increases apoptosis induced by other agents. Recent studies have demonstrated the influence of absolute configuration of this tripeptide aldehyde on its cytotoxicity, with (*L,D,L*) isomer being the most active (Mroczkiewicz et al., 2010). Since a great number of tripeptide aldehydes contain side chains of non-coded amino acids but they usually correspond to natural *L*-amino acids this finding shed a new light on the importance of peptidyl absolute configuration.

Structurally related α-ketoaldehydes exert their action via mechanism similar to this described earlier for epoxyketones (Gräwert et al., 2011). This is a cyclization mechanism, which proceeds through formation of hemiketal with threonine hydroxyl followed by Schiff base formation between the nucleophilic *N*-terminal threonine and aldehyde moiety, which finally results in the reversible formation of a 5,6-dihydro-2*H*-1,4-oxazine ring (Figure 19). The examination of the binding mode of these inhibitors serves as a new lead for the development of anticancer drugs (Fig. 19).
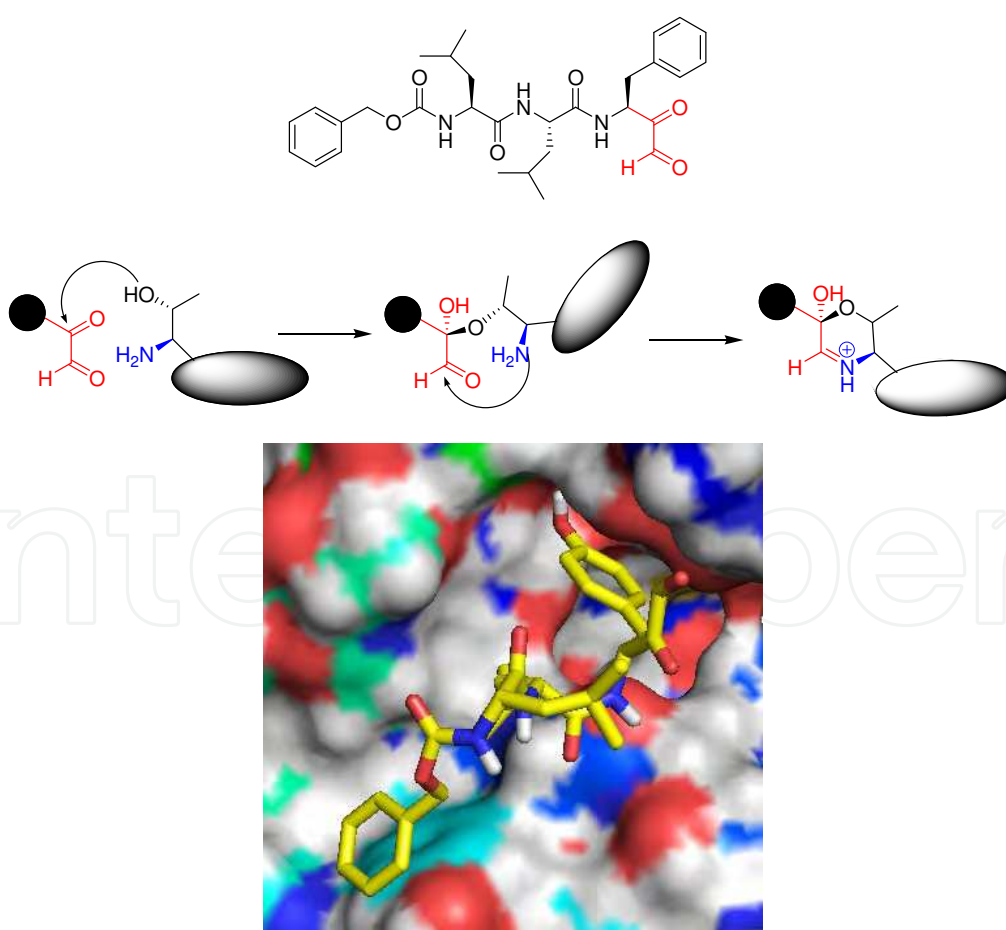

Fig. 19. Molecular mechanism of action of α–ketoaldehyde inhibitor of proteasome and the mode of its binding in the active site.

Searching for a new class of 20S proteasome inhibitors is a hot subject and to date a plethora of molecules that target the proteasome have been identified or designed (de Bettignies and Coux, 2010). Synthetic inhibitors possess a homogeneous structural profile - they are generally peptide-based compounds with a C-terminal pharmacophore function required for primary interaction with catalytic threonine of the enzyme. The peptide component seems to be important for determining specificity of the interactions with the enzymatic pockets. Essentially, most of these inhibitors act on the chymotrypsin-like activity of the proteasome although two remaining activities are also addressed.

Protection of the aldehyde moiety in a form of semicarbazone provides compounds that are more stable than counterpart aldehydes. They do not form adducts with cellular proteins and are irreversible inhibitors of proteasome requiring the action of this enzymatic complex to release inhibiting aldehyde. Thus, they may be classified as suicidal inhibitors. Recently two peptide semicarbazones, S-2209 and SC68896, were found to exert anti-melanoma and anti-glioma activities in preclinical studies (Baumann et el., 2009: Leban et al., 2008; Roth et el., 2009). For the latter one company was given an approval to start phase I/II clinical studies in 2011.

Structurally related *N*-acylpyrrole peptidyl derivatives were designed as irreversible inhibitors of proteasome. They appeared to possess unique biological profile and interact reversibly with β1 catalytic site of the proteasome also displaying good pharmacological properties (Baldisserotto et al., 2010). Molecular docking of the *N*-acylpyrrole molecule shown in Figure 20 enabled to rationalize the mode of their binding.

The vinyl sulfone group is less reactive than the aldehyde group but also binds irreversibly to the active sites. The advantage of vinylsulfone inhibitors is that they are easy to prepare. One of the most potent inhibitor - Ada(Ahx)$_3$-LLL-VFS, specifically and irreversibly inhibits both the constitutive and the induced proteasome by binding to their three active sites with approximately equal efficiencies (Kessler et al., 2001).

The screening of huge libraries of structurally variable compounds is a method for the identification of new cell-active inhibitors with novel chemical scaffolds. Such a procedure was also used in order to obtain new inhibitors of proteasome. Thus, a high-throughput screen of the Millennium Pharmaceuticals Inc. library (approximately 352,500 compounds) afforded 3015 hits, which were further optimized by applying X-ray crystallography and molecular modeling. In such manner 16 various structures were selected. They appear to exhibit high potency and selectivity towards β5 subunit of 20S proteasome. The crystal structures determined for the most active compounds (Fig. 20) enabled to determine the structural requirements of the inhibited subunit. Similar screening done on National Cancer Institute Diversity Set library composed of 1,992 compounds resulted in selection of four promising inhibitors of proteasome, with organocopper NCS 321206 (Fig.20) being the most active one (Lavelin et al., 2009).

Different approaches to the selection of new inhibitors of proteasome relayed on the use of computational tools, namely multistep structure-based virtual ligand screening strategy. First scoring engines were standardized using known inhibitors in order to obtain results similar to those found from crystallographic studies. It appeared that none of the presently developed scoring functions are fully reliable nor they fully correlate with experimental affinities. Therefore three protocols were used simultaneously - FRED, LigandFit and Surflex, to dock 300,000 compound collection (Chembridge). This enabled to select 200 molecules for further experimental testing, using MG-132 as a standard. Twenty of these molecules appeared to act as potent proteasome inhibitors showing variable profiles of

activity. Thus six of them inhibited all three activities of proteasome, eleven of them inhibited two types of enzymatic activities, whereas three inhibited only one type of activity (Basse et al., 2010). The most active and selective inhibitors against chymotrypsin-like and trypsin-like activities are shown in Figure 20.

Fig. 20. Structurally diverse, synthetic inhibitors of proteasome.
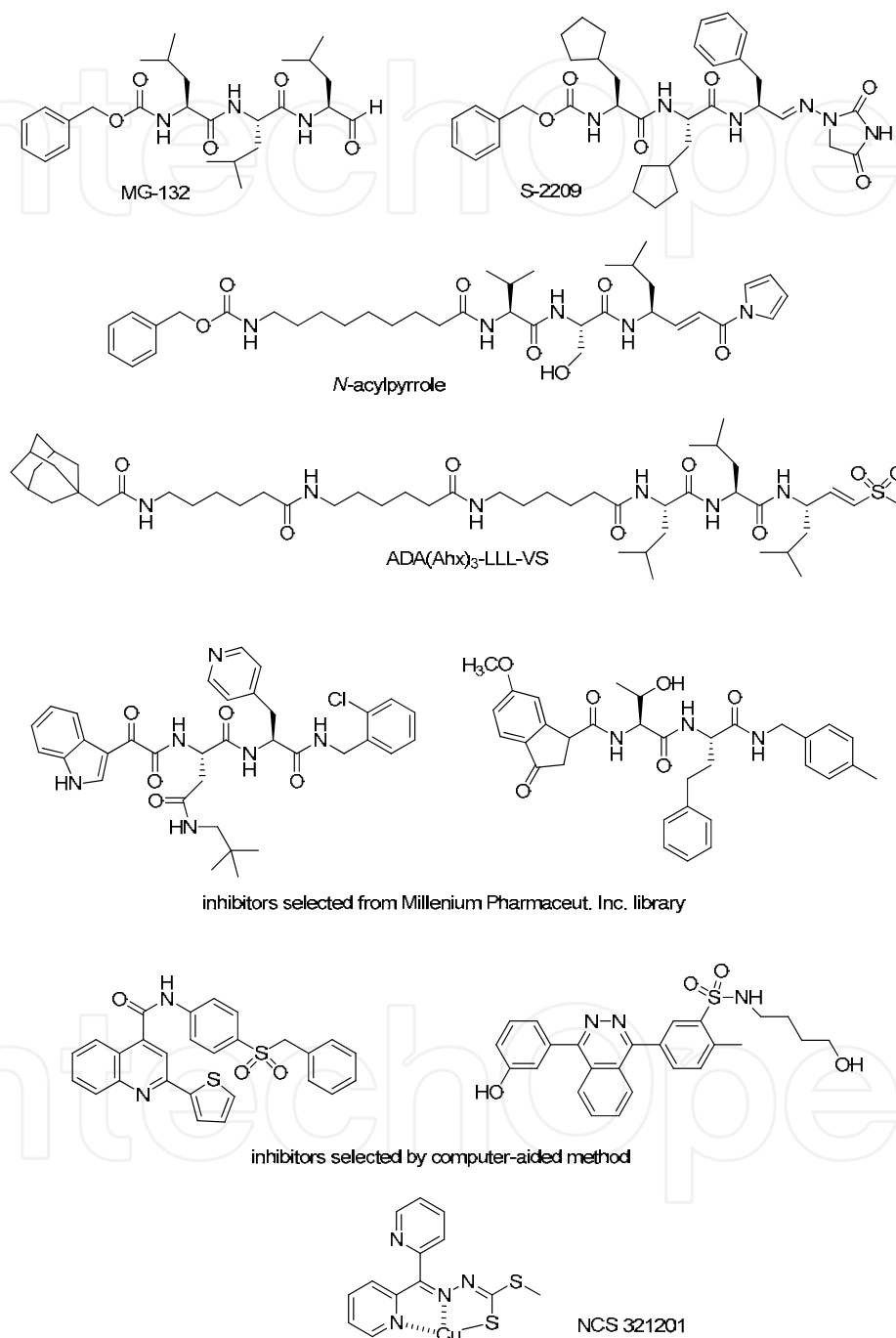
The discovery of bortezomib was followed by intensive preclinical and clinical studies on many cancer models and cancer patients. This drug was approved in 2003 for treatment of multiple myeloma as a second line of the therapy. Today it is taken by approximately 50,000 patients worldwide (Goldberg, 2007) and is still being tested clinically against other forms of

cancer. Interestingly, recent studies have indicated that this drug is a multiple inhibitor and affects also serine proteinases in cell lysates (Arastu-Kapur et al., 2011). This finding may explain better the clinical profile of this drug. Alongside with physiologic studies synthesis and evaluation of inhibitory activity of its analogues have been carried out. Although in some cases inhibitors of similar potency were obtained (Aubin et al., 2005; Vivier et al., 2005; Zhu et al, 2010) none of them was found to be better than bortezomib.

5. Aspartic proteinases

This is the smallest family of proteinases, which accounts for only 3% of them and includes several physiologically important enzymes such as pepsin, chymosin, renin, gastricsin, cathepsin D and cathepsin E. Some members of this family, in particular cathepsins D and E, have been implicated in cancer progression. High cathepsin D expression is associated with shorter disease-free and overall survival in patients with breast cancer, whereas in patients with ovarian or endometrial cancer, cathepsin E expression has been reported to be associated with tumor aggressiveness.

Quite surprisingly, the aspartic protease napsin A, expressed in lung cells, where it is involved in the processing of surfactant protein B, suppressed tumor growth in HEK293 cells in a manner independent of its catalytic activity (Ueno et al., 2008). Further insight into mechanism involved may help in producing new drugs for renal cancer.

The most extensively investigated aspartic proteinase in the context of cancer is cathepsin D, with a particular emphasis on its role in breast cancer (Benes et al, 2008). In these studies several inhibitors of this enzyme are most commonly used including peptidomimetic pepstatin (Umezawa et al., 1970) and protein inhibitors from potato and tomato (Carter et al., 2002). Search for new inhibitors of this enzyme is practically limited to peptides containing non-typical amino acid – statine. Inhibitors of this type were obtained from both natural sources as well as were synthesized basing on the crystal structure of pepstatin A (Fig. 21) complexed by this enzyme. Statine, which is a component of pepstatin A, may be considered as an analogue of tetrahedral intermediate (or transition-state) of the enzymatic hydrolysis of *L*-leucylglycine (Fig. 21). Therefore it is not surprising that most of cathepsin D inhibitors contain this amino acid or its analogue within peptidic chain (Bi et al., 2000; McConnell et al., 2003). Of special interest are grassystatins (Fig. 21) isolated from cyanobacterium *Lyngbya* cf. *confervoides*. These peptidomimetics are equally active against cathepsins D and E (Kwan et al., 2009).

The new approach to the identification of inhibitors is appropriate selection of DNA aptamers strongly interacting with chosen enzyme. This methodology was used to identify the aptamer SF-6-3, which selectively and very strongly binds cathepsin E (Naimuddin et al., 2007).

6. Metalloproteinases

Metalloproteinases are the largest class of proteinases in human genome. They are a range of enzymes possessing metal ions in their active sites. Most of them are dependent on zinc ions, which play catalytic functions. Understanding their mechanism of action is of key importance to rational design of potent and specific inhibitors of these enzymes and, consequently, to obtain drugs of improved properties. Therefore, a substantial effort has been made to study the mode of binding of their substrates and inhibitors, as well as to elucidate the three dimensional structure of these enzymes and to define the detailed

mechanisms of catalyzed reactions. Despite extensive experimental and theoretical studies the mechanism by which the catalytic center of metalloproteinases functions is still the subject of debate and several mechanism have been proposed (Mucha et al., 2010).
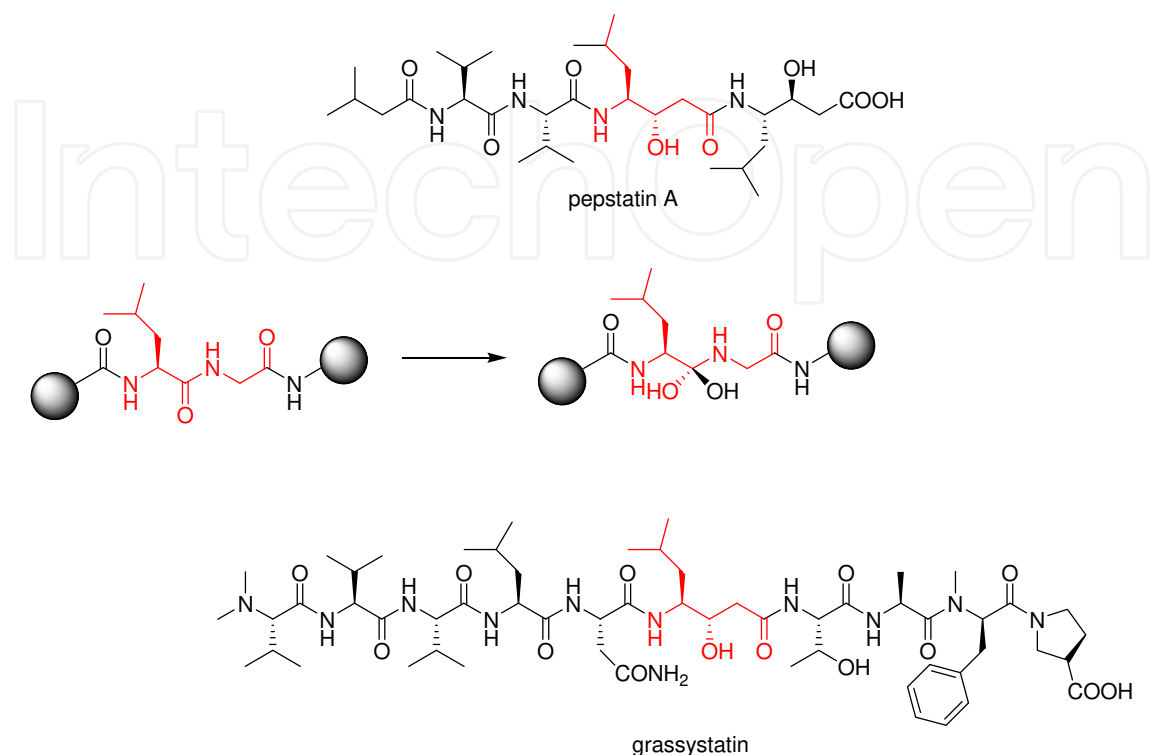

Fig. 21. Pepstatin A and grassystatin as transition-state analogues of peptide hydrolysis.

Matrix metalloproteinases (MMPs), a disintegrin and metalloproteinases (ADAMs, adamalysins) and tissue inhibitors of metalloproteinases (TIMPs) together comprise an important set of proteins that are regulatory in matrix turnover and regulate growth factor bioavailability. There are 23 MMP, 32 ADAM and 4 TIMP proteins present in humans. This shows how complex system is involved in tumorigenesis and its regulation. For example, four tissue inhibitors of metalloproteinases (TIMP1, TIMP2, TIMP3 and TIMP4) are the main endogenous inhibitors for all the metallo-endopeptidases, of which there are more than 180.

6.1 Matrix metalloproteinases

Matrix metalloproteinases (MMPs) consist of a multigene family of zinc dependent extracellular endopeptidases implicated in tumor growth and the multistep processes of invasion and metastasis, including proteolytic degradation of extracellular matrix, alteration of the cell–cell and cell–matrix interactions, cell migration and angiogenesis (Gialeli et al., 2011). These structurally and functionally related endoproteinases share common functional domains and activation mechanisms. The MMPs were the first proteinase targets seriously considered for combating cancer. After encouraging preclinical results in various cancer models several of the MMP inhibitors were tested in advanced clinical trials but all failed because of severe side effects or no major clinical benefit (Turk et el., 2006). These include: hydroxamate inhibitors batimastat, marimastat, and prinomastat and the non-hydroxamate ones such as neovastat (an extract from shark cartilage of a molecular mass up to 500kDa introduced by Aeterna) rebimastat and tanomastat (Fig. 22).
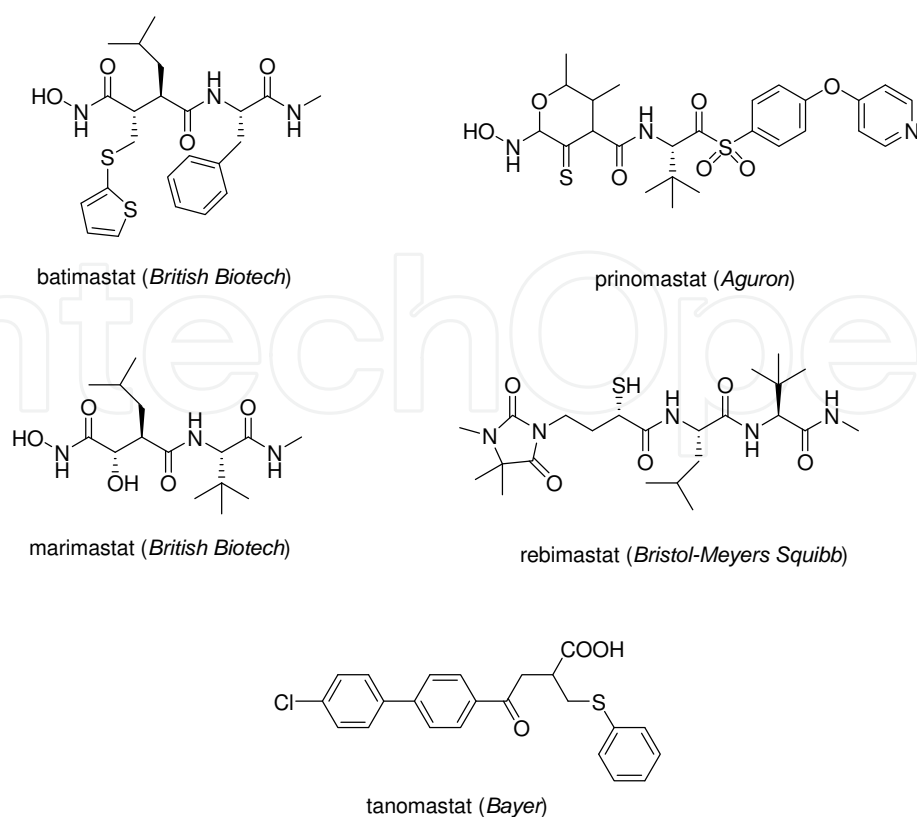
Fig. 22. Matrix metalloproteinase inhibitors, which failed in clinical studies. In parentheses companies, which introduced these compounds are given.

Clinical studies indicated that timeframe of targeting MMPs differs, depending on the stage of cancer, because the expression profile, as well as the activity of these enzymes, is not the same in the early stage compared to advanced cancer disease. As a consequence, the use of broad-spectrum inhibitors raises concerns that certain MMPs that exert anticancer effects are inhibited, which in turn may result in promotion of the disease (Gialeli et al., 2011). Thus, pharmacological targeting of cancer by the development of a new generation of effective and selective inhibitors to individual matrix metalloproteinases is an emerging and promising area of research (Devel et al., 2010; Manello, 2006). However, despite intense efforts, very few highly selective inhibitors of these metalloproteinases have been discovered up to now. This is because MMPs have catalytic domains composed of 160–170 amino acid residues that share a marked sequence similarity, with the percentage of identical residues being in the range of 33% to 90%. The three dimensional structure of the catalytic domains of 12 out of 23 human MMPs has been solved either by X-ray crystallography or NMR (Maskos, 2005), and the results supported that they are of significant similarity. The other cause of low specificity of most of MMP inhibitors is that their action relays on strong complexation of zinc ion present in the active sites of these enzymes. This is especially true in the case of hydoxamic acid-based inhibitors (Yiotakis & Dive, 2008), which are the most intensively studied so far (Attolino et al., 2010; Fisher & Mobashery, 2006; Nuti et al., 2010).

Among different zinc-binding groups, the phosphoryl moiety was thought to be the weakest binder. Indeed, it turns out that numerous peptide analogues with a phosphorus-containing moiety replacing the scissile amide bond have been found to regulate the activity of

metalloproteinases (Collinsová & Jiraček, 2000). The intense optimization of the phosphinic inhibitor structures, using parallel or combinatorial chemistry, is generally required to identify nanomolar inhibitors and to get selectivity (Dive et al., 2004). Without selective inhibitors, which are indispensable tools for studying the structure and the role of individual enzymes at different stages of complex tumorigenesis, anticancer strategies based on MMP inhibition are unlikely to provide important therapeutic benefits. Two representative inhibitors of this class of inhibitors are shown in Figure 23.

High-throughput screening of chemical libraries has also led to the discovery of unusual MMP inhibitors, selective against MMP-13. Among these, a new class of MMP inhibitors that do not possess a zinc-binding group and thus do not interact directly with the zinc active site ion is of special interest (Fig. 23) (Chen et al., 2000).

6.2 Aminopeptidases

Aminopeptidases are proteolytic enzymes that hydrolyze peptide bonds from the amino termini of polypeptide chains with the release of a single amino acid residue from polypeptide substrates. Although their involvement in tumorigenesis was well established the studies on their anticancer properties are far less developed than studies on MMPs. This may also result from the fact that physiologic role of these enzymes is far more complex.

A plethora of inhibitors of aminopeptidases have been synthesized and tested clinically against various pathological disorders, including cancers (Bauvois & Dauzonne, 2006; Mucha et al., 2010; Selvakumar et al., 2006; Wickström et al., 2011). Bestatin, a general inhibitor of aminopeptidases and aspartyl proteinases, has been the most intensively studied (Fig. 24). It was originally isolated from *Streptomyces olivoreticuli* more than 30 years ago (Umezawa et al., 1976). Bestatin studies in biological systems both *in vitro* and *in vivo*, resulted in discovery of several interesting properties of this compound such as ability to induce apoptosis in cancer cells, and anti-angiogenic, anti-malarial or immunomodulatory effects. Presently, bestatin (Ubenimex®) is on Japanese market where it is applied for treatment of cancer and bacterial infections. Examples of successful inhibition of aminopeptidases by bestatin include aminopeptidase N (CD13), leucine aminopeptidase (LAP) and aminopeptidase B. These aminopeptidases, as well as methionine aminopeptidase 2 are the most exploited targets to obtain new anticancer agents.

In contrast to MMPs selectivity of the inhibitor is not a required feature and in most cases general inhibitors of aminopeptidases are used in clinical studies. Such an example is tosedostat (Fig. 24) (Krige et. al, 2008; Moore et al., 2009), a hydroxamic acid inhibitor of M1 family of aminopeptidases (especially leucine aminopeptidase), which is now being introduced to the market by Chroma Therapeutics. In clinical studies tosedostat was well tolerated, given orally once a day, and it has produced encouraging response rates in difficult to treat patients with acute leukemia and a variety of blood related cancers. Tosedostat (CHR-2797) is a prodrug and exposure of cancer cells to this drug results in the generation of the active metabolite CHR-79888 (Fig. 24), which is poorly membrane-permeable, what limits its pharmacological activity. The use of prodrug results in intracellular accumulation of CHR-79888 and desirable physiological effect.

6.3 Carboxypeptidases

Carboxypeptidases cleave the peptide bond of amino acid residue at the carboxylic terminus of protein or peptide. Humans contain several types of carboxypeptidases, which have diverse functions ranging from catabolism to protein maturation. There is practically lack of

information about the role of carboxypeptidases in tumorigenesis. However, some of them were proposed as markers of individual tumors (Kemik et al., 2011; Lee et al., 2011). This indicates that they also might be considered as targets in anticancer therapy. Indeed, there are two reports on antitumor activity of two endogenous protein inhibitors of carboxypeptidases – latexin (Pallares et al., 2005) and retinoic acid-induced tumor suppressor retinoic acid receptor responder 1 (RARRES 1) (Sahab et al., 2011).
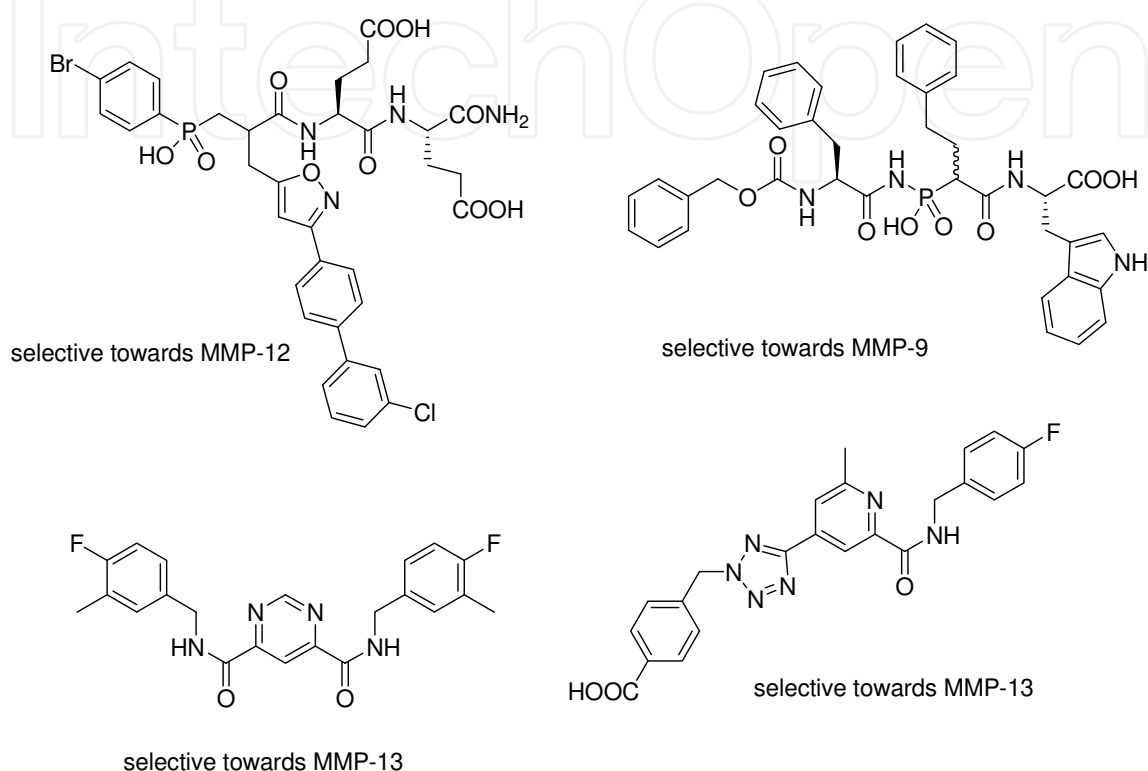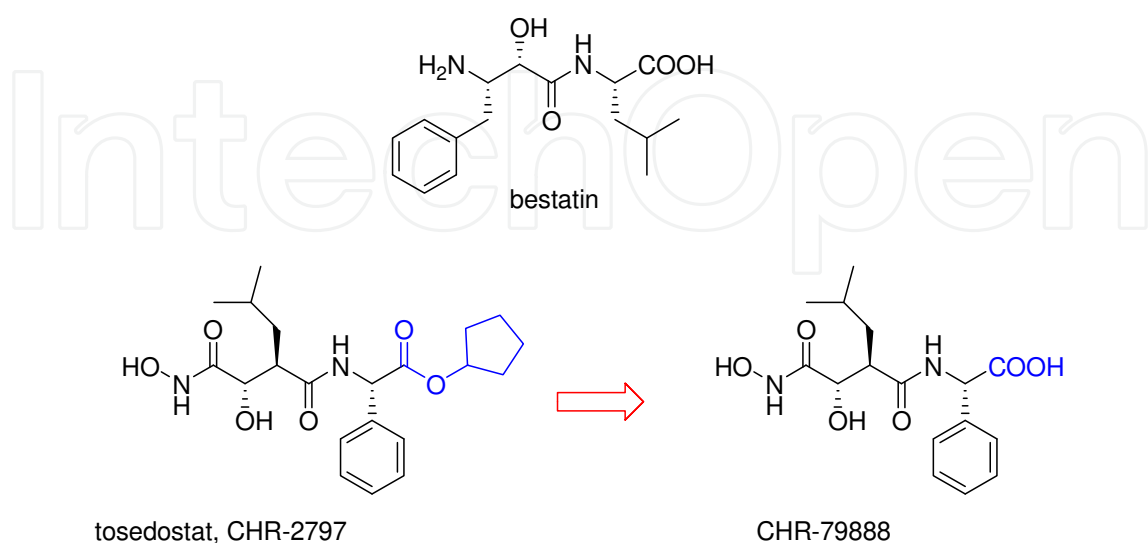

Fig. 23. Selective MMP inhibitors.

Fig. 24. Bestatin and tosedostat – general inhibitors of aminopeptidases and promising anticancer drugs.

7. Conclusion

Looking back at the progress made with anticancer therapies using inhibitors of various proteinases it is hard to consider it as particularly successful. Today the major successful areas in protease-targeted therapies are the cardiovascular, inflammatory and infectious diseases (mostly anti-HIV), however, the intensive studies on therapies of cancer and neurodegradative disorders are predicted. This is a good prognosis if taking into account that the annual spending for protease-directed drugs amounts close to US$ 10 billion annually (Turk, et al., 2006).

It is worth to note that past drug failures are not worthless. They provide not only invaluable lessons but are also a useful resource of data, which could still be used.

In order to achieve more satisfactory results, better understanding of the proteolytic network in tumor envinroment and increased knowledge in protease biology based on comprehensive analysis of protease activity in physiologically relevant conditions are required. The fact that tumor cells are only one part of the tumor environment and that extracellular matrix components and stromal cells are important contributors to the proteolytic activity of tumors should also be taken into consideration. For example, the use of transgenic animals may help in elucidation of the role of individual components of this complex networks.

Also the techniques of inhibitor design are developing significantly with *in silico* structure-based ligand design and various types of high-throughput screening being the major ones. Today's strategy in inhibitor design is to provide compounds complementary to active sites of the inhibited proteins, while the other concepts are used scarcely. One of the solutions is to design allosteric inhibitors altering proteinase activity by binding outside the enzyme active site, most likely in the cavity lacking any physiological role. The development of computer-aided methods for drug design (especially docking procedures) might be very helpful in this respect.

8. Acknowledgment

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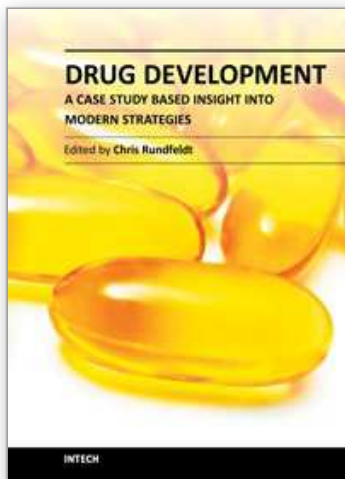
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This book represents a case study based overview of many different aspects of drug development, ranging from target identification and characterization to chemical optimization for efficacy and safety, as well as bioproduction of natural products utilizing for example lichen. In the last section, special aspects of the formal drug development process are discussed. Since drug development is a highly complex multidisciplinary process, case studies are an excellent tool to obtain insight in this field. While each chapter gives specific insight and may be read as an independent source of information, the whole book represents a unique collection of different facets giving insight in the complexity of drug development.

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