General discussion and future perspectives
INTRODUCTION

Rheumatoid Arthritis (RA) is a common autoimmune disease characterized by synovial inflammation and hyperplasia, autoantibody production, cartilage and bone destruction of which the underlying cause lies in immune regulatory factors such as the loss of tolerance. \(^1\) Current consensus among rheumatologists is to treat early onset of the disease as aggressively as possible using classical DMARD drugs, biological agents, or their combination. With this strategy, considerable response rates can be achieved, with approximately 50% of RA-patients experiencing clinical remission. \(^2,3\) However, the remaining 50% of RA-patients still have low disease activity reduction or persistent active disease. Moreover, long-term efficacy of both DMARDs and biological therapeutics in initially responding patients is not unlimited. \(^4,5\) These facts, coming along with toxicity issues at higher effective doses and high costs of biological agents, call for a continued refining of classical treatment schedules \(^6\) or the search for novel, more efficacious, less toxic and less expensive drugs for (combination) treatment.

A large portion of current investigational small molecule drugs with novel mechanisms of action originally emerged from anti-cancer drug development programs. Although treatment objectives differ in autoimmunity (dampening the immune system to avoid reactions to self-antigens) vs. cancer (boosting the immune system and eliminating malignant cells) there are overlapping targets, such as the NF-κB signaling pathway that hold relevance for both settings. The general paradigm is that drug-induced effects are either beneficial for the treatment of autoimmune diseases or beneficial for the treatment of cancer: anti-inflammatory vs. pro-inflammatory.

In this thesis we focused on investigating immune effector cell function upon targeting with drugs that impact the protein degradation pathway as a novel therapeutic modality. In particular, we explored two classes of experimental drugs: one inhibiting proteasome catalytic activity and another inhibiting aminopeptidases functioning downstream of the proteasome. Given the aforementioned loss of efficacy of most common therapeutic drugs after prolonged administration, we were not only interested in short-term effects of these drugs, but also in the impact of long-term exposure and the possible onset of drug
resistance.

**Effects of inhibition of the proteasome on immune effector cells**

Bortezomib (BTZ) is the first prototypical proteasome inhibitor that was registered for treatment of therapy-refractory multiple myeloma patients. Beyond conveying anti-cancer properties, pre-clinical and clinical studies demonstrated that BTZ also elicited immunosuppressive effects either by interfering with the NF-κB signaling pathway or inducing impaired development or depletion of specific blood cell types, including dendritic cells (DCs), macrophages, osteoclasts, T cells and B cells. In Chapter 3, 4 and 6 of this thesis we examined the impact of BTZ and next-generation PIs on DC and B-cell function.

**Pre-clinical effects on Dendritic Cells**

DCs are the most powerful professional antigen presenting cells (APCs) that also control other players and processes in immune responses through the release of regulatory or stimulatory cytokines, depending on their developmental stage and activation state. There are two major DC subsets derived from a common haematopoietic progenitor cell and abnormalities in both types, i.e. myeloid DCs (mDC) and plasmacytoid DCs (pDC) are associated with the development, maintenance and progression of several autoimmune diseases. For their development and function, DCs rely heavily on the Ubiquitin Proteasome System (UPS) and thus effects of proteasome inhibition on DC functionality has been the subject of extensive studies. In mDCs, short term proteasome inhibition by BTZ reduced the expression of DC specific subset and activation markers (CD40, CD86, CD80, HLA-DR, CD206, CD209 and CD83), induced apoptosis (upregulation and of the pro-apoptotic protein Bax), inhibited TLR receptor signaling, and suppressed cytokine release (IL-12, TNFα). Proteasome-inhibited DCs also failed to prime allogeneic T cells. The susceptibility depended on the DC maturation stage as immature DCs appeared to be much more prone to
respond to BTZ than mature DCs.\textsuperscript{17} In other pre-clinical models of pDCs, effects of proteasome inhibition with BTZ, or the related compounds carfilzomib and ONX 0914, included inhibition of TLR trafficking, IFN-α and IL-6 production and induction of apoptosis.\textsuperscript{18,19} Importantly, all these preclinical evaluations of proteasome inhibitors (PIs)-induced effects on human DCs involved a relatively short drug exposure time and models of inflammatory DC differentiation holding potential relevance from an anti-rheumatic perspective, clinical efficacy in chronic inflammation is often dependent on multiple cycles of drug administration and thus will also impact steady-state DC development.\textsuperscript{7} In our studies, described in Chapter 3, we examined the impact of long-term BTZ exposure on CD34\textsuperscript{+} DC progenitors in a sustainable MUTZ-3 cell line model\textsuperscript{20,21} of steady-state Langerhans Cell (LC) differentiation which revealed induction of early differentiation in precursor cells along with enhanced cytokine-driven LC differentiation and maturation. Assessment of nuclear NF-κB subunit levels in BTZ-adapted MUTZ-3 cells provided evidence for a role of canonical RelB/p50 activation in the enhanced DC differentiation and maturation. Translating these observations into a clinical perspective, this would imply that long term BTZ exposure may enhance DC-mediated immune functions, which could be beneficial from an antitumor immunity perspective rather than from an anti-inflammatory perspective. The impact of long-term BTZ exposure to DCs would thus argue for primary applications of this drug in cancer, in combination with cancer immunotherapeutic targeting strategies. Nevertheless, as inflammatory DCs are primarily involved in RA pathogenesis, beneficial effects on steady-state DC differentiation does not necessarily preclude application of agents in RA therapy.

Our next study on DCs, described in Chapter 6, explored short-term effects of proteasome inhibition by BTZ and the immunoproteasome inhibitor ONX 0914 on inflammatory DCs generated from monocytes in the presence of IFN-α. These studies were prompted by observations of Bancherau et al. who employed a cytokine-induced monocyte-to-DC activation model to demonstrate that inflammatory DCs arising under the influence of TNFα and type-I IFNs (IFN-α. and -β) contributed to the development of autoimmunity. Given the
pathogenic excess of IFN-α/-β in SLE, SS, dermatomyositis and early stages of psoriasis, as well as an excess of TNFα in RA, inflammatory bowel disease (IBD), Crohn’s disease and psoriasis, these cytokines may induce the generation of detrimental monocyte-derived inflammatory DCs. Targeting both the production of TNFα by mDC and type-I IFNs by pDCs in these diseases might therefore be an attractive strategy to dampen chronic inflammation, while preserving protective immunity. Our data showed that specific inhibition of the immunoproteasome by its inhibitor ONX 0914 was equally effective as BTZ in suppressing inflammatory DC development and function upon differentiation in the presence of IFN-α. Designed to have lesser unwanted side effects than BTZ, ONX 0914 may broaden the therapeutic window and provide an opportunity for clinical targeting of inflammatory DCs. Of note, its long-term effects on steady-state DC development remain to be determined.

**Pre-clinical effects on B lymphocytes**

B cells play a pivotal role in the pathogenesis of various autoimmune diseases, and are thus attractive targets for therapeutic intervention. This has been successfully achieved with B cell depleting agents such as the CD20-targeted antibody rituximab. Since the UPS regulates CD20 expression as well as the expression of the B cell Receptor (BCR) and B cell-mediated antigen presentation, we examined the impact of long-term BTZ on CD20 expression in a B cell model of human JY cells (Chapter 4). Interestingly, CD20 expression was markedly increased in BTZ-resistant JY cells, tentatively by hampered breakdown of CD20 due to proteasome inhibition. CD20 in BTZ-resistant cells had retained functional capacity to induce enhanced complement-dependent cytotoxicity (CDC). These experiments thus provide a rationale to combine rituximab and BTZ, both to overcome BTZ resistance and to overcome down-regulation of CD20 as a resistance mechanism to rituximab.
Mechanisms of PI resistance and implications for autoimmunity

Onset of drug resistance is a recurrent phenomenon associated with chronic exposure to almost any type of therapeutic drug.\textsuperscript{429} Also for BTZ, mechanisms of acquired resistance were described in human THP1 monocytic cells,\textsuperscript{30} T cells and multiple myeloma cells.\textsuperscript{31} Whether or not development of resistance to BTZ was also noticeable in DCs and B cells, was not previously explored and the subject of studies in Chapters 3 and 4 in which attempts to provoke BTZ resistance in the MUTZ-3 DC and JY cell models are described.

Intriguingly, prolonged exposure of MUTZ-3 cells to BTZ did not provoke acquired resistance to this drugs as was previously reported for many hematologic cell line models of monocyte/macrophage, T- and B-cell origin, and lung cancer cells.\textsuperscript{30–33} In all of these cell lines, BTZ resistance was associated with the upregulated expression of constitutive proteasome subunits along with the acquisition of point mutations in the PSMB5 gene encoding the β5 proteasome subunit resulting in the introduction of amino acid substitutions in the BTZ-binding pocket, which conferred diminished BTZ binding. The 1.3-fold resistance level that was reached in the MUTZ-3 cells might be considered as low for an in vitro mechanistic resistance study but might nevertheless hold clinical relevance.\textsuperscript{30,33,34} It is not readily clear why higher levels of BTZ-resistance were not observed in MUTZ-3 cells, except that these cells require addition of cytokines, provided by conditioned medium of 5637 cells, for their growth. Conceivably, this cytokine environment and activation of downstream signalling cascades interferes with the triggering mechanisms of BTZ resistance.

Other than in MUTZ-3 cells, acquired resistance to BTZ was readily induced in JY B- lymphoblastoid cells. The underlying molecular mechanism involved a point mutation in the PSMB5 gene leading to an amino acid alteration Met45Ile in the BTZ binding pocket of the β5 subunit. Of note, Met45 is critically involved chymotrypsin-like proteasome activity as it serves enzyme-substrate interactions during hydrolysis of peptide bonds harboring hydrophobic amino acids.\textsuperscript{35} It was of interest to note that levels of BTZ resistance in JY/BTZ cells
were lower (10-12 fold) than in BTZ-resistant THP1, CEM and 8226 cells selected at the same concentration of BTZ, being > 100-fold resistant to BTZ. A possible explanation for this could be that JY/BTZ lacked a common feature observed in BTZ-resistant THP1, CEM and 8226 cells, i.e. down regulation of immunoproteasome levels. This raises the question as to whether this is a property of malignant cells as compared to lymphoblastoid JY cells. The absence of alterations in immunoproteasome expression in JY/BTZ cells would also be consistent with appreciable retention of sensitivity to carfilzomib and the immunoproteasome inhibitor ONX 0914. It should be mentioned that thus far no PSMB5 mutations were recognized in blood cells of patients experiencing clinical resistance to BTZ.\textsuperscript{30-32,36-39} This could imply that genetic alterations underlying β5 mutations require prolonged exposure to BTZ and are preceded by other resistance mechanisms (discussed in Chapter 2). In nature, however, PSMB5 mutations have been reported in the marine bacterium Salinospora Tropica to generate self-resistance to the PI Salinosporamide A (Marizomib). This mutant form of β5, harbors a single amino-acid substitution identical to those found in haematological cell lines with acquired BTZ-resistance and is transiently up-regulated upon release of Salinosporamide A.\textsuperscript{31,40}

A more classical mechanism of drug resistance, mediated by Multidrug Resistance (MDR) related drug efflux transporters also appeared to apply for PIs \textsuperscript{29} (Chapter 5). Resistance to PIs was particularly conferred by the drug efflux transporter Pgp, and not by other common MDR family members such as ABCC1-5 (MRP1-5) or ABCG2 (BCRP). BTZ proved to be a relatively poor substrate for Pgp, unlike the peptide epoxyketone-based irreversible proteasome inhibitors carfilzomib, ONX 0912 and ONX 0914, which displayed diminished activity in Pgp-overexpressing cell lines. However, Pgp levels as they are present in human peripheral blood cells just modestly abrogated proteasome inhibition by carfilzomib, ONX 0912 and ONX 0914, as only a 2-fold gain in sensitivity could be achieved by Pgp blocking agents. Of note, chemical modifications can be introduced into epoxyketone-based PIs to abolish their substrate affinity for Pgp.\textsuperscript{41}
These mechanisms of acquired resistance may come on top of intrinsic mechanisms that can interfere with PI activity, including up-regulated expression of heat shock proteins,\textsuperscript{42,43} impaired inhibition of NF-κB and activation of PI3K/Akt pro-survival pathways through increased secretion of the insulin like growth factor (IGF)-1 leading to enhanced activation of the IGF-1 receptor.\textsuperscript{44,45} This latter scenario could be of particular relevance in an autoimmune disease treatment setting since IGF-1 is produced by plasma cells in the bone marrow microenvironment, and under various disease conditions.\textsuperscript{46}

Together, these multifactorial mechanisms could contribute to the (loss of) efficacy to PIs. This level of complexity calls for the design of multiple targeted strategies that may help overcome resistance or potentiate PI activity within a window of acceptable side effects. One approach, described by Niewerth et al.,\textsuperscript{47} relied on the induction of immunoproteasomes after interferon-γ exposure which significantly sensitized BTZ-resistant cells with mutated β5 subunits to the immunoproteasome inhibitor ONX 0914. Furthermore, new generations of PIs have been investigated that target α-subunits of the proteasome to avoid the impaired interaction with the mutated β5 subunit.\textsuperscript{46} Indeed, BTZ-resistant cells proved sensitive to proteasome α7 subunit inhibitors, although concentration-wise their potency was in the micromolar range whereas compounds like bortezomib and carfilzomib were found to be active at low nanomolar concentrations. In light of this, recent discoveries of natural PIs may also be of interest given that their toxicity profile proved acceptable at therapeutic dosages. Some natural PIs include green tea polyphenols and a number of flavonoids, e.g. genistein, curcumin and resveratrol, all of which showed chemosensitizing properties in several solid tumors and in combination therapies for drug-resistant tumors.\textsuperscript{49} Several natural PI-candidates were also recognized as candidate therapeutic response modifiers to prevent/treat inflammatory-related diseases through pathways involving the ubiquitin-proteasome pathway.\textsuperscript{50}
IMMUNOPROTEASOMES AND THEIR INHIBITION

The interest in immunoproteasomes and their related functions is due to the fact that in immune effector cells they outweigh constitutive proteasomes and could thus be viable targets for selective therapeutic interventions. Moreover, their expression can be further induced by exposure to inflammatory cytokines such as IFN-γ and TNFα. The structural changes in the substrate binding pockets of immunoproteasomes are believed to give an immunological benefit by altering the cleavage pattern of the multicatalytic complex, thus optimizing quality and quantity of the generated peptides for presentation on MHC class I molecules. Because of a pivotal role in class-I ligand generation, the immunoproteasome shapes the naive CD8-T-cell repertoire in the thymus and cytotoxic T-cell responses in the periphery. In inflammatory/autoimmune diseases the question remains what the exact role of immunoproteasomes is.

They have been attributed a protective function against the development of autoimmunity, displaying a markedly altered repertoire of antigenic peptides for MHC class I presentation and preventing the accumulation of physiological degradation substrates that would otherwise contribute to autoimmunity. These questions may also be of relevance in the context of prolonged exposure to BTZ and/or onset of BTZ resistance. Myeloid and B line model studies in this thesis, and those from Oerlemans et al. and Franke et al. indicated that the primary response to long term BTZ exposure is the induction of constitutive proteasome and down regulation of immunoproteasome. Upon acquisition of resistance this is accompanied with upregulation of mutated β5 subunit expression. How these alterations translate into peptide processing and antigen presentation is the subject of ongoing studies evaluating the reactivity of specific T cell clones directed against specific antigen epitopes presented to them (collaboration with Dr Mirjam Heemskerk, Leiden). In an arthritis setting post-translational modification processes such as citrullination and carbamylation of proteins adds another factor that may be altered and influence antigen presentation after BTZ exposure.
**Current clinical status of PIs in autoimmune diseases**

Unlike for malignant diseases and graft-versus-host disease (GVH), clinical application of BTZ or next-generation PIs in the treatment of autoimmune diseases is still at an early stage. Only a few case reports have described clinical experience with BTZ in immune-mediated diseases, with indications of beneficial effects in patients with autoimmune diseases such as SLE and refractory autoimmune hemolytic anemia. Currently, only two registered clinical trials for BTZ in autoimmune diseases (i.e. Refractory Cold Agglutinin Disease and IgA Nephropathy) are ongoing, registered clinical trials for autoimmune diseases with next-generation PIs (either carfilzomib, ONX 0912/oprozomib, delanzomib or ONX 0914) are yet awaited. Lessons learned from multiple clinical trials in cancer with respect to achieving optimal efficacy and minimizing toxicity will be helpful to expedite the design of clinical trials in autoimmune diseases. Although RA patients could represent an attractive target group, the availability of many therapeutic options with known efficacy and safety, could withhold clinical trials in RA. Rather, autoimmune diseases for which therapeutic options are meager, e.g. SLE, SS and sclerodema would deserve further exploration. Whether BTZ or new generation PIs may elicit their best efficacy against autoimmune diseases as single agents or in combinations with conventional DMARDs and/or biologicals, is another issue that also needs to be addressed.

**Future perspectives PIs**

In conclusion, preclinical evaluation of BTZ and next-generation PIs for the treatment of autoimmune diseases such as SLE, SS, scleroderma shows promise. PIs have the capacity not only to induce selective apoptosis (e.g. of autoantibody producing plasma cells), but also to target pro-inflammatory cytokines and their receptors and disrupt intracellular signaling pathways in pro-inflammatory
immune effector cells. Both preclinical and clinical observations with BTZ have indicated that the timing and scheduling of drug administration are key factors in optimizing treatment and minimizing BTZ-related toxicities observed in early clinical trials in cancer.\textsuperscript{60-62} These issues are obviously clinically relevant factors with regards to chronic drug treatment. Altered dose schedules did reduce side effects of BTZ, and in particular subcutaneous BTZ administration reduced side effects without losing efficacy.\textsuperscript{8} The orally delivered next-generation PIs MLN9708, ONX 0912 and carfilzomib may further limit toxicities.\textsuperscript{63} The position of natural PIs as upcoming agents is yet unclear but they might be more tolerable and possibly more effective in combination with other anti-arthritic drugs and chemotherapeutics.\textsuperscript{50} Studies described in Chapter 3 of this thesis indicated that in DCs long term BTZ exposure was accompanied by altered functional properties. Thus immunomonitoring of clinical samples of patients receiving BTZ or second generation PI treatment would be informative and recommended to establish how the immune system responds to long term exposure to these drugs.

Because of the important role of the (immuno) proteasomes in class-I peptide ligand generation, it also is responsible for shaping of the naive CD8-T cell repertoire in the thymus and inducing cytotoxic T cell responses in the periphery. These are important processes in early RA/autoimmune disease stages but also in anti-cancer immunity and immunotherapy. From the RA perspective, further investigations on the impact of long term PI exposure with respect to antigen presentation from peptides in general and of citrullinated and carbamylated peptides in particular, would be very interesting since anti-citrullinated protein antibodies (ACPA) and anti-carbamylated protein antibodies (AcarP) are associated with pathophysiology of this disease. From a cancer (immunotherapy) perspective, investigations into splicing of specific (tumor) antigens by the constitutive and immunoproteasome (and hybrids thereof\textsuperscript{52}) and how this is impacted by general and immuno-PIs, would be crucial to ultimately ensure elicitation of specific anti-tumor responses. In this context, the reactivity against specific tumor antigens presented (e.g. PRAME and MART-1) might be further tested with a range of specific T cell clones directed against specific antigen epitopes presented to them upon long- or short-term exposure to PIs of the target cells.
Aminopeptidase inhibitor prodrugs

Mechanism of action and resistance modalities

Within the cycle of protein degradation and synthesis, aminopeptidases operate downstream of the proteasome by trimming peptides either for MHC class I antigen presentation or catabolism of peptides to free amino acids that can be reutilized for protein synthesis. Obviously, for cancer cells, sufficient supply of amino acids is essential for cell proliferation, but also immune cells are critically dependent on amino acid homeostasis as illustrated by the tryptophan-metabolizing enzyme indoleamine 2,3 dioxygenase in DCs and macrophages that controls tryptophan levels and proliferation of lymphocytes.64,65 Inspired by the therapeutic efficacy of proteasome inhibition by BTZ, therapeutic intervention in the protein degradation pathway at the level of aminopeptidases has also attracted attention.

Aminopeptidases cover a broad family of proteins exerting their specialized function at the level of the cell membrane, cytoplasm and endoplasmatic reticulum (reviewed in Chapter 7). Bestatin has long been recognized as a broad spectrum inhibitor of aminopeptidases. More recently, a new generation of aminopeptidase inhibitors was designed with prodrug features allowing them to act more selectively against cells harboring prodrug-converting enzymes. Tosedostat/CHR2797 is a representative of this new class of aminopeptidase inhibitors and is currently evaluated in phase I/II clinical trials for myeloid leukemia where it shows promising activity.66 In the case of Tosedostat the prodrug properties stem from a cyclopentyl group linked to the peptide-mimic structure,67 providing the molecule with a hydrophobic nature that allows entry of cells by diffusion. Only cells that harbor esterase activity have the capacity to subsequently convert tosedostat to its active hydrophilic metabolite that will be intracellularly retained to inhibit aminopeptidase activity. Carboxylesterases (CES) are likely candidates to mediate the conversion and activation of drugs like Tosedostat. Among CES family members, there is now evidence that CES1 is selectively expressed in myelomonocytic cells (including macrophages),68 which provides a rationale to treat M4 and M5 FAB classified myeloid leukemia with Tosedostat. Beyond this, from an inflammatory/auto-immune perspective, this may also imply that Tosedostat could be an interesting drug for selective targeting
of pro-inflammatory macrophages that play a role in pathophysiology of RA. Since no information is available on the long term-efficacy and/or propensity to develop acquired resistance to drugs like Tosedostat, we addressed these issues for the first time in a model system of human U937 myeloid cells that were chronically exposed to stepwise increasing concentrations of CHR2863, a close structural analogue of Tosedostat (Chapter 8). Our studies indicated that long term exposure to CHR2863 is accompanied by the development of resistance to this drug. Unravelling the molecular basis of acquired resistance to CHR2863 revealed at least 3 mechanisms that contributed to the resistant phenotype. First, down-regulation of CES1, which leads to impaired conversion of CHR2863 to its active metabolite and thus diminished efficacy. However, since CES1 fulfils important physiological functions, a.o. in cholesterol homeostasis, loss of CES1 activity needs a compensatory response by upregulating another CES family member, CES2. Consequently, in CHR2863-resistant cells we observed collateral sensitivity to prodrugs (e.g. Irinotecan) that are preferentially activated by CES2. Second, a role for intracellular lipid droplets was indicated in sensitive cells by the association with CES1 and CHR2863 activation, and in resistant cells by CHR2863 sequestration. For a long time, lipid droplets were simply thought to play a role in lipid storage. Nowadays, lipid droplets have emerged with multiple functions related to their dynamic properties of interaction with the cytoskeleton and different cell organelles, including mitochondria, ER, peroxisomes and endosomes. Lipid droplets can take up, temporarily store and/or extrude damaged or inactivated proteins. Lipid droplets also are recognized as organelles that host leukotriene metabolism and carry cytokines as well as so alarmins, both inflammatory mediators in autoimmune diseases like RA. These links between lipid droplets and inflammation deserve further exploration. Third, it appeared that CHR2863 resistant cells had adopted a programme to reactivate the Akt/mTOR survival pathway in response to the previously reported suppression of mTOR activity by Tosedostat. Strikingly, Akt/mTOR activation was associated with a marked gain of sensitivity to the mTOR inhibitor rapamycin, which served as a good biomarker for resistance to CHR2863. These observations argue in favor of exploiting combinations of CHR2863 and rapamycin (or other rapalogs) in targeting myeloid leukemia cells or inflammatory macrophages.
Unravelling the molecular basis of CHR2863 resistance has expanded our knowledge of the mechanism of action of this aminopeptidase inhibitor prodrug. Further studies should be dedicated to examine its potential anti-inflammatory properties. In a pilot study (unpublished observations) we noted that CHR2863 could suppress the release of pro-inflammatory TNFα and increased the release of anti-inflammatory IL-10 from activated peripheral blood mononuclear cells. Given their unique mechanism of action and suitability for use in combination therapies, drugs like Tosedostat warrant further exploration in a (pre)clinical anti-inflammatory or autoimmune disease setting.

Altogether, results described in this thesis provide support for further exploration of drugs that interfere in protein degradation processes as experimental therapeutics in an autoimmune disease setting. The growing knowledge from the field of oncology on improving the clinical efficacy of proteasome inhibitors such as bortezomib and second generation PIs holds promise and merits future evaluation in autoimmune disease treatment. The same holds for aminopeptidase inhibitor prodrugs like Tosedostat which may add to greater selectivity by targeting of specific immune effector cells. However, as a natural response to foreign compounds and building a defence mode, it will be hard to fully avoid the onset of drug resistance, in particular following chronic drug administration. Therefore, studies dedicated to understand the molecular basis underlying loss of efficacy and onset of cellular drug resistance can help to improve the therapeutic window and extended therapeutic application of PIs and aminopeptidases.
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