ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Proteosome Inhibitors: A Targeted Therapy.

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ABSTRACT

Proteasome inhibition is a valid anticancer strategy. The ubiquitin proteasome pathway plays acritical role in regulating many processes in the cell which are important for tumour cell growth and survival. The proteosomal degradation is a highly complex and regulated process and is essential for cellular regulatory mechanism and maintaining homeostasis. In the ubiquitin proteasome pathway, the ubiquitin tagged proteins are recognised by 19s proteasomes and it is untagged by the same. The resultant protein substrates are unfolded by the enzyme ATPases; the substrates are fed into inner catalytic compartment of 20s proteasomes followed by the opening of molecular gate channel. These unfolded proteins are then hydrolyzed by 6 active proteolytic sites like Chymotrypsin like (CT-L), Caspase like (C-L) and trypsin like(T-L)on ß-subunits into small polypeptides. This activity of proteasomes is inhibited by the proteasome inhibitors. Development of Proteosome inhibitors shows the hope for the cancer patients. This review facilitates easy understanding of the concepts of proteasome inhibitors, dealing in brief about the ubiquitin proteasome pathway, Biological effects of Pi,Therapeutic applications andto enlighten the approved drugs for clinical practice.

Keywords: Proteosome inhibitors, NF –κB, Chymotrypsin like site(CT-L),Bortezomib, Multiple myeloma

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ISSN: 0975-8585

BACKGROUND

Novel agents like proteasome inhibitors led to the demonstration that the proteasome catalyses the degradation of transcriptional regulatory proteins which is essential for the regulation of cell growth and gene expression. The 20 and 26s Proteosomes has played an important role. Thus, the cell cycle progression is controlled by the proteasomal degradation of cyclins and inhibitors of cyclin - dependent kinases. Multiple myeloma though a rare cancer of the plasma cell with an incidence of 1-4/1 lakh people per year, and being stated as the disease that cannot be cured but can be treated remained a dooming condition until the re-emergence of thalidomide and the advent of proteasome inhibitors.Cancer treatment has thus been able to see a ray of hope since the introduction of proteasome inhibitors in clinical practice.

STRUCTURE OF PROTEOSOME COMPLEX:

The 26s proteosome:

The 26s proteosome is a multicatalytic, multisubunit proteolytic complex found in all eukaryotic cells. The degradation of most protein in nucleus and cytoplasm is via Ubiquitin –proteosome pathway[1].The 26s proteosome is 2.5 MDa molecule composed of proteolytic core particle of one 20s and two 19s regulatory particles which catalyses protein degradation. The 20s proteosome is sandwiched by two 19s cap regulatory protein[2]. The 20s proteosome is a functional proteolytic core; hollow cylindrical shaped structure made of 28 subunits arranged in 4 stacked rings which recognize ubiquitinated proteins. There are two pairs - outer ring and inner ring. The two outer ring contains 7 alpha subunits and two inner ring contains 4 beta subunits[3]. There are six catalytic enzymes involved in the activity; two chymotrypsin, two trypsin and two caspase. These three catalytic sites are major target for the proteosome inhibition[1] . The cleavage of hydrophobic, basic and acidic residues are done by the catalytic enzymes; to match the specificity of MHC I molecules. In most cells and tissues of the immune systemalso express immunoproteosomes, which contain replacements for the three catalytic subunits of standard proteosome. The standard core particle is replaced by LMP 2 (β1i) , MECL (β2i) and LMP 7 (β5i) [4]. The production of immune proteosomes in most of the cells is due to stimulus of oxidative stress and proinflammatory cytokines.

UBIQUITIN PROTEOSOME PATHWAY:

The ubiquitin – proteasome pathway(UPP) is the major proteolytic pathway for intracellular protein degradation in the cytosol and nucleus of all eukaryotic cells [5,6]. Majority of cellular proteins involved in the development of majority of deadly disease including those involved in cell-cycle transcription, DNA repair, protein quality control are degraded by this pathway. This ubiquitin– proteasome pathway has a role in tumorigenesis, Immune surveillance [7], regulation of metabolic pathways [8-10], selective removal of mutant, damaged and misfolded proteins(12). These abnormal unfolded mutated proteins are degraded by proteasome and are important causes of various genetic diseases including cystic fibrosis [13-14] and hereditary α_1 antitrypsin deficiency[15].

The process involving the degradation of proteins via the UPP is accomplished by co-ordinated action of three enzymes – E_1 ubiquitin activating enzyme that activates the ubiquitin molecule in the ATP- dependent process and transfers it to one of atleast 15 different ubiquitin carrier proteins by E_2 Ubiquitin conjugating enzyme. The Ubiquitin is then transferred to the substrate protein by Ubiquitin Proteins Affected by Inhibition of Ubiquitin-Proteasome Pathway ligase E_3[16]. There are more than 30 E_2 and more than 100 E_3 enzymes. There are three steps involved in the degradation of cellular proteins which is mediated by the three enzymes explained above. Step 1:Addition of polyubiquitinated tails to proteins destined for destruction; addition is through covalent bonding, Step 2: Identification of these ubiquitinated proteins by the intracellular proteasome complex, Step3: Degradation of identified proteins in the central portion of the proteasome complex

ISSN: 0975-8585

Proteins affected by inhibition of ubiquitin-proteasome pathway

Proteins	Effect of Proteasome Inhibition
I-κB	inhibition of NF-κB* →growth inhibition, apoptosis, decreased expression of angiogenic cytokines and adhesion molecules
p21, p27, p15, p16, p18, p19	G1-S cell-cycle arrest and apoptosis
p^{53}	Apoptosis
BAX*	Apoptosis
Damaged cellular proteins	Apoptosis
JNK*	activation of caspase-8 and caspase-3; release of cytochrome C;apoptosis
Topoisomerase Iiα	DNA torsional stress–relieving enzyme

BIOLOGICAL EFFECTS OF PROTEOSOME INHIBITORS:

Inhibition of protein breakdown and its measurement in cells:

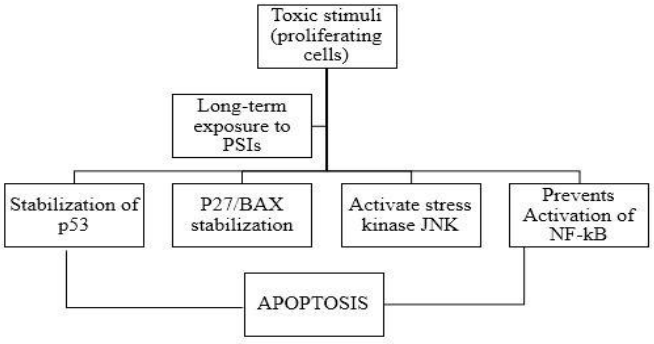
Almost the tumour cells are toxic highly proliferative and have increased need for protein synthesis which make them more vulnerable (sensitive) to proteosome inhibitors. The degree of inhibition of chymotrypsin like activity is useful qualitative measure of efficacy against proteins [17]. It is identified that there is 50% inhibition of proteolysis with radiolabelled protein. Due to this there shows doubling time of proteins. Several studies have been conducted to analyse the quantitative inhibitor efficacy by measuring the accumulation of short lived proteins [18]. Studies shown that, in any period an inhibitor is expected to have much larger effect on the levels of short lived protein than on a long lived cell protein.

Induction of heat shock response:

Due to decrease of overall rates of protein breakdown in cells that leads to rapid accumulation of short lived proteins [19,20]. Endoplasmic reticulum plays an important role in protein folding and maturation [20-22]. Due to proteosome inhibition there is accumulation and aggregation of misfolded protein in ER which results in ER stress, producing heat shock response [21]. It is pro-survival response to reduce the accumulation of unfolded proteins and restore the ER function. Due to persistent accumulation of proteins, changes in the Endoplasmic reticulum from pro-survival to pro-apoptotic phase. Malignant cells are more prone to protein aggregation they have higher protein synthesis and are more sensitive to PI's. Toxic conditions such as increased temperature or O2 radical can be protected by short exposure of PI's , thus inhibition of proteosome exhibits various changes in the cell with short-term exposure of within 30-60mins[23-25].

Cytotoxicity of proteosome inhibitors:

Proteosome inhibitors are toxic on chronic exposure. Though short term exposure prevents many toxic condition, Long term or chronic exposure results in toxicity to all cells and causes death by apoptosis[26-30]. P53 tumour suppressor gene induces apoptosis, PI's stabilizes the P53 gene and accumulation of this leads to cell death. Apoptosis may be temporarily inhibited by stabilization of the inhibitors of apoptosis Bcl-2 and IAP[27, 31-36].



Exposure of proteosome inhibitors to less than 16hrs on non-proliferating cells will lead to stabilisation of Bcl2 which is an anti-apoptotic protein [30, 37]. Thus causes temporary resistant of cells to apoptosis.

Anti - Inflammatory activity:

Initiation of inflammatory response in the rapid destruction of the inhibitory protein IκB which occurs in response to various toxic stimuli[39]. IκB is an inhibitor of transcription factor NF- κB, which activates the expression of many genes encoding inflammatory mediators (eg. TN, IL 1), enzymes, cyclooxygenase, NO synthetase) and leukocyte adhesion molecules (ICAM, VCAM) [40,41]. Thus ubiquitination of this complex leads to the degradation of IκB. Excess availability of NF-κB causes DNA transcription and release of inflammatory mediators. The production of NF-κB is suppressed by proteasome inhibitors like bortezomib. Thus it is very effective in the treatment of arthritis and reduces reperfusion injury to the brain[41-43, 46]. Cyclooxygenase II, a critical enzyme involved in synthesis of inflammatory PGs the neu which is potentially pro-inflammatory[42-45].Proteosome inhibitors blocks the DNA transcription and release of inflammatory mediators, leucocyte adhesion molecules[43,45]. Thus promotes the antiflammatory activity of proteasome inhibitors.

Anti-tumour effects:

Proteosome inhibitors are well-known for its anti-tumour effect because of its ability to supress the proliferating cell lines and selectively induce apoptosis in proliferating cells [48]. In addition to this apoptosis, it also inhibit angiogenesis[47] , these properties of proteosome inhibitors make these agents a well-effective anticancer drugs. Anti-tumour activity of Proteosome Inhibitors are due toits unique activity of NF – κBinhibition[40], Increased pro-apoptotic proteins, ER stress[21] and removal of misfolded proteins, Cell cycle arrest[38], Impairment of DNA damage response, FoxM1 inhibition and due to Inhibition of angiogenesis[47]. Growth factors such as IL-4 and IL-6 produced by NF-κB are required for certain cancer cells. The production of NF-κB is prevented by proteosome inhibitors.

Multiple myeloma

Multiple myeloma is a plasma cell malignancy predominantly localised in the bone marrow and characterised by paraproteinemia [57]. Polyubiquitination is an essential event for proteins targeted for proteasomaldegradation. Proteins degraded by the proteasome include mediators of cell-cycle progression and apoptosis, such as the cyclins, caspases, B-cell lymphoma 2 (BCL2),and NF-kB activation [49].Bortezomib can, therefore, lead to an increase in the cytoplasmic level of IkB, resulting in a blockade of NF-kB translocation to the nucleus and DNA-binding activity [50]. It also inhibits angiogenesis in the bone marrow microenvironment, which plays an important role in both multiple myeloma pathogenesis and disease progression.

Adaptation and resistance to proteosome inhibitors:

Though it is proven that chronic exposure to proteosome inhibitors is almost lethal to all cells,but 0.3% of cells escape cell death and multiply even after the treatment due to adaptation[51]. These type of cells are resistant to apoptosis.Studies showed that adaptation to proteasome inhibitors is seen in Burkitt lymphoma cell lines **.**The mechanism of adaptation is still unclear[52].

Anti- HIV effects:

Proteosome inhibitors are suggested for the treatment of HIV. Due to the depletion of pool of free ubiquitin by the proteasome inhibitors leads to the unavailablility of ubiquitins for the conjugation to viral gag polyproteins[53,54]. Thus inhibits the virus release from the cell. This unique mechanism made proteasome inhibitors to play its role in HIV.

Inhibition of antigen presentation:

Proteasomes plays a major role in antigen presentation by inhibiting the MHC Class I molecule. It has a key role in immune surveillance against viruses and cancer. The first in vivo study of proteosome inhibitors demonstrated that blocking the proteosome reduces the generation of peptides used in MHC Class I antigen presentation[19]. The role of proteasomes inhibitor reduces the presentation of majority of epitopes. At low concentration of PI's presentation of few epitopes are stimulated and at higher concentration there is inhibition of presentation[55].

PROTEOSOME INHIBITORS:

The year 2003 saw the first proteosome inhibitor from natural sources. Borteozomib was approved for third-line treatment of multiple myeloma by the FDA in 2003[1]. They are enzyme specific inhibitors of proteases; almost all the synthetic and natural inhibitors of the proteosome act predominantly on the chymotrypsin sites but also has some weaker action on trypsin and caspase sites. The rate limiting step in the protein breakdown is cleavage by chymotrypsin-like sites; the potency of inhibition is often been measured only against the chymotrypsin like activity. These enzyme specific inhibitors are usually short peptides linked to a pharmacopore, generally located at its C-terminus. Based on the pharmacopores, proteosome inhibitors can be divided into several groups like peptide aldehydes, peptide boronate . The other Non – peptide inhibitors include lactacystin and β – lactone, Peptide vinyl sulfones, Epoxyketones , synthetic proteosome inhibitors and Bivalent inhibitors. There are some newly developed natural compound inhibitors like TMC- 95A , a cyclic peptide metabolite of Apiosporamontagnei ; Gliotoxin – a fungal metabolite and Cathechin – 3- gallate derivatives of green tea[2].

APPROVED DRUGS:

Bortezomib:

Bortezomib , belongs to peptide boronate family and was the first inhibitor to enter clinical practice[1]. It is a reversible inhibitor having its own primary action on chymotrypsin – like enzymatic sites. This compound is screened against the panel of 60 cancer cell lines, on the basis of its potency and cytotoxicity. Furthermore in vivo and in vitro studies in various tumour types indicated the activity of this compound in multiple myeloma, mantle cell lymphoma, Cutaneous or peripheral T-cell lymphoma, relapsed/refractory (unlabeled use), Follicular lymphoma, relapsed/refractory (unlabeled use), Systemic light-chain amyloidosis, Waldenström'smacroglobulinemia, relapsed/refractory.Bortezomib (VELCADE) is the first proteosomal inhibitor approved in May 13, 2003.Due to upregulationof the proteasome subunits, especially for increased levels of the β5-subunit resistance to bortezomib has been observed. Thus to overcome this clinical crisis the second and third generation proteasome inhibitors have been developed. These were formulated either by adding an irreversible bond to β5-subunit (such as carfilzomib) or binding to different subunits (such as marizomib) or possibly by oral administration (such as ixazomib)[3].

Dosing schedule for the treatment of Multiple myeloma (first-line therapy; in combination with melphalan and prednisone): I.V., SubQ: 1.3mg/m^2 days 1, 4, 8, 11, 22, 25, 29, and 32 of a 42-day treatment cycle for 4 cycles, followed by 1.3 mg/^{m2} days 1, 8, 22, and 29 of a 42-day treatment cycle for 5 cycles.

Alternative first-line therapy (unlabeled dosing):

CyBorD regimen: I.V.: 1.5 mg/m^2 days 1, 8, 15, and 22 of a 28-day treatment cycle for 4 cyclesin combination with cyclophosphamide and dexamethasone.

Pharmacodynamics and Pharmacokinetics:

Distribution: widely to peripheral tissues
Protein binding: ~83%
Metabolism: Hepatic primarily via CYP2C19 and 3A4, lesser extent CYP1A2
Half-life elimination: Single dose: I.V.: 9-15 hours; multiple dosing: 1 mg/m^2: 40-193 hours; 1.3 mg/m^2: 76-108 hours

Drug Interactions: CYP3A4/2C19 inhibitors & inducers
Preferred route of administration is intravenous or subcutaneous. Any other route of administration is contraindicated.

Adverse Reactions

>10%	1 to 10%	<1%
Constitutional	Cardiovascular	ARDS
Dermatological	Endocrine & Metabolic	DIC
Gastrointestinal	Hematological	SIADH
Hematological	Local	Electrolyte imbalance
Neuromuscular & skeletal	Pneumonia	Hypersensitivity
Respiratory	Herpes zoster	Weight loss

Carfilzomib:

Carfilzomib (PR-171) is an epoxomicin-based proteasome inhibitor

Carfilzomib (KYPROLIS) irreversibly binds to and inhibits the chymotrypsin-like activity of the 20S catalytic core subunit of the proteasome, a protease complex responsible for degrading a large variety of cellular proteins. Inhibition of proteasome-mediated proteolysis lead to cell cycle arrest, induction of apoptosis, and inhibition of tumor growth. Advantage of carfilzomib over bortezomib is characterized by irreversible binding to the CT-L subunit. Carfilzomib is currently in phase III clinical trial for multiple myeloma. A phase III confirmatory clinical trial(the ASPIRE trial), comparing three drug regimen(carfilzomib, lenalidomide and dexamethasone) versus two drug regimen (lenalidomide and dexamethasone) in patients with relapsed multiple myeloma is ongoing. ASPIRE trial indicated that more patients responded significantly to the three-drug regimen compared to the two-drug regimen.

Kyprolis is indicated for the treatment of patients with relapsed or refractory multiple myeloma as a single agent. Preferred route is I.V. administration over 2-10 minutes. Flush line before and after carfilzomib with NS or D_5 W.Hazardous agent; use appropriate precautions for handling and disposal[4]. The most common adverse reactions of Carfilzomib occurring in at least 20% of patients in monotherapy trials: anemia, neutropenia, diarrhea, breathlessness, fatigue, thrombocytopenia, pyrexia, insomnia, muscle spasm, cough, hypokalemia.

Ixazomib:

U.S. FDA Approves Takeda's ixazomib (Ninlaro) the First oral Proteasome Inhibitor to Treat Multiple Myeloma[60]. Ixazomib (Ninlaro) provides a new option for cancer patients and indicated in combination with lenalidomide and dexamethasone for the treatment of patients with multiple myeloma who have received at least one prior therapy[59]. Ixazomib is administered through oral route, once-weekly on 1, 8, and 15th day of a 28-day treatment cycle. The most common adverse reactions occurring in greater than or equal to 20% of patients treated with Ixazomib were diarrhea, constipation, thrombocytopenia, peripheral neuropathy, nausea, peripheral edema, vomiting and back pain.

Others

Salinosporamide A:

Salinosporamide A (NPI – 0052) , is a novel proteosome inhibitor derived from marine organism and is structurally related to lactacystin – derived proteosome inhibitor[1]. It is also named as Marizomib. It irreversibly binds to all the three sites.

Epoxomicin:

The α′,β′-epoxyketone containepoxomicin (a natural product) was isolated from an *Actinomycetes* strain. It is selective and irreversible inhibitor of 20s proteosome. Epoxomicin potently

inhibits primarily the chymotrypsin-like activity[58]. Epoxomicin, a potent and selective proteasome inhibitor, also exhibits anti-inflammatory activity. Epoxomicin prevents IκB degradation and activation of NF-κB DNA-Binding Activity so itwould be an attractive agent for anti-inflammatory therapeutic intervention.

Lactacystin:

Lactacystin is a non-pepitde inhibitor isolated in the year 1991 from the culture broth of Streptomyces lactacystinaeus; potent,selective and irreversible proteasome inhibitor. It also Inhibits NF-κB activation by inhibiting IκB degradation, ubiquitin proteasome pathway in cell culture and cathepsin A[62]. Studies showed that lactacystin itself is not active against proteasomes in vitro but at neutral pH it undergoes transition to clasto-lactocystin-β- lactone which is active against proteasomes[61].

Mg 132:

MG132, is a peptide aldehyde, one of a group of chemicals able to inhibit different types of proteases, including serine proteases, calpain, etc [61]. MG132 and other peptide aldehydes have been shown to strongly inhibit calpain activity and multiple peptidase activities of proteasomes.

POTENTIAL THERAPEUTIC APPLICATION OF PROTEASOME INHIBITORS:

Treatment of organ transplant patients

Bortezomib provides effective therapy for antibody and cell mediated acute rejection[64].
The drug provides
i) prompt rejection reversal
ii)marked and prolonged reduction in donor specific antibody(DSA) levels
iii) improved renal allograft function
iv)suppression of recurrent rejection for atleast 5 months Bortezomib thus plays a marked role in plasma cell targeted therapy.

Auto immune diseases:

Immune proteasome are protective against the development of autoimmunity (41). The proteosome involvement in antigen presentation explores the role of PI's in auto immune disease like Rheumatoid Arthritis, Systemic Lupus Erythematosus ,Sjogren's syndrome and scleroderma. Protesome inhibitor are drugs with unique ability to

i) Inhibit the activation of NF-κB and transcriptional regulation of proinflammatory cytokine(TNF a and IL-6) release ii) induce apoptosis of activated immune cells

Anti - inflammatory activity of Proteosome inhibitors:

The key proteins modulated by proteasome are those involved in the control of inflammatory processes,thus proteasome inhibition is a potential treatment option for inflammatory condition.NF–κB is a key regulator of inflammation, it is an attractive target for anti- inflammatory therapeutic intervention[47]. Used in the treatment of arthritis, psoriasis, Bronchial asthma, ulcerative colitis.

Treatment of reperfusion injury after stroke:

The extent and progression of brain injury resulting from cerebral ischemia is due toreperfusion mechanisms; many of which involve post injury inflammatory response elements.

Stimulation of boneremodelling and hair growth:

The remodelling process in localized area for endosteal bone surface shows features common with the mammalian hair cycle. In both the process there are phases of resorption or regeneration, a transition phase and then a phase of growth termed anagen in hair follicle and formation in the bone remodelling cycle.

Fortunately both use the same molecular mechanism and specifically Hedgehog-BMP Wnt signal transduction cascade. Proteasome inhibitors are compounds that inhibit NF-κB activity that directly stimulate bone formation. Proteosome activity is necessary for NF-κB translocation. NF-KB is present in the cytoplasm bound to the inhibitory proteins IKBα and IKB β which present its translocation. Translocation occurs when kinases phosphorylate IKB β to cause its degradation by proteasome activity, thus resulting in the release for entry into the nucleus. Inhibition of proteasome activity prevents this release and inhibit NF-κB .β–catenin is known to play a role in cell – cell adhesion and growth factor signal transduction; after ubiquitination β-catenin is degraded by the proteasomes this process by proteasomes inhibition promotes growth of the hair follicle.

Proteasome Inhibitors as Anti – Infectives:

Proteasome inhibitors are used in the treatment of Mycobacterium tuberculosis. It inhibits peptide priming in the endoplasmic reticulum which is a key part of the host immune response by decreasing the pole MHCclass I molecule and they exert dose dependent mechanism based host toxicity [35]. The use of proteosome inhibitors with interferons and nucleoside analogues for treating viral hepatitis is helpful. It is also used in case of treating therapy resistant and refractory viral infections.

CONCLUSION

As rightly quoted by Martin Luther King, Jr "If you can't fly then run, if you can't run then walk, if you can't walk then crawl, but whatever you do you have to keep moving forward" the era of targeted therapy especially of proteasome inhibitors need much research and further development. The advancements in genomics and proteomics can pave the way to this by allowing the analysis of the effects of these inhibitors on global patterns of gene expression and protein composition of the cell. The Proteosome inhibitors provides ray of hope in patients suffering from multiple myeloma and other tumour likely of particular interest will be the generation of inhibitors of non-proteolytic components of the 26S proteasome and of theubiquitin conjugation enzymes. The evolution of proteasome inhibitors in cancer treatment is promising.

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