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Inhibition of ubiquitin-proteasome pathway: A possible treatment of hepatocellular carcinoma

Abdulkareem Albekairy, Abdulmalik Alkatheri and Mahmoud Mansour*

College of Pharmacy, King Saud bin Abdulaziz University for Health and Science. P. O. Box 22490, Riyadh 11426, Kingdom of Saudi Arabia.

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Alterations in ubiquitination and deubiquitination reactions have been directly implicated in the etiology of many malignancies. In general, specific cancers can result from stabilization of oncoproteins or destabilization of tumor suppressor genes. Proteasome inhibitors (PIs) represent potential novel anticancer therapy. These agents inhibit the degradation of multi-ubiquitinated target proteins, that is, cell cycle regulatory proteins such as cyclins and cyclin-dependent kinase inhibitors that regulate cell cycle progression. Following the successful application of Bortezomib as an effective treatment for multiple myeloma (MM), a number of next-generation proteasome inhibitors have been developed with the goals of improving efficacy, overcoming drug resistance, minimizing dose-limiting toxicity such as peripheral neuropathy (PN), and improving convenience of administration. The recent accelerated approval of carfilzomib exemplifies the success of this approach, with other four inhibitors currently under study both preclinically and clinically. The role of PIs in hepatocellular carcinoma (HCC) has been demonstrated for the first time in 2004 that MG-132 induced apoptosis in human HCC cells through caspase-dependent cleavage of β-catenin and inhibition of β-catenin-mediated trans-activation. In addition, effect of Bortezomib on HCC was investigated and concluded that Bortezomib induced apoptosis in HepG2 cells as a model of HCC by stimulating both the extrinsic and intrinsic apoptotic pathways. Moreover, it has been shown that treatment with MG132 in combination with celecoxib resulted in synergistic anti-proliferative rather than anti-inflammatory and proapoptotic effects against liver cancer cells, providing a rational basis for the clinical use of this combination in the treatment of liver cancer.

Key words: Hepatocellular carcinoma, ubiquitin-proteasome pathway, proteasome inhibitors.

PROTEASOME STRUCTURE AND ORGANIZATION

In structure, the intact 26S is a large multi-subunit complex, which is approximately 2000 kilodaltons (KDa) in molecular weight and it composed of one 20S core particle structure and two 19S regularity caps (Figure 1). That core is a hollow cylindrical-shaped structure that provides an enclosed chamber in which proteins are destructed. The openings at each end of the core cylinder make it possible for the target protein to enter (Wang and Maldonado, 2006). Each side of the barrel-shaped structure is attached to a cap structure which has several ATPase active sites and ubiquitin binding sites. This cap structure regulates the recognition of the polyubiquitinated targeted substrates and translocates them to the proteolytic cavity of the core particle. 11S particle, which is an alternative form of regulatory subunit, can be attached to the 20S core particle in exactly the same way as the 19S particle (Wang and Maldonado, 2006).

*Corresponding author. E-mail: mansour1960us@yahoo.com. Tel: 0096618011111, 0966502997605. Fax: 0096612521011.

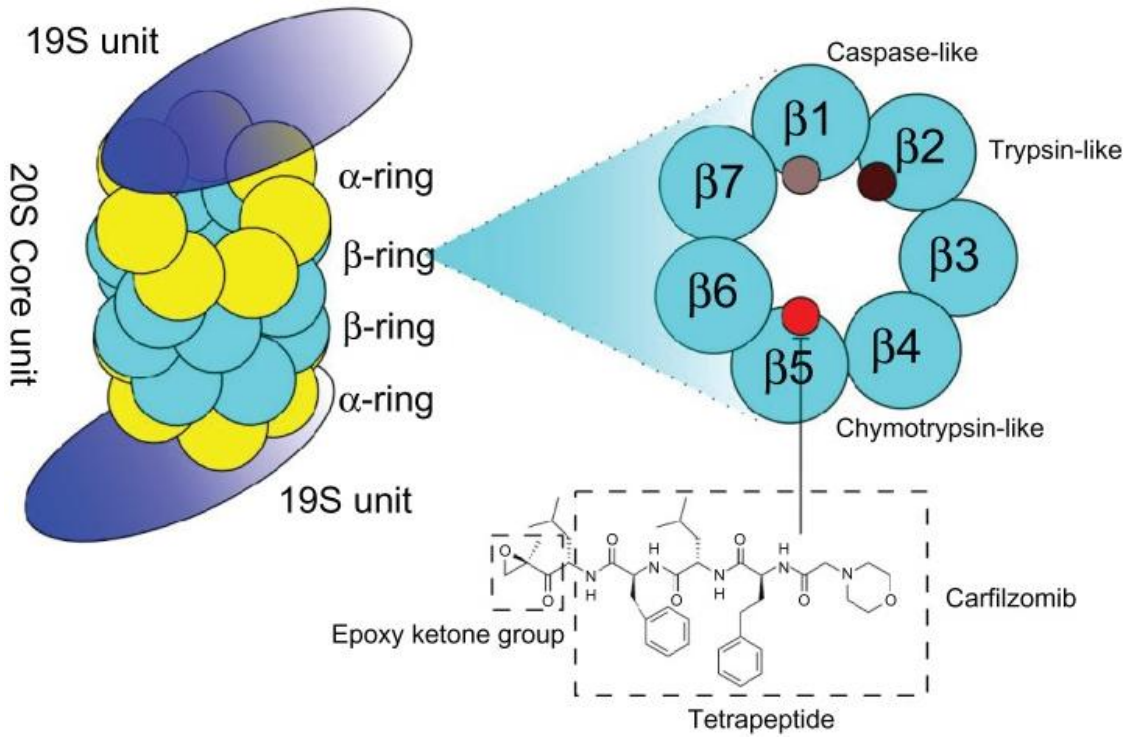

Figure 1. Composition of the 26S proteasome, carfilzomib chemical structure and it is binding site in the 20S proteasome and immunoproteasome. The 26S proteasome comprises a hollow cylindrical 20S proteolytic core and one or two 19S regulatory particles. The 19S unit recognizes poly-ubiquitynated substrates, and prepares them for proteolysis, which occurs inside the 20S cores. The 20S core comprising 2 pairs of 14 different polypeptides arranged in 4 stacked rings. Six subunits carry catalytic residues for the proteolytic sites: two are chymotrypsin-like (β5), two trypsin-like (β2), and two caspase-like (β1).

20S core particle

The type of organism is an important factor in which the number and diversity of subunits contained in the 20S core particle depends on. The distinct and specialized subunits is smaller in unicellular than multicellular organisms and smaller in prokaryotes than in eukaryotes. All 20S core particles comprise four stacked seven-membered ring structures. These structures consist of two different types of subunits: α subunits and β subunits. The α subunits have structural nature while β subunits are basically catalytic in nature. Each of the exterior two rings in the stack composed of seven α subunits. These subunits acts as docking domains for the regularity caps and the α subunits N-termini form a gate which restricts unregulated passage of proteins to the lumen of 20S core particle. Each of the interior two rings composed of seven β subunits and they have multiple catalytic sites which are responsible for the proteolysis reactions (Smith et al., 2007).

The archaebacterial 20S proteasome such as *Thermoplasma acidophilum* has just one type of α subunit in the outer two rings in the stack and one type of β subunit in the inner two rings in the stack while the 20S core particle in the eukaryotic proteasome consist of seven distinct but homologous α subunits and seven distinct but homologous β subunits. This core particle in the eukaryotic proteasome has at least three distinct proteolytic activities. Despite sharing a common mechanism, they have three distinct substrate specificities known as chymotrypsin-like (β5), trypsin-like (β2) and post-glutamyl peptide hydrolase-like (caspase-like, β1) (Heinemeyer et al., 1997; Adams, 2003). Alternative β forms denoted β1i also known as low molecular mass polypeptide 2 (LMP2), β2i also known as multicatalytic endopeptidase complex subunit (MECL1) and β5i, also known as low molecular mass polypeptide 7 (LMP7) can be shown in hemato-poietic cells mainly monocytes and lymphocytes upon stimulation with inflammatory signals like cytokines, especially interferon-gamma. The immunoproteasome is the proteasome assembled with these alternative β subunits (Altun et al., 2005; Nandi et al., 2006).

19S regulatory particle

In eukaryotes the 19S regulatory particle composed of at least 19 different subunits and can be detached further into two subcomplexes. The first is a 10-protein base that binds directly to either of the outer two rings of the 20S core particle, and the second is a 9-protein peripheral lid in which polyubiquitin chain is bound. In the base of the

19S regulatory particle six of the ten proteins are ATPase subunits which belong to the AAA family, and an evolutionary homologous of these ATPase subunits exists in archaea. It was called Proteasome-Activating Nucleotidase (PAN) (Zwickl et al., 1999). The binding of ATP to 19S ATPase subunits achieves the assembling of the 19S and 20S particles. The assembled complex degrading folded and ubiquitinated proteins need ATP hydrolysis. Note that ATP-binding alone can support all steps needed for protein destruction (e.g., complex assembly, gate opening, translocation, and proteolysis) except the unfolding of substrate required energy from ATP hydrolysis (Smith et al., 2005; Liu et al., 2006). Opening the gate in the α ring of the 20S core particle which prevents the access of substrates into the interior cavity is one role of the 19S regulatory ATPase subunits (Köhler et al., 2001). The way by which the 19S regulatory ATPase subunits opens the gate in the α ring of the 20S core particle has been recently explained (Pathare et al., 2011). 20S gate opening allows substrate entry into the proteolytic cavity of the core particle and requires the C-termini of the 19S regulatory ATPase subunits that has a certain motif known as hydrophobic-tyrosine-X (HbYX) motif. The proteasomal ATPases C-termini bind to pockets in the top of the 20S core particle. Tying these C-termini into these 20S pockets by themselves induces α ring conformational changes which subsequently lead to 20S gate opening in the same way that a key in a lock opens the door (Smith et al., 2007).

11S regulatory particle

A second type of regulatory cap that can be associated with 20S core particle is the 11S regulatory particle. It is a seven-membered structure that does not contain any ATPases. This heptameric structure is also known as PA28 or REG. It can facilitate entry and degradation of short peptides but not of complete proteins. It is supposed that this is because the proteasome assembled with this alternative regulatory particle cannot unfold larger substrates. It is binds to the 20S core particle through C-termini of its subunits and promotes α ring conformational changes which subsequently lead to 20S gate opening (Forster et al., 2005). 11S particle is induced by interferon gamma. By the union of the immunoproteasome β subunits it is responsible for the generation of certain peptides for major histocompatibility complex class I (MHC-I) presentation (Wang and Maldonado, 2006).

PROTEASOME FUNCTION: THE PROTEIN DEGRADATION PROCESS

Ubiquitylation and targeting

The proteins destined for destruction by the proteasome are marked covalently by a polyubiquitin chain. The ubiquitination of protein is carried out by the coordinated action of a cascade of enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligases (E3) (Figure 2). In the first step of this cascade, in an adenosine triphosphate ATP-dependent manner, ubiquitin is activated by an ubiquitin-activating enzyme. This step involves hydrolysis of ATP, adenylylation of an ubiquitin molecule and the covalent binding of adenylylated ubiquitin to cysteine in the active site of an ubiquitin-activating enzyme in concert with the adenylylation of a second ubiquitin (Haas et al., 1982). After activation, the first adenylylated ubiquitin is then transferred to the active site cysteine of an ubiquitin-conjugating enzyme. In the third and last step, adding ubiquitin to the target protein is catalyzed by a member of many E3s. This E3 recognizes the specific protein to be ubiquitinated and catalyzes the transfer of ubiquitin from the active site cysteine of an ubiquitin-conjugating enzyme to a lysine residue on this target protein. A target protein must be labeled with a polybiquitin chain of at least four units before it is recognized by the proteasome lid (Thrower et al., 2000). The high substrate specificity of this system lies in the diversity of different E3s that can identify a specific substrate (Risseeuw et al., 2003). The organism and cell type determine the number of E1, E2 and E3 proteins. Here we must say that in humans there are a huge number of targets for the ubiquitin proteasome system because there are many different E3 enzymes (Li et al., 2008).

It is not fully understood how a polyubiquitinated protein is shuttled to the proteasome. Recent work has shown that ubiquitin-receptor proteins contain an N-terminal ubiquitin-like (UBL) domain and one or more ubiquitin-associated (UBA) domains. The UBL domains are recognized by the 19S regulatory particle while the UBA domains tie ubiquitin by three-helix bundles (Elsasser and Finley, 2005).

Unfolding and translocation

After a protein has been tagged with a polybiquitin chain, it is recognized by the 19S regulatory cap. This occurs in an ATP-dependent binding step. After that the tagged protein must then access the interior cavity of the 20S core particle to come in direct contact with the protease active sites contained in the β-ring of the proteasome. The protein substrates must be at least partially unfolded before their entry into the catalytic cavity; that is because the 20S core particle's central channel is narrow and locked by the α subunits N-termini. The journey of the unfolded substrate to enter the proteolytic chamber of the 20S core particle is called translocation and it occurs after removing the attached ubiquitin molecules (Liu et al., 2006). Zhu et al. (2005) reported that the order in which substrates are deubiquitinated and unfolded is not yet clear. However, the specific substrate decides on which of these processes is the rate-limiting step in the

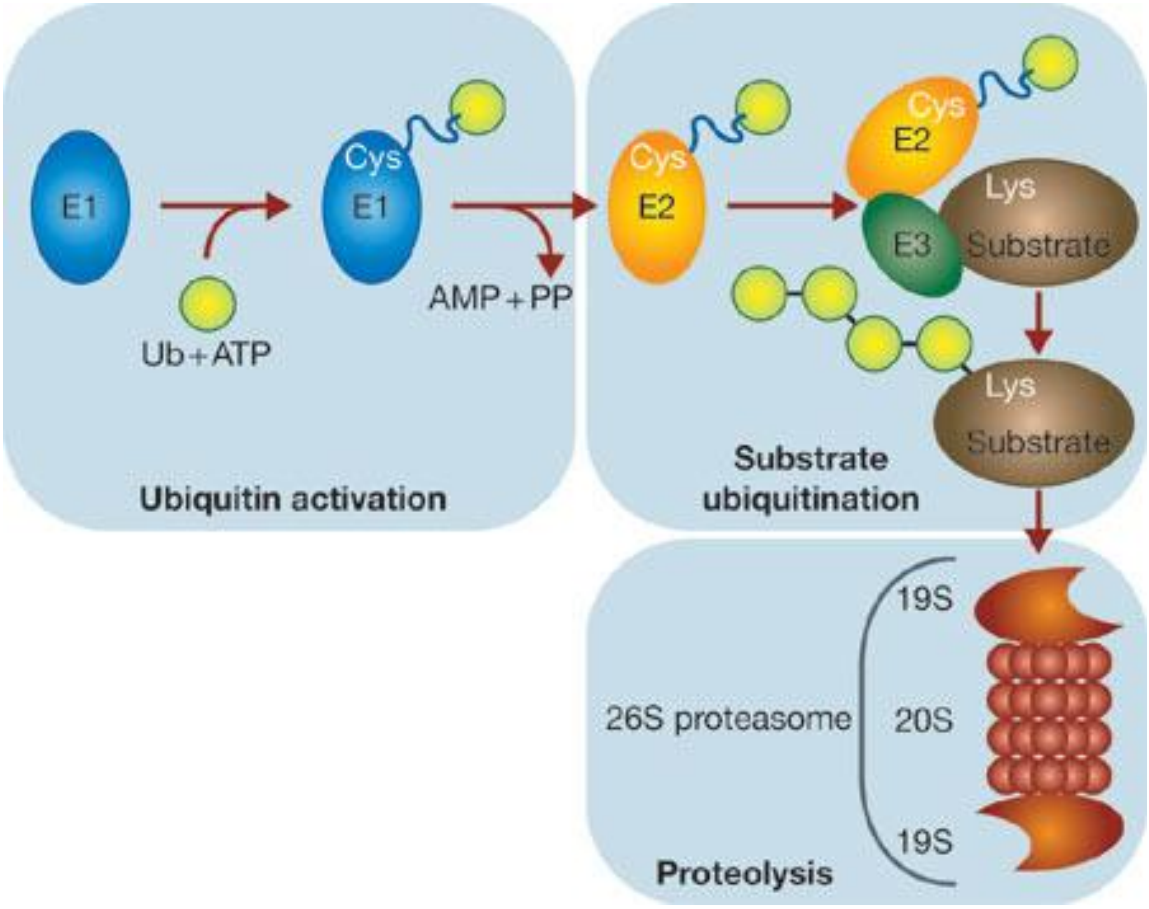

Figure 2. The protein ubiquitination pathway. A cascade of enzymatic reactions leads to ubiquitination of lysine residues of the substrate. First, the ubiquitin-activating enzyme (E1) hydrolyses ATP and forms a high-energy thioester linkage between its active site cysteine and the carboxy terminus of ubiquitin. Activated ubiquitin is then transferred to a member of the family of ubiquitin-conjugating enzymes (E2). E2 enzymes together with ubiquitin protein ligases (E3) attach ubiquitin to lysine residues of substrate proteins. In most cases, E3s function as substrate-binding factors that align the substrate and E2 in a way that facilitates ubiquitination (Meusser et al., 2005).

overall proteolysis reaction. The unfolding process for some proteins, for example, is rate-limiting while for other proteins deubiquitination is the slowest step (Smith et al., 2005).

Peptides longer than about four residues are prevented from entering the lumen of the 20S particle by the gate consisted of the α subunits N-termini. The ATP molecules, which are bound before the recognition of the tagged protein, are hydrolyzed before translocation. Energy from ATP hydrolysis is not required for translocation while it is needed for protein unfolding. The assembled complex can destruct unfolded substrates in the existence of a non-hydrolysable ATP analog. However, it cannot destruct folded substrates. This implies that energy from ATP hydrolysis is used for protein unfolding (Smith et al., 2005; Liu et al., 2006). If the 19S regulatory particle is in the ATP-bound state, the passage of the unfolded protein through the opened gate happens via facilitated diffusion (Smith et al., 2006).

Proteolysis

In protein destruction by the catalytic β-subunits of the 20S core particle, its mechanism is considered to be the threonine-dependent nucleophilic attack. Destruction happens in the middle of the core particle channel of the two β rings. In most cases, it does not generate partially destructed products, instead it cleaves protein substrate into short polypeptides of about 7 - 9 amino acids long, although they can vary between 4 to 25 amino acids in length depending on the organism and substrate protein. The biochemical process that specify the length of the peptide in the decomposition products is not fully clear yet (Voges et al., 1999). Although the catalytic activity of three β subunits shares a common destruction

mechanism, they have slightly different substrate specificities which are considered chymotrypsin-like, trypsin-like, and caspase-like. These differences in substrate specificity due to the interatomic contacts with local residues are near the active sites of each subunit. Also each catalytic β subunit contains a conserved lysine residue required for proteolysis (Heinemeyer et al., 1997; Rape and Jentsch, 2002; Adams, 2003).

PROTEASOME INHIBITORS

Proteasome inhibition treatment can induce cell cycle arrest and apoptosis. Inhibition of the proteasome by specific inhibitors has been shown to induce apoptosis in a variety of mammalian cells, just like neurons (Qiu et al., 2000), epithelial cells (MacLaren et al., 2001), aortic endothelial cells (Drexler et al., 2000), vascular smooth muscle cells (Kim, 2001) and tumor cells (Shah et al., 2001). The mechanism(s) through which proteasome inhibitors induce apoptosis is not clear and several factors seem to be important in different cells.

Tumor suppressor protein p53 is a short-life transcription factor that is normally degraded via ubiquitin-mediated proteolysis (Scheffner, 1998). Biochemically, Mdm2 acts as an ubiquitin ligase that covalently attaches ubiquitin to p53 and thus targets p53 for proteasomal degradation (Haupt et al., 1997). Accumulation of p53 will induce growth arrest, DNA repair or apoptosis. Bcl-2–associated X protein (Bax) (Chang et al., 1998) and an active form of BH3-interacting domain death agonist (tBid) (Breitschopf et al., 2000), which are examples to pro-apoptotic proteins, are also substrates of the proteasome. Inhibition of proteasomes could possibly induce apoptosis by causing an accumulation of these pro-apoptotic proteins. In addition, proteasome inhibition prevents degradation of inhibitor of kappa B (Iκ B), thereby preventing NF-κB transcriptional activity, which is involved in the induction of various anti-apoptotic Bcl-2 family members and some members of the inhibitor of apoptosis (IAP) family that directly prevent activation of caspases (Orlowski and Baldwin, 2002). Since proteasome does not only destruct pro-apoptotic proteins, but also enhance the expression of anti-apoptotic proteins, we can deal with proteasome as an anti-apoptotic factor. The accumulation of transcriptionally active p53 (Chen et al., 2000) and other pro-apoptotic Bcl-2 family members like Bax (Li and Dou, 2000), lead to the release of cytochrome c from mitochondria into the cytoplasm, which activates downstream caspase members have all been documented in the proteasome inhibitor-induced apoptosis. In addition, proteasome inhibitors caused a steady increase in activity of c-jun NH2-terminal kinase (JNK), which translocates to mitochondria and induces the release of cytochrome c and second mitochondrial activator of caspases (Smac), followed by caspase-9 activation (Chauhan et al., 2003). Besides the above-noted signaling events, endoplasmic reticulum stress and unfolded protein response, inhibition of angiogenesis and impairment of DNA-damage response have all been reported to contribute to the apoptotic affect of proteasome inhibitors in tumor cells (Crawford et al., 2011).

PIs can be classified into three groups by chemical properties and targets into: peptide boronates (Bortezomib), peptide epoxyketones (carfilzomib/PR-171), and β-lactones (NPI-0052/marizomib).

Peptide boronates

Bortezomib

Bortezomib (originally PS-341 and marketed as VELCADE by Millennium Pharmaceuticals), is the first proteasome inhibitor to enter clinical practice as a chemotherapeutic agent. It is approved by FDA for the treatment of relapsed multiple myeloma (Richardson et al., 2003), as well as mantle cell lymphoma (Fisher et al., 2006). Bortezomib is a dipeptide boronic acid analogue that reversibly inhibits the chymotrypsin-like (CT-L) and caspase-like (C-L) active sites, with minimal effect on trypsin-like (T-L) activity of the proteasome (Dick and Fleming, 2010). As a result of this approval, several second-generation proteasome inhibitors, such as carfilzomib have been developed and entered clinical trials in an attempt to overcome resistance to Bortezomib and improving safety profile.

Peptide epoxyketones

Carfilzomib

Carfilzomib (formerly known as PR-171) is a tetrapeptide epoxyketone analog (Figure 1) of the microbial natural product, epoxomicin 3, that was discovered initially as antitumor agents in animals and later on it was shown to be a potent inhibitor of the proteasome (Demo et al., 2007; Bennett and Kirk, 2008). Carfilzomib selectively binds to and inhibits the chymotrypsin-like activity of the proteasome via the β5 and LMP7 proteasome subunits, and has minimal cross-reactivity with the trypsin-like or caspase-like activities of the proteasome (Figure I). Carfilzomib exhibits mechanistically irreversible proteasome inhibition and requires new proteasome complexes synthesis for recovery of cellular proteasome activity. As a result, we can say that carfilzomib provides prolonged proteasome inhibition if we compared it with that of the slowly reversible inhibitor, Bortezomib. Also the epoxybutane pharmacophore of carfilzomib shows a high level of selectivity for the NH2-terminal threonine residue of the proteasome active sites, most potently the CT-L active sites of the constitutive proteasome (β5

subunit) and immunoproteasome (LMP7 subunit) (Arastu-Kapur et al., 2011; Jain et al., 2011). In several phase 2 clinical trials, single-agent carfilzomib has demonstrated tolerability and significant anti-tumor activity (O'Connor et al., 2009; Martin et al., 2010; Siegel et al., 2012; Vij et al., 2012).

β-lactones

Marizomib (NPI-0052)

NPI-0052 has been evaluated in a number of phase I trials in patients with advanced hematologic and solid malignancies. The initial data from dose-escalating studies of once weekly intravenous administration had shown rapid, broad and potent dose-dependent proteasome inhibition, with a favorable safety profile and some efficacy (Hofmeister et al., 2009; Spencer et al., 2004). The common adverse events include mild-to-moderate fatigue, with no significant PN, neutropenia or thrombocytopenia (Richardson et al., 2009).

The results from a phase I trial of once weekly NPI-0052 in combination with the histone deacetylase inhibitor vorinostat in solid tumor patients showed marked synergistic effect in a number of cell lines *in vitro*. Moreover, the administration in patients appeared to be safe and tolerable as well, without any drug–drug interaction (Millward et al., 2012).

PROTEASOME INHIBITORS AND HEPATOCELLULAR CARCINOMA

Treatment of human cancers is limited by the systemic toxicity of chemostatic or chemotoxic anti-cancer agents and also by the existence of drug resistance mechanisms. HCC is one of the most common malignancies in the world with an estimated annual incidence of greater than 1 million new cases per year (Schafer and Sorrell, 1999). Although several alternative therapies other than radical operation have been employed such as a transarterial embolization, there is still no satisfactory improvement in the prognosis of HCC to date (Schafer and Sorrell, 1999). One of the reasons for the poor prognosis of HCC is the high rate of recurrence. This high recurrence rate, even after curative therapy, has been shown to be due to intrahepatic metastasis or multicentric development of each respective neoplasm clone (Ikeda et al., 2000). It has been shown that the existence of liver fibrotic changes promotes hepatocarcinogenesis (Sakaida et al., 1998).

Emanuele et al. (2002) showed that MG132 reduced the viability of HepG2 cells in a time- and dose-dependent manner. The effect was in tight connection with the induction of apoptosis, and was accompanied by a remarkable increase in the production of H_2O_2 and a reduction in mitochondrial transmembrane potential ($\Delta\psi m$). In addition cell death was prevented by antioxidants such as GSH, N-acetylcysteine or catalase.

Western blot analysis showed that HepG2 cells contain a very low level of Bcl-2 and a much higher level of Bcl-XL, another antiapoptotic factor of the same family. When the cells were exposed to MG132 the level of Bcl-XL diminished, while a new band, corresponding to the expression of the proapoptotic protein Bcl-XS was detected (Emanuele et al., 2002). MG132 also caused the release of cytochrome c from mitochondria and the activation of caspase-3 with the consequent degradation of poly-ADP ribose polymerase (PARP). Cervello et al. (2004) supported the therapeutic potential of the proteasome inhibitors in HCC. He confirmed the induction of apoptosis by the proteasome inhibitor MG132 in human HCC cells by caspase-dependent cleavage of beta-catenin and inhibition of beta-catenin-mediated transactivation.

TRAIL exhibits potent anti-tumor activity on systemic administration in mice. Because of its proven *in vivo* efficacy, TRAIL may serve as a novel anti-neoplastic drug. However, approximately half of the tumor cell lines tested so far is TRAIL resistant, and potential toxic side effects of certain recombinant forms of TRAIL on human hepatocytes have been described.

Previous study have demonstrated that inhibition of proteasome function effectively sensitizes cells to TRAIL by regulating several factors, normally reduced in neoplastic cells through enhanced proteasome degradation (Zhang et al., 2007). Ganten et al. (2005) in this issue of hepatology reported that proteasome inhibitors can also sensitize hepatocellular carcinoma cells, but not primary human hepatocytes, to TRAIL-induced apoptosis.

The mechanism of increased TRAIL sensitivity in HCC cells have been investigated by Inoue et al. (2006). He examined surface expression of TRAIL and its receptors in different HCC cell lines. MG132 up-regulated both TRAIL and its receptors (TRAIL-R1 and -R2) in SK-Hep1 and HLE, parallel with down-regulated the expression of X-linked inhibitor of apoptosis protein (XIAP) in SK-Hep1 and HLE, and survivin in all three cell-types. Furthermore, MG132 down regulated phospho-AKT and its down-stream target phospho-BAD, indicating that MG132 activated the mitochondrial apoptosis pathway by inhibiting phosphorylation of AKT and BAD.

Lauricella et al. (2006) elucidated the molecular mechanism of apoptosis induced by Bortezomib in HepG2 cells and ascertain the reasons for the insensitivity to Bortezomib shown by Chang liver cells. Bortezomib induced apoptosis in HepG2 cells by stimulating both the extrinsic and intrinsic apoptotic pathways.

Moreover, Chen et al. (2008) investigated the role of Bortezomib on Akt signaling as a major molecular mechanism in determining Bortezomib-induced apoptosis in HCC cells and showed the suppression of tumor growth with down regulation of P-Akt in Huh-7 tumors but

not in PLC5 tumors.

Combinational therapy for molecular targeted therapy has been a common approach to improve responsiveness in cancer therapy. Cusimano et al. (2010) assessed the effects of celecoxib in combination with MG132 on the growth of two HCC cell lines regarding cell viability, apoptosis and ER stress response, and concluded that combination treatment with celecoxib and MG132 resulted in synergistic antiproliferative and proapoptotic effects against liver cancer cells, providing a rational basis for the clinical use of this combination in the treatment of liver cancer.

Deleted in liver cancer 1 (DLC1), a tumor suppressor gene has been identified in a primary human heaptocellular carcinoma. Luo et al. (2011) showed that, intracellular stability of DLC1 protein is regulated by the 26S proteasome in human hepatocellular carcinoma cell line Hep3B and demonstrated that DLC1 is an unstable protein that is rapidly degraded by the 26S proteasome in human hepatocellular carcinoma Hep3B cells. The protein levels of endogenous DLC1 were significantly higher in HEK293 and Hep3B cells after treatment with the proteasome inhibitor MG132. The protein levels of exogenous DLC1 were also increased by inhibition of the 26S proteasome, suggesting that both endogenous and exogenous DLC1 proteins are degraded by the 26S proteasome.

CONCLUSION

These findings suggested that proteasome inhibitors may have a pivotal role in hepatocellular carcinoma and therefore the increasing evidence of multiple roles for the Ubiquitin-Proteasome System within the pathogenesis of HCC suggests that it may prove to be fertile ground on which to develop novel therapies that will prove effective in the treatment of this most devastating disease.

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