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Inhibitors of the Ubiquitin-Proteasome System and the Cell Death Machinery: How Many Pathways are Activated?

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Abstract: Over the past decade, the promising results of UPSIs (UPS inhibitors) in eliciting apoptosis in various cancer cells, and the approval of the first UPSI (Bortezomib/Velcade/PS-341) for the treatment of multiple myeloma have raised interest in assessing the death program activated upon proteasomal blockage.

Several reports indicate that UPSIs stimulate apoptosis in malignant cells by operating at multiple levels, possibly by inducing different types of cellular stress. Normally cellular stress signals converge on the core elements of the apoptotic machinery to trigger the cellular demise. In addition to eliciting multiple stresses, UPSIs can directly operate on the core elements of the apoptotic machinery to control their abundance. Alterations in the relative levels of anti and pro-apoptotic factors can render cancer cells more prone to die in response to other anti-cancer treatments. Aim of the present review is to discuss those core elements of the apoptotic machinery that are under the control of the UPS.

Keywords: Apoptosis, caspases, IAP, IBM, bortezomib, Bcl-2, mitochondria, death receptors, apoptosome, p53, NF-kB, Noxa, Mcl-1, Bim, Bik, Bid Bcl.xL, ubiquitin, proteasome, deubiquitination.

INTRODUCTION

The control of protein turnover is commonly used to modulate many aspects of the cell's life and represents a fundamental task, which is conserved in all living organisms. A number of genes belonging to various pathways are involved in protein degradation control, in response to environmental signals or to internal cellular requirements [1]. The ubiquitin-proteasome system (UPS) is the major site for the degradation of cellular proteins. Proteins that control basic cellular processes such as transcription, replication and mitochondrial biogenesis are under the regulation of the UPS [2]. Moreover precisely timed degradation of certain substrates by the UPS plays a key role in the scheduling of complex fate decisions, that have strong implications for cancer development, such as cell cycle progression differentiation and apoptosis [3]. Thereby, polypeptide degradation by means of the UPS is a highly regulated process, through which cells integrate extracellular signals into a plethora of cellular responses.

As a direct consequence of the many functions under proteasomal control a consistent number of nuclear and cytosolic proteins are degraded *via* the UPS [4]. Furthermore, UPS-dependent degradation is not limited to nuclear and cytoplasmic proteins but it is extended to misfolded secretory and transmembrane proteins. In the last occurrence, proteins are transported back into the cytosol from the ER before poly-ubiquitination and proteasome mediated degradation, in the process known as ERAD (endoplasmic reticulum associated degradation) [5]. Not surprisingly, the discovery of the ubiquitin-proteasome system was awarded the Nobel Prize in Chemistry in 2004.

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THE UPS (UBQUITIN-PROTEASOME SYSTEM)

To fulfill the protein-degradation process two branches, operating at different levels, principally comprise the UPS. The first branch is formed by the enzymatic activities responsible for delivering the substrate to the degradative machinery: the targeting branch. The second branch is represented by the proteolytic machinery, which ultimately fragments the protein substrate into small oligopeptides. Oligopeptides are further digested to single amino acids by cytosolic proteases [2, 6].

The small 8 kDa peptide ubiquitin together with three different families of enzymes, the E1, E2 and E3, comprise the targeting branch. The final goal of the E enzymes is to create an isopeptide bond between specific lysines of the target protein and ubiquitin (Ub) [2, 7]. The conjugation occurs between the C-terminal carboxyl group of mature Ub and the ε-amino group of a lysine residue in the target protein. Ubiquitin is ligated to proteins by the sequential action of the E1 ubiquitin-activating enzyme, the E2 Ub-carrier enzyme and finally the E3 Ub-protein ligase (Fig. **1**) [3]. Hundreds of distinct E3 enzymes exist, which show specificity in substrate choice. The primary role played by dysfunctional E3 enzymes in cancer development is well documented [8, 9].

It is important to remember that conjugation of ubiquitin to a specific protein is not sufficient to determine its degradation. In fact, mono-ubiquitination or poly-monoubiquitination and in certain cases also poly-ubiquitination of proteins are post-translational modifications related to various cellular functions including DNA repair or membrane trafficking [10]. To deliver polypeptides for proteasomal degradation poly-ubiquitin chains of more than 4 ubiquitins must be assembled through lysine-48 linkages [11].

The proteolytic branch is constituted a 2.4-MDa complex, which can be further subdivided into a 20S core particle and two 19S regulatory particles. The 20S core particle is

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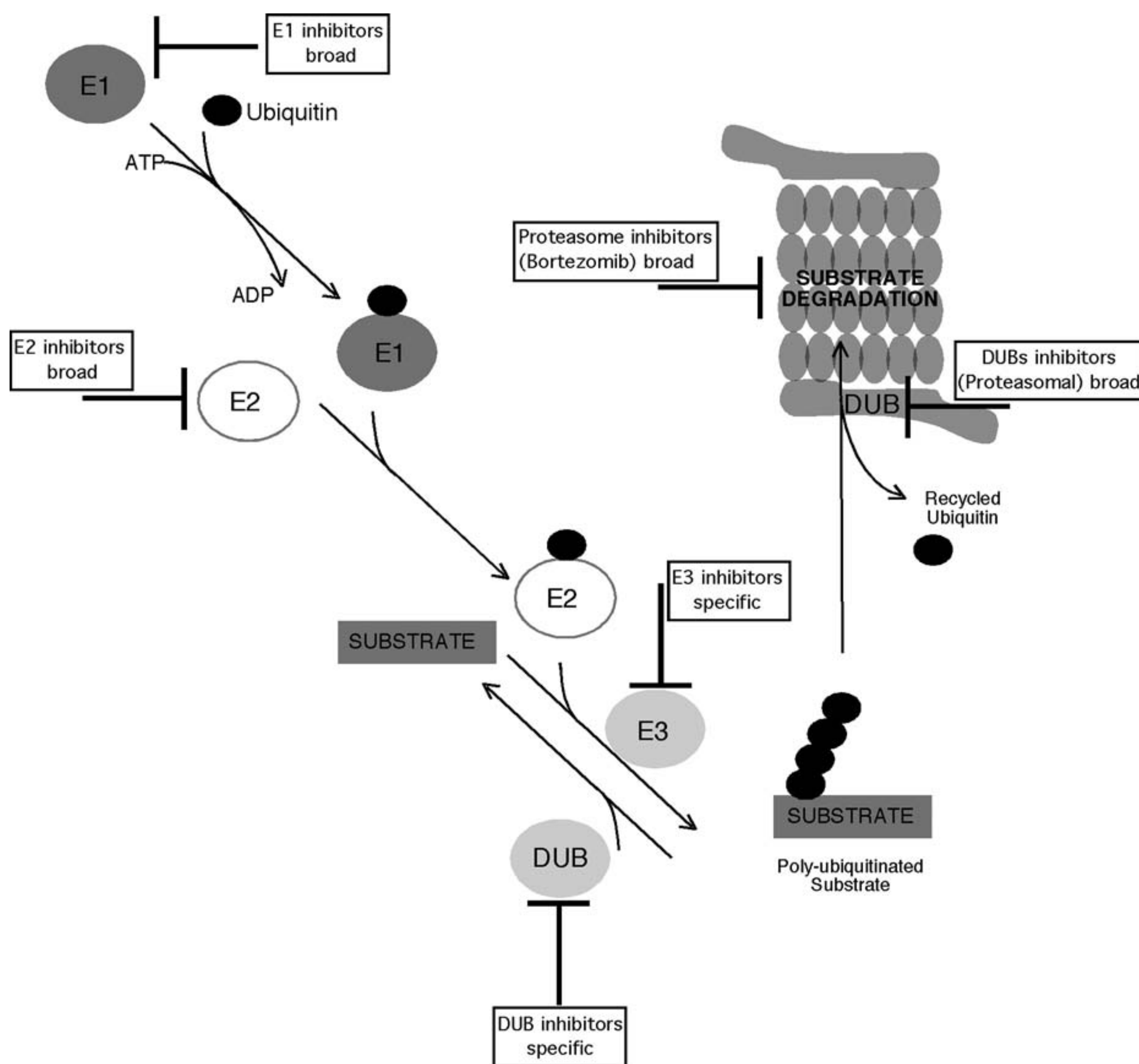

Fig. (1). Schematic representation of the UPS including the deubiquitinating enzymes (DUBs). Some of the possible pharmacological targets are indicated.

a barrel-like structure that contains the proteolytic chamber. Two identical heptameric outer α rings and two identical heptameric inner β rings form the proteolytic chamber [1, 2, 7]. The catalytic sites are embedded in the inner β rings. There are 3 catalytic sites for each β ring. These sites show specific requirements in terms of substrate specificities and catalytic activities, and they are identified as trypsin-like, which prefer to cleave after hydrophobic bonds, chymotrypsin-like, which cleave at basic residues and postglutamyl peptide hydrolase-like or caspase-like activities, which cut after acidic amino acid [1, 2, 7]. Each proteasome active site uses the side chain hydroxyl group of an NH_2-terminal threonine as the catalytic nucleophile, a mechanism that distinguishes the proteasome from other cellular proteases [12]. The presence of alternative catalytic sites guarantees the efficient processing of several different substrates. After substrate proteolysis small size peptides ranging from 3 to 22 residues are generated, whereas intact ubiquitins are released by the deubiquitinating activity of the 19S particles (Fig. **1**), and recycled for further rounds of degradation [6].

A third important branch of the UPS has recently emerged in the form of deubiquitinating enzymes (DUBs). More than 100 DUBs are present in the human genome, and they control different aspects and processes involving ubiquitin [13]. DUBs could operate as a proofreading mechanism to impede the erroneous degradation of poly-ubiquitinated proteins. In contrast, as above described, proteasomal-associated DUBs are responsible for removing bulky ubiquitin chains from substrate proteins prior to proteolysis, thus enhancing the rate of degradation. They also contribute to the reversion of non-proteasomal linked ubiquitin modifications of proteins, for example during the control of DNA repair or membrane trafficking. Finally, DUBs are also responsible for the maturation of ubiquitin precursors [13, 14].

UPS INHIBITORS

By UPS inhibitors (UPSI) we mean small molecules that share the ability to target and inhibit specific activities of the UPS, causing the accumulation of poly-ubiquitinated pro-

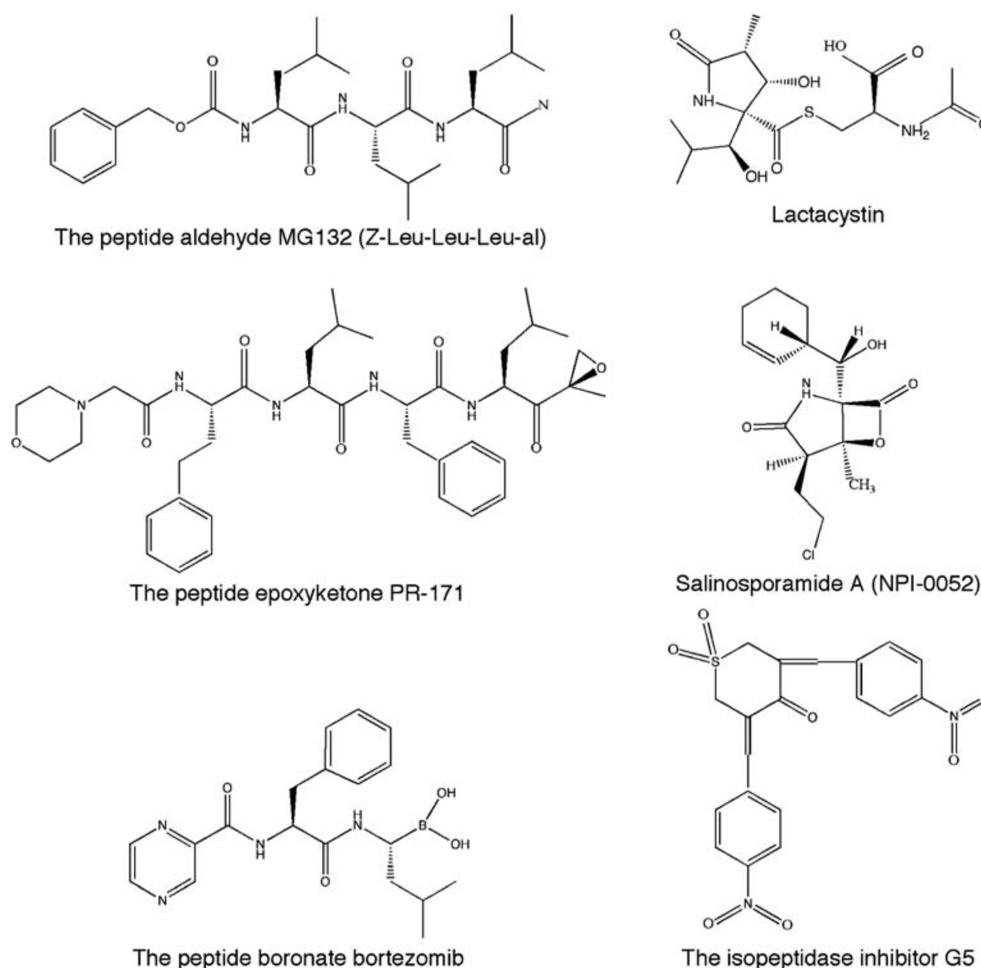

Fig. (2). Structures of the principal proteasome inhibitors and of the ubiquitin isopeptidase inhibitor G5.

teosomal substrates. UPSIs are heterogeneous compounds and among them bortezomib is the only one used in clinical practice, at the moment (Fig. **2**). The most common UPSIs can be broadly subdivided into 5 different classes: the peptide aldehydes (reversible), peptide boronates (reversible, or with slow on and off rates), the peptide vinil sulfones, the lactacystins, and the peptide epoxyketones, all irreversible inhibitors [12, 15]. The clinical utility of many of the UPSI is limited because of the broad specificity, poor metabolic stability, and irreversible binding to the proteasome [16].

Many efforts are focused on discovering new UPSIs with the hope to provide clinicians with additional drugs for anticancer therapies. Recently, these efforts have succeeded and two new UPSIs have entered clinical trials (http://clinicaltrials.gov).

Salinosporamide A (also called NPI-0052), derived from the marine bacterium *Salinispora tropica*, has an unusual bicyclic β-lactone γ-lactam structure (Fig. **2**). It is a potent inhibitor of all three proteolytic activities of the 20S particle, showing an IC50 of 1.3-3.5 nM [17-19]. NP1-0052 induces apoptosis in cancer cells in culture. This new drug is actually in phase I clinical trials to examine its safety, pharmacokinetics and pharmacodynamics using escalating doses in patients with relapsed or relapsed/refractory multiple myeloma.

PR-171, a modified peptide related to the natural product epoxomicin, is composed of two key elements: a peptide portion that selectively binds with high affinity in the substrate binding pocket(s) of the proteasome and an epoxyketone pharmacophore that stereospecifically interacts with the catalytic threonine residue and irreversibly inhibits enzyme activity (Fig. **2**). In comparison to bortezomib, PR-171 exhibits equal potency, but greater selectivity, for the chymotrypsin-like activity of the proteasome. In cell culture PR-171 is more cytotoxic than bortezomib. In mice PR-171 is well tolerated and shows stronger anti-tumor activity when compared with bortezomib [20]. Clinical studies are in progress to test the safety of PR-171 at different dose levels on some hematological cancers.

All the UPSI used up to now or tested in clinical studies inhibit the degradative function of the 20S catalytic particle. It is evident that targeting the proteolytic activities of the proteasome is a rather broad strategy to alter protein turnover. In perspective more specific compounds should be identified that counteract the degradation of specific substrates involved in the suppression of the transformed phenotype. Efforts are ongoing to identify specific inhibitors of E3 ligases that control the degradation of tumor suppressor proteins such as p53. Other interesting targets for drug development are the deubiquitinating enzymes (Fig. **1**). Here again broad inhibitors have demonstrated their efficacy in

preclinical studies by efficiently inducing apoptosis in several cancer cell lines [21, 22]. Further efforts will be needed to ascertain the most appropriate DUBs involved in the promotion/maintenance of the transformed phenotype. Once the critical DUBs are identified, it will be possible to adopt high-throughput screenings to identify more specific inhibitors.

BORTEZOMIB

Clinical validation of the proteasome as a therapeutic target in oncology has been provided by the dipeptide boronic acid bortezomib (also known as PS-341 or Velcade). Bortezomib is a covalent, slowly reversible inhibitor that primarily targets the chymotrypsin-like activity of the proteasome [23]. The FDA (United States Food and Drug Administration) and EMEA (European Medicine Evaluation Agency) granted an approval for the use of this UPSI in the treatment of relapsed multiple myeloma. In fact clinical studies have demonstrated that therapy with bortezomib, alone or in combination with dexamethasone, produces durable responses with survival benefits in patients with recurrent and/or refractory multiple myeloma [24]. Subsequently in a phase III study in patients with multiple myeloma who had a relapse after previous therapies, the UPSI yielded a better rate both for complete and partial responses compared to dexamethasone [16, 25]. Bortezomib has proven efficacious as a single agent, not only in multiple myeloma, but also for the treatment of indolent non-Hodgkin's lymphoma (NHL) and in mantle cell lymphoma [16, 26].

The most common toxicity elicited by bortezomib is thrombocytopenia. Others side effects include fatigue, myalgia, and neuropathies [16, 27]. Interestingly both *in vitro* and *in vivo* observations support the idea that bortezomib treatment can directly stimulate bone formation by multiple pathways including an increase in transcription factor Runx2/Cbfa1 activity and the reduction of dickkopf-1 in human osteoblast progenitors and osteoblasts [28, 29]. It can also counteract the abnormal balance of osteoclast regulators, namely, RANKL (receptor activator of NF-kB ligand) and osteoprotegerin, leading to osteoclast inhibition and decreased bone destruction. Promotion of bone formation is an important aspect of bortezomib activity because bone disease is one of the most debilitating manifestations of multiple myeloma [28, 29].

As demonstrated in preclinical studies bortezomib can synergise with multiple anti-tumor drugs to block proliferation and induce cell death. Treatment with gemcitabine, irinotecan, gefitinib or cetuximab (a monoclonal antibody anti-EGFR) in combination with bortezomib always strengthened anti-proliferative effects [27, 30, 31]. The wide spectrum of synergistic activities suggests that UPSIs elicit peculiar cellular responses, which cannot be replicated by the most common anti-tumor treatments. Bortezomib has also shown activity in preclinical studies of a variety of solid tumours, including breast, gastric, colon, pancreas and non-small lung cancer (NSCLC). These *in vitro* and *in vivo* studies, based on animal models, have prompted several phase I/II clinical studies with bortezomib as a single agent or in combination studies [27]. Phase I studies on prostate cancer and hepatocellular carcinoma have revealed limited and partial effects. Some phase I studies with bortezomib in combination with gemicitabine, irinotecan paclitaxel or docetaxel on advanced solid tumors have been concluded. The overall response rates are quite heterogeneous probably reflecting the different origin of tumors and the different combinatory therapies [27]. The best response rate (47%) was observed for ovarian tumor in combination with carbonplatin [32].

When tested as single agent in phase II studies bortezomib failed to show a significant clinical activity in metastatic malignant melanoma, recurrent or metastatic sarcoma, metastatic neuroendocrine tumors, colorectal cancer and metastatic breast cancer [27]. By contrast in metastatic renal cell carcinoma, a tumor highly resistant to chemotherapy, bortezomib yielded partial responses in 11% of the patients and stable disease in 38% of the patients [33].

Initial phase II studies have been performed to determine the efficacy of bortezomib in combination with irinotecan, gemcitabine or docetaxel. No benefit or modest benefits in certain patients was observed [27, 34, 35]. It is evident that further investigations are warranted to determine and optimize the bortezomib combination for the treatment of solid cancer.

Recently a new possible clinical application for bortezomib has emerged. Myelofibrosis with myeloid metaplasia (MMM) is a chronic myeloproliferative stem-cell disorder characterized by dysplastic megakaryocytic hyperplasia, ineffective erythropoiesis, extramedullary hematopoiesis, bone marrow fibrosis, and osteosclerosis. Dysregulation of the NF-kB pathway in megakaryocytes and circulating CD34 cells characterizes the MMM patients. In a murine model of MMM bortezomib significantly increased survival, reduced myeloproliferation, bone marrow and spleen fibrosis, and bone osteosclerosis [36].

CELL DEATH BY UPSI

In vitro experiments have unambiguously established that incubation of neoplastic cells with UPSIs including bortezomib triggers their death. Cell death can take place by at least 3 different mechanisms, historically divided by morphological criteria into apoptosis, necrosis and autophagy [37]. Although autophagy can serve to protect cells, it may also contribute to cell death especially when it is induced massively and for prolonged periods (type II cell death) [38]. Autophagy is a genetically regulated process and numerous *ATG* (autophagy) genes have been identified, conserved from yeast to humans.

Apoptosis or type I cell death relies on the timed activation of caspases, a group of cysteine proteases, which cleave selected cellular substrates after aspartic residues [39]. Two main apoptotic pathways keep in check caspase activation. The extrinsic pathway is initiated with the engagment of a family of death receptors at the cell surface and controls the activation of caspase-8, whereas the intrinsic pathway is triggered by the release of killer proteins such as cytochrome c and Smac/DIABLO from mitochondria [40, 41]. These killer proteins cooperate to activate caspase-9 and the effector caspases 3 and 7. Caspase-9 activation is driven by the assembly of the apoptosome, which is promoted by the binding of cytochrome c to Apaf-1. In both pathways the activation of the effector caspases, controlled by the regulatory

caspases-8 and 9, is responsible for the cleavage of selected cellular substrates to provoke cellular dismantling.

Type III cell death or necrosis is less defined. Historically necrosis was considered a passive, accidental, and unregulated cellular response; however it is now emerging that under physiological conditions necrosis is a cell-regulated process. PARP activation, mitochondrial dysfunction, ATP depletion, calpain and cathepsin proteolytic activities were observed in cells dying by necrosis and they have been used as markers of the necrotic response [37]. During death receptor-induced necrosis a signaling pathway controlled by RIP1 kinase has been identified [42]. Unfortunately the RIP1 kinase-dependent necrotic pathway is largely obscure.

The ability of UPSIs to induce apoptosis in tumor cells is well established. It has been widely confirmed that the induction of apoptosis is the critical response for the anti-neoplastic efficacy of the UPSIs [19, 22, 23, 39, 43-49]. Nevertheless we cannot exclude the possibility that the type II and III cell deaths are activated in parallel with apoptosis following inhibition of proteasomal activities.

ELEMENTS OF THE APOPTOTIC MACHINERY UNDER PROTEOSOMAL REGULATION

The turnover of a large number of cellular proteins is under the control of the UPS [4, 7]. Thus in principle any proteosomal substrate could contribute directly or indirectly to the cell death phenotype [50]. This is perfectly exemplified by two master regulators of cell life and death, p53 and NF-kB. UPSIs cause NF-kB inhibition through reduced IkB degradation and, in opposition; they promote stabilization and accumulation of p53 [16, 39]. Throughout the review I will mention p53 and NF-kB, when discussing expressional changes driven by UPSIs. However it is important to remember that cell death in response to UPSIs can occur independently from p53 activation and NF-kB inhibition [16, 39].

To limit the discussion and the length of the present review, it is reasonable to postulate that the elements of the apoptotic machinery, the levels of which are under the control of the UPS, are the principal candidates for transducing the apoptotic signals activated by UPSIs.

BCL-2 FAMILY MEMBERS

Bcl-2 family members are the leading guardians of MOM (mitochondrial outer membrane) integrity. They are divided into three different classes consistent with the specific repertoire of Bcl-2 homology domains (BH1-4). Multidomain anti-apoptototic proteins (containing BH1-4 domains) include Bcl-2, Bcl-xL, Bcl-w, Bfl-1/A1 and Mcl-1. Multidomain pro-apoptotic proteins characterized by the presence of three BH domains (BH1-3) include Bax and Bak. Finally Bad, Bik/Blk/Nbk, Hrk/DP5, Bid, Bim/Bod, Noxa, Bmf and PUMA/Bbc3 belong to the group of BH3-only pro-apoptotic proteins [51, 52].

Several models have been proposed to explain how Bcl-2 proteins regulate each other and apoptosis. A discussion of this fascinating item goes beyond the scope of the present review. However it is important to remember that two models can summarize the huge amount of sometimes conflicting experimental data [53-58]. Accordingly, in the first model the BH3-only proteins act on the anti-apoptotic Bcl-2 members to free the pro-apoptotic multidomain Bax and Bak from sequestration. The second model postulates the existence of two different groups of BH3-only proteins. A first group (named activators) can bind and activate the mutidomain Bax and Bak and a second group acts on the anti-apoptotic Bcl-2 to free the activators (BH3-only) from the inhibitory embrace [59].

UPSIs can modulate the levels of pro-apoptotic and anti-apoptotic Bcl-2 family members directly, if the protein is degraded *via* the proteasome, or indirectly, when some regulators of the member (p.e. kinases or transcription factors) are proteasomal substrates. The last condition is exemplified by p53 or NF-κB [39, 60]. The levels of these transcription factors are modulated by UPSIs, and in turn they can change the expression levels of some Bcl-2 family members in response to the USPIs.

BCL-2

Much published data excludes the possibility that Bcl-2 levels are under proteasomal control. Studies in multiple cell lines and with different UPSI treatments have demonstrated that Bcl-2 levels remain constant after inhibition of the proteasome [22, 45, 46, 48, 61, 62]. However, there are also indications that in particular cell types and under the influence of specific signals, Bcl-2 could become a proteasomal target [63, 64]. Bcl-2 phosphorylation could be the molecular switch that controls its degradation. A complex between Bcl-2 and PP2A has been isolated from ER membrane. Genetic or pharmacological inhibition of PP2A promotes the degradation of phosphorylated Bcl-2 [65]. From these studies a model has been proposed in which PP2A-mediated dephosphorylation of Bcl-2 is required to protect Bcl-2 from proteasome-dependent degradation, affecting resistance to ER stress.

In the human non-small cell lung cancer H460 cell line an increase in Bcl-2 phosphorylation, which parallels G2 arrest and apoptosis, was reported after treatment with bortezomib [66]. However it is unknown whether this modification plays a relevant role in the apoptotic response to UPS inhibitors.

BCL-XL

Similarly to Bcl-2, Bcl-xL is not under the direct control of the proteasome. Hence bortezomib does not dramatically alter Bcl-xL protein levels [45, 48]. However since i) UPSIs are potent inhibitors of NF-kB activation and ii) NF-kB is a positive regulator of Bcl-2 and Bcl-xL transcription [39, 60], treatments with UPSIs could down-regulate Bcl-2 and Bcl-xL expression, under particular conditions, such as TNF-α stimulation or in particular cell lines [67].

MCL-1

The anti-apoptotic Bcl-2 family member Myeloid cell leukemia-1 (Mcl-1) is overexpressed in many types of human cancer and is implicated in cell immortalization, malig-

nant transformation, and chemoresistance [51]. Mcl-1 is under the tight regulation of the proteasome [68]. Disappearance of Mcl-1 at the onset of apoptosis induced by DNA damage is achieved by the combination of neosynthesis blockage and continuous degradation controlled by the UPS [69, 70]. Mcl-1 is poly-ubiquitinated by the E3 ligase ARF-BP1/Mule. Interestingly ARF-BP1/Mule contains a BH3 domain that allows specific interaction with Mcl-1 [68, 71].

The modulation of Mcl-1 stability/function during cell death is versatile, depending on the type of BH3-only protein involved in the interaction. In healthy cells Mcl-1 sequesters and prevents the activation of the multidomain pro-apoptotic protein Bak. Normally during apoptosis a Noxa/Mcl-1 complex is recognized for destruction, potentially by Mule or by some other adapter molecules. On the contrary other BH3-only proteins, such as Bim or Puma, bind and stabilize Mcl-1, possibly by precluding the binding to Mule [72].

Multiple environmental signals (DNA damage, growth factors) control Mcl-1 expression [73]. Not surprisingly its turnover may experience several controls. Recent data suggested a novel mechanism responsible for the control of Mcl-1 stability, in response to growth factor deprivation and glucose catabolism [74 -76]. The kinase glycogen synthase kinase 3 (GSK-3ß) can phosphorylate Mcl-1 at three residues. This phosphorylation elicits the subsequent ubiquitination and degradation of Mcl-1 by the E3 ligase ß-TrCP. The inhibitory phosphorylation by GSK3, as mediated by the PI3K-Akt axis or by PKC, can stabilize Mcl-1 and promote cell survival [74, 76].

Mcl-1 destruction or inactivation liberates the pro-apoptotic potential of Bak. Bcl-xL can also sequester Bak [56]. Thus to potently trigger apoptosis in many cell types, it may be necessary to displace Bak from both Mcl-1 and Bcl-xL. Hence strategies aimed to kill tumor cells through the blockade of Mcl-1 function could be in sufficient if Bcl-xL is abundantly co-expressed.

Various investigators have observed the cell-line dependent pro-apoptotic effect of Mcl-1 down-regulation. For example in multiple myeloma the role of Mcl-1 is prominent in respect to other anti-apoptotic family members. Indeed whereas Mcl-1 antisense triggers a rapid induction of apoptosis, a similar antisense strategy targeting Bcl-2 or Bcl-xL is ineffective [77]. In other tumor cell lines down-regulation of Mcl-1 is not sufficient to trigger cell death, but it can synergize with UPSIs to induce apoptosis [78]. This observation is expected since, when tumor cells are forced to die by bortezomib, Mcl-1 stabilization is the undesired effect. Also the killing of melanoma cells incubated with bortezomib is enhanced after targeting Mcl-1 by using siRNA, UV light, or fludarabine [79].

BFL-1/A1

The human Bfl-1 and its mouse homolog A1 are mitochondrially localized anti-apoptotic proteins that suppress cell death in myeloma and lymphoma. Bfl-1 is capable of interacting with Bax in a two hybrid assay [80, 81]. A1 like Mcl-1 is a Noxa target. Interestingly a detailed quantitative study of the interactions among different BH3-only peptides and the anti-apoptotic Bcl-2 members has established that Noxa binds only Mcl-1 and A1 [55].

Bfl-1/A1 undergoes constitutive ubiquitin/proteasome mediated turnover [82]. Our knowledge of the enzymes controlling Bf1-1/A1 is limited and further studies will be necessary to understand its contribution to apoptosis resistance. For example it will be interesting to investigate if Noxa is involved in Bfl-1/A1 proteasomal degradation.

As for the other anti-apoptotic Bcl-2 family members, expression of Bfl-1 is regulated at the transcriptional level by NF-kB [83]. These data imply that UPSIs might exert a limited effect on the accumulation of the Bfl-1 protein, by suppressing NF-kB activity and as a consequence Bfl-1 expression.

BCL-W

Bcl-w is an anti-apoptotic Bcl-2 family member, which is widely expressed in the nervous and hematopoietic systems, colon, testis, heart and pancreas. Mouse development proceeds normally in the absence of Bcl-w with the exception of testicular degeneration. This degeneration renders male mice sterile [84]. Up to now no data have been available on the relationship between Bcl-w and the UPS or on the regulation of its protein turnover. Bcl-w is localized in several subcellular compartments including mitochondria, ER membrane, cytosol and nuclear envelope [85].

BAX AND BAK

The multidomain pro-apoptotic Bcl-2 family members Bax and Bak are fundamental and redundant regulators of the intrinsic/mitochondrial death pathway in response to a plethora of signals [86]. Bax and Bak are ubiquitously expressed and they represent the master regulators of MOM permeability. Since Bax and Bak double knockout cells are resistant to cell death in response to UPSIs, the presence of the multidomain pro-apoptotic members is required for UPSIs induced apoptosis. No overt changes of Bax and Bak protein levels have been observed in different cellular systems in response to UPSIs, thus excluding a direct involvement of the UPS in the regulation of their turnover [45, 47, 48, 61].

BH3-ONLY PROTEINS

Only certain BH3-only proteins are constitutively under the control of the UPS. However, as previously discussed for the anti-apoptotic Bcl-2 members, the levels of some BH3-only proteins can be regulated by UPSIs in an indirect manner. This is exemplified by Puma, a p53–transcriptional target, or by Bad, whose pro-death activity is normally inhibited by an Akt-dependent phosphorylation which UPSIs can inactivate the Akt-dependent pathway [87, 88]. In addition ubiquitin-dependent degradation of some BH3-only proteins seems to occur in a context dependent fashion, as reported for Bim, Puma and Bad, during Chlamydia infection. Chlamydia are obligate intracellular bacteria that need to suppress apoptosis, in order to replicate in a vacuole inside a host cell [89]. However it should be mentioned that this study does not provide evidences on the *in vivo* poly-

ubiquitination of such BH3-only proteins. At the moment the contribution of Hrk/DP5 and Bmf to UPSI-induced cell death is unknown. In contrast, Noxa, Bim, Bik/Blk/Nbk and Bid are certainly the most investigated BH3-only members in the context of the apoptotic response to UPSIs.

NOXA

Noxa was cloned using a differential display technique and it was the first BH3-only protein reported to be a p53 target. The name 'Noxa' is the Greek word for 'damage' [90].

Mouse Noxa encodes for a small protein (103 aa) containing two BH3 domains, whereas the human counterpart is shorter (53aa) and contains only a single BH3. In accordance with the p53-dependent expressional regulation, the Noxa promoter is characterized by the presence of a functional p53 responsive element [91]. As discussed previously the BH3 of Noxa binds selectively Mcl-1 and Blf-1/A1.

In many tumor cell lines the dramatic induction of Noxa expression is the common response to the UPS block [22, 45-48]. siRNA experiments have unveiled the important contribution of Noxa in UPSI-induced cell death. In some cell lines the apoptotic response to UPSI appears to depend entirely on Noxa [61], whereas in others its contribution, although important, is partial [22, 45-48].

The Noxa promoter is under intense regulation and p53-independent activation of Noxa transcription is documented [92, 93]. Not surprisingly Noxa induction in response to UPSIs is controlled at transcriptional levels in p53 dependent and independent manners [45].

Inhibition of the UPS leads to the accumulation of misfolded proteins in the ER, and the subsequent activation of a cellular response known as the Unfolded Protein Response (UPR). The UPR is a prosurvival response to attenuate the accumulation of misfolded proteins and restore normal ER function. However if protein misofolding cannot be prevented the ER stress signaling switches from pro-survival to pro-apoptotic [94].

PEK/PERK (pancreatic eIF2a kinase/RNA-dependent-protein-kinase-like ER kinase), an upstream kinase of the UPR, is activated by bortezomib-induced ER stress [95]. Once activated PERK can phosphorylate eIF2 to attenuate protein synthesis and to regulate the expression of the UPR-dependent genes. The role of this kinase and of the UPR response in the up-regulation of Noxa transcription following bortezomib treatment has been demonstrated by using MEFs that express a mutant form of eIF2 (eIF2A/A), which cannot be phosphorylated by PERK [95]. How the UPR response leads to the activation of Noxa transcription and which are the signaling elements linking ER stress to the Noxa promoter are still open questions. Certainly the detailed characterization of the Noxa promoter during different stress conditions will provide important clues to understanding Noxa induction in response to UPSIs.

BIM

The BH3-only protein Bim is expressed in three isoforms: BimEL, BimL, and BimS. In contrast to Noxa, Bim is a promiscuous BH3-only protein competent in binding many of the anti-apoptotic Bcl-2 members [53, 55].

Bim is subjected to multiple regulators; transcriptional activation, following growth factors deprivation, cytoskeletal sequestration and proteasomal degradation all can contribute to control of Bim activity [91, 96, 97]. The Ras/MAPK pathway phosphorylates Bim on serine 69 and targets it for destruction *via* the proteasome [97-101]. Within this scenario, which should be a hallmark of many tumors, USPIs cause Bim stabilization.

There are also reports indicating that the concept Bim phosphorylation = degradation can not be universal, but rather cell type dependent. In neurons Ser65-phosphorylated BimEL is bound by the peptidyl-prolyl cis/trans isomerase Pin1 and promotes apoptosis [102].

ERK-dependent degradation of Bim could also contribute to the oncogenic potential of Epstein-Barr virus. EBV can be associated with the development of malignant lymphomas including Burkitt's lymphoma, Hodgkin's lymphoma, post-transplant lymphomas, some T cell lymphomas, and nasopharyngeal carcinomas. EBV infection elicits the post-transcriptional inhibition of Bim expression, a process that involves the phosphorylation of Bim by ERKs, followed by its degradation through the UPS [103].

The protein phosphatase 2A is also implicated in the control of Bim turn-over following ER stress. PP2A can dephosphorylate Bim and inhibit its poly-ubiquitination. In this manner Bim stabilization by UPSIs can result from two different actions: a direct effect of the proteosomal blockage and an indirect effect of ER stress induction [104].

Finally UPSIs can modulate Bim expression at the transcriptional level, possibly through the induction of ER stress [95, 104]. In a neuroblastoma cell lines Bim and PUMA transcription were up-regulated in response to epoxomicin. However, subsequent studies in PUMA- and Bim-deficient cells indicated that epoxo-induced caspase activation and apoptosis were predominantly PUMA-dependent [87].

BID

The BH3-only protein Bid is an important caspase substrate. Caspase cleavage promotes the insertion of Bid into the MOM and the subsequent efflux of cytochrome c. Bid processing, when activated by caspase-8, links the death receptor pathways to the amplifying function of the apoptosome [41]. In many cell types apoptosome recruitment is fundamental for an extensive cell death response. Bid is also cleaved by caspase-2, both *in vitro* and *in vivo*, but the pathway through which this cleavage operates is still unclear [105].

UPSIs in B-cell chronic lymphocytic leukemia (B-CLL) do not alter Bid levels prior to the onset of apoptosis [106]. However it has been reported that UPSIs can favor the accumulation of tBid, the caspase-cleaved form of Bid. Over-expressed tBid is poly-ubiquitinated and mutation of the putative ubiquitin acceptor sites within tBid results in a stabilized protein. Finally over-expression of a stabilized tBid enhanced apoptosis induction approximately 2-fold when compared with wild type tBid [107]. *In vivo* tBid stabiliza-

tion by UPSIs could contribute to the well-known synergism observed when tumor cells are co-treated with death receptor ligands and bortezomib or other UPSIs [107].

BIK/BLK/NBK

Marshansky and colleagues provided the first proof of a possible involvement of the UPS in the control of Bik protein levels [96]. The proteasome-mediated turnover of Bik was confirmed by other studies using various cell lines [49, 97, 108]. Hence, Bik up-regulation is a common event occurring upon UPS inhibition. Experiments aimed to ablate Bik expression using siRNA have highlighted the contribution of Bik to UPSI induced cell death [49, 97, 108]. Bik is also important for the sensitization activity of UPSIs towards the death receptor pathway [97]. BIK binds strongly BCL-xL, but also Bcl-2 *via* its BH3 domain and inactivates their anti-apoptotic functions [55, 109]. Hence, in principle, Bik stabilization in response to UPSIs perfectly integrates with Noxa induction, which is more specific for Mcl-1 and Bfl-1/A1 to fully induce Bax and Bak-dependent MOMP, caspase activation and apoptosis.

The Noxa-Bik synergism is supported by additional observations. UPS inhibition elicits clustering of mitochondria into the perinuclear area followed by their early and dramatic fragmentation [22]. Both Noxa and Bik are BH3-only proteins capable of regulating mitochondrial fission. Bik can modulate mitochondrial fission before MOMP through ER-mediated Ca^{++} release and DRP-1 activation [110, 111], and Noxa depletion impairs mitochondrial fragmentation in response to UPSIs [22].

Another interesting consideration about the synergism between Noxa and Bik during UPSI induced cell death concerns their subcellular localization. Noxa is mainly a mitochondrially localized protein, whereas Bik resides in the ER [100, 101, 111]. As discussed previously, UPSIs induced both ER and mitochondrial stress signals; hence these two BH3-only proteins could be the critical actors to transduce these stresses into an apoptotic response.

Even though all these considerations point to Bik as a promising candidate for understanding the apoptotic pathway activated by UPSIs, the mechanisms controlling its targeting to the UPS are largely unknown.

POST-MITOCHONDRIAL EFFECTS OF UPSIS

The UPS plays an important role in the regulation of post-mitochondrial apoptotic events. After the mitochondrial release of the killer proteins citochrome c, Smac/DIABLO and OMI/HtrA2, the apoptosome is assembled and the proteolytic cascade composed of caspase-9, caspase-3 and caspase-7 is suddenly activated. However, the degradative events and cell death can still be inhibited by the action of the IAP (Inhibitor of Apoptosis Proteins) [112, 113]. It is possible that IAPs only temporarly buffer the death process and that in the presence of widespread mitochondrial damage the death process can take different roads, such as type III cell death or necrosis.

The IAPs represent the logical target of UPSIs action at post-mitochondrial levels. In fact some IAPs contain a RING domain exhibiting E3 ligase activity [11]. Auto-ubiquitination of mammalian IAPs has been demonstrated for the best characterized members of this family: XIAP, cIAP1 and cIAP2 [11]. In principle UPSIs by augmenting IAP levels should inhibit caspase activity. However, *in vivo* bortezomib can provoke the down-regulation of XIAP [45, 114, 115]. Again, as discussed above since certain IAPs are under NF-kB control, XIAP down-regulation could be the indirect consequence of NF-kB inhibition. Although we should consider that the down-regulation of XIAP, as activated by UPSIs, could be more complex. XIAP is also a caspase target and NF-kB independent down-regulation of XIAP was observed in the response to bortezomib [116].

The important anti-apoptotic function of the RING domain of DIAP, which is responsible for the ubiquitination of the regulatory caspase DRONC is well documented in *Drosophila* [117, 118]. In fact, besides the RING domain, IAPs are characterized by the presence of three BIR domains (Baculovirus IAP Repeats). The BIR domains mediate the interaction with active-processed caspase. In particular in mammals, BIR3 and BIR2 together with a linker region, bind respectively the post-mitochondrial caspase-9 and caspase-3/-7 and repress their activities [119-121]. Certainly direct inhibition of caspases through the BIRs is a simple and efficient method to suppress apoptosis. On the other hand the presence of the RING domain has supported a model in which the antagonistic activity of IAPs is driven initially by the direct inhibition of the caspase catalytic function through the BIR domain, followed by their targeting to the proteasome *via* the RING finger. In *Drosophila* this model was confirmed *in vivo*, whereas in mammals, although some *in vitro* experiments have proved that IAPs can mediate the poly-ubiquitination of caspases [43, 122-124], their poly-ubiquitination *in vivo* is still an open question.

IAP anti-apoptotic activities are antagonized by a group of heterogeneous proteins that share a small peptide sequence at the N-terminus: the IBM (IAP Binding Motif) [11]. Through the IBM the IAP antagonists are able to interact with the same BIR domains required for caspase binding/inhibition. Therefore, IBM proteins promote apoptosis by competing for caspase binding, essentially acting as IAP inhibitors [11, 112, 113].

The interaction between IBM containing proteins and IAPs is a battle for survival. However, IBMs in principle could be delivered to the proteasome by the E3-ligase activity of the IAPs. The best characterized IBMs in mammals are the mitochondrial proteins Smac/DIABLO and OMI/HtrA2, which spill into the cytosol together with cytochrome c, after MOMP.

Smac/DIABLO has been intensely investigated as a possible substrate of the IAP E3 activities. Similarly to caspase ubiquitination the picture for Smac/DIABLO is not yet fully defined, and the results of diverse experiments are sometime controversial. Some studies demonstrated that XIAP promotes poly-ubiquitination of Smac *in vitro* and *in vivo* [124-126]; others have shown that cIAP1 and cIAP2 are ubiquitin ligases for Smac/DIABLO [127]. The chimeric E2-E3 ubiquitin ligase Apollon/BRUCE/Birc6, among the different cellular targets, can bind caspase-9 and Smac and trigger their poly-ubiquitination and proteasomal degradation [128-130].

More recently, the IAP Livin, which, in addition to the RING finger contains a single BIR, has been proposed as an E3-ligase for Smac [131].

Regardless of the specific mechanism implicated in the ubiquitin-dependent degradation of Smac, a remarkable contribution to the debate on Smac degradation has been provided by the demonstration that, the levels of cytosolic mature Smac are raised when apoptosis is induced by UPSIs in comparison to other insults [22, 46]. These observations confirm that cytosolic Smac is under the control of the proteasome and that UPSIs, by increasing Smac levels, could sustain caspase activation and apoptosis. It is important to remember that the mitochondrial pathway of caspase activation is based on amplified actions that act as feed-back to rapidly induce cellular demise. For example it is well known that caspases can act back on the mitochondria to demolish its function and promote the release of additional killer factors [132]. UPSIs by altering the level of IAP antagonists and IAPs could favor caspase activation, thus reducing the minimal threshold required for their full-activation, and at the same time, enhancing the caspase-amplifying loop. It is evident that additional studies will be necessary to obtain a clearer picture of the UPS-regulation of IAP antagonists, IAP and caspase stabilities.

THE EXTRINSIC PATHWAY

A large body of evidence proves that UPSIs strongly synergize with the extrinsic/death receptor pathway to augment apoptosis [133-136]. The synergism finds molecular justification in the different elements of the death receptor pathway that are modulated, directly or indirectly, by the proteasome [39, 50]. Furthermore some preclinical data indicate that combination therapy of death ligand TRAIL and bortezomib may be an effective strategy for various types of tumors [137, 138]. Recently, a cautionary note has been raised about the synergism between the UPSIs and the death receptors. Depending on the intracellular levels of XIAP, c-FLIPs, and intrinsic proteosomal activity, UPSIs can temporarily slow death receptor induced cell death [139]. Never-

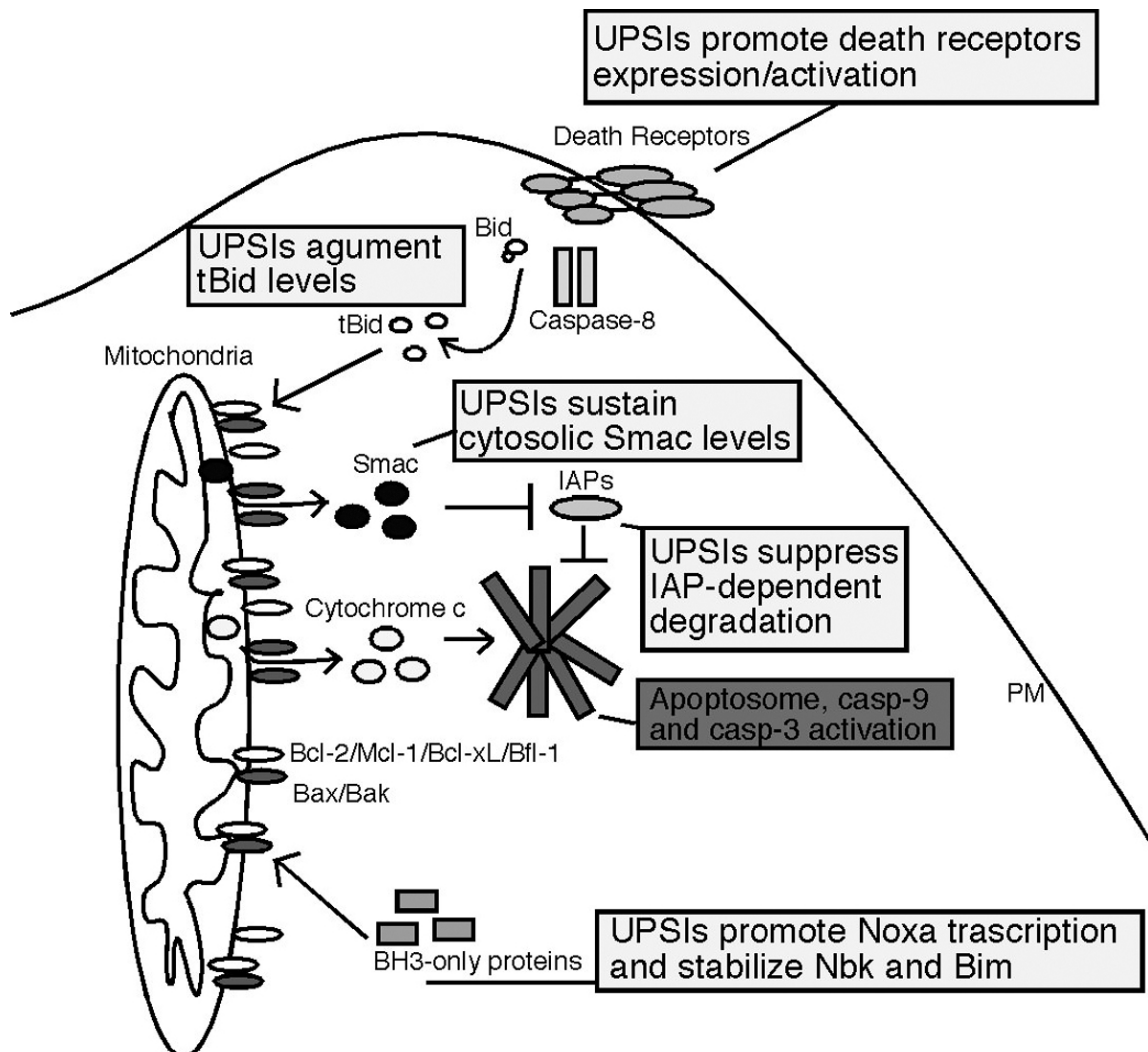

Fig. (3). Summary of the principal effects of the UPSIs on core elements of the death machinery. The scheme illustrates components of the extrinsic and intrinsic apoptotic pathways, modulation, of which by UPSIs, contributes to cell death. UPSIs can influence IAP activities indirectly, through the inhibition of NF-kB. (Caspase-cleaved Bid).

theless at later times, cell death in the presence of UPSIs was increased compared to death ligand alone.

Treatment of various cancer cell lines with UPSIs results in the up-regulation of DR4/TRAIL-R1, DR5/TRAIL-R2, and DcR2/TRAIL-R4 [137, 140]. In particular DR5 is regulated by UPSIs at multiple levels [141] including ubiquitination and increased transcription [142, 143].

UPSIs do not simply promote the accumulation of some elements of the extrinsic pathways but they also globally engage, in a still mysterious way, the pathway [22, 137, 144]. UPSI-dependent apoptosis, in certain cell lines or when the mitochondrial pathway of caspase activation is not fully functional, depends entirely on the death receptor pathway. Under these conditions, by interfering with the activation of the death receptor pathway, cell survival in the presence of UPSIs can be fully maintained [22, 137, 144].

c-FLIP is the most important element of the extrinsic pathway under the direct control of the UPS [145, 146]. Two different FLIP isoforms exist: c-FLIP$_L$ (Long) and c-FLIP$_S$ (Short). c-FLIP$_L$ is highly homologus to caspase-8 and contains two tandem repeat Death Effector Domains (DED) and a catalytically inactive caspase-like domain. c-FLIP$_S$ likewise present the DED domains at the N-terminus, but it is shorter than in c-FLIPL and the C-terminal part contains a specific motif. In a large number of cell death models c-FLIP is persistently an anti-apoptotic factor that competes for caspase-8 activation, whereas c-FLIP$_L$ can assume anti-apoptotic functions or can stimulate caspase-8 activation, depending on the relative concentration [147]. Both FLIPs can be degraded by the UPS; however they display distinct half-lives and the unique C terminus of c-FLIP$_S$ possesses a destabilizing function [148].

The regulation of c-FLIP levels in response to UPSIs is rather controversial. Some reports indicate that UPSIs can reduce c-FLIP levels and in this manner synergize with TRAIL to promote apoptosis [149]. Other studies indicate that UPSIs do not alter c-FLIP levels [135, 136, 144] or even increase levels of c-FLIP [137, 139]. The reported discrepancies could reflect variations in the basal levels and in the constitutive turnover of FLIP in the investigated cancer cell lines.

Similarly to the stabilization of Mcl-1 in the mitochondrial pathway c-FLIPs stabilization is the unwanted response to the UPSI treatment. Strategies aimed to contain such stabilization should potentiate the pro-death activity of the UPSI [78, 137].

CONCLUSIONS

UPSIs activate multiple cellular responses and different stress signals that ultimately cause cell death (Fig. **3**). For this reason they represent broad inducers of apoptosis. In addition, since many of the available UPSIs alter the proteolytic activity of the proteasome, they represent non-specific modulators of the expression/activity of various components of the apoptotic machinery. Paradoxically they can simultaneously favor the accumulation of pro- and anti-apoptotic factors. The detailed definition of molecular mechanisms controlling turnover of key pro- and anti-apoptotic proteins will be instrumental in the design of more specific inhibitors. For instance these new inhibitors could target the relative E3-ligase [9] or the relative DUB in order to obtain a more selective modulation of the specific elements and pathways leading to cancer cell death.

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