

CASPASE CONTROL: PROTAGONISTS OF CANCER CELL APOPTOSIS

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Emergence of castration-resistant metastatic prostate cancer is due to activation of survival pathways, including apoptosis suppression and anoikis resistance, and increased neovascularization. Thus targeting of apoptotic players is of critical significance in prostate cancer therapy since loss of apoptosis and resistance to anoikis are critical in aberrant malignant growth, metastasis and conferring therapeutic failure. The majority of therapeutic agents act through intrinsic mitochondrial, extrinsic death receptor pathways or endoplasmic reticulum stress pathways to induce apoptosis. Current therapeutic strategies target restoring regulatory molecules that govern the pro-survival pathways such as PTEN which regulates AKT activity. Other strategies focus on reactivating the apoptotic pathways either by down-regulating anti-apoptotic players such as BCL-2 or by up-regulating pro-apoptotic protein families, most notably, the caspases. Caspases are a family of cysteine proteases which serve critical roles in apoptotic and inflammatory signaling pathways. During tumorigenesis, significant loss or inactivation of lead members in the caspase family leads to impairing apoptosis induction, causing a dramatic imbalance in the growth dynamics, ultimately resulting in aberrant growth of human cancers. Recent exploitation of apoptosis pathways towards re-instating apoptosis induction via caspase re-activation has provided new molecular platforms for the development of therapeutic strategies effective against advanced prostate cancer as well as other solid tumors. This review will discuss the current cellular landscape featuring the caspase family in tumor cells and their activation via pharmacologic intervention towards optimized anti-cancer therapeutic modalities. This article is part of a Special Issue entitled "Apoptosis: Four Decades Later".

Key Words: apoptosis, caspase-8, proteasome inhibitors.

THE CHALLENGE IN CANCER TREATMENT

In the year 2012, the challenge for clinicians, oncologists and basic scientists remains the development of effective therapeutic strategies that block malignant cell growth, without impairing normal healthy cells. Investigative efforts focus on successful exploitation of cancer specific characteristics acquired during malignant transformation via a series of genetic and epigenetic mutations resulting in uncontrolled growth and evasion of apoptosis mechanisms [1]. Losing critical regulatory mechanisms that control normal tissue homeostasis enables tumor cells to acquire new characteristics such as tissue invasion, metastasis and angiogenesis. Some of the control pathways are activated such as cell proliferation, cell cycle progression and pro-survival pathways, while others are down-regulated, like the cell death pathways including apoptosis and anoikis.

Aberrant cell proliferation during cancer initiation and progression to metastasis is controlled by cell cycle progression. The cell cycle consists of several phases; G₀, G₁, S, G₂, and Mitosis regulated by various cyclins and CDK (cyclin dependant kinases) and

progression from one phase to another is dependent on specific checkpoints [2]. A very critical player at this checkpoint is p53 (notoriously known as the guardian of the genome) due to its role in rescuing damaged DNA, via up-regulation of downstream effectors, such as p21 (induces cell cycle arrest) and PUMA which blocks anti-apoptotic players leading to apoptosis induction [3]. Cell cycle regulation via p53 can primarily be abrogated by loss-of-function mutations, losing p53 activity allows for the cell to replicate regardless of DNA integrity and increases apoptosis resistance [4]. Additional mechanisms of p53 down-regulation involve the over-expression of MDM2, an E3 ligase that mediates the polyubiquitination of p53 resulting in its degradation. Over-expression of MDM2 ensures rapid degradation of p53 leading to diminished if not abolished p53 activity and unregulated cell cycle progression [5]. Proteasome inhibitors, agents that block protein degradation mediated by 26S proteasome, have been shown to stabilize p53 and restore p53 mediated apoptosis [6]. Established chemotherapeutic agents such as mitomycin C or doxorubicin are used to induce cell cycle arrest in a variety of epithelial cancers. These agents work to either cross link DNA (mitomycin C) or bind directly to the DNA intercalating into the double-helix strands causing the DNA to become rigid and break (doxorubicin) [7]. The major limitation however is that these drugs damage surrounding healthy normal cells, tissues, and organs such as kidney (mitomycin C) or the heart (doxorubicin) [8, 9]. Treatment of MCF-7 breast cancer xenografts with a combination of mitomycin C with

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Abbreviations used: APC – allophycocyanin; ATCC – American type culture collection; CRPC – castration-recurrent prostate cancer; DISC – death inducing signaling complex; FADD – Fas associated death domain; GST – glutathione S-transferase; HDAC7 – histone deacetylase 7; IP – immunoprecipitation; MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NB7 – neuroblastoma 7; PI – propidium iodide; TNF- α – tumor necrosis factor- α ; TRAIL – TNF-alpha related apoptosis inducing ligand.

curcumin significantly decreased mitomycin C related side effects [10].

Strategies involved with overcoming the toxic side effects of doxorubicin by changing the mode of doxorubicin delivery by encapsulating the drug in titanium nanoparticles which showed promising results [11]. Circumventing the caveats associated with systemic toxicities of these chemotherapeutics involved examining other pro-survival pathways, such as the AKT signaling pathway which can also impact cell cycle regulation and growth arrest. The AKT pathway is activated through binding of growth factors to their cognate tyrosine kinase receptors which then carry out the signal transduction through the interplay between SRC, phosphatidylinositol-3 kinase (PI3K) and phosphatidylinositol-4, 5-bisphosphate (PIP₂), and phosphatidylinositol-3, 4, 5-triphosphate (PIP₃) and a critical regulatory molecule, PTEN [12]. PTEN, a phosphatase, regulates AKT activation because it dephosphorylates and converts PIP₃ to PIP₂ thus preventing PIP₃–AKT interaction [13]. AKT is a kinase that phosphorylates several different targets such as mTOR, IKK (an inhibitory binding protein that prevents the nuclear factor of kappa B (NF-κB) activation), and cell cycle inhibitors (p21, p27). In cancer activation of the AKT pathway can become aberrant because of a variety of mutations that can occur within *PI3K*, *AKT*, and *PTEN* [14]. One of the most detrimental and oncogenic potential promoting mutations are those that render these molecules constitutively active. Constitutively active PI3K can lead to the increase in the conversion rate of PIP₂ to PIP₃ favoring PIP₃ production leading to increased AKT activation [15]. A specific *AKT* mutation, E17K in either *AKT1* or *AKT3* leads to constitutive activation and promotes increased trafficking to the plasma membrane [16]. *PI3K* and *AKT* activating mutations are deleterious for the cell; however, another mechanism that can elevate AKT activity to supra-physiological levels and contribute to oncogenesis is the loss of PTEN, a critical regulator of AKT activation. Interrogation of the signaling events dictated by AKT, mTOR, and PI3K has led to the development of a powerful class of pharmacologic inhibitors. The most promising class of AKT inhibitors are the lipid based inhibitors which essentially inhibit AKT binding to the plasma membrane. Perifosine has emerged as one of the most promising AKT inhibitors and has been through several phase II clinical trials [17–19]. The mTOR inhibitors include rapamycin and its derivatives, such as CCI-779, blocks mTOR function through similar mechanisms that primarily involve binding to the co-factor FKBP and together, rapamycin and FKBP bind to mTOR and inhibit activity [20]. PI3K inhibitors like wortmannin or its derivative, LY294002 bind covalently to PI3K to inhibit its kinase activity [21]. These agents however, lack specificity and new carefully designed inhibitors such as CAL-101 (Calistoga) have shown promising results in clinical trials [22] which are on-going at Clearview Cancer institute (2012). PI3K inhibitors exhibit higher efficacy

combination with existing chemotherapeutic agents such as, an AKT or mTOR inhibitors because these combinations block two pathways, eliminating individual pathway activity as well as preventing activation of alternate or redundant non-AKT mediated pathways activated through PI3K [23]. PTEN is the direct inhibitor of AKT because it converts AKT activating PIP₃ into PIP₂ which does not activate AKT. PTEN studies have revealed that PTEN may be down-regulated either through inactivating mutations, gene deletions, and phosphorylation of PTEN by CK2 have the same result, persistent AKT signaling that contributes to tumor formation [24]. PTEN loss has been associated with several cancers at the advanced stage of disease, including prostate cancer [25]. *PTEN* mutations have been linked with aggressive androgen dependant or androgen independent (termed castration recurrent) prostate cancer [26, 27] and evidence suggests that oncogenesis results due to the loss of AKT and cell cycle regulation [20, 28].

Prostate cancer is one of the most prevalent causes of cancer related death in males with several risk factors, such as age, race, and diet contributing towards prostate cancer development and progression [29]. Regulation of androgen signaling via the androgen receptor (AR) is critical to maintaining prostate homeostasis. The androgen axis involves conversion of testosterone into 5α-dihydrotestosterone by 5α-reductase, the active metabolite that binds to AR and the ligand-receptor complex is translocated to the nucleus to activate subsequent signaling pathways [30]. When prostate cells undergo tumorigenesis they take on different molecular characteristics, one of the more prominent changes is the up-regulation of androgen receptor either through gene amplification or through other processes leading to AR overexpression [31]. Such an event prominently activates AR pathways leading to increased proliferation and reduced apoptosis, or may further sensitize prostate cancer cells to growth factors stimuli such as EGFR [32]. Currently the most promising therapy for treating castration-recurrent prostate cancer (CRPC), involves chemically depleting androgens in the prostate by inhibitors of androgen axis such as abiraterone [33]. Additional agents are currently being tested in clinical trials such as MDV3100 which is a competitive inhibitor blocking AR–androgen signaling with therapeutic promise in prostate cancer patients [34].

Another class of chemotherapeutics are proteasome inhibitors capable of inducing apoptosis and thus with great potential as anti-cancer agents [35]. There are two types of proteasome inhibitors, natural inhibitors (lactacystin and epoxomicin) and synthetic inhibitors such as MG132 and velcade [36]. MG132 inhibits the chymotrypsin like activity of the 26S proteasome [37]. Velcade, (PS-341/bortezomib) is an FDA approved proteasome inhibitor used in treating multiple myeloma [38]. Velcade is a dipeptide boronic acid small molecule that blocks the chymotrypsin-like activity of the 20S particle [39]. Investigators have

reported that velcade has an impact on several key cellular processes such as inhibiting cell cycle and NF- κ B activation [40]. Velcade sensitizes cancer cells to apoptosis through several mechanisms, such as the down-regulation of c-FLIP, which inhibits caspase-8 activation at the DISC [41]. Several *in vitro* and *in vivo* studies have shown that velcade induces apoptosis in multiple myeloma cells [42]. However multiple myeloma patients are either initially resistant or acquired resistance to velcade during the course of treatment [43]. Attempts to overcome velcade resistance have led to development of various combinations of velcade with different chemotherapeutic agents such as, PCI-24781 (an HDAC inhibitor) which was found to synergize with velcade to induce reactive oxygen species damage as well as caspase-8 activation in non-Hodgkins lymphoma [44]. Significantly enough, Mitsiades and colleagues [45], showed that velcade in combination with doxorubicin can overcome velcade resistance in multiple myeloma. Another anti-cancer strategy involved using velcade in combination with TNF- α related apoptosis inducing ligand (TRAIL). Recent studies by Christian *et al.* suggests the ability of velcade to sensitize TRAIL-resistant prostate cancer cell lines *in vitro* and *in vivo* to TRAIL-mediated apoptosis and together TRAIL and velcade stabilize caspase-8 p18 subunit [46, 47].

A major caveat for using velcade as an anti-cancer strategy is the lack of cell type and cell signaling specificity. Velcade was designed to block the proteasome and not to discriminate between a malignant or a healthy cell therefore, all cells are impacted by velcade treatment. Velcade treatment can lead to side effects such as thrombocytopenia and peripheral neuropathy [48]. To bypass the caveats associated with velcade, while achieving apoptosis induction investigators are pursuing the activity of E3 ligases in an attempt to achieve and their involvement with the extrinsic pathway of apoptosis.

For CRPC, taxanes provide the only clinically effective chemotherapeutic approach. These agents target microtubules and the cellular cytoskeleton, thus stabilizing microtubules and preventing microtubule reorganization, towards disruption of kinetochore formation during mitosis [49]. Proposed mechanisms conferring taxane resistance involve either microtubule mutations that prevent drug binding or the cell itself pumps out the taxane through P-glycoprotein pumps [50]. Taxanes have been used against other solid tumors such as breast, lung, ovarian, and esophageal cancers [51, 52]. Alternative approaches involve inducing or restoring the apoptotic pathways through a variety of other agents such as staurosporin, etoposide, and a new emerging class of apoptosis inducing agents, the death ligands such as TRAIL.

MECHANISMS OF APOPTOSIS REGULATION

Apoptosis (programmed cell death) plays a critical role in regulating cell growth and tissue development.

Since loss of apoptosis leads to tumor initiation, growth, and progression [53], exploitation of apoptosis mechanisms can lead to developing new anticancer strategies, that can effectively impair the tumorigenic process. Each pathway of apoptosis is activated by different triggers such as cell-detachment (anoikis) mitochondrial signals (intrinsic pathway), or death ligands (extrinsic pathway) (Figs. 1 and 2).

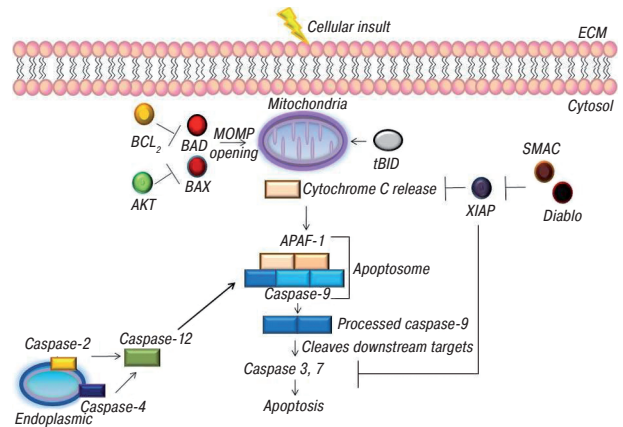


Fig. 1. Intrinsic and ER pathways of apoptosis. The intrinsic pathway of apoptosis activated by various stimuli, leads to a down-regulation of anti-apoptotic BCL-2 family members, allowing pro-apoptotic members to perturb the mitochondria. Cytochrome c release from the mitochondria leads to APAF-1 and pro-caspase-9 recruitment forming the apoptosome; caspase-9 is then activated, cleaving downstream targets, such as executioner caspase-3 and -7. The ER stress pathway induces the activation of caspase-2 or -4, leading to caspase-12 activation, which then activates the intrinsic pathway of apoptosis

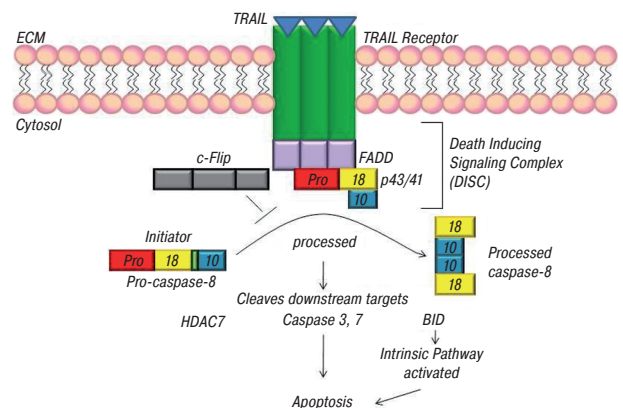


Fig. 2. TRAIL-mediated extrinsic pathway of apoptosis. Mediated through the binding of TRAIL to its cognate receptor, upon binding the receptors oligomerize within the membrane. Fas associated death domain (FADD) is recruited, followed by pro-caspase-8 which is then processed into its active p18 and p10 subunits which then can oligomerize into a heterotetramer

A mechanism designed to protect against cellular metastasis is anoikis, which is an apoptosis program that is induced upon the loss of critical protein interactions between the cell and the extracellular matrix. The major players and pathways involved with anoikis are integrin, focal adhesion, and growth factors (IGF-1) interactions as well as the JNK pathway, and caspase activation signaling events [54]. Anoikis plays a role in all tissue development and regeneration and in preventing epithelial cell detachment and migration in normal and tumor cells [55], including

shedding of colon epithelial cells [56] and mammary gland reduction [57, 58]. Anoikis is initiated when adherent cells detach from the basement membrane, more specifically, the loss of integrin (either α_5 or β_3) signaling with the focal adhesion points [59]. Apoptotic pathways are activated upon cell detachment and loss of integrin signaling in normal cells; cancer cells develop resistance to anoikis through diverse mechanisms such as over-coming the loss of focal adhesion kinase (FAK), overexpression of talin (integrin partner/focal adhesion player), acquiring mutations in FAK that trigger anoikis inhibitory mechanisms or navigating stimuli from the microenvironment signaling loss of apoptotic pathways [60].

The intrinsic pathway of apoptosis is under heavy regulation by several different types of molecules that can be separated into two main classes, anti-apoptotic proteins such as the XIAP (inhibitors of apoptosis), BCL-2 family proteins such as BCL-2, BCL- x_L or the pro-apoptotic proteins, which include BCL-2 family members; BAX, BAD, BID, SMAC, and Diablo are activated through signaling events that lead to mitochondrial outer membrane permeabilization (MOMP). Cytochrome *c* is released, binds with APAF-1 and caspase-9 to form the apoptosome [61]. Upon apoptosome formation, caspase-9 becomes catalytically active and acts on downstream targets including caspase-3 and -7 (Fig. 1) [62]. Tumor cells can inactivate apoptotic signaling programs by engaging anti-apoptotic mechanisms involving the up-regulation of apoptotic suppressors (Bcl-2, Bcl- x_L) and/or through the down-regulation of critical apoptosis inducing players such as the caspase family (caspase-2, -4, -6, -8, -9, -10, -12) [63]. Mechanisms that inhibit the intrinsic pathway of apoptosis are interconnected with activities of the AKT (Fig. 2) and NF- κ B pathways. Therefore, activated AKT pathway inhibits the intrinsic pathway of apoptosis as AKT signaling promotes BCL-2 and BCL- x_L activity while inhibiting BAX and BAD players involved with inducing apoptosis [64]. Blocking BAX and BAD activity can prevent MOMP from opening, thus preventing cytochrome *c* release, and consequentially inhibiting apoptosome formation. Another family of anti-apoptotic proteins that can inhibit both the intrinsic and extrinsic pathways is the inhibitors of apoptosis (IAP) which have two arms, the cIAP or X-linked IAP (XIAP). The IAP family consists of E3 ligases that can block apoptosome formation through binding directly to APAF-1 or caspase-9, thus inhibiting caspase-9 activation [65]. XIAP also bind directly to caspase-3 preventing its activation, and in-addition to blocking activation XIAP can facilitate the transfer of ubiquitin, thereby tagging the caspases for degradation by the 26S proteasome [66]. There are also mutations acquired in the pro-apoptotic machinery itself, the most notable mutations being those occurring in the caspase family. To that end, Srinivula *et al.*, identified caspase-9 β , a caspase-9 mutant that lacks the large active subunit and established that caspase-9 β acts in a dominant negative fashion pre-

venting caspase-3 activation [67]. Moreover, Park and colleagues identified several gene polymorphisms that give rise to altered forms of caspase-9 that impair caspase-9 activity and thereby block apoptosis induction [68]. Post-translational modification of caspase-9 phosphorylation at Thr 129 mediated as a result of CDK-1 and cyclin B in cell cycle [69] also prevents caspase-9 recruitment to the apoptosome blocking caspase-9 activation. Regardless of how caspase-9 is modified, if this caspase fails to become active then the subsequently executioner caspase-3/-7 activation is inhibited, ultimately impairing the intrinsic pathway of apoptosis activation [70, 71].

The extrinsic pathway (also referred to as the death receptor pathway) involves the induction of apoptosis through the activation of death receptors via death ligands such as tumor necrosis factor- α (TNF- α), FASL, and TRAIL [72]. While FASL and TRAIL strictly activate the extrinsic pathway mediated apoptosis, TNF- α can play two different roles, although this molecule is capable of inducing apoptosis, TNF- α is also capable of activating pro-survival pathway. TNF-activation impacts several critical cellular pathways some of which include cellular proliferation, differentiation, and apoptosis [73]. Specifically, TNF- α binding to its cognate receptor can lead to the formation of two separate complexes, complex 1 which can lead to the induction of either the NF- κ B pathway (pro-survival) [74] or complex 2 which activates the apoptotic pathway mediated primarily through Fas associated death domain (FADD) and caspase-8 activation [75]. Complex 1 mediated NF- κ B induction is initiated through the binding of TNF- α to its cognate receptor TNFR-1 which then leads to the recruitment of two adaptor proteins TNF receptor-associated protein with a death domain (TRADD) and receptor-interacting protein 1 (RIP1) [76]. Upon binding of TRADD another adaptor molecule, TNF associated factor-2 (TRAF), followed by the recruitment of cIAP (cellular inhibitors of apoptosis), as well as Ubc6 and Ubc13 (E2 ubiquitin conjugating enzymes) [77] to form complex 1. Once complex 1 is formed, TRAF2 is phosphorylated by PKC resulting in K63 link polyubiquitination [78] that leads to proteasomal degradation [79]. TRAF2, cIAP, and Ubc13 function in concert to facilitate K63 linked polyubiquitination of RIP1 [80]. K63 linked polyubiquitination of RIP-1 leads to activation of Tak1/TAB complex to activate the IKK complex [81]. The IKK complex consists of several components, IKK α , IKK β , and IKK γ , this kinase complex phosphorylates the inhibitor of kappa B molecule (I κ B- α) [82]. I κ B α , is a bound inhibitor of NF- κ B that functions to prevent nuclear import of NF- κ B into the nucleus. Nuclear translocation of NF- κ B results in binding to its respective DNA binding sites and gene activation [83]. NF- κ B up-regulates several different gene types, such as inflammatory response pro-survival genes, BCL-2 family, caspase-8 inhibitor c-FLIP, cIAP and angiogenesis players and proliferation genes, cyclin D1 and MYC [84]. Interestingly enough, proteasome

inhibition can impede the NF- κ B pathway as it prevents proteasome degradation of I κ B- α thus leading to decreased activation of NF- κ B [85].

Apoptosis induction through complex 2 of the TNF- α pathway proceeds via depletion of c-FLIP and/or c-IAP expression, as well RIP1 kinase phosphorylation [86, 87]. Once phosphorylated, RIP1, FADD and initiator caspase-8 are recruited thus assembling complex 2. Once complex 2 is formed, caspase-8 is processed and can then cleave its downstream targets, caspase-3 and -7, towards apoptosis execution [88]. Recent evidence identified the ripoptosome, a 2mD apoptosis signaling complex composed of RIP-1, FADD, and caspase-8m as a key player in apoptosis activation [89]. This complex assembles when cIAP expression levels are depleted, either through up-regulation of SMAC, a direct inhibitor of cIAP, or through SMAC mimetics or other chemotherapeutics such as etoposide, which is a topoisomerase II inhibitor used to treat solid tumors. In addition to inducing DNA breaks, etoposide can down-regulate cIAPs [89–92]. The ability to form this apoptosis inducing complex, can serve as a powerful tool for developing anti-cancer strategies because an agent (or combination of agents) could induce apoptosis, while bypassing the normal requirements for apoptosis induction. Interestingly the ripoptosome triggers necroptosis (programmed necrosis) mediated through RIP3 signaling [121].

The extrinsic pathway of apoptosis is abrogated through several mechanisms, including the up-regulation of the inhibitors of apoptosis proteins such as cIAP or XIAP. Up-regulation of these inhibitors of apoptosis molecules will drive the TNF- α pathway towards NF- κ B activation in the same manner as described above. Apart from inhibition by the IAP family, recent data indicate that IL-6/STAT3 signaling can override apoptotic signals by activating pro-survival proteins (BCL-2, BCL-x_L) as well as cyclin D [93]. TRAIL- and FAS-mediated apoptosis pathways are very similar to one another in that these trimeric ligands bind their specific cognate receptors towards apoptosis induction. TRAIL binds to the DR4/DR5 receptors which leads to receptor oligomerization in the plasma membrane, some groups suggest that these receptors oligomerize in the lipid rafts of the plasma membrane [94]. Once the receptors oligomerize there is recruitment of adaptor protein FADD. FADD binding to the TRAIL receptor leads to initiator caspase-8 recruitment to form the death inducing signaling complex (DISC). Following DISC formation procaspase-8 becomes autocatalytically active, once active caspase-8 is processed into the active p18 and p10 subunits via two cleavage events. Once processed the p18 and p10 dimers can oligomerize with other p18/p10 dimers to form active heterotetramers, that cleaves specific targets such as HDAC7 [95], and executioner caspase-3 and -7 which fully induce the apoptotic response [96]. Tumor cells utilize various mechanisms to inactivate the extrinsic pathway of apoptosis; that be down-

regulation of death receptors or up-regulation of decoy receptors [97]. For TRAIL, its cognate receptors consist of death receptor-4 or -5 (DR-4, -5), as a protective measure, the cell also expresses decoy receptors Dcr1 and Dcr2 as an effort to prevent unintended apoptosis induction through TRAIL binding to the death receptors [98]. In addition to receptor or decoy mediated inhibition, the extrinsic pathway is inhibited by over-expression of BCL-2, BCL-x_L, cIAP and XIAP anti-apoptotic proteins [99, 100]. Studies in mouse models demonstrated that TRAIL targets cancer cells and not healthy non-neoplastic cells [101]. This specificity renders TRAIL a valuable chemotherapeutic agent due to the limited side effects and TRAIL protein can be synthesized via standard protein purification methods [46]. TRAIL C-terminal conjugation can extend TRAIL half-life by 11 hours allowing it to be used for *in vitro* and *in vivo* experiments. For clinical trial and treatment applications companies, like Human Genome Sciences (Rockville MD, USA) have generated TRAIL receptor activating antibodies with the intent to extend TRAIL half-life. These companies were successful in generating TRAIL receptor antibodies as evidenced by several *in vitro* and *in vivo* studies that analyzed TRAIL antibodies, mapatumumab and lexatumumab indicating their ability to induce apoptosis [102, 103]. Additional pre-clinical studies have combined TRAIL with existing chemotherapeutic agents, including phytoingosine (impacts sphingolipid metabolism) [104], doxorubicin [105], docetaxel [106] and paclitaxel [107] all of which with much promise, suggesting that TRAIL should be investigated further as a chemotherapeutic strategy. There is a clinical trial in progress involving the combination of TRAIL and VEGF inhibitor, bevacizumab (Clinical trial identifier, NCT00508625).

CASPASES IN CONTROL OF APOPTOSIS

Caspases are a family of cysteine proteases which contain cysteine residue at their active site and cleave their substrate at position next to aspartate residue. A very unique family of enzymes which remain active right from early stages of embryonic development till the death of organism. The entire group of mammalian caspases is divided into three different groups on the basis of their prodomains and specific function they play in the in several different pathways, including, inflammatory, development, and apoptotic pathways. Although each caspase serves a different purpose there are several similarities in cleavage, the proform is cleaved into a large catalytically active subunit and a small subunit as shown for the critical apoptotic caspase-8. Caspase-1 and -5 play a role in inflammation [108, 109]. The endoplasmic reticulum (ER) stress response pathways, unfolded protein response (UPR), or ER associated degradation (ERAD) are mediated through caspase-4, eventually leading apoptosis induction through the intrinsic pathway. ER is a critical cellular organelle whose primary function is to ensure proteins are properly

folded before export into the Golgi apparatus [110]. The protein folding machinery consists of several components that work in concert to ensure proper protein folding. However, when the ER is overwhelmed with polypeptides that are incapable of being folded correctly, three sensors, IRE-1, PERK, and ATF6 trigger the UPR [111]. As high ER stress persists, CHOP expression is markedly increased, down-regulating the pro-survival BCL-2 family members, BCL-2 and BCL-x_L allowing for BAX and BAD expression and activity to increase [112]. Alternative intrinsic pathway induction mechanisms involve the activation of caspase-2, -4, and -12 [113], contributing to caspase-9 processing either through disrupting the mitochondria [114] or through APAF-1 independent mechanisms [115]. Caspase-2 induces the intrinsic pathway of apoptosis either through Bid cleavage thus leading to intrinsic pathway activation via the opening of the MOMP releasing cytochrome *c* facilitating apoptosome formation or through caspase-2, PIDD, and adaptor protein RAIDD bind and form PIDDosome [116]. Compared to healthy cells, tumor cells have a marked increase in protein synthesis as well as an over-production of misfolded or unfolded protein in the ER, resulting in extremely high ER stress levels. Therefore treatment strategies focus on apoptosis induction via elevating ER stress using chemotherapeutics such as velcade [117]. Velcade is designed to block the 26S proteasome and once inhibited, UPR response mechanisms become impaired leading to apoptosis induction [118]. To circumvent proteasome inhibition, cells activate lysosomal pathways as an alternate mechanism for protein clearance. Exploitation of this mechanism as an anti-cancer strategy involves the use of velcade in combination with lysosomal inhibitor, tubacin (an HDAC6 inhibitor that block aggresome formation), that results apoptosis [119]. The major players involved with the intrinsic pathway of apoptosis induction, are cytochrome *c*, APAF-1, and caspase-9 which form the apoptosome. Caspase-9 is subsequently processed into its active p37 and p17 subunits [120] and capable of cleaving executioner caspase-3/-7. Cancer circumvents the intrinsic pathway activation by engaging various mechanisms, such as loss of caspase-9 activation via the involvement of the BCL-2 family members, BCL-2, BCL-x_L or XIAP binding, or by blocking apoptosome formation through pro-survival signals, preventing the opening of the mitochondria and blocking the release of cytochrome *c* [121]. Of direct clinical significance is evidence that tumors from patients with colorectal, lung, or gastric cancer, harbor different point mutations in caspase-9, that render it inactive and incapable of inducing apoptosis [122].

The mechanistic landscape of caspase activation during the tumor cellular demise, takes intriguing functional turns during cancer initiation and progression (Fig. 3). Executioner caspase-3 and -7 (effector caspases) are processed into active subunits and responsible for the execution of the apoptosis program through the cleavage of caspase-activated DNase

which then translocates to the nucleus and cleaves DNA [123]. Caspase-3 propagates and amplifies the apoptosis signal through a loop that leads to caspase-9 cleavage thus further propagating the apoptotic cascade [124]. Loss of caspase-3 expression promotes tumorigenesis [125], while caspase-7 is down regulated in cancer [126]. Caspase-10 is an initiator caspase recruited to the DISC like caspase-8 and is capable of inducing apoptosis in certain caspase-8 deficient tumor cells [127] however, caspase-10 is not sufficient to induce apoptosis in the absence of caspase-8 in other cancer types [128].

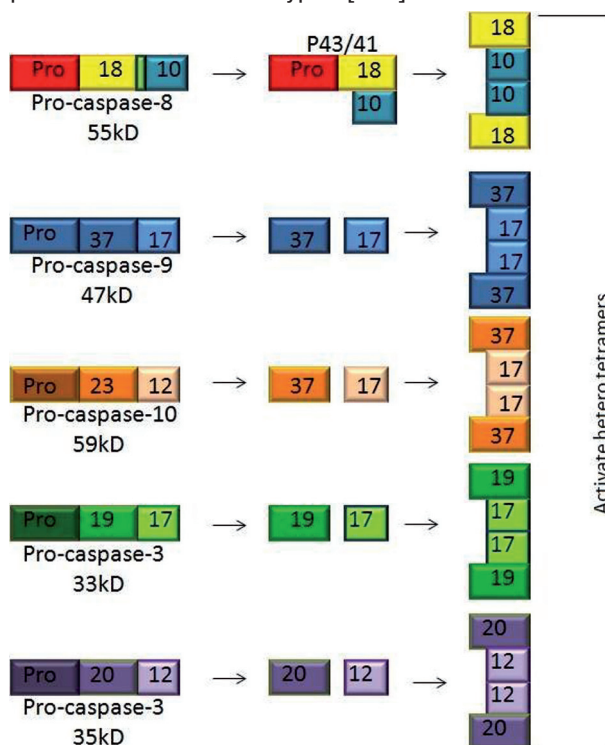


Fig. 3. General cleavage events for critical apoptotic related caspases. Caspase-8 and -10 are larger than other caspases because they contain DED domains responsible for binding to the DISC complex

CASPASE-8 IN DEATH RECEPTOR INDEPENDENT AND DEPENDENT PATHWAYS

Intriguing new evidence supports a role for caspase-8 in non-apoptotic signaling pathways. Stupack *et al.* (2009) reported that caspase-8 in neuroblastoma cell lines plays a role in mediating focal adhesion complex formation and cellular migration [129]. Earlier studies defined a pathway, similar to anoikis phenomenon, termed intergrin mediated death [130]. Caspase-8 is phosphorylated on tyrosine residue 380 via SRC kinase and is associated with FAK and CNB2 and upon its recruitment activates the calpain family of proteases that cleave talin [131]. Significantly enough, the N-terminal cleavage product of talin, the FERM, is an integrin binding domain which facilitates cell migration [129], indirectly implicating caspase-8 in mediating metastasis via the focal adhesion complex [129]. Moreover, Rab5, a critical modulator of caspase-8 action in cell migration [132], is function-

ally involved in migration, either through lamellipodia formation [133], $\beta 1$ integrin binding, or through actin cytoskeleton [134].

Several lines of evidence support the involvement of caspase-8 in EGF signaling pathways inducing ERK activation through the incorporation of caspase-8 in SRC containing complexes. This work identified through a RXDLL motif found within the DED of caspase-8 pro-domain, this allows caspase-8 to associate with SRC although the data did not show any evidence that caspase-8 phosphorylation was required for SRC association and EGF pathway activation [135]. Caspase-8 as a critical player for extrinsic pathway activation has long been considered a tumor suppressor molecule. Indeed caspase-8 deficient cells are insensitive to death ligand stimulus and cannot induce apoptosis through the extrinsic pathway, thus facilitating tumorigenic transformation and conferring therapeutic resistance [136]. Human cancer cells regulate caspase-8 activity through a variety of mechanisms, one mechanism is caspase-8 partial or whole gene deletion, [137] or gene methylation. For example, medulloblastoma pediatric neuroblastoma tumors down-regulate caspase-8 expression through methylation of the caspase-8 promoter thereby inhibiting caspase-8 transcription thus preventing protein translation and expression [138]. Studies involving a screen across multiple cancer types identified frame shift and missense mutations in caspase-8 [139], which altered amino acid compositions in the DED domain, a domain absolutely critical for caspase-8 recruitment to the DISC and initiating cleavage events [139]. Moreover, mutations were found in the p18 catalytically active subunit and the p10 regions validation of the screen results revealed that most of the mutants severely diminished apoptosis induction in gastric carcinomas [139]. Upon recruitment to the DISC caspase-8 undergoes two cleavage events, the first cleavage event occurs at aspartic acid residue 384 in the p10 subunit, giving rise to the p43/41 intermediate which is bound at the DISC. This cleavage event is followed by a second cleavage at aspartic acid residues 210, 216 which then release caspase-8 from the DISC into the cytosol (Fig. 2). Pioneering work by Dr. Marcus Peter defined how the DISC components were assembled at the plasma membrane through TRAIL and/or FAS receptor and FADD palmitoylation [140, 141]. Further studies provided evidence towards DISC mediated caspase-8 processing [142, 143]. Additional studies focusing on DISC formation by Marcus Peter's lab identified that c-FLIP was a specific inhibitor of caspase-8 DISC recruitment and activation [144]. Subsequent work identified c-FLIP isoforms that block gene induction as well as processing of caspase-8 [145]. Besides c-FLIP, XIAP and cIAP are also capable of blocking caspase-8 activation [146]. Emerging evidence by two independent groups, Jin *et al.* [147], and Peng *et al.* [148], provided new insights regarding caspase-8 polyubiquitination. Jin and colleagues provided evidence indicating that E3 ligase CUL3 me-

diated polyubiquitination led to caspase-8 incorporation into an aggresome. Caspase-8 polyubiquitination however in the context of EGR signaling, is mediated through RSK6 activity [148], engaging two E3 ligases, Siah2 and POSH, in prostate cancer cells [149], thus exerting a regulatory role on caspase-8 activity downstream of DISC and caspase-8 processing [149]. The combination of proteasome inhibition with TRAIL takes an all-lethal impact, as it induces apoptosis in TRAIL-resistant prostate cancer cells *in vitro* and *in vivo* [47]. Moreover, combination of TRAIL and velcade leads to caspase-8 p18 subunit stabilization [46, 47, 150], implicating caspase-8 degradation being controlled by the 26S proteasome.

In summary, strategies to circumvent therapeutic resistance by restoration of apoptotic pathways, utilizing single apoptosis inducing agents such as TRAIL, separately or in combination with other chemotherapeutics, provide new promise in the clinical management of cancer patients. These apoptosis inducing agents may also be capable of inducing apoptosis, regardless of the tumor hormonal milieu and driven by the cellular interactions with the tumor microenvironment. In that regard combination of proteasome inhibitor and TRAIL is capable cleaving and activating caspase-8 in either androgen-dependent, or androgen independent prostate cancer (CRPC). The clinical knowledge of microtubulin-targeted chemotherapy (taxanes) as the only effective treatment for CRPC, calls for the need to understand the mechanisms of action of this drug in order to augment its therapeutic efficacy and overcome the therapeutic resistance to its antitumor actions in a large number of prostate cancer patients. Profiling the caspase activation status in response to taxane-based chemotherapy (in combination with apoptosis-driven agents) and in the context of cytoskeleton organization, provides exciting new platforms for therapeutic optimization driving apoptosis to its full execution in a subset of tumors and ultimately impacting patient survival.

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