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Procaspase-3 Overexpression in Cancer: A Paradoxical Observation with Therapeutic Potential

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Abstract

Many anticancer strategies rely on the promotion of apoptosis in cancer cells as a means to shrink tumors. Crucial for apoptotic function are executioner caspases, most notably caspase-3, that proteolyze a variety of proteins, inducing cell death. Paradoxically, overexpression of procaspase-3 (PC-3), the low-activity zymogen precursor to caspase-3, has been reported in a variety of cancer types. Until recently, this counterintuitive overexpression of a pro-apoptotic protein in cancer has been puzzling. Recent studies suggest subapoptotic caspase-3 activity may promote oncogenic transformation, a possible explanation for the enigmatic overexpression of PC-3. Herein, the overexpression of PC-3 in cancer and its mechanistic basis is reviewed; collectively, the data suggest the potential for exploitation of PC-3 overexpression with PC-3 activators as a targeted anticancer strategy.

Graphical Abstract

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Author Contributions

M.W.B. and P.J.H. wrote the manuscript with input from J.P. M.W.B. and J.P. conducted literature reviews of the concepts outlined with input from P.J.H.

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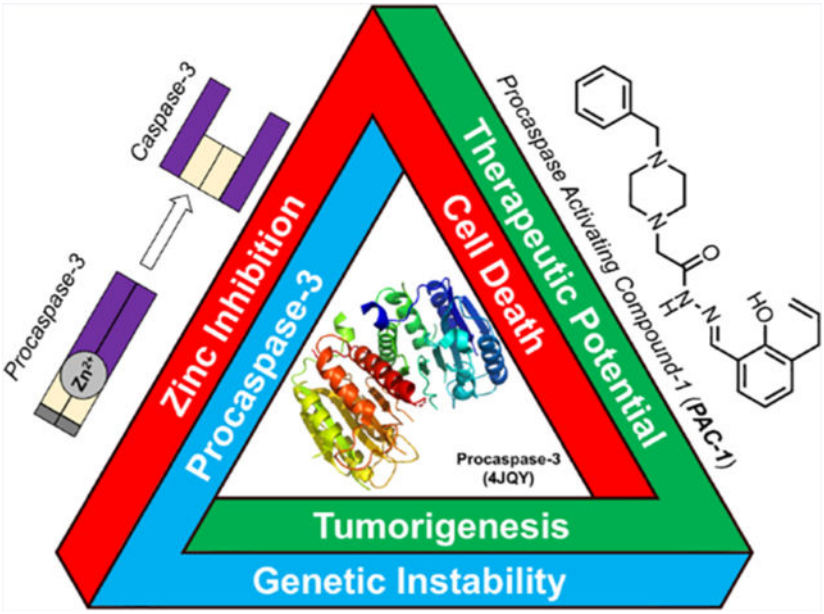

Keywords

Proteolysis: the enzyme-catalyzed hydrolysis of peptide bonds found in a protein; **Zymogen:** refers to a precursor protein that upon an activating stimulus (e.g., proteolytic cleavage, liberation of inhibitory entities) forms a more active enzyme; often synonymous with the term proenzyme; **Apoptosis:** a process of programmed-cell death that culminates in the activation of executioner caspases (caspase-3 and −7) and the cleavage of essential proteins; **Procaspase-3 (PC-3):** the zymogen form of caspase-3 that has minor proteolytic activity and can autoactivate to form caspase-3; **Caspase-3:** cysteine protease that cleaves a variety of cellular substrates, leading to phenotypes associated with apoptosis; **Labile zinc:** the amount of “free” zinc ions within a given system; these ions are in equilibrium with their binding partners and not sequestered in proteins; **Genomic instability:** a cellular state where the machinery to repair damaged DNA has been compromised (e.g., BRCA mutations) and/or sustains high levels of DNA damage (e.g., double-strand DNA breaks); this landscape is considered a hallmark of cancer as it promotes mutation, a major driver of cancer; **Overexpression:** an abnormally high level of an entity in a cell, in the context of this review is through the comparison of noncancerous and cancerous tissue; **PAC-1:** acronym for the first procaspase-activating compound; a small molecule that binds labile zinc, promoting autoactivation of PC-3 to caspase-3 and apoptosis of cancer cells

CANCER, APOPTOSIS, AND PROCASPASE-3

Apoptosis is a central pathway used in organismal development and maintenance of homeostasis, with a crucial role in eliminating genetically unstable or aberrantly growing cells. First postulated in 1972[1,2] and later evidenced by recognition of the Bcl-2 oncogene,[3,4] apoptosis represents a major barrier for the development and progression of cancer. This inhibitory relationship has led to the canonical view that cancers must evade apoptotic induction to root themselves as a developing tumor, with evasion of apoptosis classified as a major hallmark of cancer.[5,6] Cancer cells employ a variety of strategies to evade apoptosis, as has been extensively reviewed.[5,6] Classically, these strategies follow the dogma that

cancers overexpress antiapoptotic proteins or have mutated/downregulated pro-apoptotic proteins, consistent with the notion that apoptosis is tumor suppressive.

Induction of apoptosis results from a variety of intrinsic or extrinsic signals. Halting intrinsic apoptosis can be the result of multiple mechanisms including (l) shunting pro-apoptotic signals (e.g., p53 loss-of-function mutations),[7,8] (2) increased expression of antiapoptotic proteins (e.g., Bcl-2 overexpression),[9] or (3) decreased expression of pro-apoptotic proteins (e.g., APAF-1).[10] Extrinsic apoptosis is often prevented via perturbation of death receptors (e.g., decoy receptors)[11,12] or employment of the altered expression patterns described for intrinsic apoptotic evasion. Most importantly for this review, these alterations of apoptosis almost always lie upstream of the proteolytic cleavage of executioner caspases, namely the activation of zymogen procasapase-3 (PC-3) to active caspase-3 (Figure 1).[13,14] Of note, loss-of-function mutations of PC-3/caspase-3 are rarely observed in tumors.[13,15–17]

Interestingly, recent studies suggest there are also oncogenic roles for pro-apoptotic machinery,[18–24] including PC-3. In this review, we analyze the multidisciplinary work surrounding the study of PC-3 expression and its role in oncogenesis, the biochemistry and cellular biology of PC-3 regulation, and therapeutic development seeking to utilize PC-3 overexpression as a target for selective anticancer therapy. Specifically, the evidence for overexpression of PC-3 in multiple cancers is summarized, and the landscape of PC-3 gene and enzymatic regulation is detailed. These data provide an emerging explanation for PC-3 overexpression in cancer, and this common aberration in cancer suggests a broadly leverageable therapeutic target.

Procaspase-3 Activation to Caspase-3.

PC-3, the precursor to caspase-3, consists of a prodomain, a large subunit, and a small subunit (Figure 2A). PC-3 activation to caspase-3 results from proteolysis at Asp9, Asp28, and Asp 175.[26–28] Caspase-3 is a cysteine protease that cleaves over 200 proteins and ultimately leads to apoptotic cell death.[29–31] The conversion of PC-3 to caspase-3 is a crucial node of apoptosis and is often considered as a “point of no return” for a cell. While PC-3 is generally regarded as the inactive zymogen form of caspase-3, multiple groups have demonstrated that PC-3 does have proteolytic activity, albeit at least 200-fold less active than caspase-3[26,28,32] (although experimental care must be taken to ensure observed activity is not due to small amounts of contaminating caspase-3[33]). This is perhaps best evidenced by experiments in which proteolysis was observed with a noncleavable mutant of PC-3, which is unable to form caspase-3.[28] While in canonical apoptosis PC-3 is cleaved to caspase-3 via activity of caspase-8/−9, PC-3 activation can also be the result of an autocatalytic mechanism in which PC-3 or caspase-3 cleaves another equivalent of PC-3 (Figure 2B).[28,33] This autocatalysis enables minimal activity of PC-3/caspase-3 to propagate, having profound effects in cells (Figure 2C). As such, analyses that implicate caspase-3 proteolysis are complicated by intrinsic activity of PC-3, and small perturbations in basal PC-3/ caspase-3 levels and activity can lead to significant outcomes as demonstrated by engineered overexpression of PC-3/caspase-3. experiments[34–36].

Procaspase-3 Overexpression in Cancer.

PC-3 overexpression in cancer has been reported in a variety of contexts, summarized in Table 1. There are a few caveats to this compiled data set. First, in studies on this topic it is not always reported if the antibodies used are specific for caspase-3, PC-3, or both. We have excluded references that are vague in their antibody descriptions, such as reports that solely measure active caspase-3 levels in tumors. Another note is defining the term overexpression. "Overexpression" in Table 1 is noted

when a report describes abnormally high expression of PC-3 as compared to matched normal tissue (the ideal case) or increased expression when comparing clinical stages of cancerous tissues. "Underexpression" is defined as the opposite case. Finally, there are numerous cancer types that do not appear in Table 1. This is simply because there are no published data about those missing cancers, and as such absence from Table 1 does not imply any information on the PC-3 expression.

Table 1 summarizes a growing body of work suggesting that the overexpression of PC-3 is common across a wide range of cancer types. There are still conflicting reports within some cancers, likely due to insufficient data. For example, in colorectal cancers, Yeatman and co-workers highlighted the correlation of PC-3 expression with the mutational status of APC, a critical tumor suppressor that is mutated in 80% of colorectal cancers.[68,95] However, other reports demonstrate robust overexpression of PC-3 in colorectal cancer patient samples with no mention of APC mutational status.[32,64–67] Such inconsistencies notwithstanding, the totality of the studies in Table 1 demonstrate strong evidence for the near-ubiquitous overexpression of PC-3 in cancers. These data further demonstrate the continued need for robust tumor samples along with matched normal tissue to empower the understanding of cancer s proteomic landscape.

Transcriptional Regulation of Procaspase-3.

CASP3 (the gene encoding PC-3) is one of the target genes for the E2F family of transcription factors.[96] In the absence of growth signals, E2F forms a complex with the retinoblastoma (Rb) family of proteins, specifically pRb,[97] which silences its transcriptional activity (Figure 3A). When growth signals are present, CDK4/6 kinases are not inhibited by $p16^{INK4a}$ (encoded by *CDKN2A*) and form a complex with cyclin D to phosphorylate pRb. Phosphorylated-pRb dissociates from the pRb–E2F complex, liberating E2F to turn-on transcriptional activity (Figure 3B). Interestingly, the pRb/E2F signaling nexus is often dysregulated in many cancers (for example, through overexpression of CDK4/6 and cyclin D), leading to unfettered transcriptional activity of E2F (Figure 3C).[98–103] This common occurrence of pRb/E2F pathway dysregulation in multiple cancers, which funnels to the eventual upregulation of *CASP3* transcription, is a possible explanation for the prevalence of PC-3 overexpression across numerous cancers.

Post-Translational Regulation of Procaspase-3 and Caspase-3 with Inhibitory Zinc.

It is important when discussing overexpression of a protein to also consider key post-translational regulation of an enzyme's activity in cells. Specifically for PC-3 and caspase-3, inhibitory zinc plays a vital regulatory role. Labile zinc pools have been studied in a variety

of contexts,[104–108] and the labile zinc concentration in cells is estimated to be in the high picomolar to low nanomolar range, suggesting a tightly regulated system of zinc transport.[109–112] Preventing aberrant apoptosis is closely tied to maintaining labile zinc,[113–115] and there are multiple reports of zinc inhibition of caspase-3[16 – 20] and PC-3[28] in biochemical assays.

A recent detailed report from Hardy and co-workers[121] demonstrates the importance of zinc to modulate the activity of caspases and further establishes the stoichiometry of zinc ion binding, and their experimental values for zinc inhibition of caspase-3 are in agreement with previous experimental methods,[118,120] as are their caspase-3/zinc stoichiometries.[122] These data suggest zinc inhibits caspase-3 with an IC_{50} of 12.5 nM, and caspase-3 binds three zinc ions.[121] Interestingly, caspase-3 binds these zinc ions even in the presence of a covalent caspase inhibitor (zVAD-FMK). This result suggests that one zinc ion inhibits the active site of caspase-3 while leaving the reactive cysteine unperturbed, consistent with a prior investigation.[120] Hardy et al. hypothesize that one zinc ion binds the catalytic histidine (inhibiting proteolysis) while the two other zincs bind in exosites outside the active site of caspase-3 (Figure 4A).[121] While studies of inhibitory zinc often focus on caspase-3, PC-3 proteolytic activity and autoactivation to caspase-3 are also inhibited by zinc;[28] overall, it appears that zinc plays a significant role in regulating both PC-3 and caspase-3 function (Figure 4B).

Modulation of zinc levels can be a powerful means to alter caspase activity in a given cell type. Regardless of the absolute zinc levels in cancer cells,[123–127] the overexpression of PC-3 across many cancers results in a perturbation of the labile zinc/ PC-3 ratio. This ratio is important for controlling the basal caspase-3 activity in cells, as an increase in PC-3 concentration favors PC-3 activation (Figure 5). Further, this ratio in normal cells (low expressers of PC-3) is a differential that provides a basis for targeting cancer cells specifically with PC-3 activators.

Other Modulations of Caspase-3 Activity.

Post-translational modifications (PTMs) that enhance or inhibit caspase activity can be a mechanism for regulation of caspase activity in cells (comprehensively reviewed by Lavrik and co-workers).[128]Specifically for caspase-3, there are multiple examples of PTMs that inhibit the enzymatic function of caspase-3, including S-nitrosylation on the catalytic Cysl63,[129–33] glutathionylation of cysteine residues,[134] and phosphorylation of Seri50 by p38-MAPK.[135,136] The overexpression of PC-3 could counteract inhibitory PTMs found on caspase-3 and promote apoptosis in these diseased cells.

The overexpression of the X-linked inhibitor of apoptosis (XIAP) protein is a direct mechanism to inhibit caspase-3 activity and is reported in a variety of cancers. XIAP is an E3 ligase that mediates the ubiquitination of caspase-3,7,9.[137,138] However, XIAP does not inhibit PC-3 activity, since the LAP recognition motif is only revealed upon PC-3 cleavage to caspase-3.[137,139,140] Therefore, XIAP acts to prevent cytotoxic caspase-3 activity, but this expression does not alter proteolytic events facilitated by PC-3. Perturbing XIAP activity to promote apoptosis has been demonstrated with SMAC mimetics, and there are ongoing explorations of these anticancer agents.[138]

Proteolysis through Caspase-3 and Its Role in Cancer.

While sufficiently high caspase-3 activity leads to apoptotic death, it now appears that PC-3 and caspase-3 activity may have nonapoptotic roles and broader effects on a cell population (Figure 6A).[141] The possibility of PC-3/caspase-3 activity as pro-tumorigenic has significant implications for basic and translational research, and these single cell and tumor-microenvironment mechanisms have been the focus of recent reviews.[18–24] Here, we consider implications of minimal caspase-3-like activities (either from PC-3 or caspase-3), providing a possible advantage for cancer cells that overexpress PC-3.

Sub-Lethal Caspase-3 Activity Leads to Genomic Instability.

The elucidation of cellular substrates for caspase-3 reveals that proteins involved in DNA repair are preferentially cleaved by caspase-3 during apoptosis.[30] These perturbations in DNA repair protein levels may lead to genomic instability, ultimately enhancing carcinogenesis. For example, Li and coworkers demonstrated that sublethal doses of ionizing radiation led to profound DNA damage (Figure 6B),[142] but these genomic instabilities were not observed in cells lacking PC-3 expression or lacking catalytically competent caspase-3. In a follow-up study, the Li group reported sublethal activation of caspase-3 promoted DNA damage as a result of Myc-induced oncogenesis in breast epithelial cells, MCF10A, suggesting caspase-3-dependent oncogenic transformation.[143] CRISPR/Cas9-mediated knockout of PC-3 led to reduced DNA damage and abolishment of carcinogenic effects. In both of these studies,[142,143] DNA damage was the result of endonuclease G (EndoG), a DNase that is liberated from the mitochondria following caspase-3 activation. EndoG activity has been shown in the presence of pan-caspase inhibitors,[144] implying that caspase-3 activation may not be solely responsible for EndoG-mediated DNA damage. Importantly in both studies, tumor formation in mice was compromised when cancer cells had PC-3 knocked down, implying a dependency on PC-3 expression for tumor formation and maintenance (Figure 6C). Thus, the overexpression of PC-3 may not merely be a passive effect but could be a true driver for oncogenic transformation, strengthening the case for PC-3 as a therapeutic target.

In a separate report, Tait and co-workers found that sublethal concentrations of ABT-737, a Bcl-2/Bcl-xL inhibitor, led to minority mitochondrial outer membrane permeablization (MOMP), caspase-3-mediated CAD (caspase-activated DNAase) activation, and extensive DNA damage (Figure 6D,E).[145] Treatment with a pan-caspase inhibitor led to no DNA damage upon ABT-737 treatment. Pretreatment of cells with ABT-737 increased tumor formation as compared to pretreatment with the inactive enantiomer of ABT-737 (Figure 6F), consistent with studies by Li and co-workers[142,143] and suggesting again that caspase-3 is critical for tumorigenesis. Further validating these reports, other work has revealed that sublethal antimitotic[146] and extrinsic-apoptotic agents[147] induce DNA damage. Taken together, it appears that caspase-3 promotes DNA damage and oncogenic transformation when sublethal levels of PC-3 activation are induced by a variety of environmental stresses.

These data suggest that there may be an advantage to cancer cells that overexpress PC-3, resulting in higher levels of genetic instability. Given PC-3 autoactivation, a lower barrier to sublethal caspase-3 activity afforded by a decreased zinc/PC-3 ratio (Figure 5) may be

sufficient to promote DNA damage and oncogenesis. Whether this lower level of proteolysis is the result of direct PC-3 mediated cleavages resulting from aberrantly high concentrations of PC-3 or low levels of caspase-3 accessed via PC-3 autocatalysis remains unanswered. Regardless, genomic instability resulting from PC-3 overexpression represents a possible explanation for the paradoxical overexpression of PC-3, a canonical pro-apoptotic protein, in cancer. There are other possible pro-oncogenic roles of PC-3/ caspase-3 activity, including promoting a tumorigenic proliferative state, as has been extensively discussed.[18–20,22,23,148]

Leveraging Procaspase-3 Overexpression for Selective Anticancer Therapy.

The majority of the current anticancer arsenal (both conventional cytotoxins and targeted therapies) relies on robust activation of apoptosis via processes upstream of PC-3 for their antitumor effect.[25,149] Due to a variety of mechanisms, these therapies can fail to elicit levels of caspase-3 activity sufficient for apoptotic death, diminishing their efficacy.[149] As described above, a low level of caspase-3 activity may actually benefit cancers, which has led to the suggestion of caspase inhibition as a therapeutic strategy.[19,20] However, given (l) the downstream location of PC-3 in the apoptotic cascade relative to frequently mutated proteins (Figure 1),[150] (2) the low frequency of PC-3 loss-of-function mutations in cancer, [15,17] (3) the robust overexpression of PC-3 in a number of cancer types (Table l), and (4) the dependency of tumorigenesis on maintained PC-3 expression (Figure 6), therapeutic interventions that directly activate PC-3 leading to robust caspase-3 activity represent a strategy to overcome apoptotic evasion and synergize with the current suite of anticancer drugs.

Procaspase Activating Compound 1 (PAC-1).

With this backdrop, a first-in-class small molecule activator of PC-3, the first procaspase-activating compound (PAC-1), was reported in 2006.[64] PAC-1 treatment leads to robust activation of apoptosis in multiple cancer cell lines and patient-derived tumor cells, while having a minimal effect in normal cell lines and matched normal tissues.[64,151,152] Through multiple mechanistic studies, it has been shown that PAC-1 leads to PC-3 activation via chelation of the inhibitory zinc of PC-3 (Figure 7A).[28] The affinity of PAC-1 for zinc *(K_d =* 1.28nM)[153] allows for chelation of inhibitory zinc from PC-3 but is not strong enough to disrupt proteins containing essential zinc ions.[42,15] PAC-1 chelation of intracellular labile zinc has been demonstrated using genetically encoded zinc-selective sensor proteins,[155] and this unique mode of action of PAC-1 has been validated via utilization of caspase-specific inhibitors[156] caspase-specific substrates[151] in work with Bax/Bak double knockout cells[157,158] as well as explorations using a PAC-1 derivative in caspase-3/caspase-7 knockout cell lines.[42]

The relationship between PAC-1 and other therapeutically relevant metal chelators has been extensively described as has evidence suggesting PAC-1 is not acting through a pan-assay interfering mechanism.[25,28,153] Targeting transition metal homeostasis is known to be a viable drug strategy.[25,153,159,160] PAC-1 is stable at temperatures and pH values well outside of the physiological range[161] and the N-acyl hydrazone functional group is found in other drugs (e.g., rifampicin, eltrombopâ). Over 1000 PAC-1 derivatives have been synthesized,

[162–169] as recently reviewed.[25] In particular, the derivatives S-PAC-1, B-PAC-1, and SM-1 appear to have clinical promise (Figure 7B).[25,47,170–174]

PAC-1 has been used as a tool for exploring the effect of direct PC-3 activation in a variety of contexts, highlighted by the recent work of Fuchs and co-workers that elucidated a key role for caspase-3 activity in regulating organ size.[175] PAC-1 has also been utilized for the induction of apoptosis[176–182] for the direct activation of PC-3 downstream of the mitochondria[157,183–189] and has been the subject of detailed preclinical studies.[161,190–194] PAC-1 is part of several pharmacological reagent kits (e.g., Sigma LOPAC bioactives library, SCADs inhibitor kit) and has been evaluated in multiple large-scale drug/cell line screens.[195–197] The half-life of PAC-1 is ~25 min in mice[153] and ~2.1 h in dogs[198] but markedly longer in humans (discussed below), suggesting differences between the rodent and human metabolism of PAC-1.

Combination Studies with PAC-1.

Given that PC-3 activation is crucial for apoptotic-inducing agents, investigation of PAC-1 treatment as a means to synergistically enhance the activity of a variety of chemotherapeutics has been explored.[51,199] Of interest, PAC-1 + doxorubicin has efficacy in animal models of osteosarcoma, including in canine cancer patients.[199] PAC-1 + doxorubicin treatment induced shrinkage of pulmonary macrometastatic lesions in canines with metastatic osteosarcoma (trial size $n = 6$), and in another small trial PAC-1 + doxorubicin showed impressive results for the treatment of canine lymphoma (trial size $n = 4$).[199]

PAC-1 is a blood–brain barrier penetrant[155] suggesting the possibility for treating CNS cancers, namely glioblastoma (GBM). PAC-1 induces synergistic apoptosis with the standard-of-care drug temozolomide (TMZ) in many glioma cancer cell lines and *in vivo* intracranial rodent models.[51] Again, a small clinical trial enrolling canine cancer patients with spontaneous glioma allowed for further valuable preclinical assessment of PAC-1, given the outstanding evidence that canine glioma mirrors its human counterpart.[49,200–202] Three canine glioma patients were treated with a protocol that mimics human treatment protocols, i.e., PAC-1, radiation, and TMZ cycling. Marked responses were observed with tumor regressions of 43%, 60%, and 100% observed in these three patients[51].

As stated by Thornbury and Lazebnik in 1998, proteolysis of a protein represents an irreversible post-translational modification,[203] and the ability to degrade a target rather than inhibit its function is an emerging therapeutic strategy.[204,205] PAC-1 treatment in combination with a variety of clinically approved kinase inhibitors leads to synergistic caspase-3 activity, resulting in increased apoptotic cell death and delayed onset of resistance.[206,207] MEK1/2 are the gatekeeper kinases for ERK½ phosphorylation, and MEK1/2 reactivation is a major driver of resistance to MAPK-pathway targeted agents (Figure 7C).[208] Direct PC-3 activation with PAC-1 leads to caspase-3 dependent proteolysis of MEK1/2, irreversibly removing the necessary cellular machinery to reactivate MAPK signaling (Figure 7D). As shown, PAC-1 combinations lead to persistent inhibition of MEK1/2 phosphorylation (Figure 7E), resulting in minimal resistance (Figure 7F).[207] This study highlights the ability to leverage caspase-3's proteolytic substrate scope[29,31] to cleave

proteins within cells and utilize cancer s overexpression of PC-3 to selectively direct this degradation only to cancer cells.

On the basis of compelling data in mouse models of cancer[64,199,206,209] and m canine cancer patients,[51,199] a phase 1 clinical trial of PAC-1 for late-stage cancer patients was initiated (). Thus far, it has been reported that PAC-1 has excellent pharmacokinetics in human cancer patients (e.g., a half-life of ~20 h[210]), and PAC-1 has been dosed as high as 750 mg (oral tablet, once-a-day for 21 days) with signs of efficacy in these late-stage cancer patients.[211,212] The FDA granted PAC-1 orphan drug designation for the treatment of GBM, and based on the demonstration that PC-3 expression is increased in glioma samples,[48–51] and data showing efficacy of PAC-1 + TMZ in rodent models of glioma and canine glioma patients,[51] the combination of PAC-1 and TMZ is being assessed in recurrent human GBM patients. Initial reports of this trial () suggest that the PAC-1/TMZ combination is well-tolerated and that PAC-1 has outstanding pharmacokinetics in this combination study.[213]

CONCLUSIONS AND OUTLOOK

Apoptosis-inducing drugs are a mainstay of many anticancer regimens. Cancers must evade apoptosis to proliferate, and it appears that certain subapoptotic processes may be advantageous for cancers (e.g., through genetic instability). The zymogen precursor of executioner caspase-3, PC-3, is overexpressed in multiple malignances, and this abnormal expression suggests the possibility for crucial roles of canonical apoptotic machinery in oncogenic transformation. PC-3 presents an opportunity to target cancer through weaponization of PC-3 overexpression to selectively generate cytotoxic caspase-3 in cancer cells. PAC-1 is a first-in-class drug that chelates the inhibitory zinc from PC-3/caspase-3, restoring their enzymatic function. PAC-1 has been useful as a tool to explore procaspase-3 function, and the translational potential of PAC-1 is being evaluated in human cancer patients (, ,). Continued efforts to unravel the overexpression of PC-3 and studies that capitalize on this paradoxical observation will further our understanding of the complex relationship between normal cell growth, apoptosis, and cancer.

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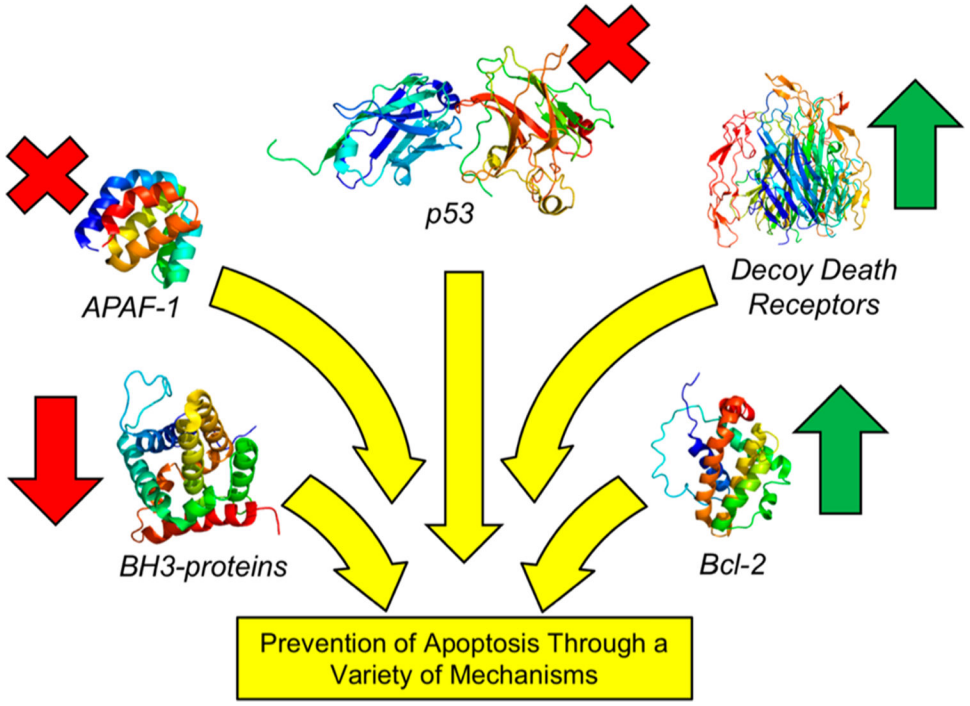

Figure 1.

Diverse upstream mechanisms employed by cancers to prevent apoptosis. Increased expression of antiapoptotic proteins (green arrows, e.g. Bcl-2, decoy death receptors) or decreased expression or mutation of proapoptotic proteins (red arrows, x's, e.g. APAF-1, BH3 proteins, p53) drive the net effect of preventing downstream activation of PC-3 to caspase-3 and avoiding apoptotic cell death. Protein structures displayed: p53 (PDB: 4QOl), APAF-1 (PDB: 1CY5), BH3-proteins (Bax, PDB: 1F16), decoy death receptor (Death Receptor 4, PDB: 5CIR, no reported decoy death receptor crystal structures), and Bcl-2 (PDB: 1G5M). For comprehensive reviews on the upstream signaling that results in apoptosis, see refs 14 and 25.

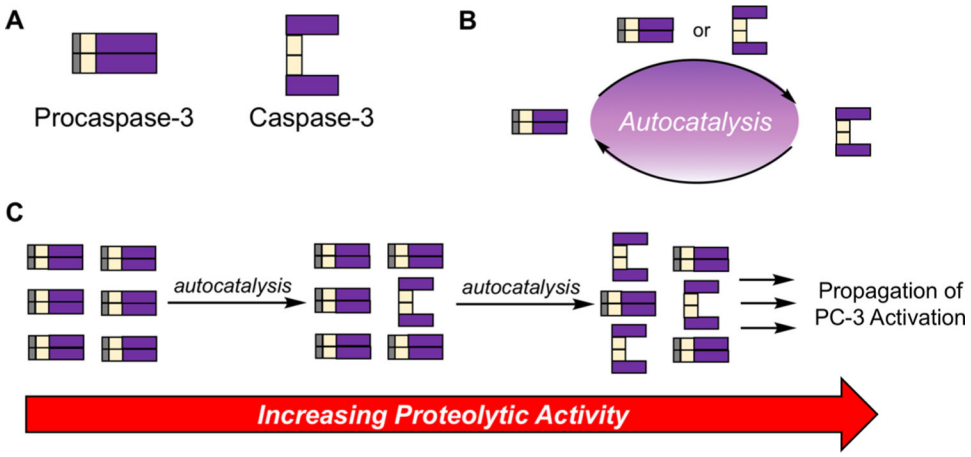

Figure 2.

Autocatalytic PC-3 activation, resulting in large effects from minor initial activity. (A) Pictorial representation of PC-3 and caspase-3 domains. (B) Reaction scheme for PC-3 activation to form caspase-3. While PC-3 is canonically cleaved by caspase-8 or caspase-9, caspase-3 can be formed by PC-3-mediated proteolysis of another PC-3 protein, or caspase-3 mediated cleavage of PC-3. (C) Initial PC-3 activation to form caspase-3 leads to a proteolytic cascade to form increased levels of caspase-3, in turn increasing proteolysis.

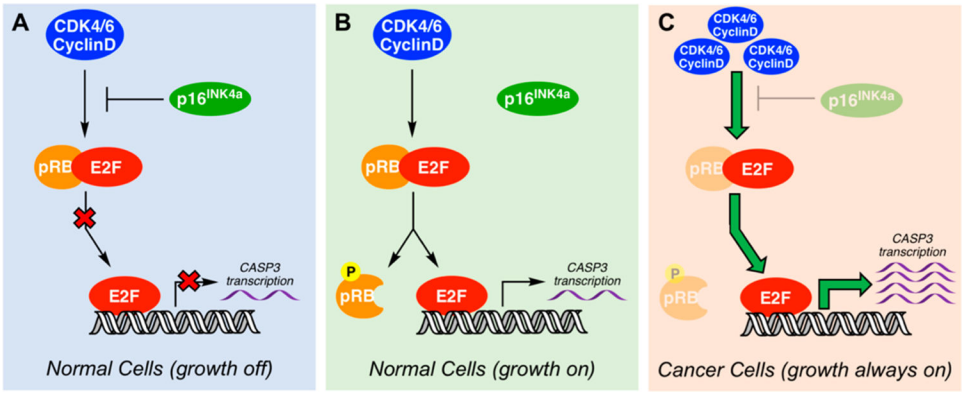

Figure 3.

pRB/E2F pathway dysregulation leading to unrestricted *CASP3* gene transcription. The pRb/E2F pathway regulation of transcription and cell cycle progression. (A) The transcriptional activity of E2F is inhibited through binding with pRb with further regulation of the pathway via $pl6^{INK4a}$. (D) In the presence of growth signals, cells turn on E2F transcription via CDK4/6 activation, resulting in phosphorylation of pRB and E2F translocation to the nucleus, turning on transcription of target genes (i.e., *CASP3).* (C) In cancer cells, the loss of $pl6^{INK4a}$, pRb, or overexpression of CDK4/6 lead to relief of E2F inhibition, resulting in unregulated transcription. For a comprehensive review of this pathway in cancer, see refs 99 and 101.

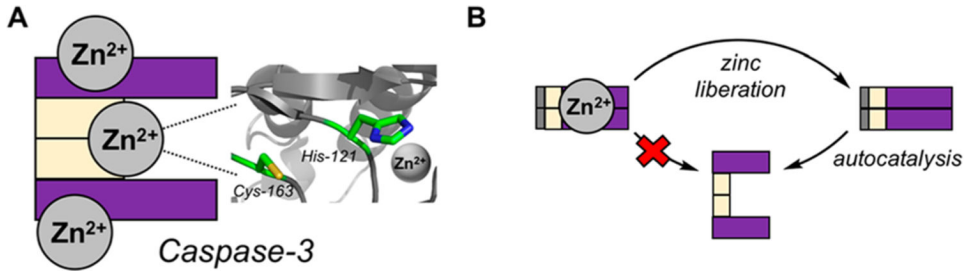

Figure 4.

Post-translational regulation of caspase-3 and PC-3 activity via inhibitory zinc. (A) Graphical representation of inhibitory zinc on caspase-3.[121] Protein structure displayed is PDB: 2XYG. (B) Zinc binding inhibits PC-3 autocatalysis to form caspase-3. Upon zinc liberation, PC-3 can autoactivate to form caspase-3. Adapted with permission from ref 28. Copyright 2009 Elsevier.

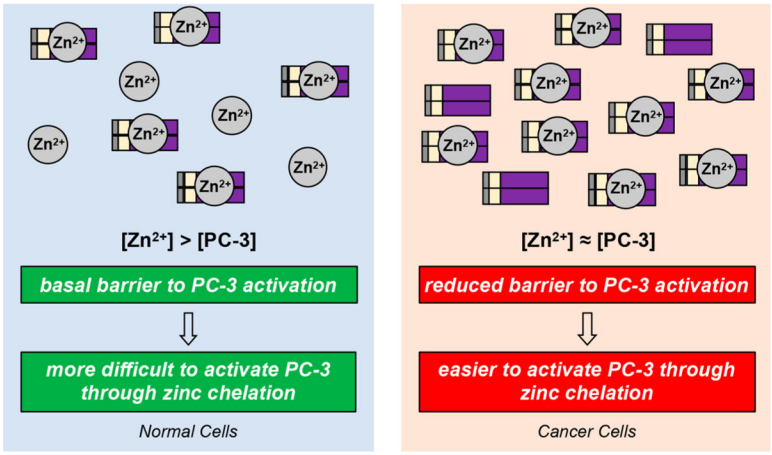

Figure 5.

Perturbation of zinc regulation via increased PC-3 levels in cancer cells. Cancer cells overexpress PC-3, making regulation of PC-3 activity through inhibitory zinc less effective. Given the autocatalytic nature of PC-3 autoactivation and amplification, these changes in zinc/PC-3 ratio may prove sufficient to alter basal PC-3 (and ultimately caspase-3) activity within a cell. Zinc (Zn^{2+}) circles represent the labile zinc pool.

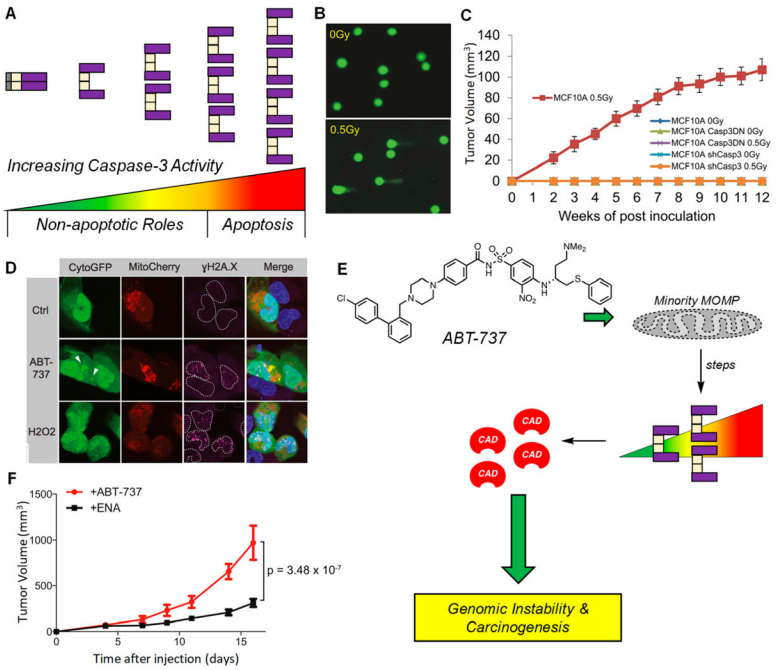

Figure 6.

Promotion of DNA damage and genomic instability by subapoptotic caspase-3 activity. (A) Graphical representation of the spectrum of PC-3/caspase-3 activity. Sublethal levels (spanning from green to yellow/orange) represent proteolytic levels not sufficient for apoptotic induction, but sufficient for cleavage of a variety nonlethal protein substrates. Lethal levels (spanning from orange to red) signify irreconcilable caspase-3 activities and result in cell death via apoptosis. (B) Sublethal irradiation of MCF10A induces DNA damage as per the comet tail assay. A larger tail moment indicates increased DNA damage. Reprinted with permission from ref 142. Copyright 2015 Elsevier. (C) Irradiated MCF10A cells form tumors in mice, while shRNA of *CASP3* leads to a dramatic reduction in tumor formation (n = 10 per arm, all arms are displayed in the panel). These results indicate an active role of caspase-3 in tumorigenesis. Casp3DN: dominant-negative caspase-3. Reprinted with permission from ref 142. Copyright 2015 Elsevier. (D) Treatment of U20S cells, transiently expressing CytoGFP/Mitocherry, with ABT-737 (5 *μM)* leading to increased /H2AX foci; H_20_2 is the positive control. Increases in yH2AX foci are indicative of heightened DNA damage. Reprinted with permission from ref 145. Copyright 2015 Elsevier. (E) Treatment with ABT-737 causes minority MOMP (displayed as a perforated mitochondria), leading to sublethal levels of caspase-3, resulting in CAD activation and genomic instability and carcinogenesis. (F) Primary pi9^{Arf} null MEF cells were treated with ABT-737 (10 *μM)* or its inactive enantiomer (ENA, 10 *μM)* for 10 passages, then inoculated into mice. Tumor formation is increased when cells were pretreated with ABT-737 *(n* = 15 per treatment), suggesting that ABT-737 treatment leads to caspase-3 mediated genomic instability that ultimately increases tumorigenicity. Adapted with permission from ref 145. Copyright 2015 Elsevier.

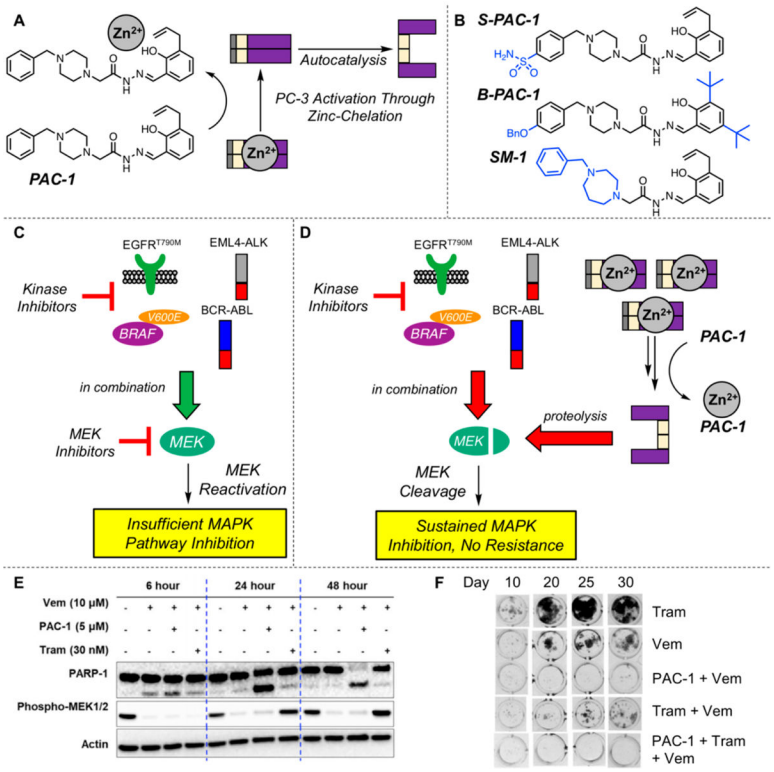

Figure 7.

Activation of PC-3 by PAC-1 and its derivatives via chelation of labile inhibitory zinc. (A) PAC-1 binds zinc, alleviating inhibition of PC-3, allowing for autocatalytic formation of caspase-3. (B) Chemical structures of reported derivatives of PAC-1. Deviations from PAC-1 are highlighted in blue. (C) A variety of kinase inhibitors that target mutant proteins found in cancer are effective as single agents and in combination with MEK inhibitors. However, this initial efficacy is short-lived, and resistance invariably occurs through a variety of mechanisms surrounding MEK reactivation.[208] (D) PAC-1 treatment synergizes with targeted kinase inhibitors and leads to robust activation of PC-3. These increased levels of caspase-3 activity lead to dramatic reduction of MEK levels via caspase-3 mediated cleavage. This protein degradation strategy sustains inhibition of MEK and the MAPK pathway and delays the onset of resistance.[207] (E) Time course of phosphorylated MEK1/2 levels upon treatment of vemurafenib ($BRAF^{v600E}$ inhibitor, Vem), PAC-1, trametinib (MEK1/2 inhibitor, Tram), and combinations as indicated. Experiment was conducted with A375 cells ($BRAF^{v600E}$ cell line). PAC-1 combined with vemurafenib leads to persistent phosphorylated MEK1/2 suppression and increased apoptosis as measured by PARP-1 cleavage. Reprinted with permission from ref 207 (some blots have been removed for simplification). Copyright 2018 Elsevier. (F) Long-term incubation of A375 cells with PAC-1 (l *μM),* vemurafenib (10 *μM),* trametinib (3 nM), and combinations thereof. PAC-1 in combination with kinase inhibitors dramatically decreases the occurrence of resistant cell growth. Reprinted with permission from ref 207 (the orientation of the figure is rotated from the original). Copyright 2018 Elsevier.

Table 1.

Procaspase-3 Expression Levels in Cancer

	cancer type[a]	PC-3 expression levels	refs
blood	ALL	overexpressed	37, 38
	AML	overexpressed	38, 39
	BL/BLL	overexpressed	40
	CLL	overexpressed	41, 42
	DLBCL	overexpressed	43
	NHL	overexpressed	41, 44
		underexpressed	45
	childhood NHL	overexpressed	46
	multiple myeloma	overexpressed	47
brain	astrocytomas	overexpressed	48, 49
	glioblastoma	overexpressed	48–51
	meningioma	overexpressed	49, 52, 53
	neuroblastoma	overexpressed	54
	oligodendrogliomas	overexpressed	49
solid tumors	breast	overexpressed	55–60
		underexpressed	61
	cervical	overexpressed	62, 63
	colorectal	overexpressed	32, 64–67
		underexpressed	68
	esophageal	overexpressed	69, 70
	gallbladder	overexpressed	71
	gastric	overexpressed	72
	hepatocellular	overexpressed	73
		underexpressed	74, 75
	NSCLC	overexpressed	76–81
	melanoma	overexpressed	82, 83
	pancreatic	similar	84
		overexpressed	85, 86
	prostate	overexpressed	87
		underexpressed	88–90
	SCC	overexpressed	91–93
	stomach	overexpressed	94

[a]ALL, Acute Lymphocytic Leukemia; AML, Acute Myeloid Leukemia; BL/BLL, Burkitt Lymphoma/Burkitt-Like Lymphoma; CLL, Chronic Lymphocytic Leukemia; DLBCL, Diffuse Large B-Cell Lymphoma; NSCLC, Non-Small Cell Lung Cancer; SCC, Squamous Cell Carcinoma.