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PRECISION THERAPY TO TARGET APOPTOSIS IN PROSTATE CANCER

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Androgen-independent prostate cancer shows limited response to existing systemic therapies. Recent advances in prostate-selective targeting of small molecule inhibitors and bacterial toxins have created opportunities to design a new generation of therapies for advanced prostate cancer. Yet prioritizing targets for these therapies remain challenging, since multiple mechanisms contribute to the pathophysiology of androgen-independent prostate cancer. This review explores the possibility of targeting the apoptosis regulatory network as most direct approach to efficient treatment of advanced androgen-independent prostate cancer.
***Key Words*: prostate cancer, apoptosis, prostate-selective therapies, PSA-activated pro-drugs, PSMA-targeting toxins.**

REDUNDANT MECHANISMS OF ANDROGEN INDEPENDENCE IN ADVANCED PROSTATE CANCER POSE CHALLENGE FOR CURRENT THERAPIES

Androgen ablation remains the most effective systemic therapy for advanced prostate cancer, that delays cancer progression 90% of cases. Still, the disease invariably recurs as androgen-independent cancer, for which no curative treatment is available. A critical role of the androgen signaling axis in prostate cancer has been unequivocally demonstrated, as androgen independence has been connected with activation of the androgen receptor (AR) despite androgen ablation therapy. Mutations in AR that lead to hypersensitivity to low concentrations of androgen, or ligand-independent activation of AR have been identified [1]. New therapies, including abiraterone acetate (an inhibitor of androgen biosynthesis), and MDV3100 (an AR antagonist that prevents nuclear translocation and chromatin binding) can achieve "complete androgen blockade". Still, even with complete inhibition of AR signalling, prostate cancer will eventually progress [2–4].

Broader analysis of androgen independence that extends beyond the AR axis connected the signaling pathways from EGFR (HER) family, GPCRs, non-receptor tyrosine kinases of the Src family, PI3K, NF-kB, myc, and other regulatory molecules with advanced androgen-independent prostate cancer [5, 6]. Of these, the PI3K pathway emerged as most prominent, since mutations leading to its activation of PI3K pathway were almost invariably detected by whole-genome sequencing of advanced prostate cancer [7, 8]. Yet unlike inhibitors of AR signaling that extend survival of patients with advanced metastatic prostate cancer up to 16–18 months, inhibitors of other signaling pathways, including PI3K/mTOR inhibitors and inhibitors of receptor tyrosine kinases, did not show significant survival benefit [9–11].

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Abbreviations used: AR – androgen receptor; PSA – prostate-specific antigen; PSMA – prostate-specific membrane antigen.

This modest anticancer efficacy is not unique to prostate cancer. Over the last 20 years, numerous inhibitors of signal transduction enzymes have been developed. These inhibitors proved invaluable as experimental tools, yet with the notable exception of Gleevec, most failed to meet high expectations to substantially increase patient survival [12]. Several reasons may account for inefficiency of signal transduction inhibitors in prostate cancer.

First, although complete androgen blockade affects other tissues and has a range of metabolic and behavioral side effects, prostate cells are the most sensitive to androgen ablation and die first [6]. In contrast, AR-independent signaling pathways play important roles in normal physiology of cells and tissues outside of prostate glands. Therefore, complete inhibition of these pathways is likely to induce morbid side effects that limit doses of inhibitors and do not allow complete inhibition of the signaling pathway in prostate tumors.

Second, there is significant variability between individual tumors and patients in signaling pathways that contribute to androgen independence. As a result, few patients will show the expected response to inhibitors of a specific pathway. When results of all treated patients are combined in a clinical trial, the differences between experimental and control groups may still did not show a statistically significant difference despite few responsive patients.

Third, analyses of topologies of signal transduction networks show a substantial level of redundancy, especially at the "top" at the receptor level [13, 14]. Thus, even complete inhibition of apical protein kinase or other signaling molecules will leave alternative pathways intact and will have little effect on critical effector molecules that determine the extent of phenotypic response. It appears that to accomplish an effective anti-tumor response, several signaling pathways must be inhibited.

Androgen ablation triggers apoptosis in differentiated prostate epithelial cells, yet in advanced androgen-independent prostate tumors, cells no longer die upon androgen deprivation. In androgen-independent prostate cancer, the default response of prostate cells to androgen ablation (i.e. apoptosis) is diverted by other

signaling pathways. Following this logic, targeting anti-apoptotic signals should complement androgen ablation therapy. Because apoptosis is most common evolutionary refined mechanism of elimination of cancer cells by intrinsic organismal responses and anti-cancer therapies [15], treatment modalities that aim at apoptosis induction are likely to be most efficient.

BAD AND MCL-1 ARE KEY NODES OF APOPTOSIS REGULATORY NETWORK

What is known about apoptosis regulation in prostate cancer cells, and how it could be induced most efficiently in prostate tumors? Seminal experiments by Charles Huggins that showed involution of prostate glands after androgen ablation in beagles were reproduced in other animal models with similar results [16]. In 1988, a group led by John Isaacs reported increased apoptosis in response to androgen ablation in rats [17]. Apoptosis in the prostate gland after androgen ablation therapy has been confirmed by others, yet the precise mechanism of apoptosis induction remained unknown [18, 19]. As the mechanism of apoptosis regulation became better understood, the critical role of Bcl2 protein family in mitochondrial outer membrane permeability and commitment to apoptosis was established. Transgenic mice with increased expression of Bcl2 showed delayed prostate involution after androgen ablation, suggesting that common principles of apoptosis regulation were also true for prostate glands. Still, no convincing data on changes in Bcl2 family proteins (or other apoptosis-regulatory molecules) in prostate glands after androgen ablation were reported [20].

Most studies on the mechanisms of apoptosis regulation used tissue culture models of cells derived from prostate tumors: LNCaP, PC3, and DU145 [21]. Of these cell lines, only LNCaP cells remain androgen-responsive, although they are no longer depend on androgen for survival [22]. Apoptosis regulation in LNCaP cells was triggered by the loss of PTEN phosphatase, which negatively regulates the PI3K pathway [23, 24]. As a result, PI3K signaling is constitutively active and protects LNCaP cells from apoptosis, whereas inhibition of the PI3K pathway induces apoptosis in these cells. Extensive analysis of apoptosis regulation in LNCaP and in C42 cells (derived from LNCaP cells by passaging in immunodeficient mice) identified several signal transduction pathways that inhibit apoptosis [13, 25].

These pathways activated by EGFR and GPCR agonists phosphorylate BAD and protect cells from apoptosis induced by PI3K inhibitors that triggered dephosphorylation of BAD. BAD knockout rendered LNCaP and C42 cells insensitive to apoptosis induction by PI3K inhibitors and, conversely, expression of BAD with mutated phosphorylation sites disabled anti-apoptotic signaling by EGFR, GPCRs, and constitutively active PI3K [13, 26]. Thus, BAD phosphorylation was established as a convergence point for anti-apoptotic signaling pathways in prostate cancer cells. However, the principal role of BAD was questioned when dynamics of apoptosis induction and BAD dephosphorylation were compared. In cells treated with PI3K inhibitors, BAD was dephosphorylated within 3–4 hours, but apoptosis was not detected until 12 hours. In contrast, the combination of a PI3K inhibitor and a protein synthesis inhibitor induced apoptosis in 6 hours, yet dynamics of BAD dephosphorylation in cells treated with PI3K alone or with a combination of a PI3K inhibitor and a protein synthesis inhibitor were similar, despite dramatic differences in timing of apoptosis induction [27]. These observations prompted the search for another regulatory molecule that commits prostate cancer cells to undergo apoptosis.

Earlier reports on interaction preferences between members of Bcl2 family identified BclXL, Bclw and Bcl2 as binding partners of BAD, whereas another anti-apoptotic protein Mcl-1 does not bind BAD [28]. Thus, in a dephosphorylated state, BAD will bind and neutralize anti-apoptotic effects of BclXL, Bclw, and Bcl2, but not of Mcl-1. Consequently, in cells that express Mcl-1, BAD dephosphorylation will have only a mild pro-apoptotic effect. Mcl-1 is indeed expressed in LNCaP cells, and treatment with PI3K inhibitors predictably induces only delayed apoptosis, since expression of Mcl-1 is not affected. In contrast, the combination of a PI3K inhibitors and a protein synthesis inhibitor decreased both BAD phosphorylation and Mcl-1 expression and induced rapid apoptosis [27]. Experiments with shRNA-mediated knockdown of Mcl-1 and expression of phosphorylation-deficient mutants of BAD confirmed the essential role of simultaneous BAD dephosphorylation and Mcl-1 loss in inducing rapid apoptosis [27].

Mcl-1 is characterized by rapid turnover, with over 80% of protein degraded within 3 hours after protein synthesis is inhibited. Rapid turnover of Mcl-1 is dictated by ubiquitination-mediated targeting to proteasomes. Ubiquitination in turn is regulated by phosphorylation, which — depending on the specific site — may either stimulate or inhibit Mcl-1 ubiquitination [29, 30].

Several signaling mechanisms can induce transcription and/or delay Mcl-1 phosphorylation and increase overall expression of Mcl-1 protein. The transcriptional factors STAT, CREB, HIF-1, and TCF, as well as micro-RNA, were implicated in increased synthesis of Mcl-1 protein. On the other hand, phosphorylation by the ERK pathway at Thr92 and Thr163 prolongs Mcl-1 half-life, whereas phosphorylation at S169 by GSK3b accelerates degradation. Thus, signaling pathways emanating from Ras/MAPK, PI3K/Akt/Gsk3b, GPCR/PKA/CREB and RTK/STAT could upregulate Mcl-1 expression [30, 31]. Analysis of BAD phosphorylation identified PKA, RAS/MAPK, Rac/PAK and PI3K/Akt pathways as responsible for anti-apoptotic effects of GPCR and EGFR agonists and loss of PTEN in prostate cells [13, 25, 26]. In summary, levels of Mcl-1 expression and BAD phosphorylation are dynamically regulated by overlapping signal transduction pathways, most of which could be inhibited with existing drugs.

The decisive roles of BAD dephosphorylation and Mcl-1 loss depend on the expression pattern of other members of the Bcl2 family. For example, the PC3 prostate cancer cell line, with high expression of BclXL, shows less apoptosis when BAD dephosphorylation and loss of Mcl-1 is induced. Likewise, less apoptosis is observed in DU145 cells with diminished expression of BAX. However, when BclXL expression in diminished and BAX expression is restored, both cells lines show comparable apoptosis compared to LNCaP cells. Taken together, these results identify BAD and Mcl-1 as critical nodes of apoptosis regulatory networks, yet their impact on apoptosis depends on the status of other network members [27].

To translate this information into improved therapies for advanced prostate cancer, several issues must be addressed. 1) A systems approach to the analysis of Bcl2 family proteins is needed, to identify prostate tumors that may respond to BAD dephosphorylation and Mcl-1 loss. 2) Signaling pathways that control BAD phosphorylation and synthesis of Mcl-1 play important physiological roles in other tissues, and inhibiting these pathways is likely to induce prohibiting side effects. A possible way to minimize these side effects is to use prostate-selective inhibitors. As advanced prostate cancer affects mostly older men, preservation of a functional prostate gland is seldom a priority for these patients [32].

PROSTATE-SELECTIVE THERAPIES THAT TARGET BAD PHOSPHORYLATION AND MCL-1

Designers of prostate-selective drugs have utilized two approaches: making a pro-drug activated by prostate-specific antigen (PSA) cleavage, or linking active toxins to prostate-specific membrane antigen (PSMA)-targeting antibodies.

The approach using PSA-activated pro-drugs pioneered by John Isaacs' group at Johns Hopkins University by linking a PSA substrate peptide to thapsigargin, an inhibitor of the Ca^{2+} channel pump in the endoplasmic reticulum. Apoptosis was induced in prostate cancer cell upon 24 h exposure. This approach was expanded by this group and others by generating PSA-activated pro-drugs of doxorubicin, vinblastine, thapsigargin, paclitaxel and proaerolysin [33–38].

However, no pro-drug inhibitors of protein kinases that can be activated by PSA cleavage and inhibit their targets only in prostate cells have been made. Since PI3K is the most frequently activated signaling pathway in advanced prostate cancer, and our earlier publications identified PI3K as the major signaling pathway responsible for constitutive BAD phosphorylation in prostate cancer cells, it was reasonable to test whether a PI3K inhibitor pro-drug could be created. As we have shown recently, LY294002, a widely used PI3K inhibitor, can be converted into inactive pro-drug that selectively inhibits PI3K in prostate cancer cell lines (C42 and LNCaP) cells that secrete PSA, but not in breast cancer cells, which do not secrete PSA [39]. Future experiments will tell whether more potent inhibitors that block PI3K activity at nM concentration can be generated, and whether these pro-drug PI3K inhibitors can selectively block PI3K activity in prostate tumors *in vivo* [40]. Should these experiments succeed, it will open the door for design of prostate-selective inhibitors of protein kinases like EGFR, MEK, PAK or PKA that were identified as upstream kinases of BAD in prostate cancer cells.

A different approach to producing prostate-targeted drugs is based on fusing toxins to antibodies against PSMA. The most attention has been generated by J591 monoclonal antibodies developed by Neil Bander, now used for both therapeutic and diagnostic applications [41]. Another set of monoclonal antibodies has been raised by a German group that fused variable light chain of these antibodies with Pseudomonas Exotoxin A [42], which ribosylates elongation factor 2 and inhibits the translation step of protein synthesis. A recent analysis of synergistic apoptosis induction in prostate cancer cells by PI3K and protein synthesis inhibitors identified Mcl-1 as a critical regulatory protein responsible for pro-apoptotic effects of protein synthesis inhibitors in prostate cancer cells [27, 43].

In summary, both BAD phosphorylation and Mcl-1 expression are controlled by several convergent signal transduction pathways [30, 31, 44, 45]. When Mcl-1 expression is decreased simultaneously with BAD dephosphorylation (triggered by PI3K inhibition), robust apoptosis in prostate cancer cells is induced within 3–4 hours. In contrast, when either BAD phosphorylation or Mcl-1 expression alone was inhibited, apoptosis was evident only after 12–24 hours [27]. Thus, monitoring BAD phosphorylation and Mcl-1 expression along with immediate targets of signaling inhibitors allows prediction of whether prostate-selective inhibitors will have intended effects. In contrast, monitoring only inhibition of immediate targets will have less predictive power because of multiple redundant signals that converge on Mcl-1 and BAD.

Analysis of protein expression and phosphorylation is relatively straightforward for hematopoietic cancers, yet for solid tumors it poses a substantial challenge. Repeated biopsies are needed, which is especially problematic for metastatic tumors. To circumvent the need for tumor biopsies, surrogate markers have been proposed. For example, analysis of EGFR phosphorylation in hair follicles has been used to monitor efficacy of EGFR kinase inhibitors [46]. However, this approach may not be applied for monitoring BAD and Mcl-1, since signaling pathways that regulate these proteins are likely different in prostate than in other tissues. Furthermore, considering the high level of heterogeneity observed within prostate tumors, several biopsies may be required to confirm that Mcl-1 expression and BAD phosphorylation throughout the tumor tissue are changing. Recently, circulating tumor cells attracted significant attention as a possible replacement for tumor biopsies. But it remains unproven whether, taken out of the microenvironment, tumor cells can

adequately represent signaling events inside solid tumors [47]. One possible alternative is using primary xenografts of tumor biopsies to select optimal combination of therapies without subjecting cancer patients to highly invasive procedures [48]. In fact, a panel of primary xenografts established from tumor biopsies could be used to test whether monitoring BAD phosphorylation and Mcl-1 expression better predicts a curative response than monitoring immediate targets of signal transduction inhibitors.

AUTHOR CONTRIBUTIONS

GK conceived and wrote the manuscript.

COMPETING INTERESTS

Author declares no competing interests.

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