BBA - Molecular Cell Research 1867 (2020) 118731

Contents lists available at ScienceDirect

BBA - Molecular Cell Research

journal homepage: www.elsevier.com/locate/bbamcr

Deregulated PTEN/PI3K/AKT/mTOR signaling in prostate cancer: Still a potential druggable target?☆

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ARTICLE INFO

Keywords:
Prostate cancer
PTEN/PI3K/AKT/mTOR signaling
Targeted therapy
Resistance
PIK3CA mutation
PTEN deletion

ABSTRACT

Although the prognosis of patients with localized prostate cancer is good after surgery, with a favorable response to androgen deprivation therapy, about one third of them invariably relapse, and progress to castration-resistant prostate cancer. Overall, prostate cancer therapies remain scarcely effective, thus it is mandatory to devise alternative treatments enhancing the efficacy of surgical castration and hormone administration.

Dysregulation of the phosphoinositide 3-kinase pathway has attracted growing attention in prostate cancer due to the highly frequent association of epigenetic and post-translational modifications as well as to genetic alterations of both phosphoinositide 3-kinase and PTEN to onset and/or progression of this malignancy, and to resistance to canonical androgen-deprivation therapy.

Here we provide a summary of the biological functions of the major players of this cascade and their deregulation in prostate cancer, summarizing the results of preclinical and clinical studies with PI3K signaling inhibitors and the reasons of failure independent from genomic changes.

1. Introduction

Through physiological activation by a plethora of growth factors, the phosphoinositide 3-kinase (PI3K) and its downstream effectors exert crucial regulatory roles over almost all cellular functions from cell cycle progression, survival, migration and angiogenesis to metabolism [1]. It is therefore regarded as a key molecular hub in the cell, characterized by an intense cross-talk with other signaling nodes. Given this background, it is not surprising that disturbance of PI3K, and/or of its effectors and regulators causing constitutive activation of the pathway, has been involved in a wide array of diseases, including cancer [1–3]. Given the pro-oncogenic function and the druggability of most of its key members, this pathway has been exploited for drug discovery since the ′90s [4]. As a result, a massive number of drugs has been available to preclinical studies [5].

Prostate cancer is a clinically heterogeneous malignancy representing the most frequently diagnosed cancer in men and a leading cause of cancer-related men death. As most prostate cancers are androgen-dependent, pharmacological or surgical castration is the standard treatment. Androgen deprivation extends survival and improves the quality of life of patients. It is not sufficient to eradicate cancer, though, thus also patients with localized prostate cancer and good prognosis after castration surgery almost invariably relapse, and develop into castration-resistant metastatic prostate cancer [6–8].

In prostate cancer, PI3K pathway deregulation due to genetic alterations, such as activating mutations or deletion of PIK3CA, AKT1 and PTEN, as well as epigenetic and post-translational modifications, is always associated with cancer progression, rendering this signaling axis an attractive target for therapy also in this malignancy [9].

Regrettably, the success of these efforts has been often frustrated mostly by the emergence of resistance due to acute reactivation and/or cross-talk to the androgen receptor (AR) or other signaling networks.

Here we summarize the functions of the major players of the PI3K signaling cascade and their deregulation in prostate cancer. Furthermore, we review the results of preclinical and clinical studies with PI3K signaling inhibitors, focusing on the drugs and drug targets suggested to impact on prostate cancer, highlighting the importance of combination therapies.

1.1. PI3K/AKT/mTOR

Although three classes of the PI3K lipid kinase exist in mammalian

☆ This article is part of a Special Issue entitled: GSK-3 and related kinases in cancer, neurological and other disorders edited by James McCubrey, Agnieszka Gizak and Dariusz Rakus.

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https://doi.org/10.1016/j.bbamcr.2020.118731
Received 31 March 2020; Received in revised form 21 April 2020; Accepted 23 April 2020
Available online 29 April 2020
0167-4889/

cells, class I PI3Ks represent a crucial node integrating multiple signals, including oncogenes, and are clearly implicated in cancer. This class includes several paralogs, namely PI3Kα, PI3Kβ, PI3Kγ and PI3Kδ. Each PI3K is a dimer of one regulatory subunit and one catalytic subunit (collectively called p110). Active receptor tyrosine kinases, Ras and small GTPases (PI3Kα) or G-protein coupled receptors and RAC1/CDC42 small GTPases (PI3Kβ) [1,4,10] recruit the regulatory subunit in close proximity of the plasma membrane, therefore releasing the block operated on the catalytic subunit. Thus p110 begins to convert phosphatidylinositol-4,5-bisphosphate (PIP2) into phosphatidylinositol-3,4,5-triphosphate (PIP3) through phosphorylation of the 3′-hydroxyl group. In turn, PIP3 acts as a powerful amplifier of PI3K signal, docking pleckstrin homology (PH) domain-containing proteins to specific subcellular locations. Both the phosphoinoside–dependent kinase 1, PDK1, and the main PI3K effector v-akt murine thymoma viral oncogene homolog, (AKT), also called protein kinase B (PKB), are indeed recruited to the membrane by binding of their PH domains to PIP3. Engagement by PIP3 acts as a switch that relieves AKT autoinhibition, allowing a conformational change that primes AKT itself for phosphorylation by PDK1 at T308 and by the mechanistic target of rapamcyin (mTOR) complex 2, mTORC2, at S473. These modifications support binding and phosphorylation of AKT substrates. It is worth mentioning here that allosteric control of AKT activity by the PH domain was exploited to design small molecules, such as AKT inhibitor VIII, designed to pack the PH domain to the kinase domain in a “PH-in” inactive conformation [11,12].

Importantly, AKT mutant forms with stable membrane binding, such as the PH domain mutant E17K or myristoylated AKT, are constitutively active and oncogenic. Whether anchoring to PIP3 is rate-limiting also for maintaining AKT activity is still debated [13]. Remarkably, also recruitment and activation of mTORC2 has been suggested to depend on PIP3 binding to a member of the complex, namely SIN1. According to this hypothesis, upon PI3K activation and PIP3 production SIN would bind to PIP3, therefore releasing its inhibitory action on mTOR, while relocalizing the complex to the membrane in proximity of AKT [14]. However, it has also been reported that mTORC2 can be directly recruited in the membrane in a PI3K-independent mechanism [13], therefore this point needs to be settled. To complicate the picture further, it has been recently reported that AKT can phosphorylate SIN1 at T86, resulting in increased mTORC2 activity and AKT S473 phosphorylation [15].

Importantly, phosphorylation of AKT by mTORC2 at T450, in the turn-motif, is required for proper folding [16]. In the regulatory domain, AKT can be phosphorylated at S477 and T479 by the cyclin A-CDK2 complex in a cell-cycle dependent manner [14], by the mTOR-related kinase DNA-dependent protein kinase (DNA-PK) in a DNA-damaging context [17], and by mTORC2 upon growth factor stimulation [14]. Other sites include S129, targeted by CK2 to increase catalytic activity [18], and T312, an inhibitory site phosphorylated by GSK3α [19]. Many other sites have been mapped by phosphoproteomics, whose significance and regulatory mechanisms are unknown.

In physiological conditions, the signal from PI3K is abrogated by inactivation of PI3K together with dephosphorylation of PIP3 at 3′ position by the phosphatase and tensin homolog chromosome 10, PTEN, which antagonizes PI3K by means of its lipid phosphatase activity [4] (Fig. 1). Moreover, while INPP4B 4-phosphatase, which dephosphorylates both PI3,4,5P3 and PI3,4P2, has been recently identified as a suppressor of PI3K/AKT signaling [20], the role of phosphatases that dephosphorylate the 5-phosphate position, such as INPP5s and SHIP1/2, in modulating this pathway is less clear [21].

AKT has been the first discovered *bona fide* effector of class I PI3K. Although the three highly homologous isoforms, with both redundant and specific functions, Akt1 (PKBα), Akt2 (PKBβ) and Akt3 (PKBγ) are encoded by distinct genes [22], they share more than 80% protein identity and a modular architecture with an N-terminal Pleckstrin Homology (PH) and a C-terminal regulatory domain flanking a central catalytic core [12,23]. AKT can undergo a number of post-translational modification, such as acetylation, methylation and glycosylation [1,23–25], however the above mentioned PI3K-dependent phosphorylations by PDK1 at T308 and by mTORC2 at S473 remain rate-limiting for complete activation. It should be noted that PDK1 and mTORC2 can, in parallel, phosphorylate other A-G-C kinases at similar motifs, such as the serum and glucocorticoid-regulated kinase SGK, with the exception of p70S6 kinase that is phosphorylated by mTORC1 [1].

Active AKT phosphorylates serine or threonine residues in the R-x-x-R-x-S/T-φ motif (where X is any amino acid while φ indicates hydrophobic residues) [26]. A strikingly high number of AKT substrates containing this motif have been identified over the years [1,27–29] and many of them were confirmed *in vivo* [30]. Directing its activity towards a plethora of substrates, AKT can carry out very different functions in the cells. Although phosphorylation can either activate or inactivate AKT substrates, inactivation is more common. In the canonical axis, AKT relays the signal downstream to mTORC1. Then, this kinase can activate key downstream members of the pathway such as 4EBP1 and p70S6K. The complexity of the interconnections among the members of the PI3K signaling pathway is exemplified by the AKT cross-talk with mTOR. mTORC1 activity is controlled by the tuberous sclerosis complex, TSC, formed by the heterodimer TSC1 and TSC2, and by the Proline-rich AKT Substrate of 40 kDa (PRAS40). TSC2 contains a GTPase-activating protein (GAP) domain able to activate the Ras homolog enriched in brain small G-protein (Rheb). Once converted in its GDP-bound form, Rheb becomes inactive. Phosphorylation of TSC2 by AKT at multiple sites triggers its GAP function and inactivates Rheb. Because Rheb is a strong activator of mTORC1, through TSC2 phosphorylation AKT impairs Rheb control over mTORC1 ultimately enhancing its activity [31]. Regulation of mTORC1 by AKT entails also phosphorylation of the mTORC1 inhibitor protein PRAS40 at T246 [1], which in turn dissociates from mTORC1, thus releasing inhibition and favoring access of mTORC1 to substrates. However, while loss of the TSC2/Rheb leads to full AKT-independent activation of mTORC1, loss of PRAS40 yields only partial mTORC1 activation [1].

The first AKT substrate to be identified was the glycogen synthase kinase 3 (GSK3) [32], a Ser/Thr kinase encoded by two paralogous genes. GSK3α (51 kDa) and GSK3β (47 kDa) are highly similar in terms of protein sequence (98% identity in the kinase domain) and possess largely redundant functions, with the exception of neural tissue and, limited to GSK3β, some type of cancer stem cells. GSK3 is a phosphate-directed hierarchical kinase, even though it can also phosphorylate substrates in a proline-directed manner [30,33]. Opposite to AKT, GSK3 is usually active in the absence of a PI3K/AKT-driven stimulus. However, phosphorylation at S21/S9 of GSK3α/β induces the binding of the substrate priming phospho-residue leading to a pseudosubstrate structure that precludes phosphorylation of the consensus motif by the catalytic site on the primed substrate [34,35]. While for some time GSK3 has been regarded as a key enzyme only in metabolism and metabolic disorders, its role has been now widely expanded, and its druggability for cancer therapy is under investigation [36].

GSK3-mediated phosphorylation is recognized by specific E3 ubiquitin ligases that enable proteasome-mediated degradation of GSK3 substrates. GSK3 substrates include the pro survival BCL-2 family member MCL-1 and c-Myc. Thus, these proteins are stabilized following AKT activation and phosphorylation of GSK3.

Regulation of cell proliferation by GSK3 can be achieved through a key substrate, namely β-catenin, the central effector of the canonical Wnt pathway. In quiescent cells, β-catenin undergoes phosphorylation by CK1 at S45, acting as priming site, and by GSK3 at S41, S37 and S33, followed by ubiquitination and proteasomal degradation [37–40]. Conversely, inhibition of GSK3β results in β-catenin stabilization and nuclear relocation. In the nucleus, β-catenin interacts with the Tcf/Lef family of transcription factors, which in turn transactivate genes involved in proliferation, apoptosis, and invasion and can therefore impact cancer progression [41–45]. Importantly, however, while

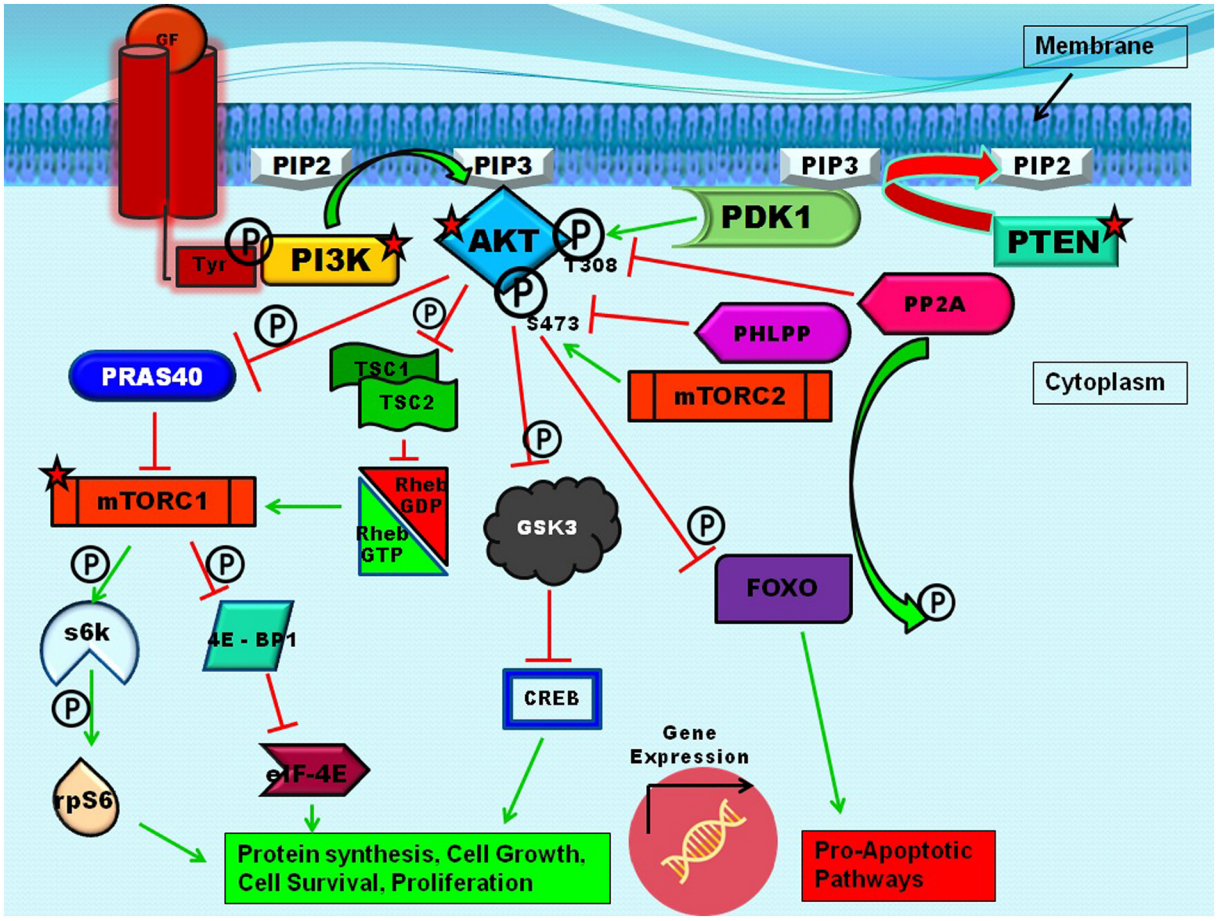

Fig. 1. Overview of the PI3K-AKT/mTOR signaling axis. Red star indicates proteins with common mutation in cancer. For details, see the text. Abbreviations: mTORC1, mechanistic target of rapamycin complex 1; mTORC2, mechanistic target of rapamycin complex 2; PDK1, phosphoinositide-dependent kinase 1; PI3K, phosphatidylinositol 3-kinase; PRAS40, Proline-rich AKT substrate of 40 kDa, FoxO, Forkhead Box O; GSK3, glycogen synthase kinase; 4E-BP1, eIF4E-binding protein; p70S6K1, 70-kDa ribosomal S6 kinase 1; rpS6, S6 ribosomal protein; TSC1, tuberous sclerosis 1; TSC2, tuberous sclerosis 2. PTEN, phosphatase and tensin homolog chromosome 10; PP2A, protein phosphatase 2; PHLPP, PH domain Leucine-rich repeat Protein Phosphatase; CREB, cyclic-AMP response element binding protein; PIP2, phosphatidylinositol-4,5-bisphosphate; PIP3, phosphatidylinositol-3,4,5-triphosphate; GF, growth factor; AKT, v-akt murine thymoma viral oncogene homolog; Rheb, Ras homolog enriched in brain.

phosphorylation at Ser21/Ser9 by AKT inhibits GSK3, such inactivation does not always affect β-catenin levels in the cell nor does completely inhibit GSK3. Consequently modulation of GSK3 in the WNT/β-catenin axis seems to follow a mechanism parallel but distinct from that acted by growth factor dependent AKT signaling.

Forkhead Box O transcription factors (FoxO1, 3, 4 and 6) represent crucial substrates of AKT. Phosphorylation of three conserved residues creates a recognition motif for 14-3-3 proteins, which in turn sequester FoxO proteins in the cytosol. This prevents FoxOs from entering the nucleus and transactivate target genes involved in apoptosis, such as Bim and Puma, in cell-cycle arrest, such as p21 and p27, in catabolism and growth inhibition such as Sestrin3, MAP1LC3B and BNIP3, and tissue specific metabolic changes, such as PEPCK and G6PC [46].

1.2. PTEN

Termination of AKT signaling is primarily achieved through dephosphorylation of Thr308 by protein phosphatase 2 (PP2A), and Ser473 by PH domain Leucine-rich repeat Protein Phosphatase (PHLPP1/2) [47]. AKT activity is also buffered by the dual lipid and protein phosphatase PTEN, a tumor suppressor gene mapping to chromosomal region 10q23. PTEN decreases PIP3 cellular content by dephosphorylation of the 3′-position of PIP3, ultimately terminating AKT activity and downstream function.

Besides its role as a negative regulator of PI3K signaling, however, it should be reminded that PTEN maintains also some lipid phosphatase-unrelated functions [48]: through direct dephosphorylation of FAK, SHC and Fyn kinases indeed PTEN can impact cell migration [49,50].

Fine tuning of PTEN activity is achieved through both translational and post-translational regulation. Several positive or negative regulators of PTEN transcription have been reported, such as p53, IGF-2-stimulated EGR1 and PPARγ [51], while SNAIL, c-Jun and NF-kB are negative regulators of PTEN transcription [50,52–54]. Moreover, at a post-transcriptional level its expression can be modulated by miR-25 downstream of the PI3K/AKT and the MEK/ERK pathways [53], as well as by MYC, which downregulates PTEN through the miR-19, besides the frequently upregulated miR-21 [48]. Moreover, miR-153 has been shown to enhance cell proliferation of human prostate cancer cells *via* downmodulation of PTEN expression [55].

It is also important to emphasize that the PTEN pseudogene 1 (PTENP1) displays decoy functions towards miR targeting PTEN, therefore stabilizing PTEN mRNA and increasing its protein levels [56].

At a post-translational level, PTEN can undergo phosphorylation as well as acetylation, oxidation and ubiquitination. Of peculiar importance is the phosphorylation of a cluster of residues in the C-terminal tail because they stabilize PTEN protein while hampering its lipid phosphatase activity.

Interestingly, the phosphatase PHLPP1, which dephosphorylates

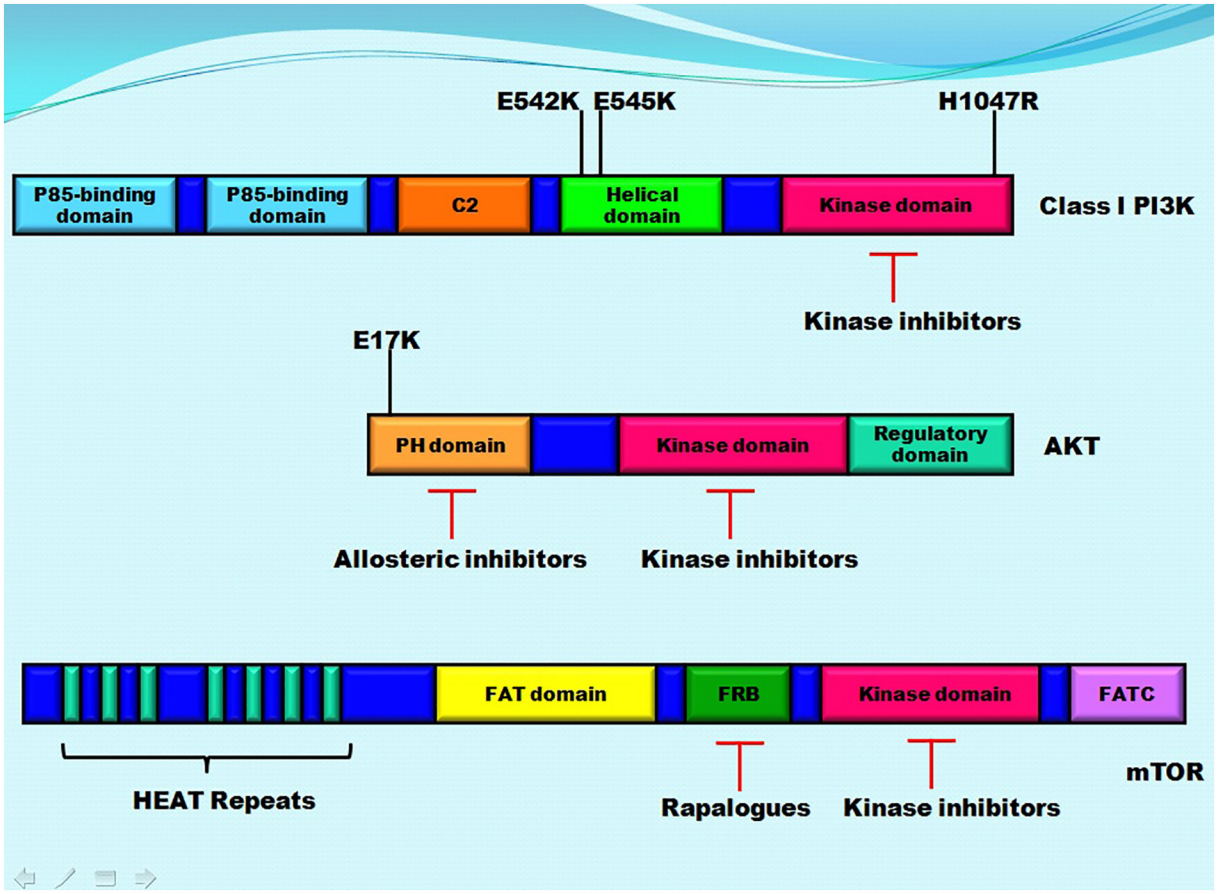

Fig. 2. Schematic diagram depicting frequent mutations of PIK3CA, AKT and PTEN. For details, see the text. Abbreviations: PI3K, phosphatidylinositol 3-kinase; PTEN, phosphatase and tensin homolog chromosome 10; AKT, v-akt murine thymoma viral oncogene homolog.

AKT S473, has been reported to act as a prostate tumor suppressor, while PHLPP1-loss induces neoplasia prostate carcinoma in mice with partial PTEN deletion [57]. In line with this report, our group has recently highlighted the existence of an AKT-driven PTEN-PHLPP cross-regulation circuit in epithelial prostate cells [58].

2. PTEN/PI3K/AKT/mTOR signaling and prostate cancer

Aberrantly active PI3K signaling has been described in 40% early diagnosed and more than 70% advanced prostate cancer disease [59–62]. PTEN loss occurs in approximately 30% of primary and 60% of castration-resistant prostate tumors, is associated with high Gleason score and is regarded as a key feature in hormone-naïve and castration-resistant prostate cancer [61]. Accordingly, activation of signaling downstream of PI3K correlates with resistance to androgen ablative treatment, progression to hormone refractory disease and poor outcome [62].

In line with the importance of this pathway in cancer, since the 1990s a large number of small molecule inhibitors of AKT, PI3K and/or mTOR are being developed, which target either single or multiple kinases. The trailblazer compounds wortmannin and LY294002, the first PI3K inhibitors, have been revolutionary tools to decoding the complexity of the PI3K pathway since their discovery in the early '90s [1]. Wortmannin covalently binds the ATP-binding site of p110 catalytic subunit, resulting in irreversible inhibition of PI3K. LY294002, like the majority of the other inhibitors, is a reversible ATP-competitive synthetic compound that displaces ATP from the ATP-binding pocket. Although both molecules were very helpful in *in vitro* studies, their toxicity was far too high for clinical use. Based on the structure of these two compounds and thanks to the crystal structures of the catalytic p110 isoforms, many highly selective small molecules have been generated. They are ATP-competitive pan-PI3K inhibitors or compounds selectively targeting one or two isoforms with activity in low nanomolar range, and many have entered clinical trial for cancer. The clinical efficacy of pan isoform-inhibitors has been modest despite high toxicity, possibly because of concurrent inhibition of all isoforms. Studies on the diverse tissue localization of p110s and on the phenotypes resultant from isoform-specific genetic alteration suggested different biological functions of each PI3K isoform, driving the efforts towards the synthesis of isoform-specific inhibitors [63]. In particular, the efficacy of specific PI3Kα and β inhibitors in prostate cancer will be discussed below.

Similarly, several generations of mTOR inhibitors have become available in the last decades and all of them have been extensively investigated both at preclinical and clinical level. They comprise both ATP-pocket and allosteric mTOR binding compounds. The bacteria-derived macrolide rapamycin is an allosteric inhibitor displaying selectivity for mTORC1, although it can inhibit also mTORC2 upon long-term exposure. Rapamycin and its analogs, the so-called rapalogs, RAD001/everolimus, and CC1-779/temsirolimus, form a complex with FKBP12 which in turn binds mTORC1. Decreased phosphorylation of mTORC1 substrates, especially p70S6K and to a lesser extent 4EBP1, is the readout of inhibition. RAD001/everolimus and CC1-779/temsirolimus are FDA and EMA approved [63]. Several ATP-competitive mTOR inhibitors blocking both mTORC1 and mTORC2, or DNA-PK/mTOR, such as vistusertib and sapanisertib, are in Phase II oncological clinical trial [63,64]. To overcome rapamycin resistance, a new generation of bivalent mTOR inhibitors, the so-called rapalink, are being developed combining rapamycin with an ATP-binding inhibitor of mTOR, namely MNL0128. Although of high interest, this drug has not been evaluated in prostate cancer yet [65].

Most AKT inhibitors bind to the ATP site of AKT. However, allosteric inhibitors have also been available, that mimic the allosteric regulation of AKT activity by PI(3,4,5)P3 binding to the PH domain. The alkylphospholipid perifosine, a lipophilic choline analogue that prevents the translocation of AKT to the plasma membrane and its subsequent phosphorylation, has been the first of this class of compounds tested *in vitro* and *in vivo*. Unfortunately, despite promising results from preclinical studies, no study of perifosine as monotherapy demonstrated significant efficacy for a wide range of tumor types. Thus, in the following paragraph we will restrict our attention to the drugs that have shown promising effects in preclinical models of prostate cancer and entered clinical trials.

2.1. PTEN/PI3K and PI3K/mTOR deregulation and therapeutic targeting in prostate cancer

Gene amplifications, deletions, insertions and missense mutations are mainly present in PIK3CA encoding Class IA p110α catalytic subunit [9]. Up to 4% of prostate cancer cases carry PIK3CA mutations and 66% carry PIK3CA copy number gain/amplification [66]. Two major hotspots mutations are exon 9 E542K and E545K in the helical domain and exon 20 H1047R in the kinase domain (Fig. 2) [66]. In particular, recent findings demonstrated that PIK3CA H1047 induce invasive prostate cancer *in vivo* and can therefore be regarded as a genetic driver of prostate cancer [66]. Although mutations of PIK3CB have not been described, PI3Kβ is tightly linked to tumorigenesis because of a complex PI3K isoform cross-regulation. Although PI3Kα is indeed the dominant isoform in prostate epithelial cells, deletion of PTEN is reported to stimulate oncogenic signaling and tumor growth through PI3Kβ [67–69]. In good agreement with the above observations, in a PTEN-deficient mouse model PI3Kβ was shown to be essential for prostate tumor growth [70]. These studies indicated the existence of a therapeutic window to target PI3K with isoform-specific inhibitors, such as GSK2636771, SAR260301, and AZD8186 [67]. However, the data that followed were not up to expectations. Indeed, the block of AKT and mTOR activity downstream to PI3Kβ inhibition triggered a rebound activation of PI3Kα, most probably *via* a FoxO and/or p70S6K/rpS6 upregulation of IR/IGF1R [71]. Conversely, drugs targeting both PI3K isoforms abrogated downstream signaling and reduced tumor growth. The efficacy was limited to mouse models of prostate cancer expressing wild-type PTEN, tough [71]. Recently this feature was analyzed further in mice carrying prostate specific biallelic PTEN-deletion/PIK3CA-H1047R *versus* matched controls carrying only PTEN-deletion [66]. These elegant study models allowed to investigate whether PTEN expression can affect PI3K activity through modulation of isoform-specific upstream activators, namely RAS for PI3Kα and RAC1 for PI3Kβ. Sustained activation of RAS/pERK was observed both in mice with coexisting PTEN-deletion/PIK3CA-H1047R mutation and in mice carrying only PTEN deletion. Conversely, high level of RAC1 activity was detectable only in PTEN-null/wild-type PIK3CA, suggesting that persistent activation of RAC1/PI3Kβ can account for cancer progression in this model. Considering the scarce efficacy of drugs blocking PI3Kβ, the authors of this study suggested inactivation of RAC in combination with PI3Kβ inhibitors as a therapeutic strategy for PTEN-deleted patients [66].

Alternative strategies that underwent thorough investigation are represented by inhibition of PI3Kα/β or PI3K/mTOR. In experiments performed *in vitro* in PTEN-null PC3 and BT549 cell lines, combined inhibition of PI3Kβ by AZD8186 and of mTOR by vistusertib effectively prevented reactivation of signaling, as shown by very low AKT and rpS6 phosphorylation even after long-term exposure, whereas treatment with each drug as monotherapy failed to control reactivation [67]. Furthermore, this drug combination achieved similar results in HCC70 and PC3 tumor xenografts, although *in vivo* downregulation of rpS6 phosphorylation was less effective [67].

Moreover, BKM120, an oral pan PI3K reversible inhibitor, and the dual PI3K/mTOR inhibitors NVP-BEZ235 (ATP-competitive) and PP242 (mTOR allosteric) were claimed to possess antitumor activity in *in vitro* and *in vivo* preclinical studies [72], thus confirming that hyperactivation of this pathway is involved not only in the onset but also in the progression of the disease [70–73]. In particular, in PTEN-null mice models of high-grade prostatic intraepithelial neoplasia, administration up to eight weeks of either BKM120 or BEZ235 induced dramatic reduction of tumor growth and apoptosis, and reversal of the above-mentioned phenotype, supporting the rationale for PI3K targeted therapy in the clinic. As a readout of PI3K and/or mTOR inhibition, prolonged dephosphorylation of AKT and rpS6 were monitored. Efficacy of BEZ235 was tested also in a more severe model of *PTEN*-null castration-resistant prostate cancer. Unexpectedly, this model was less sensitive to BEZ235. The explanation suggested by the authors is that concomitant hyperactivation of PI3K, MAPK and AR signaling conferred resistance to therapy [74]. Nevertheless, association of BEZ235 with the MAPK inhibitor AZD6224 effectively circumvented resistance, leading to the conclusion that patients with PTEN-deleted prostate tumors can benefit from drugs blocking PI3K signaling [74].

Regrettably, hitherto administration to patients of either BEZ235 or BKM120 as monotherapy has been limited by excessive toxicity and, in the case of BKM120, off-target effects due to tubulin-binding. In non-solid tumors [75], the novel dual PI3K/mTOR PQR 309 has been proposed as a substitute for BKM120. In preclinical studies. PQR 309 showed promising results and is currently in phase II for lymphoma, but no data are available about its efficacy in prostate cancer yet.

Because of the reciprocal control described above between the PI3K and the AR signaling, recently the administration of BKM120 together with the association prednisone + abiraterone acetate, a potent inhibitor of androgen synthesis which blocks AR-dependent transcriptional activity and is currently used to treat advanced castration-resistant prostate cancer, was investigated [76]. Even though initial results were promising, this trial was terminated because of slow accrual (NCT01741753).

The association of BEZ 235 to abiraterone was tested in a rat model of androgen-dependent prostate cancer. The results showed that in rats this drug combination impaired the inflammatory response and abrogated disease progression from premalignant to malignant stage [77]. Moreover, the efficacy of BEZ235 in association with abiraterone acetate has been assessed in castration-resistant patients in a phase 1b clinical trial. In this trial, the combination was poorly tolerated, possibly because of excessive pathway inhibition leading to on-target and off-target negative effects [78]. A second study in men affected by castration-resistant prostate cancer trialled both BEZ235 and BKM120 [79]. This study failed due to low efficacy in spite of high toxicity. In patients with the same phenotype, BKM120 in a phase II trial as monotherapy or in combination with the second generation AR inhibitor enzalutamide, was also not satisfactory due to poor tolerance [80]. A recent preclinical study explored the effect of the dual PI3K/mTOR inhibitor X480 on the bone metastases formation, osteoclast activation, and osteoblast function *in vitro* and in a xenograft model of prostate cancer bone metastases. Interestingly, blockade of PI3K/mTOR reduced tumor growth in primary and bone metastatic sites, and significantly prolonged survival in animal models of prostate cancer [81].

2.2. PTEN

The already delicate balance described above has been complicated further by the discovery of an intricate cross-talk between PTEN-sustained PI3K signaling and the AR. PTEN inhibits phosphorylation of AKT which, in turn, stimulates AR phosphorylation and activity in prostate cancers. In addition, PTEN also interacts directly with the AR DNA-binding domain/Hinge domain and inhibits AR nuclear translocation and AR-mediated transcriptional activity in prostate cancer cells [82,84]. By means of an *in silico* analysis of microarray data the existence of the reverse mechanism, whereby AR regulates PTEN, has also

been uncovered. AR association with the PTEN promoter indeed represses PTEN transcription (opposite to breast cancer where AR binding to the PTEN promoter enhances its expression). Such a mechanism of direct regulation of PTEN thus revealed that AR can drive PI3K/AKT signaling in prostate cancer [85].

In prostate stem cells, PTEN loss triggered cell growth, de-differentiation, and evolution to intraepithelial neoplasia [83–86]. While PTEN null mice are embryonic lethal, PTEN+/− mice display high incidence of prostate cancer, particularly in presence of other genetic alterations. [87–90].

In vivo a similar result was observed following prostate-specific biallelic deletion of PTEN [91]. Moreover, the incidence of tumorigenesis in PTEN−/− transgenic mice was decreased by coexisting knockout of mTOR [92], as well as of the mTORC2 member RICTOR [93]. Guertin and colleagues have previously shown that RICTOR is required for the PTEN-null human PC3 prostate cancer cells to form tumor xenografts, and that bi-allelic deletion of RICTOR prevents prostate cancer formation driven by PTEN loss in mice by reducing proliferation and AKT phosphorylation at Ser473 [93].

Preclinical studies pointed out that dual inhibition of PI3K and mTOR by drugs such as BEZ235 in prostate-specific PTEN-null mice was able to decrease the size of the tumor. However, BEZ235 was poorly tolerated in patients with concomitant PTEN loss and castration-resistance [78]. This failure might be explained in part by both off-target effects, that can limit efficacy, and by on-target toxicity. It is worth to remind that PI3K indeed controls many cellular functions, thus its inhibition can cause severe damage, hyperglycemia among others [94]. Moreover, as already pointed out, in cancer cells excessive inhibition of mTOR can relieve physiological negative feedback regulation thereby activating AKT and therefore limiting drug efficacy [71,95].

The dissection of these feedbacks is extremely complex. A key effector of AKT is the transcription factor FoxO 3a, whose phosphorylation precludes its entry into the nucleus and transactivation of genes such as those encoding PI3K physiologic activators IR and IGF-1R [96–98]. Moreover, phosphorylation of PRAS40 and TSC2 by AKT sequentially activates mTOR, which acts on p70S6K-rpS6 to trigger IRS1/2 degradation while increasing Grb10 stability (Fig. 3). Therefore active AKT hinders both expression and stability of PI3K key activators while promoting stabilization of PI3K inhibitor Grab10. By releasing such tight control, AKT inhibition triggers PI3K signaling and ultimately restrain drug efficacy. [71,95]. Because of this paradoxical effect, subsequent work focused on drug combinations designed to block adaptation evoked by unsettling compensatory mechanisms. In particular, in PTEN-null prostate cancer where selective targeting of PI3Kβ produced only transient inhibition of downstream signaling, as described above, addition to the therapy of inhibitors of PI3K-activating receptor tyrosine kinases, such as IGF1R/IR, interrupts the circuit [71]. Furthermore, since these receptors selectively activate the α isoform this explains why this is the dominant isoform in prostate cancer, and explains also why PI3Kα/β inhibition displayed higher efficacy than PI3Kβ inhibition alone. Unfortunately this association showed excessive on-target toxicity [71].

2.3. AKT

Being the main effector of PI3K, AKT represents a promising target also in prostate cancer therapy. Hyperphosphorylation of AKT correlates positively with high Gleason grade and poor survival rate of castration-resistant prostate cancer patients [99]. Constitutive downstream signaling triggered by ectopic expression of AKT in prostate epithelial cells has been shown to elicit prostate cancer [100]. It is therefore not surprising that AKT has been targeted with both allosteric and ATP-competitive inhibitors also in prostate cancer. In cell lines the allosteric inhibitor perifosine abrogated AKT S473 phosphorylation and slowed proliferation while increasing apoptosis. However, this drug was abandoned because of modest clinical activity as monotherapy in a phase II trial [101,102].

On the other hand, the pan, ATP-competitive inhibitor AZD5363 potently blocked proliferation and induced apoptosis not only in cell lines but also in an LNCaP xenograft model [103]. In a very recent phase I/II trial AZD5363 combined to enzalutamide in the same clinical setting mentioned above showed good response and high safety and

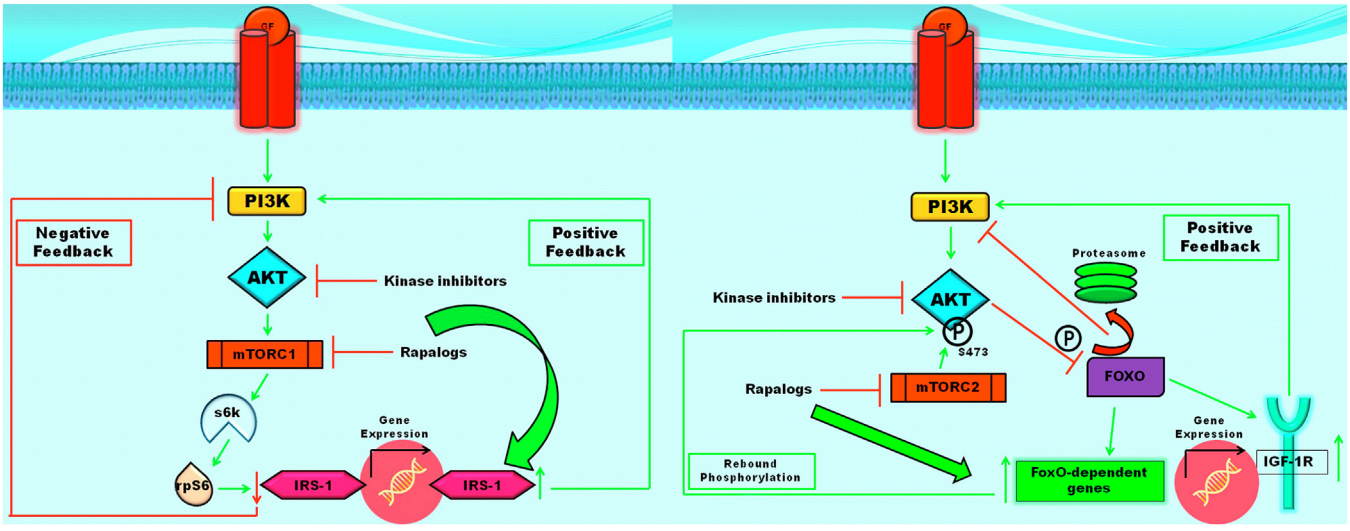

Fig. 3. Feedback regulation of the PI3K pathway. In the canonical pathway, upon activation by membrane receptor (RTK and GPCR) PI3K relays the signal to its intracellular downstream effectors, such as AKT. Signal intensity is controlled by positive and negative feedback loops. Negative feedbacks attenuates signaling in cancer cells with hyperactivation of PI3K: activated AKT phosphorylates its substrate FoxO priming it for degradation and thus limiting transactivation of FoxO-driven genes, such as the PI3K activator IGF1-R. In the same context, the mTORC1-p70S6K-S6RP axis downregulates IRS-1. As a result, both mechanisms attenuate PI3K activity. However, prolonged inhibition of mTORC1 by rapalogs or of AKT relieves the feedback inhibition upon IRS-1 and triggers rebound activation of PI3K signaling. Furthermore, sustained inhibition of AKT or mTORC2 strongly inactivates AKT resulting in stabilization of FoxO and in turn transactivation of FoxO-dependent genes, inducing rebound phosphorylation of AKT S473 and PI3K signaling which can lead to resistance. Abbreviations: GPCR, G-protein-coupled receptor; RTK, receptor tyrosine kinase; PI3K, phosphoinositide 3-kinase; mTORC1, mechanistic target of rapamycin complex 1; mTORC2, mechanistic target of rapamycin complex 2; FoxO, Forkhead Box O; p70S6K1, 70-kDa ribosomal S6 kinase 1; S6RP, S6 ribosomal protein; IS-1, insulin receptor substrate 1; AKT, v-akt murine thymoma viral oncogene homolog.

tolerability (NCT02525068). Remarkably, enzalutamide did affect AKT activity, as monitored by the phosphorylation of AKT well-known substrates GSK3β and PRAS40, indicating that combined blockade of AKT and AR can be a new therapeutic strategy in patients characterized by PTEN loss and castrate-resistant prostate cancer [104]. It is however worth noting that, although well tolerated, AZD5363 can interfere with glucose metabolism causing hyperglycemia.

Preclinical work showed that the ATP-competitive AKT inhibitor GDC-0980/ipatasertib abrogates AKT downstream signaling activation. Moreover, this compound displayed dose-dependent inhibition of tumor growth in xenograft models of PTEN-deficient prostate cancer [105]. Interestingly, this drug was also well tolerated as monotherapy in a phase I study [105]. On this premises, recently an international, multicenter, randomized phase study II of abiraterone acetate investigated the efficacy of the association of abiraterone with GDC-0068 in metastatic prostate cancer patients with wild-type PTEN *versus* PTEN loss. The results of this trial were extremely encouraging: extension of progression free survival was observed in the abiraterone plus ipatasertib cohort *versus* abiraterone alone, that was further increased in the patient population carrying PTEN loss. Importantly, the combination was also well tolerated [106]. On this basis, a randomized phase III trial is in progress to evaluate GDC-0068 together with abiraterone and prednisone in patients with metastatic castration-resistant prostate cancer that did not receive previous treatment (NCT03072238).

3. Conclusion and perspectives

Although the PI3K/AKT/mTOR pathway has been long recognized as one of the three major signaling pathways in cancer, central to cancer cell growth, survival, and proliferation, and considerable effort has been dedicated to the design and preclinical and clinical studies of specific inhibitors, its ever-growing complexity is constantly humbling the struggle to devise effective therapeutic targeting. Indeed, despite the availability of a number of potent and selective compounds, the road to PI3K signaling therapeutic targeting in cancer has been discouraging, as most compounds did not reach phase II. Preclinical studies in the last decade have unraveled the root causes of this failure providing a rationale for the design of the next generation drugs. Indeed, while the PI3K/AKT/mTOR pathway is often outlined as a linear cascade where each member relays the signal to a downstream effector, it is now clear that frequent network branching and intense cross-talk with other signaling circuits can override the effect of drugs-mediated inhibition and restore paradoxical active signaling through a number of mechanisms. These include, but are not limited to, up-regulation of receptor tyrosine kinases [107,108], excessive inhibition, signaling redundancies, activating point mutations in PI3K such as the PIK3CA H1047RK, loss of function deletions of PTEN, disruption of negative feedback loops limiting the efficacy of the therapy, such as the rapamycin-induced activation of AKT [109]. As a result, it is not surprising that the clinical outcome of drugs targeting PI3K signaling as monotherapy in prostate cancer has been thus far disappointing. Despite the availability of many allosteric or active-site inhibitors, pan- or isoform-specific and also dual kinase inhibitors, there are indeed only few drugs FDA and EMA approved, none of which for prostate cancer.

Conversely, combination with other compounds appears a more promising strategy, although the addition of drugs with similar adverse effects can lead to excessive toxicity, as in the case of PI3K and mTOR inhibitors association with conventional chemotherapy.

However, very encouraging results have been recently obtained in a phase Ib/II study trialling the combination of the ATP-active site AKT inhibitor GDC-0068/ipatasertib with the androgen synthesis inhibitor abiraterone acetate in metastatic castration-resistant prostate cancer [106]. These patients are characterized by constitutive activation of PI3K/AKT/mTOR and AR signaling, associated with very poor prognosis. Although this trial produced overall good results, slowing of disease progression and treatment tolerability were particularly positive in patients with loss of PTEN, paving the way for a new randomized phase III study in PTEN-null castration-resistant prostate cancer.

Although the results of the abovementioned phase III clinical trial are not yet available, the association of GDC-0068/ipatasertib to abiraterone acetate could turn out a landmark study for the therapy not only of PTEN-null castration-resistant prostate cancer patients, but also for treatment of early-stage prostate cancer patients with the same genetic background.

Therefore, to characterize the genetic background as well as the signaling circuitry is mandatory for the appropriate design of therapeutic intervention in prostate cancer.

CRediT authorship contribution statement

Luca Braglia: Conceptualization, Writing - original draft. **Manuela Zavatti:** Data curation. **Marco Vinceti:** Supervision. **Alberto M. Martelli:** Supervision. **Sandra Marmiroli:** Conceptualization, Writing - review & editing.

Declaration of competing interest

The authors declare no financial or commercial conflict of interest.

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