Pharmacological Research 156 (2020) 104806

Contents lists available at ScienceDirect

Pharmacological Research

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

Akt-targeted therapy as a promising strategy to overcome drug resistance in breast cancer – A comprehensive review from chemotherapy to immunotherapy

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ARTICLE INFO

Keywords:
Akt
Protein kinase B
Breast cancer
Drug resistance
Targeted therapy
Hypoxic conditions

ABSTRACT

Breast cancer is the most frequently occurring cancer in women. Chemotherapy in combination with immunotherapy has been used to treat breast cancer. Atezolizumab targeting the protein programmed cell death-ligand (PD-L1) in combination with paclitaxel was recently approved by the Food and Drug Administration (FDA) for Triple-Negative Breast Cancer (TNBC), the most incurable type of breast cancer. However, the use of such drugs is restricted by genotype and is effective only for those TNBC patients expressing PD-L1. In addition, resistance to chemotherapy with drugs such as lapatinib, geftinib, and tamoxifen can develop. In this review, we address chemoresistance in breast cancer and discuss Akt as the master regulator of drug resistance and several oncogenic mechanisms in breast cancer. Akt not only directly interacts with the mitogen-activated protein (MAP) kinase signaling pathway to affect

Abbreviations: ABC, ATP-binding cassette; ADR, Adriamycin; AI, Aromatase inhibitor; ARE, Anti-oxidant response element; BAX, BCL-2-associated X protein; BBB, Blood-brain barrier; BCSC, Breast cancer stem cell; BRD4, Bromodomain-containing protein 4; BRCA1, Breast cancer tumor suppressor 1; CaM, Calcium-dependent calmodulin; CBP, cAMP response element-binding protein (CREB)-binding protein; CCN5, Cysteine rich 61/connective tissue growth factor; CHP2, Calcineurin B homologous protein 2; CIP2A, Cancerous inhibitor of PP2A; c-Jun, AP-1 transcription factor subunit; CSC, Cancer stem cell; CSN, COP9 signalosome complex; DNMT, DNA methyl transferase; DUBs, Deubiquitinating enzymes; EGFR, Epidermal growth factor receptor; EMT, Epithelial to mesenchymal transition; ER, Estrogen receptor; ESR, Estrogen receptor gene; ETC, Electron transport chain; FIH-1, Factor-inhibiting HIF-1; FoxO1/3, Forkhead box O1/3; G-6-P, Glucose-6-phosphate; GNRHR, Gonadotropin-releasing hormone (GnRH) receptor; GRP75, Glucose-regulated protein 75 chaperone; GSK-3, Glycogen synthase kinase 3; HAT, Histone acetyl transferase; Her2, Human epidermal growth factor receptor 2; HDAC, Histone deacetylase; HIF-1α, Hypoxia-inducible factor-1α; HK2, Hexokinase 2; HM, Hydrophobic motif; HO-1, Heme oxygenase-1; HRE, Hypoxia-responsive element; HSF1, Heat shock factor 1; HSP90, Heat shock protein 90; I2PP2A, Oncogenic inhibitor of PP2A (SET); IGF-1, Insulin-like growth factor 1; IKK, Inhibitor of NF-κB kinase; IP3R, Inositol trisphosphate receptor; IRF1, Interferon regulatory factor 1; JAK, Janus Kinase; KD, Kinase domain; Keap1, Kelch-like ECH-associated protein 1; KLF5, Kruppel like factor 5; LC3, Microtubule-associated protein 1A/1B-light chain 3; LDHA, Lactate dehydrogenase A subunit; MAM, Mitochondria-associated endoplasmic reticulum membranes; MEK, Mitogen-activated protein kinase kinase; MRP1, Multidrug resistance protein 1; mTORC1, Mammalian target of rapamycin complex 1; mTORC2, Rapamycin-insensitive companion of mTOR complex; NADD4, Neural precursor cell expressed developmentally down-regulated protein 4; NHE1, Na^+/H^+ exchanger; NF-κB, Nuclear factor kappa-light-chain; NQO1, NAD(P)H quinone dehydrogenase 1; Nrf2, Nuclear factor erythroid 2-related factor 2; PAK1, p21 (RAC1) activated kinase 1; PD-L1, Protein programmed cell death-ligand; PDH, Pyruvate dehydrogenase; PDK1, Pyruvate dehydrogenase kinase 1; PDPK1, 3-Phosphoinositide dependent protein kinase 1; PHD, Pleckstrin homology domain; PI3K, Phosphoinositide 3-kinase; PKB, Protein kinase B (Akt); PP2A, Protein phosphatase 2; PPARg, Peroxisome proliferator-activated receptor gamma (PPAR-γ); PTEN, Phosphatase and tensin homolog; Rictor, Rapamycin-insensitive companion of mTOR; RTK, Receptor tyrosine kinase; S6K, S6 ribosomal protein kinase; SALL2, Spalt-like transcription factor 2; SIRT1, NAD^+-dependent deacetylase sirtuin-1; Skp2, S-phase kinase associated protein 2; SOD, Superoxide dismutase; STAT, Signal transducer and activator of transcription; SUMO, Small Ubiquitin-like Modifier; TCA, Tricarboxylic acid cycle; TIM, Translocase of inner membrane; TKI, Tyrosine kinase inhibitor; TKT, Transketolase; TOM, Translocase of outer membrane; TRAF, Tumor necrosis factor (TNF) receptor associated factor; TRPC1, Transient receptor potential cation channel subfamily C (canonical) member 1; TRPM2, Transient receptor potential cation channel subfamily M member 2; UEV1A, Ubiquitin-conjugating enzyme E2 variant 1; USP18, Ubiquitin specific peptidase 18; VDAC, Voltage-dependent anion-selective channel; VHL, von Hippel–Lindau protein

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https://doi.org/10.1016/j.phrs.2020.104806
Received 27 March 2020; Received in revised form 4 April 2020; Accepted 5 April 2020
Available online 12 April 2020
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PD-L1 expression, but also has crosstalk with Notch and Wnt/β-catenin signaling pathways involved in cell migration and breast cancer stem cell integrity. In this review, we discuss the effects of tyrosine kinase inhibitors on Akt activation as well as the mechanism of Akt signaling in drug resistance. Akt also has a crucial role in mitochondrial metabolism and migrates into mitochondria to remodel breast cancer cell metabolism while also functioning in responses to hypoxic conditions. The Akt inhibitors ipatasertib, capivasertib, uprosertib, and MK-2206 not only suppress cancer cell proliferation and metastasis, but may also inhibit cytokine regulation and PD-L1 expression. Ipatasertib and uprosertib are undergoing clinical investigation to treat TNBC. Inhibition of Akt and its regulators can be used to control breast cancer progression and also immunosuppression, while discovery of additional compounds that target Akt and its modulators could provide solutions to resistance to chemotherapy and immunotherapy.

1. Introduction

The incidence of breast cancer has been increasing, particularly in young women [1]. Breast cancer is the most common cause of cancer-related mortality in women and is the fifth leading cause of deaths due to cancer [2]. Despite progress in reducing the mortality rate, many existing treatments benefit only a subset of breast cancer patients [3]. Breast cancer tumors that do not respond to hormone therapy and Her2-targeted therapy are termed triple-negative breast cancer (TNBC). TNBC has the poorest prognosis among breast cancer types and chemotherapy is still the only available treatment for these patients. In a cohort of 5903 breast cancer patients from Singapore comprising three major Asian ethnic groups of Chinese, Malay, and Indian, TNBC subtype accounted for 13 % of all breast cancers [4].

Akt is a key component of the phosphatidylinositol-3-kinase (PI3K)/Akt and mammalian target of rapamycin (mTOR) signaling pathway, which contributes to cellular signaling that regulates fundamental cellular processes including survival, proliferation and differentiation [5]. Akt activation is modulated by numerous upstream signaling proteins that then regulate multiple downstream effectors. Various receptors such as receptor tyrosine kinases (RTKs), G-protein coupled receptors (GPCRs), cytokine receptors, and integrin can trigger the PI3K/Akt pathway by recruiting the phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit α (PIK3CA) to phosphorylate PIP2, which generates PIP3. PIP3 then activates Akt to initiate a range of signaling events. Under normal conditions, the level of PIP3 is subject to tight regulation by phosphatase and tensin homolog (PTEN) that converts PIP3 back to PIP2 [6].

Chemotherapy including tyrosine kinase inhibitors (TKIs) and CDK4/6 inhibitors in combination with immune checkpoint inhibitors are now seen as promising therapies for TNBCs [7]. Recently, atezolizumab, a programmed death-ligand 1 (PD-L1) inhibitor, was approved for use in combination with paclitaxel to treat PD-L1^{+} TNBC patients [8]. However, resistance to paclitaxel has been shown to occur in TNBC

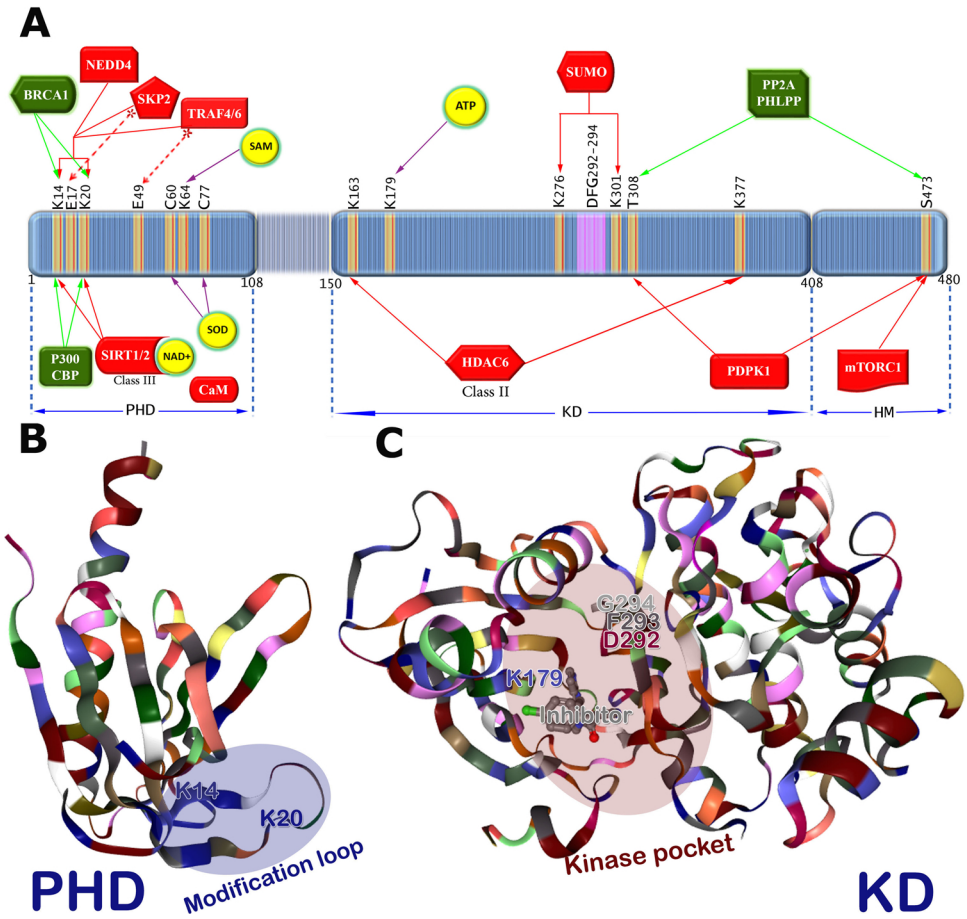

Fig. 1. Akt structure and crucial interactions. (A) Akt modifiers. Akt has three domains. Pleckstrin Homology Domain (PHD) binds PIP3 for recruitment of Akt to the plasma membrane. Histone acetylases (P300 and CBP) dissociate Akt from PIP3, while class III Histone Deacetylases (SIRT1/2) activate Akt for PIP3 binding. Class III Histone Deacetylases require NAD^{+} for activation. K63-linked ubiquitination is also required for plasma membrane localization whereas K48-linked ubiquitination leads to proteasomal degradation of Akt. E3 ligases, particularly Skp2 and TRAF4/6, are required for ubiquitination. BRCA1, a tumor suppressor, facilitates Akt degradation. Akt Kinase Domain (KD) is targeted by phosphoinositide-dependent protein kinase 1 (PDPK1) for phosphorylation at T308, and by SUMO and HDAC6 for further modifications. K179 interacts with ATP and Akt inhibitors. To activate Akt kinase, phosphorylation at T308 and S473 is required. PDPK1 phosphorylates both residues whereas mTORC2 targets S473 located in the Hydrophobic C-terminal regulatory Motif (HM). Phosphatases (PP2A and PHLPP) inactivate Akt. Calcium-dependent calmodulin (CaM) and ROS, which target PHD, are also involved in Akt activation. Akt is a cellular sensor that can sense oxidative stress and calcium levels through interactions with CaM and ROS, which are both necessary for Akt oncogenic activity. **(B)** Pleckstrin Homology Domain (PHD) interacts with the plasma membrane and is targeted by numerous protein modifiers. K14 and K20 located in the modification loop are important for PHD ubiquitination and deacetylation. **(C)** The kinase domain (KD) includes a DFG motif (residues 292-294) and upon assuming a suitable conformation allows K179 to bind ATP, the key substrate for kinase activity. Competitive Akt inhibitors can bind to this kinase pocket. Images are from the RCSB PDB (rcsb.org) [17] using PDB IDs 1UNR [18] and 3OCB [19] and were created using NGL viewer [20]. * Dotted arrows: Mutations in Akt: Mutations in E17 and E49 residues (E17 K and E49 K) lead to sustained activation of Akt via increased ubiquitination.

through overactivation of Akt signaling and hypoxic conditions [9–11]. On the other hand, interaction of PD-L1 and programmed death 1 (PD-1) causes resistance to chemotherapy through activation of PI3K/Akt and mitogen-activated protein kinase (MAPK) pathways, both of which are under regulation by RTKs [12]. Interestingly, a recent study reported that treatment of TNBC cells with lapatinib, a dual epidermal growth factor (EGF) receptor (EGFR) and Her2 inhibitor overactivated Akt (compared to untreated cells) and induced resistance to TKI [13]. Accordingly, there are conflicting opinions about the role of Akt, which makes selection of treatments for TNBC more challenging.

Therefore, there is an urgent need to highlight the challenges in treating breast cancer, especially TNBC, and to define the characteristics that contribute to the role of Akt as a master regulator of chemoresistance and immunotherapy. This review discusses the role of Akt-targeted therapy in breast cancer initiation and progression, as well as the mechanisms of resistance to therapy to examine the crucial therapeutic role of Akt inhibitors in combinatorial therapies for breast cancer, and the most incurable type of breast cancer, TNBC. We also extensively discuss how hypoxic conditions and resistance to immunotherapy impact Akt signaling.

2. Akt structure and regulators

Akt, or protein kinase B (PKB), is a serine/threonine kinase that describes three closely related proteins, termed Akt1, Akt2, and Akt3. Based on Ensembl (www.ensembl.org) assessment, the genes for Akt1, Akt2, and Akt3 are located at 14q32.33, 19q13.2, and 1q44 respectively. Akt isoforms are involved in gene transcription, protein synthesis, cell survival and proliferation, genome stability, glucose metabolism, and cytoskeletal organization [6]. However, the metabolic role of Akt3, which is restricted to only a few organs such as the brain and kidney, has not been well-recognized [14]. Although both Akt1 and Akt2 are ubiquitously expressed throughout the human body, Akt1 is particularly well-characterized in breast cancer cells [15]. Akt isoforms have conserved structure comprising three domains [16]: Pleckstrin homology domain (PHD), kinase domain (KD), and hydrophobic C-terminal regulatory motif (HM) (Fig. 1).

PHD is a 100 aa region located at the N terminus of Akt that interacts with PIP3 generated by the upstream kinase PIK3CA. PHD/PIP3 binding recruits Akt to the plasma membrane, which, in ER^- breast cancer cells, occurs via a mechanism that requires calcium-dependent calmodulin (CaM). CaM antagonists that inhibit Akt promote apoptosis of ER^- breast cancer cells [15]. PHD contains seven N-terminal β-sheets (β1-β7) and one C-terminal helix. Recent studies showed that, in contrast to our previous understanding of CaM function that involved interactions with helices of its substrate, the first 42 residues (β1-β3) of PHD in Akt interact with both CaM and PIP3 [16].

PHD also interacts with the KD to facilitate phosphorylation of T308 [18]. Full activation of Akt thus depends on its interaction with plasma membrane phospholipids after which phosphorylation of T308 (of the KD) and S473 (of the HM) are mediated by the phosphoinositide-dependent protein kinase 1 (PDPK1) and the mTOR Complex 2 (mTORC2), respectively [16,21]. In contrast, protein phosphatase 2A (PP2A) and pH domain leucine-rich repeat protein phosphatase (PHLPP) dephosphorylate T308 and S473, which lead to Akt deactivation [22,23].

To activate Akt for binding to substrates including glycogen synthase kinase (GSK)-3β, residues 150–408 of the Akt KD acquire a conformation that allows binding of ATP, the main substrate of all kinases. D292, F293, and G294 (DFG motif) play a role as part of the well-known DFG gate [24], which consists of a conserved trio of amino acid residues within most kinases. DFG conformation is important for kinase activation [25]. In the appropriate conformation, ATP binds to K179. There are two main categories of Akt inhibitors: ATP-competitive and allosteric, which target KD and PHD respectively [13,24]. Ipatasertib, an ATP-competitive inhibitor, is the only Akt inhibitor for TNBC that is under clinical investigation [26].

2.1. Akt post-translational modifications

2.1.1. Akt PH domain deacetylation

Some post-translational changes are required for Akt activation. Residues K14 and K20 within the Akt PHD are acetylated by various histone acetyl transferases (HATs) such as p300 and cAMP-response-element-binding protein (CREB) binding protein (CBP) when Akt is dissociated from membranes [27]. The NAD^+-dependent deacetylase sirtuin family (SIRT) is a group of seven proteins that can deacetylate both histone and non-histone proteins. To recruit Akt to membranes including the plasma membrane, deacetylation of K14 and K20 by SIRT1 and SIRT2 is required, particularly for regulation of Akt activation in breast cancer cells including TNBC cells [28,29]. Histone deacetylase (HDAC) 6 is also reported to be involved in deacetylation of Akt in human neural progenitor cells, likely by deacetylating K163 and K377 [21]. Acetylation of lysine residues in PHD promotes Akt binding to the plasma membrane, and to respond to PIP3, both PDPK1 and Akt must undergo SIRT-mediated deacetylation [30,31].

2.1.2. Akt: an oxidative stress-sensing oncoprotein

The association of cysteine oxidation with Akt signaling has also been shown in adipocytes. Su et al. (2019) identified C60 and C77 in the Akt PHD as an oxidation site that facilitates localization of Akt to the plasma membrane via PIP3 binding. Increased amounts of reactive oxygen species (ROS) promote Akt activation through the formation of disulfide bonds between C60 and C77 of Akt. They also showed that Akt T308 phosphorylation could be regulated by C77 oxidation [32]. On the other hand, as mentioned earlier, Akt activation depends on the NAD^+-dependent and Class III HDACs, SIRT1/2. NAD^+ is an oxidized by-product of mitochondria following inhibition of both the tricarboxylic acid (TCA) cycle and electron transport chain (ETC) [33]. Thus, Akt activation requires high levels of the reactive molecules ROS and NAD^+. Remarkably, Akt can sense oxidative stress of cancer cells in which Akt is highly activated and in response Akt may be transferred to mitochondria to activate anaerobic respiration.

2.1.3. Ubiquitination of the Akt PH domain

Ubiquitin is a protein that has two crucial lysine residues, K48 and K63. K48 tumor suppressor ubiquitination is associated with proteasomal degradation of substrate proteins, and K63-linked ubiquitination is involved in DNA repair, protein trafficking, autophagy, inflammation, and immunity. K63-linked ubiquitination of Akt K14 and K20 is also involved in Akt regulation rather than Akt degradation. K63-linked ubiquitination is associated with insulin-like growth factor 1 (IGF-1) and interleukin (IL)-1 through activation of tumor necrosis factor (TNF) receptor associated factor (TRAF) 6 and neural precursor cell expressed developmentally down-regulated protein 4 (NEDD4), also known as E3 ubiquitin-protein ligase [34]. NEDD4 E3 ligase seems to be involved in K63-linked ubiquitination of Akt [35] whereas TRAF6 also promotes Akt activation and phosphorylation [36]. Akt requires the S-phase kinase-associated protein 2 (Skp2) and TRAF4/6 to induce K63-linked ubiquitination in EGFR-mediated Akt activation.

Moreover, Skp2 inhibitors may sensitize $Her2^+$ breast cancer cells to Her2-targeted therapy [37]. Ubiquitination may be reversed by deubiquitinating enzymes or compounds (DUBs) [38]. K63-linked Akt ubiquitination is essential for T308 phosphorylation [39]. Moreover, Skp2 phosphorylation mediated by 5'5′ AMP-activated protein kinase (AMPK) has a substantial role in Skp2 activation by which Akt undergoes K63-linked ubiquitination [40]. E17 K and E49 K mutations in bladder cancer have been reported to increase the likelihood of K63-linked ubiquitination on Akt, which, in turn, increases the likelihood of PIP3 binding by Akt on the plasma membrane [41].

In addition to its role in Akt binding to PIP3, PHD also terminates Akt activities associated with K48-linked ubiquitination that lead to Akt degradation. K48-linked ubiquitination is regulated by E3 ubiquitin ligases and BRCA1, which facilitates ubiquitin-dependent destruction of

activated Akt [42–44]. K48-linked ubiquitination of Akt has been observed after S473 phosphorylation that leads to immediate proteasomal degradation. In fact, K48-linked ubiquitination is a turn-off switch for Akt activation [45].

2.1.4. Akt SUMOylation and methylation

In addition to acetylation and ubiquitination, Akt residues are reported to be covalently modified mainly at K276 and K301 by Small Ubiquitin-like Modifier (SUMO) proteins. This process is called SUMOylation, which, in the context of Akt, is not well understood [46]. Furthermore, Akt K64 methylation performed by the SET Domain Bifurcated Histone Lysine Methyltransferase 1 (SETDB1) is required for transformation and oncogenic behavior of Akt E17 K mutant cancer cells [47]. It should be noted, however, that Akt activities and corresponding post-translational modifications may be cell-specific [48].

3. Targeting Akt regulation

3.1. mTORC2 and Rictor

Inhibition of mTORC1/2 by mTOR kinase inhibitors effectively suppresses S473 phosphorylation of Akt. mTOR kinase interacts with Rictor to form mTORC2, which is not sensitive to rapamycin and rapalogs such as everolimus, and instead phosphorylates Akt at S473 (Fig. 2). Therefore, Rictor inhibitors can also suppress Akt. The mTOR inhibitors PP242 and OSI-027 are known to target mTOR active sites to suppress S473 phosphorylation of Akt in MCF-7, MDA-MB.231, and Bcap-37 breast cancer cells [49]. However, as an allosteric mTOR inhibitor, rapamycin, which was first considered as a drug that had anticancer activity through targeting of mTOR signaling, inhibits mTORC1 but not mTORC2, and thus does not have an effect on Akt [50]. Accordingly, to suppress breast cancer cell migration and proliferation, mTORC2 inhibition is urgently required, either using mTORC2/Rictor inhibitors or Akt inhibitors in a TNBC model [51]. A positive feedback loop of mTORC2/Akt has also been observed in Her2 amplified breast cancer cells that are resistant to lapatinib [52].

mTORC2 activators such as Selenoprotein W (SelW) and the Runt-related transcription factor RunX2 also elevate Akt activity in invasive MDA-MB-231 (TNBC), SUM-159-PT (TNBC), MCF-7 (invasive ductal carcinoma) and T47D (invasive ductal carcinoma) breast cell lines that have ectopic expression of mTORC2 [53,54]. PRICKLE and Rictor are required for mTORC2 activity, and PRICKLE overexpression is also associated with metastasis in basal breast cancer [55]. As mTORC2 is still active and can activate Akt, the first generation of mTOR inhibitors were not effective against the PI3K/Akt/mTOR pathway, whereas the mTORC2/Akt feedback loop should be suppressed by mTORC2 inhibitors [56]. However, downregulation of mTORC2 expression does not affect Akt T308 phosphorylation [53]. In addition to Akt, mTORC2 phosphorylates protein kinase C (PKC) δ to enhance breast cell morphogenesis and migration [57,58]. Rictor phosphorylation together with Integrin-linked kinase (ILK) is also required for Transforming Growth Factor (TGF)-β-dependent epithelial to mesenchymal transition (EMT) in Her2$^+$ breast cancer cells [59]. In MDA-MB-231 and T-47D cells, Rictor interacts with PKC signaling to promote metastasis [60].

3.2. Protein phosphatase 2 A

Protein Phosphatase 2 A (PP2A) is a tumor suppressor with scaffold (PPP2R1A and PPP2R1B), catalytic (PPP2CA, PPP2CB) and regulatory (PPP2R2A, PPP2R5A, PPP2R2B, PPP2R5B, PPP2R5C, PPP2R2D, PPP2R5D,and PPP2R5E) subunits that dephosphorylate several targets including p-Akt, estrogen receptor (ER), Bad, E2F, and Cdc25 [61] (Fig. 3). PP2A dysregulation is seen in 60 % of basal breast cancers [62]. Furthermore, TNBC cells are sensitive to PP2A activators indicating that PP2A inhibition may play a key role in resistance to therapy that occurs due to Akt overactivation [63]. PP2A, however, affects phosphorylation of Akt at T308, not S473 [64] and PP2A dysregulation is associated with poor prognosis in both ER$^-$ and ER$^+$ breast cancer [65,66].

I2PP2A (SET), an oncogenic inhibitor of PP2A, is involved in tumor progression [67]. A study of 218 patients with ER$^+$ breast cancer who received post-surgery tamoxifen showed that I2PP2A, an oncogenic inhibitor of PP2A, was an independent prognostic predictor in ER$^+$ breast cancer with tamoxifen adjuvant therapy. PP2A and Akt are also associated with poor prognosis in ER$^+$ breast cancer [65]. Tamoxifen was shown to elevate PP2A activity in ER$^-$ HCC1937, MDA-MB-231, MDA-MB-468, MDA-MB-453, and SK-BR-3 breast cancer cells. In addition, ectopic expression of the cancerous inhibitor of PP2A (CIP2A), another PP2A inhibitor that might be related to tamoxifen resistance, inhibits PP2A and activates Akt [68]. The use of CIP2A inhibitors such as arctigenin and bortezomib, a proteasome inhibitor that also targets CIP2A, is therefore effective for PP2A activation and ultimately Akt dephosphorylation in TNBC [69,70].

Ectopic overexpression of CIP2A is also associated with resistance to lapatinib, which reduces Akt activation and expression in SK-BR-3 cells, in Her2$^+$ breast cancer as downregulation of CIP2A leads to RTK inhibition [71]. The effects of CIP2A on TNBC progression were observed in MDA-MB-231 and BT-549 cell lines [72]. Moreover, downregulation of CIP2A and I2PP2A expression increased the level of PP2A in different breast cancer cell lines including MDA-MB-231, MDA-MB-468, HCC1937, Hs-578-T, and BT-20 TNBC [73]. Upregulation of CIP2A and I2PP2A expression is also associated with drug resistance in TNBC. CIP2A inhibition improves resistance to lapatinib in SK-BR-3 cells, although fingolimod (FTY720), a PP2A activator, reduces sensitivity to lapatinib through Akt dephosphorylation [74]. The addition of FTY720 to doxorubicin and paclitaxel therapy reduces resistance to these two agents [75].

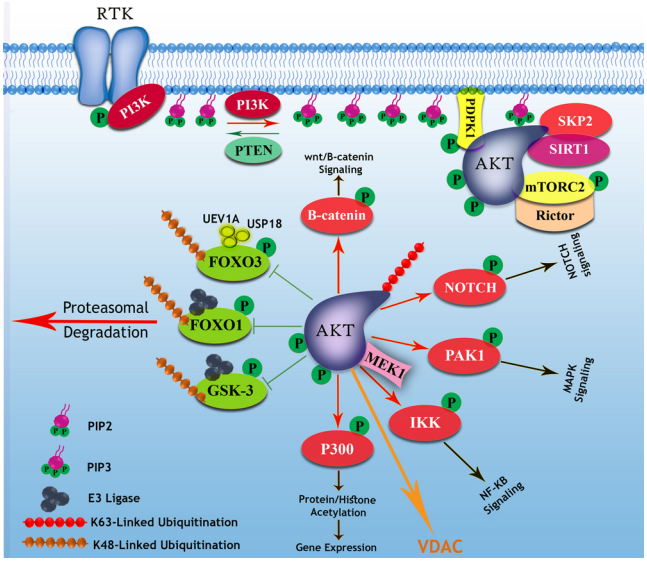

Fig. 2. Akt signaling and downstream pathways. Receptor Tyrosine Kinases (RTK) such as EGFR and Her2 are activated by their ligands to activate PI3K that in turn converts PIP2 to PIP3 that leads to PDPK1 activation and finally Akt phosphorylation at T308. Akt is fully activated by mTORC2 through S473 phosphorylation. For membrane recruitment of Akt, K63-linked ubiquitination (e.g., SKP2) and deacetylation (by HDACs, e.g., SIRT1) are also required. Upon activation, Akt phosphorylates numerous targets in the cytoplasm, nucleus, and mitochondria. Phosphorylation of FoxOs and GSK-3 leads to K48-linked ubiquitination and subsequent proteasomal degradation of these tumor suppressors. On the other hand, Akt participates in crosstalk with other pathways including Wnt/β-catenin signaling, Notch signaling, MAPK signaling, and NF-κB signaling. Akt not only promotes tumorigenesis, but also inhibits apoptosis via NF-κB signaling. In addition, Akt interacts with mitochondrial proteins including VDAC, which increases mitochondrial calcium levels.

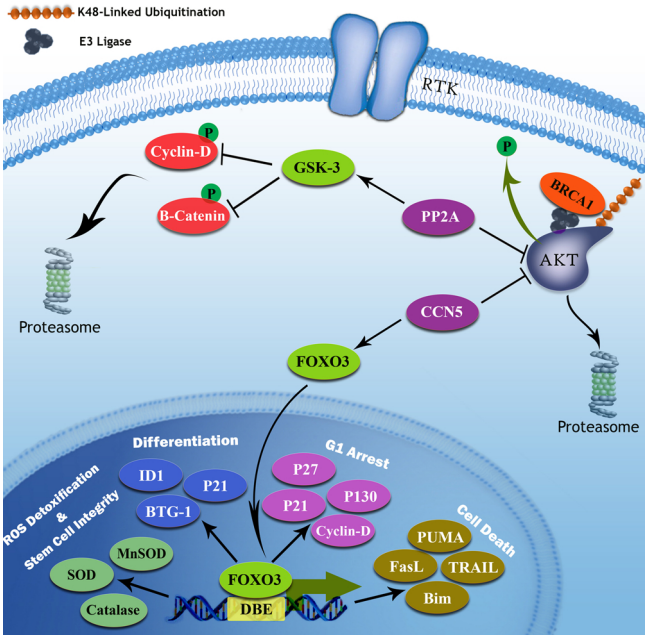

Fig. 3. Akt degradation and activation of GSK-3β and FoxO3. Akt is dephosphorylated and deactivated by PP2A phosphatase. Two crucial targets, GSK-3β and FoxO3, are converted into an active form by dephosphorylation. GSK-3β promotes proteasomal degradation of oncogenic proteins such as β-catenin, Cyclin D, and HIF-1. FoxO3 regulates genes related to detoxification, apoptosis, and tumor suppression. Deactivated Akt is a target for K48-Linked ubiquitination that promotes proteasomal degradation. CCN5 and BRCA1 facilitate Akt dephosphorylation and ubiquitination, respectively.

As mentioned above, activation of PP2A inhibitors may cause resistance to chemotherapy leading to Akt activation. In the case of PP2A inhibition, the use of Akt inhibitors may have benefits for overcoming resistance to therapy. PP2A activators such as FTY720 decrease cell viability and effectively inhibit Akt phosphorylation in MDA-MB-231 and BT-474 cell lines. Upregulation of CIP2A and I2PP2A expression as well as downregulation of PP2A due to PP2A hyperphosphorylation occur, however, at the posttranslational rather than transcriptional level [75].

3.3. Forkhead box O (FoxO) transcription factors

FoxO3 and FoxO1 are transcriptional factors that are involved in regulation of tumor suppressor-mediated (e.g. p21 and p27) apoptosis, and diminish cancer cell invasiveness and metastasis. These proteins are suppressed and degraded following Akt phosphorylation such that Akt activity negatively affects apoptosis and caspase activities via FoxO3 downregulation [76].

Drugs that activate FoxO3 suppress TNBC cell growth whereas Akt inhibitors that suppress S473 phosphorylation at Akt promote FoxO3 activation. FoxO3 has been shown to have negative effects on c-Myc, Kruppel Like Factor 5 (KLF5), and the dopamine receptor DRD2 in TNBC. These proteins are involved in producing cancer stem cells (CSC) [77]. Recently, Zhao et al. (2018) showed that calcineurin B homologous protein 2 (CHP2), which is an essential part of the Na^+/H^+ exchanger (NHE1), is overexpressed in breast cancer, and this overexpression suppresses FoxO3 to promote proliferation of breast cancer cells [78]. FoxO3 activation by inhibitors Akt S473 phosphorylation leads to activation of Bim1, a BH3 domain-containing pro-apoptotic protein [79].

Akt can also phosphorylate FoxO1 to promote its degradation that is mediated by Skp2 E3 ligase. In contrast, high levels of FoxO1 cause not only apoptosis, but may also activate Sox2 expression, and eventually may lead to the development CSCs. Therefore, FoxO1 is a double-edged sword in breast cancer signaling [80]. Ubiquitin-conjugating enzyme variant 1 (UEV1A) is also required for regulation of Akt. In MDA-MB-231 and MCF-7 breast cancer cells, UEV1 activates Akt signaling and leads to FoxO1 and Bim1 downregulation. Under stress conditions, UEV1 may cause resistance to therapy in breast cancer cells [81]. FoxO1 downregulation is associated with adriamycin (ADR) resistance in MCF-7/S and MCF-7/ADR cells, and treatment with the Akt inhibitor LY294002 sensitized MCF-7/S cells to ADR [82]. On the other hand, Procaccia et al. (2017) observed that Akt crosstalk with the MAP kinase pathway promotes degradation of FoxO1. They showed that Akt has direct interaction with phosphorylated mitogen-activated protein kinase kinase (MEK) 1 and MEK2, which then induce FoxO1 phosphorylation. Interactions between Akt and MEK are related to cell migration and metastasis, and not proliferation, thus showing the role of FoxO1 in suppression of metastasis [83].

In addition to promoting apoptosis in breast cancer cells, FoxO3 and FoxO1 regulate expression of tumor suppressors such as p27 (Kip1) and p21, respectively [84,85]. FoxO3 circular RNA also forms a ternary structure with p21 and CDK2 to suppress cell cycle progression [86]. Akt-induced phosphorylation of FoxO3 is targeted by ubiquitin signaling enzymes such as Skp2, UEV1A, and USP18 [87]. In TNBC, FoxO3 phosphorylation at S318, S253, and T32 is blocked by CCN5 (connective tissue growth factor), which can reverse EMT and inhibit cell proliferation [88]. CCN5 also inhibits Skp2 to promote FoxO3 nuclear relocalization [85]. Skp2- and Akt-mediated FoxO3 degradation has also been shown to be associated with p27 (Kip1) downregulation [89].

Resistance to apoptosis can be caused by inhibition of FoxO transcription factor activity that inhibits Bim1 and PTEN [79]. On the other hand, Akt re-activation is associated with resistance to PI3K and tyrosine kinase inhibitors [90]. Akt activation has recently been shown in lapatinib-treated MDA-MB-231 TNBC cells [91]. In this regard, HDAC (e.g. entinostat) or Akt inhibitors in combination with lapatinib have been shown to suppress Akt phosphorylation at S473 [13,92].

3.4. Glycogen synthase kinase (GSK)-3β

GSK-3 activation promotes apoptosis in several cancer types including breast cancer and can also induce cancer progression in other cases depending on GSK-3 regulators. GSK-3 has two isoforms, α and β, that localize to the nucleus/cytoplasm and cytoplasm, respectively. Akt inactivates both GSK-3 isoforms by phosphorylating N-terminal serine residues and this inactivation leads to induction of protein synthesis [93]. Inhibition of GSK-3 may stabilize β-catenin in the canonical Wnt pathway to promote cell proliferation. Indeed, GSK-3 inactivation is a function that connects Akt to Wnt/β-catenin signaling.

GSK-3β is involved in ubiquitin-dependent degradation of key proto-oncoproteins such as β-catenin, c-Myc, and cyclin D and is known as a tumor suppressor [94]. GSK-3 overactivation also induces mTORC1 in MCF-7 breast cancer cells [95]. P-gp associated with drug resistance is expressed when GSK-3 is inhibited through Wnt/β-catenin signaling in brain cells of the blood-brain barrier (BBB) whereas the GSK-3 inhibitor, 9-ING-41, sensitizes patient-derived xenograft tumor models of breast cancer cells to the topoisomerase inhibitor irinotecan. However, GSK-3β overexpression has been associated with poor prognosis and survival of patients with breast cancer [94].

3.5. Akt: oxidative stress and chemoresistance

3.5.1. Nuclear factor erythroid 2-related factor 2 (Nrf2)

DNA damage can occur from oxidative stress and ROS through epigenetic changes, which can also reduce expression of superoxide dismutase (SOD) family members, cause genomic instability, activate nuclear factor-κB (NF-κB) signaling, and promote inflammation that can eventually lead to cancer. Nrf2 is a transcription factor that protects cells against various toxic compounds through expression of anti-oxidant enzymes and ATP-binding cassette (ABC) transporters in various cell types, including breast cancer. Nrf2 has been shown to target serval genes, including anti-oxidant response elements (AREs) [24] (Fig. 4).

Thus, cancer cells that have a high level of ARE activity often show resistance to anti-cancer agents [96] and drug inhibitors of this transcription factor can sensitize cancer cells to chemotherapy [97]. The Kelch-like ECH-associated protein 1 (Keap1)-Nrf2 system is thought to be a potential therapeutic target for cancer. Keap1 binding to Nrf2 causes Nrf2-polyubiquitination, which targets Nrf2 to the 26S proteasome for degradation. Therefore, Keap1 inhibitors can prevent Nrf2 degradation and increase Nrf2 levels [98].

On the other hand, glycolysis is an aerobic process that provides sufficient energy for growth and proliferation of breast cancer cells. In MDA-MB-231 and MCF-7 breast cancer cells, expression of Nrf2 and Hypoxia-inducible factor (HIF)-1α is increased. The effects of Nrf2 on glycolysis are not well understood, although Nrf2 downregulation inhibits expression of key genes involved in glycolysis. In breast cancer cells, Nrf2 also co-activates HIF-1α expression [99] that can in turn regulate expression of glycolytic enzymes such as glycolysis-associated glucose transporter-1 (GLUT1), hexokinase-2 (HK2), pyruvate dehydrogenase kinase-1 (PDK1), and lactate dehydrogenase A [100].

Nrf2 can reduce increases in ROS levels, but in MDA-MB-231 cells Nrf2 also activates cell growth and may also induce Akt activation [101]. Transcriptional co-activator amplified in breast cancer 1 (AIB1) reduces ROS via Nrf2 activation, and induces Bcl-2 expression through activation of the Akt pathway. AIB1 also facilitates expression of ABCC2 and ABCG2, two targets of Nrf2, leading to resistance to chemotherapy [102].

Estrogen (E2) increases Nrf2 levels in ER^+ MCF-7 breast cancer cells via activation of PI3K/GSK-3β signaling. The PI3K inhibitors LY294002 and wortmannin suppress Nrf2 activation. E2 is also reported to play a role in Nrf2 activation by increasing levels of p-Akt and p-GSK-3β [103]. Patients carrying mutations in breast cancer 1 tumor suppressor gene ($BRCA1^-$) have reduced levels of antioxidant signaling that result in part from lower levels of Nrf2. In contrast, E2 induces ER^+ breast cancer cell survival by inducing Nrf2 expression. E2-induced Nrf2 accumulation is associated with Akt signaling and thus Akt inhibitors may be beneficial for patients with ER^+BRCA^- breast cancer [104]. Nrf2 is also reported to be expressed in $Her2^+$ ovarian cancer cells, suggesting that Nrf2 inhibitors could be used to suppress Her2 signaling in patients that have resistance to TKIs and docetaxel [105].

In addition to Nrf2-dependent expression of ABC transporters and anti-oxidant enzymes, Akt is involved in biosynthesis of glutathione (GSH) through a pathway that involves Nrf2 activation [106]. Reactive aldehydes increase GSH and Nrf2 levels, EMT markers, and accordingly, elevate resistance to chemotherapy. The frequency of breast CSCs (BCSC) as the main cause of metastasis and chemoresistance is associated with GSH and Nrf2 levels [107].

Moreover, Akt increases the population of BCSCs. The Notch signaling pathway functions in survival of stem-like cells survival and plays a role in drug-resistance in TNBC patients. Jagged1, the ligand for multiple Notch receptors, initiates NF-κB-dependent, as well as mitochondrial and nuclear Notch1, signaling that leads to phosphorylation of Akt at S473 in TNBC cells. Proteins downstream of Notch signaling are responsible for development of BCSC development from TNBC cells. Meanwhile, the Akt inhibitor, MK-2206, in combination with the γ-secretase inhibitor PF-03084014, which is responsible for Notch cleavage, prevent secondary mammosphere formation from CSCs in TNBC cases having Notch1 and wild-type PTEN expression [108].

3.5.2. Hypoxia-inducible factor (HIF-1α)

Akt increases translation of HIF-1α mRNA under hypoxic conditions. Under normal oxygen concentrations (normoxia), the HIF-1α C-terminal is hydroxylated and HIF-1α is degraded through proteasomal degradation [109] (Fig. 5). In contrast, when oxygen concentrations fall below 5% (hypoxia), HIF-1α is stabilized by phosphorylationm. Such stabilization is associated with chemoresistance of TNBC xenografts to docetaxel and doxorubicin [110]. Factor-inhibiting HIF-1 (FIH-1) and prolyl hydroxylase hydroxylate the C-terminal transactivation domain of HIF-1α in invasive breast cancer, which leads to HIF-1α removal [111]. Hypoxia inhibits HIF-1α hydroxylation by suppressing the activity of prolyl hydroxylase [112]. The Von Hippel-Lindau (VHL) tumor suppressor binds to hydroxylated proline residues P402 and P564 to facilitate HIF-1α ubiquitination via VHL-dependent HIF-1α degradation [113] that involves GSK-3β-dependent phosphorylation of HIF-1α at T498, S502, S505, T506, and S510 [114].

HIF-1α can be modified by two types of phosphorylation. GSK-3-dependent phosphorylation results in HIF-1α degradation, and extracellular signal-regulated kinase (ERK)1/2-dependent phosphorylation of HIF-1α at S641 and S643 promotes nuclear accumulation and subsequent increases in HIF-1α-mediated transcription by masking the nuclear export signal (NES) (I637-L638-I639) [115]. HIF-1α that does not undergo ERK1/2 phosphorylation can bind to glucose-regulated protein 75 (GRP75) and the HIF-1α/GRP75 complex is exported from the nucleus to the cytoplasm. Under hypoxic conditions, most HIF-1α localizes to the nucleus and very little is detectable in the cytoplasm [116,117].

Meanwhile, Akt signaling participates in crosstalk with the MAPK pathway through direct binding to phosphorylated MEK1/2 [83]. In chemoresistance, PI3K/Akt and MAPK/ERK pathways can be hyperactivated to facilitate HIF-1 activity by increasing rates of HIF-1α protein synthesis and HIF-1α phosphorylation, respectively [118]. ERK1/2-dependent HIF-1α activation also leads to angiogenesis through vascular endothelial growth factor A (VEGF-A) expression [119]. Akt signaling is also required for both and HIF-1α mRNA transcription transcriptional and protein synthesis that occurs through P300 activation and ribosomal protein activation of eukaryotic initiation factor-4E (eIF4E) as well as ribosomal protein S6 kinase (P70S6K) [120]. Taken together, the use of Akt inhibitors might be effective for inducing HIF-1α downregulation in breast cancer cells [121].

Furthermore, HIF-1α controls expression of the stemness-specific proteins OCT4 and Nanog that contribute to BCSC integrity. Akt inhibitors also can inhibit HIF-1 and in turn decrease drug resistance by decreasing BCSC populations [122]. Brachyury, a T-box family transcription factor, stimulates EMT and HIF-1α expression through Akt signaling that increases proliferation and metastasis of breast cancer *in*

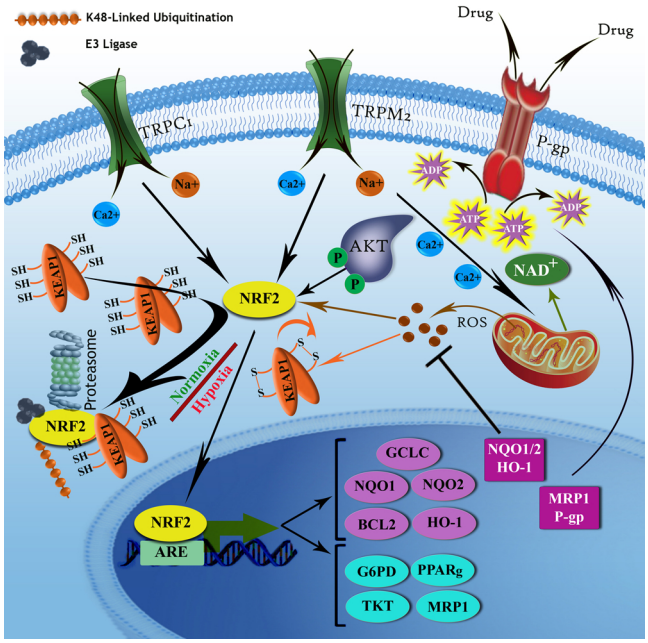

Fig. 4. Nrf2 activity based on oxygen availability. In hypoxia (5% oxygen), Akt can inhibit aerobic transpiration whereupon ROS released from the mitochondria can activate Nrf2, a transcription factor that regulates expression of anti-oxidant genes and genes related to drug resistance. Nrf2 is marked for proteasomal degradation by Keap1 under atmospheric oxygen (20 %). Cysteine residues in Keap1 are oxidized by ROS under hypoxic conditions that promote Nrf2 dissociation. P-gp is a multi-drug resistance protein that exports drugs from cancer cells. Multi-drug resistance proteins require ATP for activity.

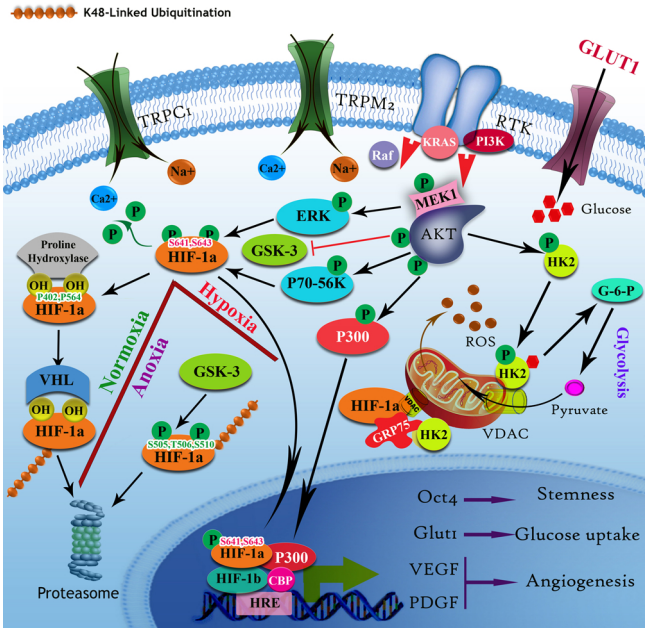

Fig. 5. Collaborative effects of PI3K/Akt and KRAS/Raf/MAPK pathways on HIF-1 activity. HIF-1α is expressed and synthesized in hypoxia. Both KRAS/Raf/MAPK and PI3K/Akt signaling pathways are involved in HIF-1α activation. ERK1/2 phosphorylates HIF-1α at S641 and S643, while Akt promotes HIF-1α synthesis by activating ribosomal protein S6. Akt also activates P300, a histone acetyl transferase (HAT), which, along with CBP, is a co-activator for HIF-1α binding. Akt binds MEK1/2 to promote crosstalk in MAPK signaling pathways. Phosphorylation of HIF-1α at S641 and S643 traps HIF-1α in the nucleus and facilitates interactions with P300, CBP, and HIF-1β. Unmodified HIF-1α interacts with GRP75, VDAC, and HK2 on mitochondrial outer membranes. HIF-1α drives the expression of genes related to cancer stem cells, glucose metabolism, and angiogenesis. HIF-1α is phosphorylated by GSK-3β at T498, S502, S505, T506, and S510 residues, which leads to HIF-1α proteasomal degradation. Akt suppresses GSK-3β activity under hypoxic conditions, although GSK-3β can mediate auto-activation in anoxia to induce HIF-1α degradation. Under normoxic conditions, HIF-1α is hydroxylated at P402 and P564 that promotes VHL-dependent degradation of HIF-1α.

vitro and in tumor xenografts [123]. HIF-1α activity is also associated with lapatinib resistance in Her2$^+$ breast cancer cells, particularly that which occurs under hypoxic conditions [124].

3.5.3. Akt and autophagy

Hypoxic conditions promote chemoresistance and cell survival through activation of HIF-1α and Nrf2. Proteins in the mitochondrial electron transport chain (ETC) are also affected by hypoxia that can increase ROS production [125] and in turn inhibit DNA repair and promote autophagy-related death of cancer cells. For activation of Nrf2, calcium influx triggers anti-oxidant signaling that reduces ROS levels. The transient receptor potential subfamilies C1 (TRPC1) and M2 (TRPM2) are cation ion channels that regulate calcium influx required for antioxidant signaling in breast cancer cells [126]. TRPC1 regulates HIF-1α activity in PTEN$^-$ MDA-MB-468 and HCC-1569 breast cancer cell lines by activating Akt [127]. HIF-1α signaling facilitates autophagy through expression of microtubule-associated protein 1A/1B-light chain 3 (LC3)-BII in MCF-7 breast cancer cells [128].

Induction of autophagy-related death is an anticancer cellular response to oxygen starvation and is also associated with increased likelihood of resistance to chemotherapy. Autophagy can be inhibited by Akt activation and corresponding Nrf2 activation that ultimately leads to decreased ROS levels. Breast cancer patients treated with pharmorubicin often develop resistance related to heme oxygenase-1 (HO-1) expression in addition to autophagy proteins such as LC3-II, LC3-I, and Beclin-1. Chemoresistance induced by HO-1 results in Akt-dependent autophagy [129] (Fig. 6).

Inhibition of Akt signaling sensitizes ER$^+$ breast cancer cells to tamoxifen and fulvestrant by enhancing autophagy-related death [130], whereas in MDA-MB-231 TNBC cells, inhibition of autophagy increases sensitivity to doxorubicin. Akt activation leads to impaired autophagy via the ELK3 transcription factor, which can enhance progression and metastasis in breast cancer [131]. Together these findings show that the effects of Akt signaling on autophagy-associated chemoresistance in breast cancer cells is dependent upon the type of chemotherapy, mutations, and breast cancer subset.

HIF-1 induces mitophagy and partial autophagy to provide nutrients required for cancer cell growth. In contrast, mitochondrial Akt induces HIF-1 activity that suppresses autophagy-related death that can support cell survival under hypoxic conditions. Akt also suppresses ROS-dependent apoptosis in breast cancer through GSK-3β inhibition [132].

Voltage-dependent anion-selective channel (VDAC) 1 phosphorylated by GSK-3β has increased affinity for Bax, a pro-apoptotic protein, and diminished interactions with HK2. Based on these results, elevated ROS, calcium overload in mitochondria, and cytochrome c release from mitochondria collapse the mitochondrial membrane potential leading to apoptosis [133]. In contrast to the role of HIF-1-dependent

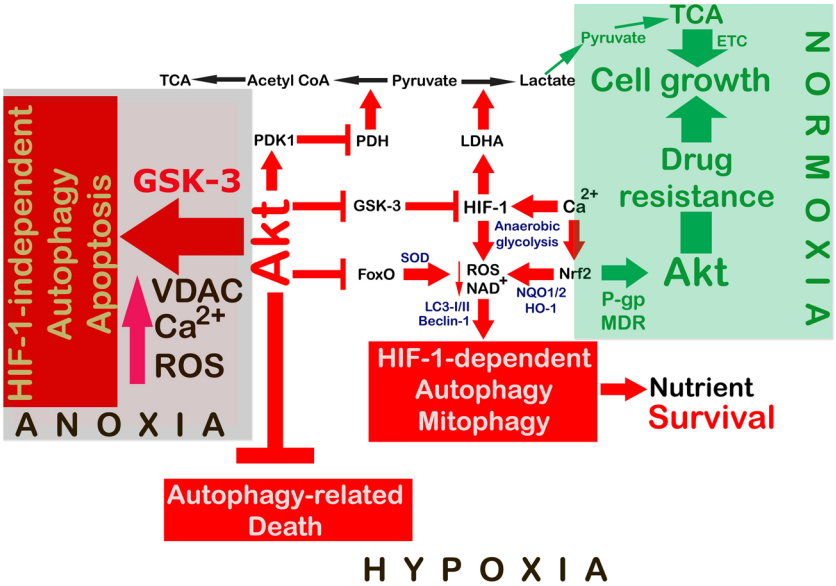

Fig. 6. The relationship between Akt signaling and autophagy. Under hypoxic conditions (0.1-3% O_2), the mitochondrial electron transport chain (ETC) produces ROS and NAD$^+$, instead of NADH and ATP that in turn leads to HIF-1 and Nrf2 activation and subsequent ROS reduction. Akt signaling induces HIF-1 and Nrf2 expression. HIF-1 activity can provide nutrients and energy (including glucose uptake) from anaerobic glycolysis and HIF-1-dependent autophagy. Some organelles, including mitochondria (mitophagy), are digested to provide nutrients. Thus, HIF-1-dependent autophagy is critical for cellular survival. Under anoxic conditions (< 0.01 % O_2), HIF-1-independent autophagy occurs to induce autophagy/apoptotic-related death. Akt signaling suppresses autophagy-related death and apoptosis by GSK-3β inhibition. Superoxide dismutase (SOD), heme oxidase-1 (HO-1), and NAD(P)H-dehydrogenase Quinone 1/2 (NQO1/2) are crucial enzymes that protect cells against oxidative stress and ROS. GSK-3β activation under anoxic conditions promotes HIF-1α degradation and drives VDAC activation and oligomerization, and finally apoptotic death.

autophagy that contributes to cell survival under hypoxic conditions, the role of HIF-1-independent autophagy/apoptosis is to promote cell death due to anoxia [134]. These results suggest that Akt highjacks cancer cell metabolism and that Akt inhibitors could reverse cell metabolism from that seen for a hypoxic state to one associated with normoxic conditions. However, the role of mitochondrial Akt in breast cancer requires additional investigation.

3.5.4. Mitochondrial Akt and metabolic remodeling

Under hypoxic conditions, ROS accumulate in cells and pyruvate is then converted to lactate during anaerobic respiration. HIF-1 increases glycolysis rates and lactate concentrations under hypoxic conditions. In cancer cells, however, Akt is the main protein that promotes metabolic reprogramming from aerobic respiration to anaerobic glycolysis [135]. HIF-1 is also associated with chemoresistance through hypoxia

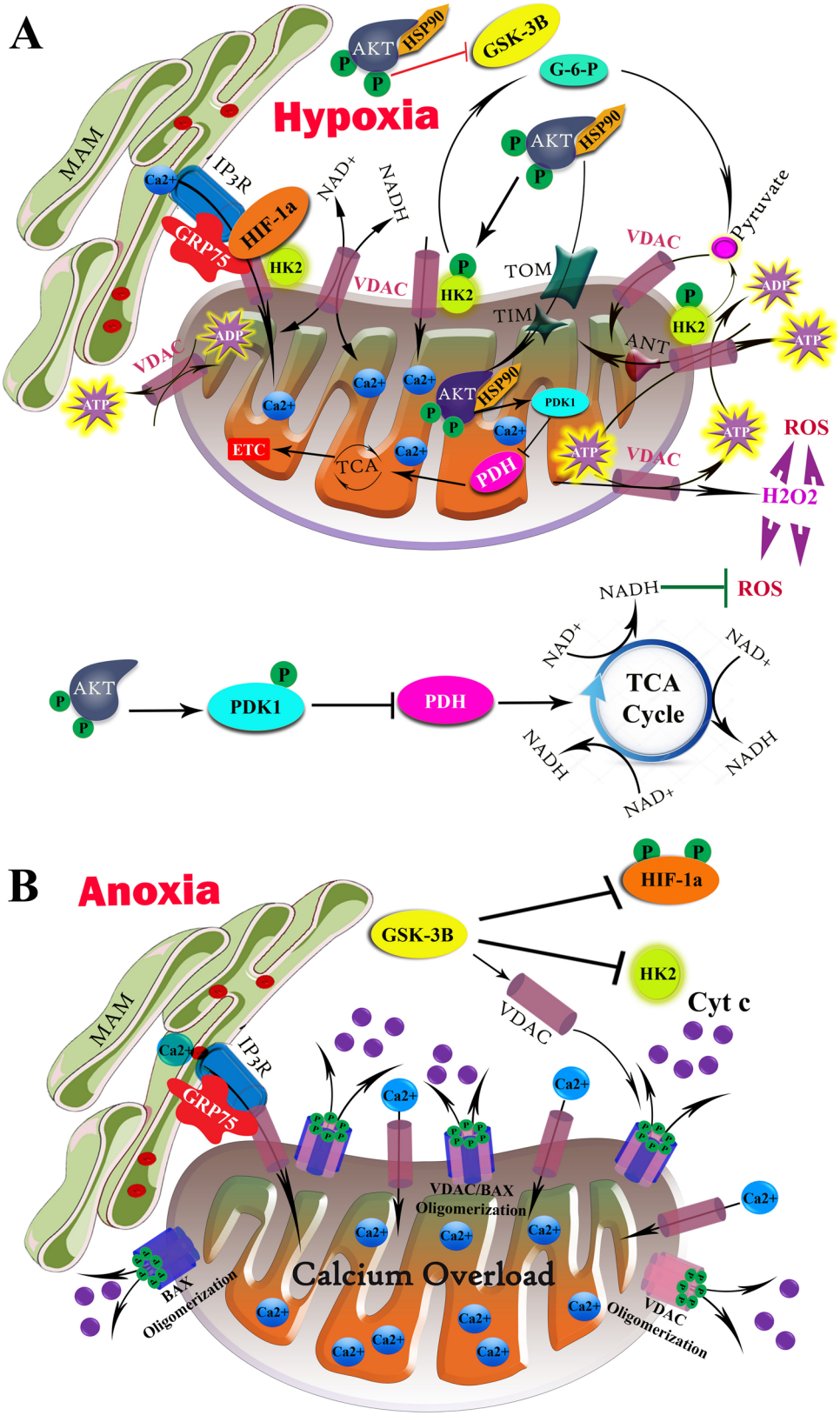

Fig. 7. Effects of Akt signaling and metabolic remodeling. (A) Akt inhibits GSK-3β under hypoxic conditions to promote metabolic remodeling. Akt is transferred into mitochondria through TOM and TIM complexes located in the outer and inner membranes, respectively. Akt phosphorylates PDK1 to inhibit PDH, which is involved in activating the TCA cycle by converting pyruvate into acetyl Coenzyme A (CoA). HK2 is also activated by Akt and is then recruited by VDAC to the outer mitochondrial membrane. Monomeric and dimeric VDACs transfer metabolites in and out of the mitochondria. VDAC binds IP3R on the MAM to facilitate Ca^{2+} transfer from the endoplasmic reticulum to the mitochondria. Suppression of PDH and the TCA cycle in turn block the electron transport chain (ETC). In the TCA cycle NAD^+ is converted into NADH that promotes ATP synthesis under normoxic conditions. Suppression of TCA cycle by Akt induces anaerobic respiration and oxidized molecules such as H_2O_2 and ROS are produced. HIF-1α is also expressed under hypoxic conditions to increase glucose uptake and metabolism. **(B)** GSK-3β is activated in anoxia that inhibits HIF-1α and HK2. GSK-3β activates VDAC via VDAC phosphorylation that induces VDAC oligomerization. VDAC can also oligomerize with pro-apoptotic BAX to form a channel that facilitates cytochrome c release to induce apoptosis via Caspase 9 activation. At the same time, higher levels of calcium influx into mitochondria promote mitochondria degradation.

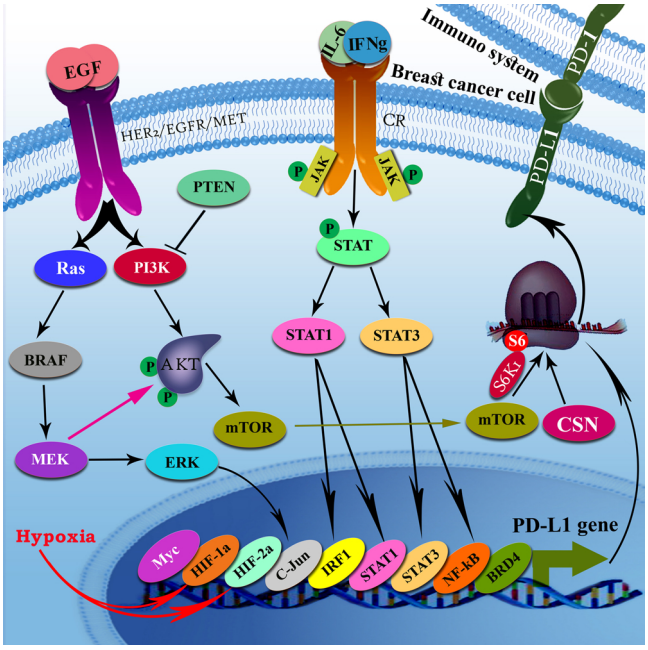

Fig. 8. PD-L1 expression and upstream regulation. PD-L1 expression in cancer cells suppresses anti-cancer immunity. Under hypoxic conditions, PD-L1 production coordinates three signaling pathways: (1) PI3K/Akt/mTOR, (2) RAS/BRAF/MAPK, and (3) JAK/STAT. PI3K/Akt and RAS/MAPK pathways are driven by receptor tyrosine kinases (RTKs) such as EGFR and Her2 that lead to activation of NF-κB and HIF-1. On the other hand, cytokine receptors (CR) are targeted by IFNγ and IL-6, which is secreted by TNBC cells. IL-6 and IFNγ secretion activate JAK/STAT signaling to promote DNA binding of IRF1 and STAT1/3. Eventually, several transcription factors and co-activators are recruited upstream of the PD-L1 promoter to activate PD-L1 expression in breast cancer cells. These upstream complexes not only activate PD-L1 transcription to suppress T-cell immunity, but also activate tumorigenesis in breast cancer cells. PD-L1 expression in breast cancer cells increases resistance to therapy. Moreover, Akt/mTOR signaling activates ribosomal protein S6 to activate protein synthesis. CSN is Component of the eukaryotic translation initiation factor 3 (eIF-3) complex, which is involved in protein synthesis. Akt signaling thus has a crucial role in expression and translation of PD-L1 in breast cancer cells.

signaling. Treatment with TKIs such as lapatinib induces hypoxia in breast cancer cells leading to acquired TKI resistance through Akt and HIF-1α activation [136]. To produce lactate, pyruvate dehydrogenase is inactivated through phosphorylation mediated by PDK1, which is regulated by phosphorylation on T346 driven by Akt. Suppression of the TCA cycle, and the ETC by Akt results in accumulation of pyruvate and NAD^+. Meanwhile, HIF-1α increases expression of PDK1 and lactate dehydrogenase A that then converts pyruvate to lactate [33]. As such, both Akt and HIF-1α are involved in anaerobic metabolism in cancer cells [137].

Chae et al. (2016) showed that the effect of Akt on PDK1 is independent of HIF-1. Although cytoplasmic Akt levels under normoxia and hypoxic conditions are similar, Akt accumulates in mitochondria to a greater degree under hypoxic conditions. Akt translocation into mitochondria is mediated by the heat shock protein 90 (HSP90), and in SH-SY5 neuroblastoma cells, both HSP90 and Akt translocation can be inhibited by the HSP90 inhibitor geldanamycin [138]. Meanwhile, HIF-1 silencing did not affect mitochondrial Akt levels, indicating the HIF-1-independence of Akt activity toward mitochondrial enzymes [33]. Akt accumulation in mitochondria is also associated with activation of the stem cell markers OCT4 and Nanog. Under hypoxia, inhibition of aerobic respiration increases stem cell features in embryonic stem cells; however, oxidative phosphorylation in the mitochondrial inner membrane leads to stem cell differentiation into specialized cells [139].

3.5.5. GSK-3β versus HIF-1 in apoptotic death

Mitochondria-associated endoplasmic reticulum membranes (MAM) are linked to mitochondria via interactions between IP3 receptors (IP3R) and VDAC-1, which allows calcium ions to flow from the endoplasmic reticulum to mitochondria. Calcium required for mitochondrial enzymes can also be imported from the cytoplasm. Akt/mTORC2 signaling, which responds to oxygen availability, has been identified at the MAM and controls glucose metabolism in mitochondria [140]. In addition to its negative and positive effects on apoptosis and cell survival, Akt crucially affect glucose metabolism via HIF-1 activation, GSK-3β inhibition, and pyruvate dehydrogenase inhibition under hypoxic conditions. HK2 is an enzyme that triggers glycolysis via glucose phosphorylation and is also phosphorylated by Akt. Phosphorylated HK2 can bind VDAC and activate glycolysis. Meanwhile, inhibition of HK2 activity by GSK-3β reduces glycolysis rates in MDA-MB-231 and MCF-7 cells [141] (Fig. 7).

VDACs (VDAC1, VDAC2, and VDAC3 isoforms) located in the outer membrane of mitochondria allow passage of ions and metabolites. VDAC1, which is expressed to higher levels than the other VDAC isoforms, self-oligomerizes and interacts with Bax to form VDAC1/Bax oligomers. This VDAC oligomerization provides a channel for release of cytochrome c from mitochondria during apoptosis [142]. VDAC1 monomers or dimers recruit HK2 phosphorylated by Akt to the mitochondrial outer membrane and facilitate metabolite transport into or out of mitochondria and also glucose phosphorylation during glycolysis. GSK-3β-mediated VDAC1 phosphorylation suppresses binding of HK2 to the mitochondrial membrane. Given that Akt inhibits GSK-3β, Akt inhibitors can increase GSK-3β activity that in turn represses HK2 activity while increasing VDAC oligomerization to induce apoptotic cell death [143].

GRP75 is a stress molecule in the MAM that regulates mitochondrial calcium influx. Mylonis et al. (2017) showed that unmodified HIF-1 (i.e., without hydroxylation and phosphorylation) is transcriptionally inactive, but can interact with GRP75 in the nucleus. The HIF-1/GRP75 complex is exported from the nucleus so that it can bind to VDAC1 and HK2 at the outer mitochondrial membrane. However, association of VDAC1 with HIF-1 can promote its partial activation under hypoxia that leads to resistance to apoptosis [144]. Akt suppresses apoptotic death under hypoxic conditions and activates glycolysis through HK2 phosphorylation and HIF-1-dependent gene expression that eventually leads to partial autophagy/mitophagy. In contrast, Akt inhibitors phosphorylate GSK-3β to facilitate HIF-1α degradation in a VHL-independent manner, and instead trigger apoptosis through VDAC oligomerization.

3.6. Epigenetic regulators of Akt signaling

3.6.1. DNA methyl transferases (DNMTs)

DNA methylation involves epigenetic modification of promoters that can negatively affect gene expression. In case of resistance to tamoxifen, expression of Spalt-like transcription factor 2 (SALL2), which upregulates genes expression of ERα (ESR1) and PTEN, is downregulated by hypermethylation that leads to Akt activation [145]. In addition, upregulation of DNA-(cytosine-5)-methyltransferase (DNMT)-1 has been shown in tamoxifen-resistant MCF-7 cells. DNMT1 targets the PTEN promoter that activates Akt [146]. The combinatorial use of DNMT inhibitors with tamoxifen sensitizes breast cancer cells to tamoxifen [145]; meanwhile, HDAC inhibitors downregulate expression of DNMTs (DNMT1, DNMT3b, and DNMT3a) as well as Akt signaling in MDA-MB-231 TNBC cells [147].

3.6.2. Histone deacetylases (HDACs)

HDACs remove acetyl functional groups from the lysine residues of histone and nonhistone proteins. The human genome has 18 HDACs that can be categorized in four classes based on the use of zinc- or NAD^+-dependent mechanisms to deacetylate acetyl lysine substrates

Table 1
Akt inhibitors tested on breast cancer subjects or solid tumors including breast tumors.

Inhibitor(s)	Treatment (Targets)	Experiments	Clinical trials			References
			Phase(s)	1st endpoint	2nd endpoint	
MK-2206	Akt	T47D, HEK293 T, MDA-MB-468 ZR7530, BT474 cell lines Breast cancer Tumors	2	OR, AEs	PFS Median response duration Safety	NCT01277757* [175,176,177,178,179,180]
Capivasertib (AZD5363)	Akt	ER^+ breast cancer cell lines (MCF7, HCC1428, T47D, ZR75.1) ER^+ invasive breast cancer	1, 2	Safety, Tolerability, PFS	PK, Toxicity OR, AEs	NCT02077569 NCT01226316 [178,181,182,183]
ARQ092	Akt	MCF10A, NCI-N87 cell lines Solid tumors	1	Safety Tolerability	PK, PD, RP2D	NCT01473095 [184]
Uprosertib (GSK2141795)	Akt	Panel of 22 HER2(+) breast cancer cell lines carrying wild type or mutant PIK3CA Solid tumors	1	RP2D	Efficacy Metabolite profile, AEs	NCT00920257 [185]
GSK2110183	Akt	Solid tumors	2	AEs, PK		NCT01531894
M2698 (MSC2363318A)	Akt	TNBC (MDA-MB-468) and $Her2^+$ breast cancer cell lines (MDA-MB-453 and JIMT-1) Solid tumors	1	DLT	PD, PK, BOR, CBR, AEs	NCT01971515 [186]
MK-2206 + Lapatinib Ditosylate	Akt+ EGFR (combination)	Breast tumors	1	MTD, AEs	OR, PFS, DLT, PFS, Safety, PK, Response	NCT01281163 NCT01245205 [187]
Paclitaxel +/- Ipatasertib	Microtubule + Akt (combination)	Breast tumors	2	pCR, PFS	OR, Efficacy, Safety, PK OS, DOR	NCT02301988 NCT02162719
GSK2141795 + Trametinib	Akt + MEK1/2 (combination)	Breast carcinoma	2	OR	CBR, DOR, AEs, SAEs, PFS	NCT01964924
Capivasertib + Paclitaxel	Akt + Microtubule (combination)	Breast tumors	2	PFS		NCT02423603
MK-2206 + Paclitaxel + Trastuzumab	Akt + Microtubule + Her2 (combination)	$HER2^+$ solid tumors	1	MTD	BOR	NCT01235897 [188]
MK-2206 + KRIBB11	Akt + HSF1 (combination)	Orthotopic xenograft mouse model of breast cancer				[189]
MK-2206 + Anastrozole + Fulvestrant	Akt + Aromatase + ER (combination)	$ER^+/HER2^-$ breast cancer	1, 2, 3	MTD, RPTD, Toxicity	CRR, AEs, RRR	NCT01344031 [190]
Capivasertib + Fulvestrant	Akt + ER (combination)	ER^+ breast cancer cell lines (MCF7, HCC1428, T47D, ZR75.1) adapted to long-term estrogen deprivation (LTED) or tamoxifen (TamR) Breast cancer formalin-fixed paraffin-embedded (FFPE) samples ER^+, $HER2^-$, metastatic or locally advanced inoperable breast cancer who had relapsed or progressed on an aromatase inhibitor	2	AEs, PFS		NCT01992952 [181,191,192]
Capivasertib + Fulvestrant + paclitaxel	Akt + ER + Microtubule (combination)	HR^+ and $HER2^-$ advanced breast cancer				[193]
Capivasertib + AZD8931	Akt + Her2 (combination)	HCC1954 xenograft model				[173]
Capivasertib + AZD8835	Akt + PI3KCA (combination)	MAP3K1-deficient MCF7 tumors				[194]
MK-2206 + Capivasertib	Akt + PI3K (combination)	breast cancer with tumors containing AKT1(E17 K) mutations				[174]
Capivasertib, MK-2206, LY294002, Wortmannin, Perifosine, Rapamycin, Everolimus, Temsirolimus	Akt + PI3K + mTORC1 (combination)	AZD5363-sensitized Hs578 T breast cancer cells				[195,196]
Uprosertib + Trametinib	Akt + MEK1/2 (combination)	TNBC	1	AEs		[197]

*Clinical trial data obtained from https://clinicaltrials.gov.
OR: Overall Response; **PFS**: Progression Free Survival; **OS**: Overall Survival; **PK**: Pharmacokinetics; **PD**: Pharmacodynamics; **RP2D:** Recommended Phase 2 trial Dose; **AEs**: Adverse Events; **MTD**: Maximum Tolerated Dose; **DOR**: Duration of Response; **DLT**: Dose-Limiting Toxicity; **BOR:** Best Overall Response; **SAEs**: Serious Adverse Events; **CBR**: Clinical Benefit Rate; **pCR**: Pathological Complete Response**;** **RPTD**: Recommended Phase Two Dose**;** **CRR:** Clinical Response Rate; **RRR:** Radiological Response Rate.

[148]. As mentioned above, mTORC2 and SIRT1/2 deacetylases can induce Akt activation. Sapanisertib (MLN0128), a pan-mTORC (mTORC1 and mTORC2) inhibitor, in combination with trichostatin A (TSA), a pan-HDAC inhibitor, inhibits proliferation of malignant breast cancer cell lines independent of hormone receptor (HR)- and Her2 status [149]. On the other hand, HDAC inhibitors suppress TNBC stem cell populations by downregulating β-catenin signaling [150].

Regarding resistance to therapy, entinostat (SNDX-275), a class I HDAC (HDAC1, HDAC2, HDAC3, and HDAC8) inhibitor, sensitizes her2$^+$ breast cancer cells to trastuzumab by downregulating Akt. Entinostat may interfere with Her2/Her3 interactions required for PI3K/Akt signaling [151]. Multi-targeting drugs including indole derivatives and berberine, which suppress both Akt signaling and HDACs, are more suitable options for treatment of breast cancer [132,152]. The HDAC inhibitor vorinostat was also shown to suppress EGFR-related pathways including Akt signaling in ER$^-$ breast cancer cells [153].

3.7. Akt and the estrogen receptor

3.7.1. Aromatase inhibitors (AIs)

Endocrine therapy is a common treatment for HR$^+$ breast cancer, although this approach can fail over if resistance develops. Aromatase inhibitors (AIs) are used to treat hormone-dependent breast cancer through inhibition of the aromatase enzyme that catalyzes E2 biosynthesis. Acquired resistance to AIs used to treat ER$^+$ breast cancer can also occur. Treatment of MCF-7 breast cancer cells that overexpress aromatase with the AI anastrozole led to the development of an AI-resistant breast cancer cell line Res-Ana that has an ER-independent reduction in sensitivity to both ERα inhibitors and AIs. High levels of Akt activation and Her2 dysregulation are seen for AI-resistant cells. Overactivation of Akt signaling was also reported in patients who relapsed under anastrozole adjuvant therapy. The activation of Akt was recognized as a main factor in anastrozole resistance. Interestingly, the crucial effect of Akt on anastrozole resistance was shown using the Akt inhibitor, MK-2206 [154]. Akt inhibition combined with fulvestrant was reported to decrease resistance to endocrine therapy in ER$^+$ Her2$^-$ breast cancer cells and in breast cancer xenografts [155].

3.7.2. The human gonadotropin-releasing hormone (hGnRH)

The human gonadotropin-releasing hormone (hGnRH) regulates the secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary [156]. hGnRH can mitigate side effects of therapies for hormone-dependent breast cancer. Palbociclib, a CDK4/6 inhibitor, in combination with exemestane, an aromatase inhibitor, in addition to hGnRH analogues, effectively decrease estrogen levels in ER$^+$ breast cancer [157]. To overcome resistance to tamoxifen in breast cancer cells that have higher levels of Her2 expression and Akt activation, treatment with hGnRH analogues sensitized tamoxifen-resistant ER$^+$ breast cancer cell lines (MCF-7-TR and T47D-TR) to tamoxifen [158].

4. Akt and the programmed death-ligand 1 (PD-L1)

PD-L1 is expressed on the surface of cancer cells. Interactions between PD-L1 and PD-1 expressed on the surface of T-cells suppress T cell-mediated anticancer immunity. PD-L1 inhibitors can block immune suppressive factors to enhance T cell activity [159]. In addition, PD-L1 and PD-1 interactions not only suppress anticancer T-cell immunity, but also induce P-gp expression and chemoresistance in breast cancer cells. Furthermore, PD-L1 expression can activate Akt signaling in breast cancer that is associated with doxorubicin resistance [12]. PD-L1 is associated with OCT4A, Nanog, and BMI1 stemness markers through activation of Akt signaling, whereas self-renewal characteristics of CSC depend on PD-L1 expression in BCSCs [160]. PD-L1 expression enhances chemoresistance in breast cancer and its regulatory activity that contributes to breast cancer stemness can be ablated by PD-L1 inhibitors in MCF-7 and MDA-MB-231 cells [161].

Table 2
Important compounds discussed in the article and their PubChem Compound IDs (CIDs).

Inhibitor	PubChem CID
MK-2206	24,964,624
Capivasertib (AZD5363)	25,227,436
Uprosertib	51,042,438
GSK2110183 hydrochloride	92,044,396
Ipatasertib	24,788,740
Berberine	2353
Lapatinib	208,908
Paclitaxel	36,314
Fulvestrant	104,741
Wortmannin	312,145

Wang et al. (2018) showed that p-Akt, p-STAT3 and p-ERK levels are significantly correlated with PD-L1 expression in non-small cell lung cancer (NSCLC) [162]. They further showed that association of Akt, STAT, and ERK proteins does not depend on EGFR mutations, but instead PD-L1 expression is regulated by EGFR/Her2-dependent pathways in NSCLC [163]. Several transcription factors including Myc, STAT3, AP1, NF-κB, and HIF-1α are activated by PI3K/Akt, JAK/STAT and ERK pathways that regulate PD-L1 expression in TNBC [164] (Fig. 8). We previously mentioned that epigenetic modifiers such as DNMT1 upregulate Akt signaling. DNMT1 is also associated with PD-L1 upregulation. As such, the use of DNMT1 inhibitors in addition to PD-L1 antagonists and Akt inhibitors can be effective for downregulating PD-L1 activity [165]. On the other hand, interferon-γ, which activates the JAK/STAT pathway, decreases the susceptibility of cancer tumors to Natural Killer (NK) cells through PD-L1 upregulation [166].

Other cytokines including IL-27, IL-17, and TNF-α regulate PD-L1 expression through STAT1/NF-κB, and ERK pathways. Zhang et al. (2018) showed that the cytokine receptor CXCR3 expressed in gastric cancer cell lines upregulates PD-L1 expression via activation of STAT and Akt signaling pathways [167]. Insulin and EGF could also elevate PD-L1 expression through Akt signaling. Dactolisib, a dual PI3K/mTOR inhibitor, could reduce levels of PD-L1 on the surface of colon cancer stem cells. Accordingly, inhibition of insulin and EGFR signaling as well as inhibition of Akt signaling might be effective in combination with PD-L1 inhibitors [168]. Sustained expression of PD-L1 results in activation of Akt signaling in PTEN-impaired cancer cells, although PD-L1 inhibitors decrease Akt phosphorylation [169]. Based on a recent study, downregulation of PD-L1 using atezolizumab also downregulates expression of genes related to metastasis, EMT, cell growth, and hypoxia in MDA-MB-231 cells [8]. MCF-7 and MDA-MB-231 cells strongly suppress anticancer immunity by virtue of the high levels of PD-L1 expression in these cells [170]. In MDA-MB-231 cells, PD-L1 expression is inhibited by hesperidin through inhibition of Akt and NF-κB signaling [171]. The crucial factors involved in PD-L1 expression as well as the modifications of PD-L1 are not well-understood, although suppression of PD-L1 expression, which may be directed by Akt signaling, seems to be an effective strategy to treat chemoresistance in breast cancer [172].

5. Akt-targeted therapy and future directions

Akt can be regulated at different levels to suppress oncogenic activity in breast cancer and lies at the intersection of several oncogenic pathways in breast cancer cells. Akt-mediated signaling is activated by EGFR, anti-oxidants, ER, the Wnt/β-catenin pathway, and PD-L1. On the other hand, breast cancer cells treated with anticancer agents such as tamoxifen and lapatinib can develop resistance, whereas PD-L1/PD-1 immunotherapy may can increase BCSC populations to contribute to chemotherapy resistance through Akt activation. Akt inhibitors such as MK-2206 and capivasertib (AZD5363) are a beneficial combination with EGFR/Her2 inhibitors such as geftinib, lapatinib, AZD8931, and

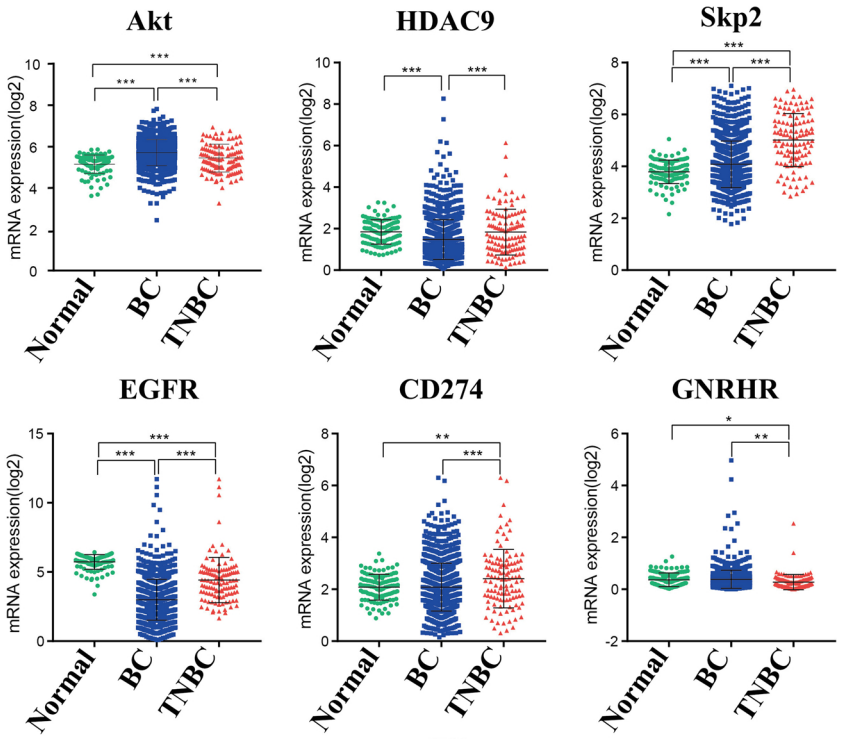

Fig. 9. Gene expression analysis of Akt and related proteins in breast cancer patients. Akt and Skp2 expression is higher in breast cancer (BC) and TNBC patients than in healthy individuals. EGFR and CD274 (PD-L1 gene) exhibit a broad range of expression among breast cancer and TNBC patients depending on the genetic profile. Therefore, use the effectiveness of EGFR and PD-L1 antagonists used to treat breast cancer could vary However, both Akt and Skp2 inhibitors have potential for successful treatment of breast cancer and TNBC patients. GNRHR is highly expressed in ER^+ breast cancer, but its levels in TNBC are very low. Thus, HDAC inhibitors could also be effective to treat this type of breast cancer. **Case numbers:** Breast cancer: 1094; TNBC: 115; Normal: 113.

trastuzumab for treatment of ER^- and ER^+ breast cancer (Table 1) (Table 2) [91,173,174].

We compared RNA expression levels of several genes including Akt, EGFR, PD-L1 (CD274), Skp2, GnRH receptor (GNRHR), and HDAC9 for breast cancer and TNBC listed in The Cancer Genome Atlas (TCGA) (Fig. 9). The expression of Akt and Skp2 in cancer patients, including those with TNBC, is significantly higher than that seen in healthy individuals. Skp2 is not only involved in Akt activation, but also proteolysis of Akt substrates such as FoxO1/3. GNRHR is a crucial regulator of E2 signaling in ER^+ breast cancer, and is highly expressed in breast cancer samples relative to TNBC. In contrast, overall, PD-L1, a suppressor of anticancer immunity, is not strongly expressed in breast cancer patients, and the range of expression levels for PD-L1 in breast cancer cases suggested that the efficacy of PD-L1 inhibitors will be patient-specific. Meanwhile, Akt is a marker of breast cancer and is highly expressed in almost all breast cancer subsets. EGFR expression levels exhibit a broader range than that for Akt in breast cancer, although, like PD-L1, EGFR inhibitors will likely not be effective for a majority of breast cancer cases.

Akt also has a role as a hypoxia-sensing oncoprotein that modulates signaling based on oxygen availability. When oxygen availability is lowest, ROS and NAD^+ levels, as well as those of calcium ions, are high in the cytoplasm of cancer cells. Inhibition of the ETC and TCA cycle in mitochondria results in release of large amounts of oxidized molecules such as ROS and NAD^+. Akt requires NAD^+-dependent deacetylation of SIRT1/2 and high levels of NAD^+ could increase the amount of SIRT activation that in turn promotes increased Akt activation. As such, Akt regulation is literally a NAD^+-dependent process. When oxygen levels are low, the amount of NAD^+ and ROS increases. The effects of Akt on the transcription factor Nrf2 are not well understood, although these two proteins do affect one another under hypoxic conditions such that Nrf2 can drive expression of anti-oxidant genes. In addition, both are highly activated in breast cancer cells that are resistant to chemotherapy.

In addition to its involvement in breast cancer cell growth and metastasis, Akt acts in different ways under different conditions. Akt suppresses autophagy in hypoxia but also activates Ca^{2+} flow into the mitochondria by mediating VDAC phosphorylation. Akt itself is translocated into mitochondria to suppress TCA cycle. Accordingly, Akt activity can promote high concentrations of mitochondrial calcium and further generation of ROS and NAD^+, which, together with GSK-3β, lead to mitochondrial collapse, cytochrome c release, and apoptosis under hypoxic conditions. Importantly, Akt can remodel metabolic profiles from aerobic to anaerobic. According to results from earlier investigations, Akt can behave not only as an oncoprotein, but also as a reprogrammer of tumor metabolism.

6. Conclusion

Akt participates in crosstalk with other signaling pathways including those involving MAPK, Notch, Snt/β-catenin, NF-κB and Nrf2. In addition, Akt reprograms the metabolism of tumor cells and its mitochondrial concentration is increased under hypoxic conditions. The reprogramming function of Akt suppresses autophagy-related death and apoptosis that in turn leads to cancer cell growth under hypoxia, while the detailed effects of Akt in breast cancer cells under anoxic conditions requires further research. Akt responds to oxygen availability through reprogramming of cell metabolism under hypoxic conditions and can respond to anti-oxidant signaling to reduce the sensitivity of breast cancer cells to therapy. In conclusion, the use of Akt inhibitors in combination with other types of therapy, including immunotherapy, can be an effective strategy to control cancer cell metabolism.

Declaration of Competing Interest

The authors declare no conflicts of interest.

Acknowledgement

This work was supported by Southwest Medical University (SWMU) grants 05/00040129 and 2019ZQN001 that were awarded to PJK.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.phrs.2020.104806.

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