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Review Article

Chemoresistance in breast cancer: PI3K/Akt pathway inhibitors vs the current chemotherapy

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Abstract: Breast cancer is the most prevalent type of cancer among women. Several types of drugs, targeting the specific proteins expressed on the breast cancer cell surface (such as receptor tyrosine kinases and immune checkpoint regulators) and proteins involved in cell cycle and motility (including cyclin-dependent kinases, DNA stabilisers, and cytoskeleton modulators) are approved for different subtypes of breast cancer. However, breast cancer also has a poor response to conventional chemotherapy due to intrinsic and acquired resistance, and an Akt fingerprint is detectable in most drug-resistant cases. Overactivation of Akt and its upstream and downstream regulators in resistant breast cancer cells is considered a major potential target for novel anti-cancer therapies, suggesting that Akt signalling acts as a cellular mechanism against chemotherapy. The present review has shown that sustained activation of Akt results in resistance to different types of chemotherapy. Akt signalling plays a cellular defence role against chemotherapy and (1) enhances multi-drug resistance, (2) increases reactive oxygen species at breast tumor microenvironment, (3) enhances anaerobic metabolism, (4) inhibits the tricarboxylic cycle, (5) promotes PD-L1 upregulation, (6) inhibits apoptosis, (7) increases glucose uptake, and more importantly (8) recruits and interconnects the plasma membrane, nucleus, endoplasmic reticulum, and mitochondria to hijack breast cancer cells and rescue these cells from chemotherapy. Therefore, Akt signalling is considered a cellular defence mechanism employed against chemotherapeutic effects. In addition, interfering roles of PI3K/Akt signalling on the current cytotoxic and molecularly targeted therapy as well as immunotherapy of breast cancer are discussed with a clinical approach. Although, alpelisib, a PIK3CA inhibitor, is the only PI3K/Akt pathway inhibitor approved for breast cancer, we also highlight well-evaluated inhibitors of PI3K/Akt signalling based on different subtypes of breast cancer, which are under clinical trials whether as monotherapy or in combination with other types of chemotherapy.

Keywords: PI3K/Akt/mTOR inhibitor, breast cancer, drug resistance, targeted therapy, stress conditions

Introduction

Breast cancer is the most prevalent type of cancer among women and the second most prevalent type of cancer worldwide [1]. Endocrine therapy is the first-line therapeutic strategy for oestrogen receptor-positive (ER^+) breast cancer patients and uses one of two main drug classes: aromatase inhibitors (AIs) and ER antagonists. Since ER signalling has crosstalk with growth factor signalling, resistance to endocrine therapy is the main barrier for effective treatment of ER^+ breast cancer patients [2]. On the other hand, approximately 20% of invasive breast cancer cases are diagnosed as human epidermal growth factor receptor (EGFR) 2-positive ($Her2^+$), making anti-Her2 therapy a second therapeutic strategy against breast cancer [3]. As successful treatment of $Her2^+$ breast cancer is mostly dependent on targeting the specific proteins expressed on the cell surface, several reports have shown that $Her2^+$ breast cancer cells can transform into metastatic and/or breast cancer stem cells (BCSCs) with a poor response to Her2 antagonists [4-6]. However, the triple-neg-

ative breast cancer (TNBC) has wider range of chemotherapy is mostly dependent on targeting the specific proteins expressed on the cell surface, such as receptor tyrosine kinases (RTKs) and immune checkpoint regulators, as well as targeting proteins mediating cell cycle, including cyclin-dependent kinases, DNA repair, and cytoskeleton modellers [7].

The phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt) signalling in cancer cells mediates chemoresistance in the tumour microenvironment by shielding the immune responses and activating survival signalling pathways in a multitude of human cancers. Overexpression of the PI3K/Akt signalling pathway has active crosstalk with several pathways in different molecular subtypes of breast cancer, including mitogen-activated protein kinase (MAPK), Notch, wnt/β-catenin, and nuclear factor-κB, which leads to chemotherapeutic resistance [8, 9]. Multidrug resistance (MDR) proteins are a crucial barrier against chemotherapy by which drugs that have entered cancer cells are pumped out through an ATP-dependent process. The expression of MDR proteins, especially p-glycoprotein (P-gp), and breast cancer resistance protein (BCRP), as well as the phosphorylated Akt, have been detected in paclitaxel- and adriamycin-resistant MDA-MB-231 and MCF-7 breast cancer cell lines [10, 11]. In addition, resistance to doxorubicin as a first-line drug is also associated with the expression of P-gp and BCRP. Akt leads to an increase in the cellular level of calcium and a decrease in mitochondrial tricarboxylic acid (TCA) cycle, which eventually increases the level of reactive oxygen species (ROS) as end products of breast cancer cell metabolism [12]; however, ROS activate ROS-dependent apoptosis in breast cancer [1, 13]. On the other hand, Akt activates antioxidant signalling and inhibits apoptosis by activating nuclear factor erythroid 2-related factor 2 (Nrf2) [14]. Nrf2 activated by Akt is a cellular response against chemotherapy through the expression of several genes related to antioxidant signalling, MDR, and cell survival [15]. On the other hand, hypoxia-inducible factor-1α (HIF-1α) is a transcription factor whose activation is regulated by both PI3K/Akt and MAPK pathways. HIF-1α upregulates several genes related to glucose metabolism and cancer stemness [16]. Akt enhances hypoxic conditions and ROS levels by activating the main regulators of chemoresistance, HIF-1α and Nrf2 (**Figure 1**). Therefore, HIF-1α activation by Akt is another cellular response against chemotherapy [17, 18]. This releases small molecules, such as calcium, pyruvate, ATP, ADP, and citrate, into the cytoplasm, enhancing the stress condition of cancer cells [19]. Furthermore, HIF-1α leads to cell survival and cancer cell migration as well as ROS creation for ROS scavenger molecules, like glutathione (GSH) [20]. On the other hand, inhibition of ROS generation and autophagy, as well as direct inhibition of Akt, are effective therapeutic strategies for overcoming Akt-promoted drug resistance [21].

Previously, we reviewed the molecular structure of Akt as well as the detailed mechanisms of Akt signalling and its effects on breast cancer cell metabolism [15]. In the present review, we focus on targeted therapy of breast cancer and the clinical roles of the whole PI3K/Akt signalling pathway inhibitors compared with the current available chemotherapies. Furthermore, we seek to explore the evidence related to the role of three categories of compounds targeting the PI3K/Akt signalling pathway (PIK3CA inhibitors, Akt inhibitors, and mTOR inhibitors) on the future of chemotherapy and immunotherapy in breast cancer.

How resistance to the current chemotherapy is connected to PI3K/Akt signalling?

PI3K/Akt pathway and cytotoxic drugs

CDK4/6 inhibitors: Approximately 20% of breast cancer patients possess impaired p53 tumour suppressors, which in turn have impaired expression of p21, another tumour suppressor; therefore, these cells are unable to check the accuracy of the cell cycle [22]. These cancer patients suffer from uncontrolled cell division due to the overactivation of CDKs. The use of CDK inhibitors, such as palbociclib, ribociclib, and abemaciclib, is critical for inhibition of the cell cycle in ER^+ breast cancer cells. As the co-alteration of cell cycle and PI3K pathways have been reported in ER^+ breast cancer, the use of CDK inhibitors combined with PI3K inhibitors was rationally recommended for ER^+ breast cancer patients [23]. However, Akt activation and expression of MDR genes have been shown in palbociclib-resistant MCF-7 ER^+ cells. Everolimus, an mTOR inhibitor, reversed resis-

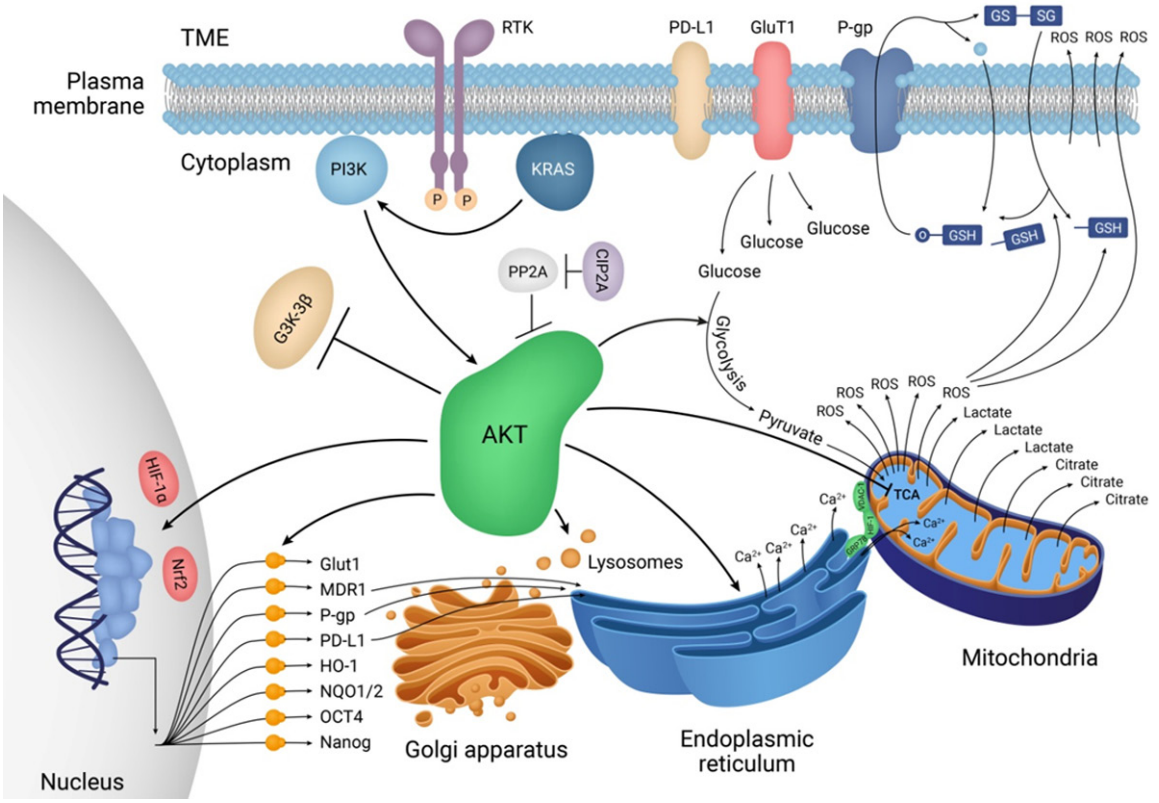

Figure 1. Akt as the master regulator of chemoresistance in breast cancer cells. Akt and its metabolic roles in breast cancer. PI3K activates Akt through Akt recruitment at the plasma membrane. In the case of RAS overactivation, KRAS alternatively activates Akt signalling in an RTK-independent manner. KRAS-dependent activation of Akt upregulates HIF-1α and Nrf2 transcription factors by which numerous genes related to glucose metabolism (e.g., glucose transporter 1), MDRs (e.g., BCRP, MDR1, and P-gp), antioxidants (e.g., HO-1 and NAD(P)H dehydrogenase 1/2), cancer stemness (e.g., OCT4 and Nanog), and immune checkpoints (e.g., PD-L1) are upregulated.

tance to palbociclib in MCF-7-P cells [24]. The use of palbociclib for TNBC cells with downregulated retinoblastoma tumour suppressor protein (Rb) is beneficial, but Akt signalling was also induced in palbociclib-treated TNBC cells [25]. Therefore, combined chemotherapy of CDK inhibitors and PI3K/Akt/mTOR pathway inhibitors are recommended for TNBC and ER^+ breast cancer patients.

Microtubule inhibitors: Microtubule inhibitors are an important category of anti-cancer drugs whose resistance to chemotherapy critically decreases the efficacy of current chemotherapy. For patients suffering from $Her2^+$ and ER^+ breast cancers showing resistance to endocrine and anti-Her2 therapies, and for patients suffering from $Her2^-$ and ER^- breast cancers, microtubule inhibitors, such as paclitaxel and Vinca alkaloids, are considered first-line chemotherapeutics. The actin-binding protein CapG has been reported to induce resistance to paclitaxel in breast cancer by activating Akt signalling through interaction with p300/cAMP-response element binding protein binding protein at the PIK3 regulatory subunit 1 promoter [26]. Moreover, inhibition of CapG and p300/cAMP-response element binding protein binding protein has been shown to attenuate PI3K/Akt signalling, resulting in a higher sensitivity to paclitaxel. In addition, suppression of PI3K/Akt signalling reverses resistance to paclitaxel when breast cancer cells are co-cultured with mesenchymal stem cells [27]. Another study also showed that transgelin-2, another actin-binding protein in breast cancer cells, promotes resistance to paclitaxel through Akt activation [26]. The Akt inhibitor MK-2206 has been shown to reverse resistance to paclitaxel in paclitaxel-resistant ER^+ cell [28]. It seems actin-binding proteins are necessary for migration and metastasis of cancer cells, thus the

activation of actin polymerization as well as enhancement of Akt-induced chemoresistance [29]. Moreover, paclitaxel-resistant ER^+, $Her2^+$, and TNBC cells have upregulated PI3K/Akt signalling and microfilament formation, which leads to metastasis and invasiveness. Since Akt is involved in Nrf2 activation, MDR proteins are also upregulated in paclitaxel-resistant breast cancer cells [30, 31].

Since TNBC cells usually have impaired PTEN, they also have overactivated PI3K/Akt signalling. Eribulin is a non-taxane microtubule inhibitor approved for metastatic breast cancer in 2010 [32]. Resistance to eribulin in breast sarcomas has been reported and might be due to increased levels of phoshpo-Akt. PTEN-deficient TNBC cells show higher activity of the PI3K/Akt/mTOR pathway. Accordingly, a combination of an mTOR inhibitor, TAK228, with eribulin was evaluated in such cells. TAK228 is a dual mTORC1/2 inhibitor [33], and mTORC2 has positive feedback on Akt; therefore, TAK228 can inhibit both Akt (indirectly) and mTOR (directly) activation in different TNBC cells, which shows therapeutic effects similar to those of Akt inhibitors. In addition, TAK228 has been shown to synergistically sensitize TNBC cells to eribulin [34, 35].

Nucleoside analogues: Antimetabolites are a category of anti-cancer agents that interfere with DNA/RNA metabolism of cancer cells by suppressing DNA replication, transcription, and protein synthesis. The pyrimidine analogue 5-Fluorouracil (5-FU) inhibits thymidylate synthetase. Akt overactivation has been reported in 5-FU-resistant MDA-MB-453 $Her2^+$ breast cancer cells, and the combination of apigenin with 5-FU reverses 5-FU resistance through downregulation of Her2 and Akt [36]. It seems that overexpression of thymidylate synthetase is the main reason for resistance to 5-FU; however, the overactivation of Akt by 5-FU has been shown to lead to 5-FU-related chemotherapy resistance [37]. Furthermore, cellular stress enhanced by activation of chaperones, such as GRP78, located at/in the endoplasmic reticulum recruits Akt to the endoplasmic reticulum and mitochondrial membranes, triggering hypoxia and metabolic reprogramming in TNBC breast cancer cells. As 5-FU induces endoplasmic reticulum stress, GRP78 is upregulated, which then upregulates Akt expression and OCT4. Therefore, targeting Akt and OCT4 as well as inhibition of GRP78 may sensitize breast cancer cells to 5-FU [38].

Gemcitabine is another antimetabolite that inhibits thymidylate synthetase. To decrease the risk of recurrence in breast cancer, anthracyclines are topoisomerase inhibitors used as adjuvant therapy [39]. Patients who do not respond to anthracyclines can receive gemcitabine combined with paclitaxel [40]. Resistance to gemcitabine has also been reported in breast cancer cells with elevated Akt and Src activities. In addition, Akt regulates BCSC migration and resistance of breast cancer cells. Accordingly, Akt and Src inhibition sensitize breast cancer cells to gemcitabine and downregulate BCSC markers, such as CD44 and OCT4 [41]. The stemness of breast cancer cells can be reversed with everolimus [24].

PI3K/Akt pathway and molecularly-targeted therapy

ER antagonists: More than 80% of breast cancer patients have an ER^+ subtype, among which 65% are also progestrone receptore-positive (PR^+). Howlader *et al.* (2018) found that hormone-receptor-positive breast cancer subtypes have higher survival compared with other subtypes of breast cancer based on grade [42]. Although ER^+ breast cancer cases are treatable with ER antagonists and AIs, poor pathological complete response rates may be due to a change in the histological subtype. Molecular changes in breast cancer subtypes (up to 43%) may lead to a conversion from ER^+ or $Her2^+$ to TNBC, thus leading to an increase in the resistance to tamoxifen or trastuzumab after several years of molecularly-targeted therapy. Therefore, a poor pathological complete response does not mean poor prognosis, but can be due to acquired resistance to chemotherapy. In addition, a pathological complete response is useful in accelerating the approval of drugs (**Table 1**) [43, 44]. Downregulation of ER-α is the main reason for resistant ER^+ breast cancer cells to tamoxifen. Spalt-like transcription factor 2 (SALL2) is a transcription factor that directly targets ESR1, which upregulates ER and PTEN by targeting ER-α and PTEN promoters followed by downregulation of PI3K/Akt signalling. Hypermethylation of the SALL2 promoter has also been observed in tamoxifen-resis-

Table 1. Common chemotherapy and combination/adjuvant therapies clinically under investigation for breast cancer patients

Category	Sub-Category	First-line	1st Target	CID/SID[a]	Combination/Replacement (The 2nd target)	Clinical trial No.[b]	Clinical phase
Cytotoxic agents	CDK inhibitor	Palbociclib[i]	CDK4/6	5330286	Capecitabine (TS)	NCT03322215	II
					Anastrozole (Aromatase)	NCT01723774	II
					Exemestane (Aromatase)	NCT02549430	II
					Letrozole (Aromatase)	NCT04318223	II
					Fulvestrant (ER)	NCT01684215[c]	I/II
					Tamoxifen (ER)	NCT03220178	IV
					Trastuzumab (Her2)	NCT02384239	II
						NCT03304080	I/II
	Anti-microtubule	Paclitaxel	Microtubule	36314	Dasatinib (Src)	NCT01306942[c]	I/II
					Trastuzumab (Her2)	NCT03179904	II
					Laniquidar (P-gp)	NCT00028873	II
					PSC 833 (P-gp)	NCT00001383	I
					Ipatasertib (Akt)	NCT03853707	I/II
					Atezolizumab (PD-L1)	NCT03515798	II
					Pembrolizumab (PD-1)	NCT02365805	II
					Bevacizumab (VEGF-A)	NCT01095003	III
					5-FU (TS)		
					Cyclophosphamide (DNA)		
		Eribulin	Microtubule	11354606	Atezolizumab (PD-L1)	NCT03202316	II
	Anti-metabolite	5-FU	TS[d]	3385	Paclitaxel (Microtubule)	NCT03515798	II
					Pembrolizumab (PD-1)		
		Gemcitabine	TS	60750	GB1275 (CD11b)	NCT04060342	I/II
Molecularly-targeted agents	Endocrine therapy	Tamoxifen	ER	2733526	Ralimetinib[e] (MAPK)	NCT02322853	II
					Palbociclib (CDK4/6)	NCT02384239	II
					Everolimus (mTOR)	NCT01298713	II
		Fulvestrant	ER	104741	Anastrozole (Aromatase)	NCT00738777	II
					Palbociclib (CDK4/6)	NCT04318223	II
					Vandetanib (VEGFR)	NCT02384239	II
					Apitolisib[f] (PI3K)	NCT02530411	II
					Everolimus (mTOR)	NCT01437566	II
						NCT01797120[c]	II
		Exemestane	Aromatase	60198	Bevacizumab (VEGF-A)	NCT00240071[c]	II
					Fulvestrant (ER)	NCT03575260	-
					Everolimus (mTOR)	NCT03695341	-
		Anastrozole	Aromatase	2187	Fulvestrant (ER)	NCT00738777	II
					Bevacizumab (VEGF-A)	NCT00240071[c]	II
					Palbociclib (CDK4/6)	NCT01723774	II
					Ribociclib (CDK4/6)	NCT04256941	II
					Abemaciclib (CDK4/6)	NCT01791985	I/II
					AZD4547 (FGFR)		

		Letrozole	Aromatase	3902	Bevacizumab (VEGF-A) Palbociclib (CDK4/6) Ribociclib (CDK4/6) Abemaciclib (CDK4/6) AZD4547 (FGFR) Pilaralisib[g] (PI3K) Voxtalisib[h] (PI3K) Everolimus (mTOR) Lapatinib (EGFR/Her2)	NCT00240071[c] NCT04256941 NCT01791985 NCT01082068 NCT01231659 NCT01275859	II II I/II I/II IV II
	RTK-targeted therapy	Lapatinib	EGFR/Her2	208908	Epirubicin (DNA) Trastuzumab (Her2) Letrozole (Aromatase)	NCT00753207 NCT01875666 NCT01275859	I I II
		Trastuzumab	Her2	135301230	Pertuzumab (Her2) Lapatinib (EGFR/Her2) Paclitaxel (Microtubule) Imetelstat[i] (Telomerase) Capecitabine (TS) Pyrotinib (EGFR/Her2) Everolimus[j] (mTOR) Dasatinib (Src) Pembrolizumab[k] (PD-1) Rapamycin (mTOR) Deforolimus (mTOR) Vinorelbine (Tubulin)	NCT01875666 NCT02073487 NCT01265927 NCT04001621 NCT00317720[c] NCT01306942[c] NCT02129556[c] NCT00411788[c] NCT00736970 NCT01007942[c]	I II I II I/II I/II I/II II II III
		T-DM1	Her2/Tubulin	135353969	Lapatinib (EGFR/Her2) Abraxane (Microtubule)	NCT02073487	II
Agents targeting immuno-signalling	Immune checkpoint inhibitor	Atezolizumab	PD-L1	249565671	Ipatasertib (Akt) Paclitaxel (Microtubule) Eribulin (Microtubule)	NCT03853707 NCT03202316	I/II II
	Cytokine pathway inhibitors	Ruxolitinib	JAK1/2	25126798	Capecitabine (TS)	NCT01562873[c] NCT02120417[c]	II II

[a]CID/SID: PubChem Compound ID/Substance ID; [b]This table prepared based on the data collected from www.clinicaltrial.gov; [c]Clinical trial with results; [d]Thymidylate Synthetase; [e]Ralimetinib is also known as LY2228820; [f]Apitolisib is also known as GDC-0980; [g]Pilaralisib is also known as SAR245408 or XL147; [h]Voxtalisib is also known as SAR245409 or XL765; [i]Imetelstat is also known as GRN163L; [j]Everolimus was formerly known as RAD001; [k]Pembrolizumab is also known as MK-3475; [l]Palbociclib is also known as PD-0332991.

tant breast cancer cells [45]. Tumour-associated macrophages, which release CC-chemokine ligand 2, can also increase resistance to tamoxifen through activation of PI3K/Akt signalling [46]. Besides, Ras and other EGFR-associated proteins, like Nogo-B receptor, which recruits Ras to the plasma membrane, also enhance endocrine therapy resistance. Nogo-B receptor regulates oestrogen-induced activation of Akt signalling. Activated ER crosstalks with Akt, and Akt activation is the leading cause of resistance to endocrine therapy [47, 48].

Besides, other proteins are also associated with resistance to tamoxifen through activation of PI3K/Akt signalling, such as peptidyl-prolyl isomerase 1, which stabilizes Akt and is overexpressed in breast cancer. Inhibition of peptidyl-prolyl isomerase 1 leads to the downregulation of MAPK/extracellular signal-regulated kinase 1/2 and PI3K/Akt pathways, thereby reducing the proliferation and viability of tamoxifen-resistant breast cancer cells [49]. On the other hand, the eukaryotic regulator 14-3-3ζ has been reported as a prognostic marker in ER^+ breast cancer patients; 14-3-3ζ regulates autophagy by interacting with beclin-1 in hepatocellular carcinoma [50, 51]. Moreover, sustained activation of Akt upregulates 14-3-3ζ through expression of nicotinamide phosphoribosyl transferase, resulting in resistance to tamoxifen in ER^+ breast cancer cells [52]. Akt activates HIF-1α-dependent autophagy in breast cancer, which leads to expression and activation of autophagy-related proteins, such as LC3-I/II and beclin-1 [53, 54]. Therefore, 14-3-3ζ may serve as another player, hand-in-hand with Akt signalling to promote hypoxia, autophagy, and resistance in ER^+ breast cancer.

The inhibitory effects of oestrogen on paclitaxel-induced apoptosis have been shown in ovarian cancer, which expresses the oestrogen receptor. The PI3K inhibitor LY294002 decreased the negative effect of oestrogen on paclitaxel-related apoptosis in Caov-3 human ovarian cancer cell line; however, transfection of this cell line with ASK1S83A, a mutated Akt with the attenuated ability of phosphorylation, reduced the inhibitory effect of oestrogen [55]. Therefore, as oestrogen promotes breast cancer progression through Akt activation [48], the combinatorial usage of Akt inhibitors would have great potential for treating ER^+ breast cancer patients [56, 57]. Finally, alpelisib, which sensitizes ER^+ breast cancer cells to tamoxifen and fulvestrant [58, 59], is the first PI3K inhibitor approved by the US FDA to treat $Her2^-$ breast cancer cells possessing mutated PI3K [60].

Aromatase inhibitors: Aromatase is an adrenal enzyme involved in the conversion of androstenedione and estrone to oestrogen. Acquired resistance to AIs, such as anastrozole and letrozole, has been observed in ER^+ breast cancer patients. Crosstalk between PI3K/Akt and ER-α signalling has a crucial role in acquired resistance to letrozole. Letrozole-resistant cells have elevated PI3K/Akt signalling levels, which might be due to elevated levels of the catalytic subunit of PIK3CA (P110α) and not Akt [61]. Resistance to AIs is not only dependent on ER, but a higher level of activated Akt has also been detected in AI-resistant patients, possibly due to dysregulation of Her2 [62]. Interestingly, treatment with MK-2206, rapamycin, Akt, and mTORC1 inhibitors has been shown to sensitize ER^+ breast cancer cells to AIs [63]. Furthermore, inhibition of Akt signalling has a beneficial role in reversing resistance to Her2 and ER antagonists in $Her2^+$ and ER^+ breast cancer cells. Metastatic breast cancer patients who are $Her2^+$ and ER^+ and carry a PIK3CA mutation are resistant to AIs and anti-HER2 therapy. However, the PI3K mutation in resistant $Her2^+$ and ER^+ breast cancer cells triggers aberrant activation of upstream proteins involved in Akt activation [64].

In addition, exemestane (a steroidal AI) induces autophagy as a cellular defence against chemotherapy, which is associated with acquired resistance in ER^+ breast cancer cells [65]. As autophagy is regulated by Akt signalling, Akt and mTOR inhibitors combined with exemestane reverses exemestane-dependent acquired resistance in $Her2^-ER^+$ breast cancer [62]. Moreover, autophagy induces resistance to AIs, which has been shown to enable anti-mTOR therapy combined with AIs to sensitize AI-resistant cells to tamoxifen and AIs [66]. However, mTORC2, which has a positive feedback on Akt, does not respond to some mTORC inhibitors, such as rapamycin, which is more specific to mTORC1. Citi *et al*. (2018) showed that cells with mutated PIK3CA and defective

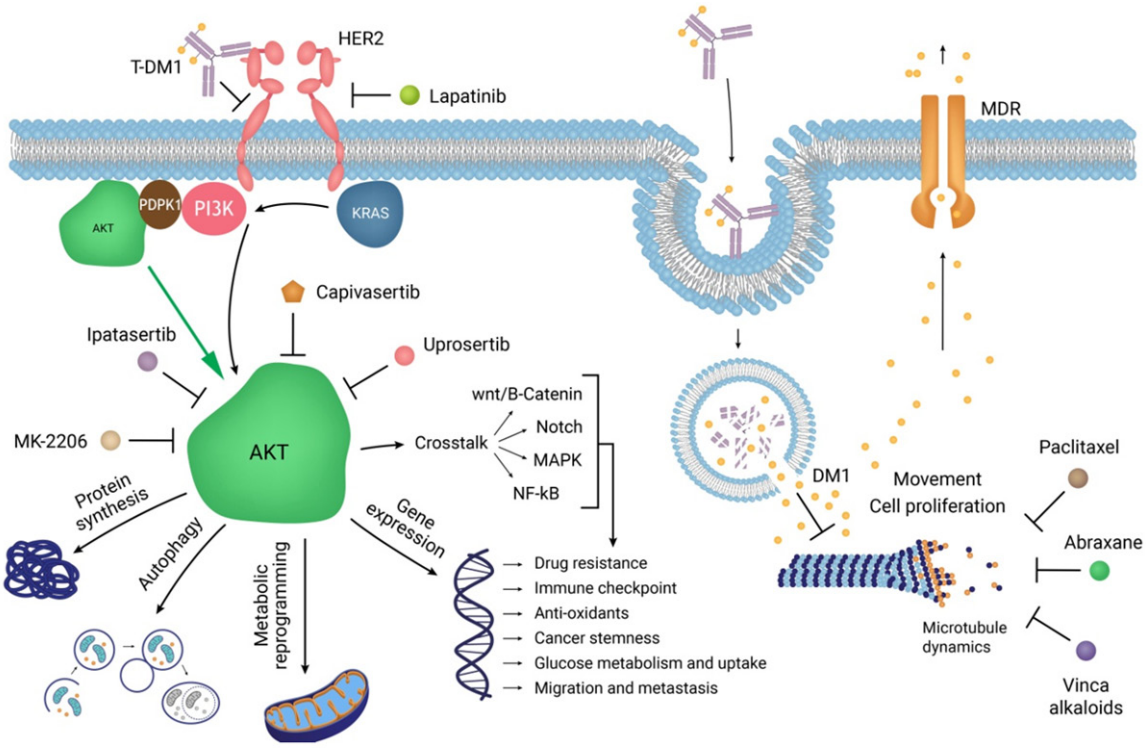

Figure 2. Akt inhibition compared with Her2/EGFR-targeted therapy in breast cancer cells. The mechanisms of actions of different drugs targeting Her2$^+$ breast cancer cells. Akt interconnects different cellular organelles to hijack cellar metabolism, forcefully alters the TME, and promotes breast cancer progression and metastasis. T-DM1, a monoclonal antibody (trastuzumab) conjugated with a small molecule (DM1), is an innovative drug against Her2$^+$ breast cancer, which targets both Her2 signalling and tubulin polymerization. However, internalized DM1 can still be targeted by MDRs and GSH/ROS-mediated recycling systems. Direct targeting of Akt in combination with common chemotherapy reverses resistance to chemotherapy. MK-2206, ipatasertib, capivasertib, and uprosertib are well-studied Akt inhibitors.

PTEN have different responses to anti-mTOR therapy. Everolimus effectively decreased the proliferation of tumorigenic cell lines with different potency and efficacy. ZR-75-1 was the most sensitive, and T-47D was the most resistant cell line to everolimus. Additionally, no significant changes in phospho-Akt levels have been found in sensitive breast cancer cell lines, MCF-7 and HCC1500, while the resistant cell line, T-47D, had higher levels of phospho-Akt [67]. Petrossian *et al.* (2018) also showed Akt is overactivated in HR$^+$/Her2$^-$ cells, which were resistant to letrozole and everolimus [68]. Therefore, three factors affect the response to anti-mTOR therapy as a replacement for anti-Akt therapy: (1) mutations inducing PIK3CA, (2) mutations inactivating PTEN, and more importantly (3) cellular levels of phospho-Akt (S473/T308) and phospho-extracellular signal-regulated kinase (ERK) 1/2 (T202/T204).

Her2/EGFR antagonists: Lapatinib is a dual TKI against both Her2 and EGF receptors. Lapatinib activates Akt phosphorylation in Her2$^+$ breast cancer and TNBC, resulting in resistance to EGFR therapy [8, 69]. Conclusively, overexpression of HIF-1α alters the TME, making it hypoxic, and reprograms the metabolism of breast cancer cells from aerobic to anaerobic [70]. These stressful conditions managed by Akt enable breast cancer cells to be more aggressive and resistant to chemotherapy. A decrease in lapatinib efficacy was also observed in EGFR expressing MDA-MB-231 TNBC cells with induced hypoxia, and downregulation of HIF-1α reversed the response to EGFR-targeted therapy in such a condition [71] (**Figure 2**).

Furthermore, inhibition of serine/threonine protein phosphatase 2 A, a negative regulator of Akt, by overactivation of cellular inhibitor of serine/threonine protein phosphatase 2 A, which stabilizes Myc oncoprotein, has been observed in lapatinib-resistant Her2$^+$ breast cancer cells and is overcome by combined treatment with Akt inhibitors [72]. However, the inhibitory

effects of lapatinib on Akt phosphorylation and MDR expression in doxorubicin-resistant MDA-MB-231 TNBC cells has also been reported [73]. Overall, mutations in ERBB2 and Ras have been shown to affect the response to TKIs through overactivation of Akt signalling. Apart from normal Ras, which directs the MAPK pathway, studies have shown that KRAS causes inhibition of EGFR and alternatively overactivates Akt signalling, leading to EGFR inhibitor resistance [74]. Additionally, ERBB2 mutations at P780-Y781 enhance Akt upregulation and induce lapatinib resistance in breast cancer cells [75].

Trastuzumab, a monoclonal antibody that targets the Her2 receptor, is another first-line medicine for $Her2^+$ breast cancer. Overactivation of Akt has also been observed in trastuzumab-resistant patients with $Her2^+$ metastatic breast cancer [76]. Overexpression of carboxyl-terminal modulator protein, a positive regulator of Akt, is another reason for Akt activation [77]. The insulin receptor, which phosphorylates insulin receptor substrate 1, can activate PI3K/Akt signalling and induce resistance to trastuzumab [78]. Ankyrin repeat domain 44 is a protein-coding gene expressing a subunit of protein phosphatase 6 that is another negative regulator of cell cycle and is involved in several critical cellular processes, such as metabolism, transcription, and apoptosis. The suppression of ankyrin repeat domain 44 increases S473 phosphorylation on Akt in $Her2^+ER^-PR^-$ breast cancer cells. The level of glycolysis and lactate dehydrogenase B activity is also increased in these cells, demonstrating the role of Akt in metabolic reprogramming as mentioned earlier [79]. Therefore, the downregulation of PP6 is associated with partial resistance to trastuzumab.

Finally, in the case of intrinsic and acquired resistance to trastuzumab, trastuzumab emtansine (T-DM1; an antibody-drug conjugate) can be used as a replacement for trastuzumab [80]. T-DM1 not only binds to the Her2 receptor and inhibits downstream MAPK and PI3K/Akt pathways, but is also internalized through receptor-mediated endocytosis. T-DM1 is released in the cytoplasm and inhibits tubulin polymerization by the same mechanism as Vinca alkaloid suppression of the cell cycle [81] (**Figure 2**). However, resistance to trastuzumab, lapatinib, and T-DM1 has also been observed during multi-Her2 targeted therapy. Recently, a study reported that two $Her2^+$ patients who did not respond to several Her2 antagonists successfully responded to a combination of pembrolizumab (PD-1 inhibitor) and albumin-bound paclitaxel. Another study also reported the use of pembrolizumab combined with trastuzumab has positive effects on the treatment of trastuzumab-resistance [82, 83]; however, the sample size of these studies was small, and further elucidation with a higher number of cases is required.

PI3K/Akt pathway and immunotherapy

Cytokine (JAK/STAT) pathway inhibitors: Janus kinase (JAK)/signal transducers and activators of transcription (STAT) signalling plays a crucial role in the development of malignant tumours as well as inflammation and immune responses through interactions with cytokine receptors. Transphosphorylation of JAK kinases leads to phosphorylation of STATs, which translocates into the nucleus after dimerizing and subsequently induces their target genes. JAK/STAT signalling responds to several cytokines and growth factors, including interleukin (IL)-6 and interferon (IFN)-γ, and activates various genes related to cell proliferation, lipid metabolism, differentiation, and anti-apoptosis. However, it also crosstalks with the PI3K/Akt pathway, which promotes metabolic reprogramming, the cell cycle, cell survival, and metastasis in breast cancer cells [84].

In addition, JAK/STAT signalling is a key pathway in response to cytokines secreted via the anti-cancer immune responses, which leads to expression of PD-L1 in breast cancer cells. Research conducted on BT-549, MDA-MB231, and MCF-7 breast cancer cells has indicated that the level of PD-L1 expression in response to IFN-γ is highly elevated whereas 1 μM Akt inhibitor (wortmannin) and EGFR/Her2 dual inhibitor (lapatinib), as well as a higher concentration (50 μM) of MAPK kinase (MEK) inhibitor (PD98059), decreased PD-L1 expression showing engagement of both Akt and MAPK pathways in PD-L1 expression. This study also showed that treatment with IFN-γ also increased the level of STAT1 and JAK2, but not JAK1 and JAK3, indicating the close association of JAK2/STAT signalling with PD-L1 expres-

sion in breast cancer cells [85]. In contrast, another study recently reported that increased MAPK/ERK signalling in MMTV-Neu cells lowers activation of STATs, and inhibition of MAPK signalling with MEK inhibitors activated STAT1, STAT3, and STAT5, thus leading to expression of PD-L1 in a murine model and TNBC cells [86].

Several JAK inhibitors, such as ruxolitinib, itacinib, and BSK805, have been tested in preclinical and clinical studies to treat breast cancer, among which ruxolitinib as a dual JAK inhibitor targeting both JAK1 and JAK2 is of greater interest. However, a study conducted on several TNBC cell lines showed that JAK-expressing cells that are resistant to MEK inhibitors, including MDA-MB468, do not respond to JAK inhibitors, indicating that an alternative mechanism of JAK activation may be involved in such cells [87]. Consistent with these results, other researchers have observed that MDA-MB468 TNBC cells that acquire resistance to ruxolitinib overexpress Akt. They also showed that Akt knockdown decreased resistance to ruxolitinib [88]. In cases where TNBC cells develop resistance to ruxolitinib, Akt has a compensatory role in decreasing this resistance and supporting the survival of breast cancer cells. Mutated JAK2 can activate Akt and MAPK pathways as well as STAT5 signalling [89]. Crosstalk between STAT5 and Akt has also been reported [90]. However, TNBC cells containing mutated JAK2 with higher Akt and MEK activations may have dual resistance to MEK and JAK2 inhibitors and needs further elucidation (**Figure 3**).

In order to evaluate the effects of JAK inhibitors on women who have breast cancer, several clinical trials have initiated, among which only two phase II studies have published the results. One study was conducted on TNBC patients [NCT01562873], while the other was on ER^+Her2^- breast cancer patients [NCT02120417]. Both of these studies were discontinued because they resulted in a poor improvement in survival rate. In the first study, the safety and efficacy of ruxolitinib were evaluated [NCT01562873] in patients with refractory, metastatic TNBC. Researchers first evaluated the activation of JAK/STAT signalling by immunostaining of phosphorylated STAT3 (N = 171). In 39.2% (67/171) of cases, availability of JAK/STAT signalling was confirmed, among which 21 patients received ruxolitinib. After treatment with 40 cycles of ruxolitinib (1-5 cycles per patient), a different level of toxicity was observed, including fatigue, anaemia, and reduced number of different blood cells such as neutrophils and platelets. As the disease worsened, the treatment was terminated [91]. Lin *et al*. (2017) showed that ruxolitinib could effectively decrease JAK2/STAT3 signalling, but concluded this compound might be cytostatic, thus failing to reduce tumour burden; however, the number of $CD8^+$ T cells in the TME was decreased, showing diminished anti-cancer immunity of the host during treatment with ruxolitinib [NCT01562873]. The small number of TNBC patients was a limitation of the study. JAK/STAT signalling has a close association with various cellular and extracellular biomarkers. The study did not evaluate the combined administration of ruxolitinib with checkpoint inhibitors and/or other drugs targeting cytokines (e.g., IL-6, IFN-γ, *etc*.), their receptors, or Akt pathway inhibitors.

Another randomized, double-blind, phase II clinical trial conducted on ruxolitinib as a single agent and in combination with capecitabine in ER^+Her2^- breast cancer patients (N = 149) showed that while the combination of ruxolitinib and capecitabine was tolerable and showed a greater objective response rate, overall survival rate and PFS were not improved compared with capecitabine alone [92]. The strength of this study was the lower risk of mortality in 40% of cases receiving the combination therapy. Thirty-nine patients (51.3%) who received combination ruxolitinib and capecitabine and 38 (52.1%) who received a combination placebo and capecitabine had died by the final data cutoff time [NCT02120417]. These two phase II studies mentioned here did not support the positive results obtained from preclinical studies. Moreover, both clinical studies ignored the associations of JAK/STAT-linked pathways.

Immune checkpoint (PD-L1) inhibitors: Atezolizumab (a PD-L1 inhibitor) has recently approved for use in combination with paclitaxel for treating TNBC. Atezolizumab affects PD-L1 expression and protein synthesis. Apart from its role in CTL inhibition, atezolizumab downregulates epithelial-mesenchymal transition and cell proliferation and negatively affects

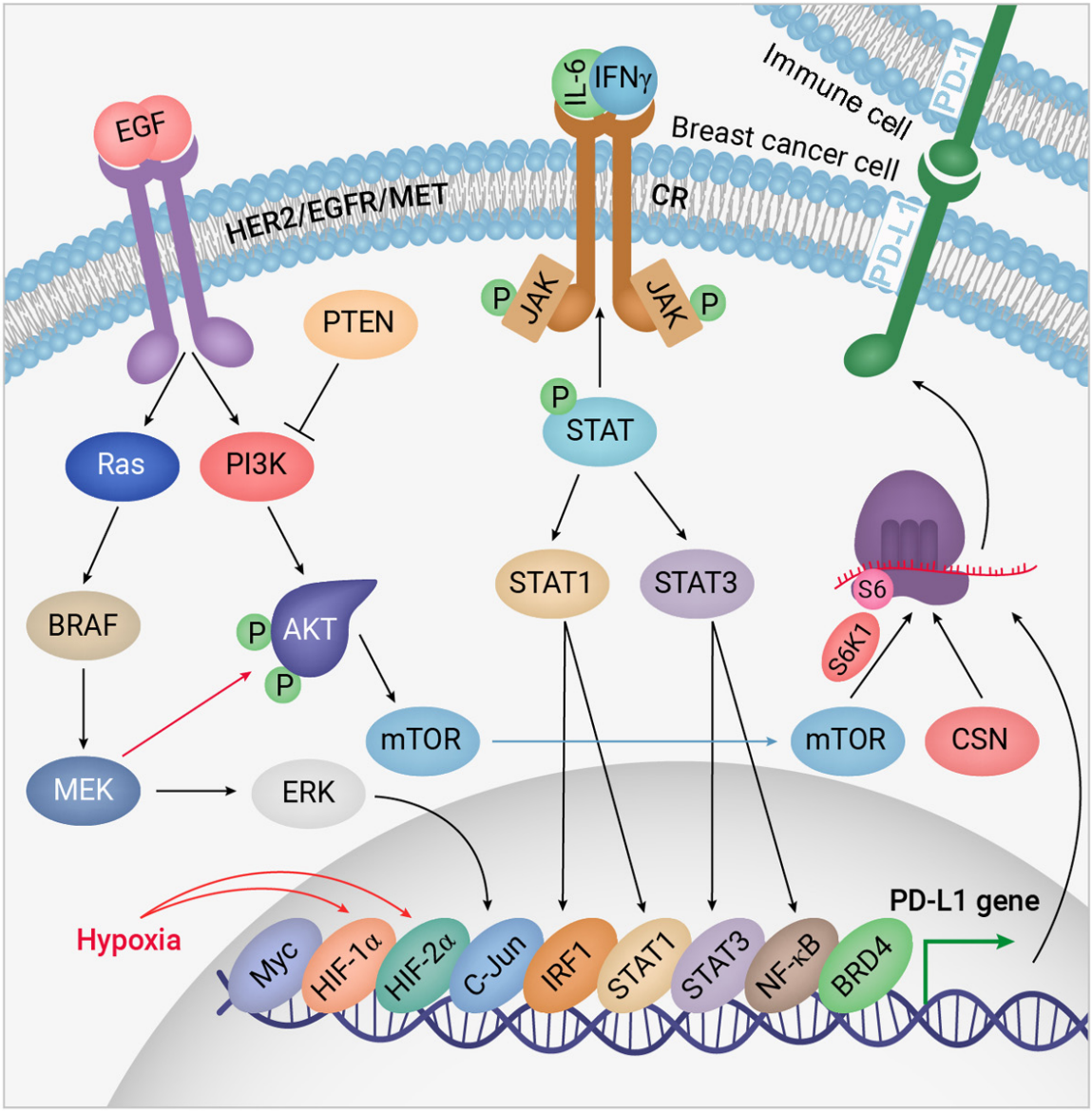

Figure 3. Collaborations of Akt, MAPK, and JAK/STAT pathways in suppressing the anti-cancer immune system. Under hypoxic conditions provided by Akt signalling, PD-L1 is expressed by breast cancer cells to inhibit T-cells. Three main signalling pathways are involved. RTKs triggers PI3K/Akt and MAPK pathways, whereas cytokine receptors (CR) are activated by cytokines released into the TME. JAK/STAT signalling is then activated to recruit STAT1/3 and other transcription factors on some specific gene promoters including the PD-L1 promoter. Simultaneously, proliferation and metastasis are activated and anti-cancer immunity inactivated. S6 and CSN are ribosomal protein S6 and components of the eukaryotic translation factors 3 (eIF-3) complex, respectively, involved in protein synthesis. "Redesigned from Pharmacological Research, Volume 156, Jabbarzadeh Kaboli *et al.*, Akt-targeted therapy as a promising strategy to overcome drug resistance in breast cancer-A comprehensive review from chemotherapy to immunotherapy, 104806, Copyright (June 2020), with permission from Elsevier".

hypoxia. Atezolizumab also downregulates Akt signalling in MDA-MB-231 cells [93]. In the phase III clinical trial IMpassion132, the combination of atezolizumab with a first-line chemotherapy, such as gemcitabine, capecitabine, carboplatine, or nab-paclitaxel, in TNBC patients was studied [NCT03371017]. The IMpassion130 clinical trial was designed only for atezolizumab combined with nab-paclitaxel in TNBC patients [NCT02425891]. In IMpassion130, atezolizumab (840 mg) was given for 15 d (from day 1 to 15 in a 28-d cycle) and nab-paclitaxel (100 mg/m^2) was given on days 1, 8, and 15 to advanced or metastatic TNBC

patients until progression. Significant effects of combined treatment of atezolizumab and nab-paclitaxel were observed on progression-free survival (PFS) and overall survival of TNBC patients, which ultimately led to final approval of this combination by the US FDA [94, 95].

Activation of MAPK signalling is associated with a lower level of tumour-infiltrating lymphocytes and leads to evasion of breast tumour cells from the immune system. Hence, MEK inhibitors combined with PD-L1 antagonists enhance the anti-cancer immune response in TNBC mouse models [96]. Accordingly, MAPK signalling has two opposing effects on JAK/STAT signalling. The mechanism by which MEK inhibition activates JAK/STAT has not been elucidated yet. The use of the JAK1/2 inhibitor (ruxolitinib), JAK1 inhibitor (itacinib), and JAK2 inhibitor (BSK805) did not affect JAK/STAT activity induced by MEK inhibition, showing that MEK inhibition uses a different mechanism for JAK/STAT activation that cannot be reversed by JAK inhibitors. On the other hand, combined treatment of EGFR inhibitors, erlotinib and lapatinib, could partially or fully reverse JAK/STAT activity induced by a MEK inhibitor (trametinib) in $Her2^+$ breast cancer cells [86].

One subset of TNBC cells expresses PD-L1. Therefore, if TNBC cells have lower expression of PD-L1, then treatment with MEK inhibitors combined with a PD-L1 inhibitor may be helpful since MEK inhibition promotes PD-L1 expression and makes it a potential target for adjuvant therapy. Measurements of PD-L1, JAK2, STAT1, STAT3, p-STAT1, and p-STAT3 in 11 TNBC cell lines show that cells possessing somatic amplification of the 9p24.1 locus express PD-L1 in response to IFN-γ. Thus, inducible PD-L1 expression was reported, which can be reversed by JAK2 knockdown and a JAK1/2 inhibitor (ruxolitinib) [97]. Furthermore, regarding MEK and Akt inhibition, a study has divided TNBC cells into three categories: (1) MEK inhibition resistant (MDA-MB468, HCC70, and BT20), (2) Akt inhibition resistant (MDA-MB231, MDA-MB435s, and HCC1806), and (3) MEK and Akt inhibition dual resistant (MDA-MB436, SKBR7, and SUM-159PT) [87]. TNBC cells resistant to Akt inhibitors have lower phosphorylated Akt levels; however, metastatic TNBC cells require activation of integrin β1, myosin light chain kinase, and myosin IIA as well as activation of Akt after MEK inhibition. Reports have also shown that higher levels of KRAS might be associated with resistance to tyrosine kinase inhibitors (TKIs) and Akt overactivation [98].

Several cytokines secreted in breast TME also affect the response of breast tumour cells to immunotherapy. Interleukin (IL)-22 is secreted by immune cells, such as T-helper 17, γδ T, and natural killer cells, which are involved in anti-cancer immunity and enhance Janus kinase/signal transducer and activator of transcription (JAK/STAT)-3/MAPK/Akt signalling pathways, thus promoting PD-L1 expression and inducing resistance to paclitaxel in TNBC [15, 99, 100]. Stress causes chemokine release into the TME and leads to the accumulation of immune cells. Stimulants of tumour necrosis factor-α and IL-6 induce secretion of IL-8 from mesenchymal cells and phagocytes whose interactions with C-X-C chemokine receptors type 1/2 enhance PI3K/Akt signalling, resulting in the upregulation of PD-L1 [101]. Furthermore, interferon-α, which induces paired-like homeodomain transcription factor 2, is also involved in breast cancer progression. Interferon-α induction of paired-like homeodomain transcription factor 2 activates Akt in letrozole-resistant breast cancer cells [102]. As mentioned above, stress conditions are closely related to resistance to chemotherapy. Therefore, paclitaxel resistance should be taken into account when combined with atezolizumab, and overall, targeting TME stress conditions may be beneficial for reversing resistance to chemotherapy.

The use of JAK/STAT inhibitors is like a double-edged sword. These compounds not only suppress the proliferation of breast cancer cells and inhibit PD-L1 expression, but they may also activate alternative pathways such as Akt signalling, which accelerates the survival of cancer cells. The preclinical studies mentioned in the current article also demonstrate negative results and show resistance to ruxolitinib [88]. However, the above mentioned clinical trials ignored the negative results of preclinical studies and only focused on the positive results. In cases where alternative pathways that have crosstalk with JAK/STAT signalling are taken into account in clinical studies, even negative results increase our knowledge about the behaviour of JAK inhibitors, which will help us better design clinical trials.

PI3K/Akt pathway inhibitors and breast cancer subtypes

The present review has shown that sustained activation of Akt results in resistance to different types of chemotherapy as Akt signalling plays a cellular defence role against chemotherapy that: (1) enhances MDR activation, (2) increases TME ROS levels, (3) enhances anaerobic metabolism, (4) inhibits the TCA cycle, (5) promotes PD-L1 upregulation, (6) inhibits apoptosis, (7) increases glucose uptake, and more importantly (8) recruits and interconnects the plasma membrane, nucleus, endoplasmic reticulum, and mitochondria to hijack breast cancer cells and rescue them from chemotherapy. Therefore, Akt signalling is considered a cellular defence mechanism employed against chemotherapeutic effects. Overall, PI3K/Akt signalling can be inhibited at three main points: (1) PI3K, located upstream, (2) Akt, centrally located, and (3) mTOR, located downstream. However, future comprehensive molecular and cellular investigations need to focus on the roles of Akt signalling in breast cancer. Thus, future research paths on Akt signalling can be divided and summarized in the following categories (**Table 2**).

PI3K inhibitors

Almost 40% of patients with HR^+Her2^- breast cancer have mutations in PIK3CA. In a phase III trial, alpelisib combined with fulvestrant was studied in HR^+Her2^- breast cancer with mutated PIK3CA [NCT02437318]. Two cohorts were designed based on whether patients are PIK3CA-mutants or PIK3CA-wild-type, and then each cohort was sub-grouped into alpelisib plus fulvestrant or placebo plus fulvestrant. As the primary endpoint, PFS was evaluated in both cohorts. The study showed that the PFS doubled (11 months) with combination alpelisib and fulvestrant compared to fulvestrant monotherapy (5.7 months), and overall response rate (ORR) was also higher in the PIK3CA-mutant cohort that received combination alpelisib and fulvestrant. In this study, the most frequent adverse reactions were hyperglycaemia, rash, and gastrointestinal disorders, including diarrhoea, which were also reported with combination therapy in PIK3CA-mutant patients [103].

Consistent with the above trial, a phase II trial was conducted on 131 HR^+Her2^- breast cancer patients with PIK3CA mutations receiving letrozole combined with alpelisib [NCT01923168]. In contrast with the previous trial, ORR and pathologic complete response were not significantly improved in the alpelisib-treated PIK3CA-mutant cohort of this study. Similarly, two cohorts were studied, PIK3CA-mutant and PIK3CA-wild-type, but with a larger sample size (N = 164 and N = 176, respectively), and two treatment groups in each cohort were created similar to the previous trial: one sub-group received combination therapy (letrozole plus alpelisib) and the other received letrozole monotherapy (letrozole plus placebo). The number of patients in each of the four groups was approximately 60. Comparison of ORRs showed a lower ORR for both PIK3CA-mutant treatment groups. Moreover, 52% of PIK3CA-mutated patients completed the full combination therapy in 24 weeks, and almost half of the patients stopped treatment because of adverse reactions or doctor/patient decision. Therefore, the results were affected by the continuation/discontinuation of therapy. Gastrointestinal problems, hyperglycaemia, fatigue, and skin rashes were the most common adverse events of HR^+Her2^- breast cancer patients treated with letrozole and alpelisib [104].

Several PI3K inhibitors have been studied for breast cancer so far, including alpelisib, apitolisib, pilaralisib, and voxtalisib. Among them, alpelisib has been approved by the US FDA for the treatment of ER^+ breast cancer patients. PI3K inhibitors are effective for patients diagnosed with mutated PI3K and PTEN. Otherwise, Akt can also be activated in an RTK-independent manner. Therefore, RTK signalling (e.g., Her2/EGFR signalling) can be bypassed by RTK-independent activation, and the use of PI3K inhibitors might be less effective than Akt and mTOR inhibitors.

Akt inhibitors

Akt has crosstalk with several oncogenic pathways, such as MAPK, Notch, and wnt/β-catenin, resulting in the structural reformation of cellular metabolism (e.g., mitochondria), cellular migration, BCSC maintenance, and cancer progression. Furthermore, most drugs work against ER^+ and $Her2^+$ breast cancer subtypes, and higher mortality rates are also due to ER^+ and $Her2^+$ breast cancer patients. In particular, the use of Akt inhibitors is more effective

Table 2. Inhibitors of PI3K/Akt/mTOR signalling clinically started or completed as monotherapy or combination therapy for breast cancer

Compound	1st Target	CID/SID[a]	Combination (The 2nd target)	Condition Breast Cancer (BC)	Clinical trial No[b]	Clinical phase
MK-2206	Akt	24964624	Paclitaxel (Microtubule)	Locally advanced	NCT01263145	I
			Anastrozole (Aromatase)	Metastatic solid tumours Metastatic BC	NCT01277757	II
			Fulvestrant (ER)	Postmenopausal women with metastatic BC	NCT01344031	I
			Lapatinib (EGFR/Her2)		NCT01245205	I
			Trastuzumab (Her2)		NCT01042379	I
			Ridaforolimus (mTOR)		NCT01295632	I
Capivasertib[c]	Akt	25227436	Fulvestrant (ER)	Locally advanced (inoperable)	NCT04305496	III
			Paclitaxel (Microtubule)	metastatic $HR^+/HER2^-$ BC Metastatic TNBC	NCT03997123	III
			Durvalumab (PD-L1)	Refractory Solid tumours	NCT03742102	I/II
				ER^+ BC	NCT02465060	II
				Advanced BC	NCT02077569	II
				ER^+ BC, previously treated with fulvestrant	NCT03182634	II
					NCT03310541	I
Uprosertib[d]	Akt	51042438	Trametinib[e] (MEK1/2)	Metastatic TNBC	NCT01964924	II
					NCT00920257	I
					NCT01138085	I
M2698	Akt	89808643	Trastuzumab (Her2)	Advanced malignancies	NCT01971515	I
			Tamoxifen (ER)			
Alpelisib[f]	PI3K	56649450	Fulvestrant (ER)	Prior endocrine therapy, in $PIK3CA^{mutant}$ BC	NCT03056755	II
			Letrozole (Aromatase)	HR^+ locally-advanced Unresectable or metastatic BC	NCT01923168	II
			Goserelin (GnRH)	Premenopausal Patients	NCT01870505	I
			Leuprolide (GnRH)	AR^+ $PTEN^+$ BC	NCT02058381	I
			Exemestane (Aromatase)	$PIK3CA^{mutant}$ $PTEN^{mutant}$	NCT02051751	I
			Tamoxifen (ER)	anthracycline refractory	NCT02088684	I
			Paclitaxel (Microtubule)	TNBC	NCT02038010	I
			Ribociclib[g] (CDK4/6)		NCT03207529	I
			T-DM1 (Her2/Microtubule)		NCT04216472	II
			Enzalutamide (Androgen Receptor)		NCT01300962	I
			Abraxane[h] (Microtubule)		NCT02077933	I
			Capecitabine (TS)			
			Everolimus (mTOR)			
Alpelisib	PI3K	56649450	Fulvestrant (ER)	Postmenopausal women, HR^+ $Her2^-$ $PIK3CA^{mutant}$ advanced or metastatic BC	FDA approval	
Apitolisib[i]	PI3K	25254071	Paclitaxel (Microtubule)	Locally recurrent or metastatic BC	NCT01254526	I
			Bevacizumab (VEGF-A)	Advanced BC	NCT01437566	II
			Fulvestrant (ER)			

Pilaralisib[j]	PI3K	56599306	Paclitaxel (Microtubule)	Metastatic BC previously treated with trastuzumab	NCT01042925	I/II
			Trastuzumab (Her2)		NCT01082068	I/II
			Letrozole (Aromatase)			
Voxtalisib[k]	PI3K	16123056	Letrozole (Aromatase)	Locally advanced/metastatic solid tumours	NCT01082068	I/II
			Pimasertib (MEK1/2)		NCT01390818	I
Rapamycin[l]	mTORC1	5284616	Trastuzumab (Her2)	$Her2^+$ metastatic BC	NCT00411788	II
Everolimus[m]	mTOR	6442177	Cisplatin (DNA)	Metastatic BC	NCT01031446	I/II
			Paclitaxel (Microtubule)	$Her2^+$ metastatic BC	NCT00411788	II
			Trastuzumab (Her2)	postmenopausal women with metastatic or advanced BC	NCT01007942	III
			Vinorelbine (Tubulin)	HR^+ $Her2^-$ BC	NCT01231659	IV
			Letrozole (Aromatase)		NCT00317720	I/II
			Anastrozole (Aromatase)		NCT02291913	II
			Exemestane (Aromatase)		NCT01298713	II
			Fulvestrant (ER)		NCT01797120	II
			Tamoxifen (ER)			
Deforolimus	mTOR	11520894	MK-2206 (Akt)	Advanced BC	NCT01295632	I
			Exemestane (Aromatase)	ER^+ BC	NCT01605396	II
			Dalotuzumab (IGF1R)	$Her2^+$ trastuzumab-refractory metastatic BC	NCT01234857	II
			Trastuzumab (Her2)		NCT01220570	I
					NCT00736970	II

[a]CID/SID: PubChem Compound ID/Substance ID; [b]This table prepared based on the data collected from www.clinicaltrial.gov; [c]Capivasertib is also known as AZD5363; [d]Uprosertib is also known as GSK2141795; [e]Trametinib is also known as GSK1120212; [f]Alpelisib was formerly known as BYL719; [g]Ribociclib is also known as LEE011; [h]Abraxane is also known as nab-paclitaxel or albumin-bound paclitaxel; [i]Apitolisib is also known as GDC-0980; [j]Pilaralisib is also known as SAR245408 or XL147; [k]Voxtalisib is also known as SAR245409 or XL765; [l]Rapamycin is also known as sirolimus; [m]Everolimus is also known as RAD001.

against $Her2^+$ and $Her2^-$ breast cancers with overactivated KRAS, and against ER^+ breast cancer in which there is ER crosstalk with Akt signalling. A few Akt inhibitors are undergoing clinical trials for breast cancer, including MK-2206, capivasertib, and uprosertib. MK-2206 and uprosertib are being investigated for ER^+ breast cancer and TNBC subtypes, respectively, whereas capivasertib is being studied for both ER^+ breast cancer and TNBC subtypes. However, there is a lack of clinical trials studying the effects of Akt inhibitors for $Her2^+$ breast cancer patients. Treatment of $Her2^+$ breast cancer is strongly dependent on the trastuzumab and lapatinib and other Her2 inhibitors. Since activated PI3K/Akt signalling also causes resistance to anti-Her2 therapy, Akt inhibitors are recommended for $Her2^+$ breast cancer, at least in combination with Her2 inhibitors (**Figure 4**).

In a phase I trial, the effects of MK-2206 were studied in combination with trastuzumab in $Her2^+$ breast cancer patients. The maximum tolerated dose of MK-2206 was used combined with trastuzumab four times per day (45-60 mg), (Cohort 1, *N* = 15) and once a week (135-200 mg), (Cohort 2, *N* = 19) [NCT00963547]. The authors observed that the pharmacokinetics of MK-2206 were not affected by trastuzumab treatment. This study also indicated that MK-2206 combined with trastuzumab has therapeutic efficacy in $Her2^+$ patients; however, treatment was stopped because adverse effects and disease progression were observed. Researchers also found that PIK3CA mutations were not detected in circulating DNA of patients who responded well to treatment, and three patients who had PIK3CA mutations did not respond to treatment with MK-2206 [105].

Furthermore, a phase II trial also studied the impact of PIK3CA mutations on treatment with the Akt inhibitor MK-2206 [NCT01277757]. This trial included PIK3CA-mutant and Akt1-mutant breast cancer patients with different subtypes. Experiments were not only performed on pre-treatment and on-treatment biopsies, but also on peripheral blood mononuclear cells and platelet-rich plasma. Significant reduction in phosphorylation was observed in both residues S473 and T308 of Akt in blood samples, but such a decrease was not observed in tumour biopsies. The researchers categorised patients into two cohorts: PIK3CA/Akt1-mutant and PTEN-loss. Overall, they recruited 27 patients; 18 were included in the first cohort and nine in the second. Most patients were HR^+ (*N* = 15), nine had TNBC, and three were $Her2^+$. One PIK3CA-mutant patient (out of 13) with the $ER^+PR^-Her2^-$ phenotype and PIK3CA and KRAS mutations partially responded to MK-2206. Researchers concluded that $Her2^-$ patients with KRAS mutations have a better response to Akt monotherapy [106]. However, the number of cases (only one case with a partial response) was too small to convincingly make such an important conclusion. Nonetheless, the conclusion is consistent with other research showing that KRAS-mutant TNBC cells have the potential to alternatively activate Akt signalling [74]. The clinical limitation of this study was due to the small number of cases (*N* = 27), tumour heterogeneity, and the different breast cancer subtypes included.

The researchers of the above-mentioned trial [NCT00963547] also completely ignored any purposefully targeted therapy of breast cancer patients using MK-2206 treatment. In our opinion, the combination of MK-2206 with other therapeutics in certain subtypes of breast cancer, in which previous treatments with other drugs have failed, is more reasonable than MK-2206 monotherapy in such a diverse range of breast cancer subtypes. Nevertheless, considering the original function of these drugs, drugs that block PI3K/Akt signalling proteins warrant further investigation in the clinic to reach final approval.

Although most studies focused on Akt1, other Akt isoforms (Akt2 and Akt3) have shown opposing activities in different physiological and pathological conditions. Akt1 has crucial initiating effects on breast cancer, while Akt2 promotes breast cancer progression and cell migration [107]. Regarding the differences observed in the activities of other Akt isoforms, knockdown studies have indicated that Akt1 and Akt3 are responsible for clonogenic and DNA double-strand break repair activities, which lead to radio resistance in KRAS-mutated breast cancer cells; however, Akt2 does not have stimulating effects on the repair of double-strand break [108]. On the other hand, Akt3 is upregulated in breast cancer cell models showing resistance to Akt inhibitors such as

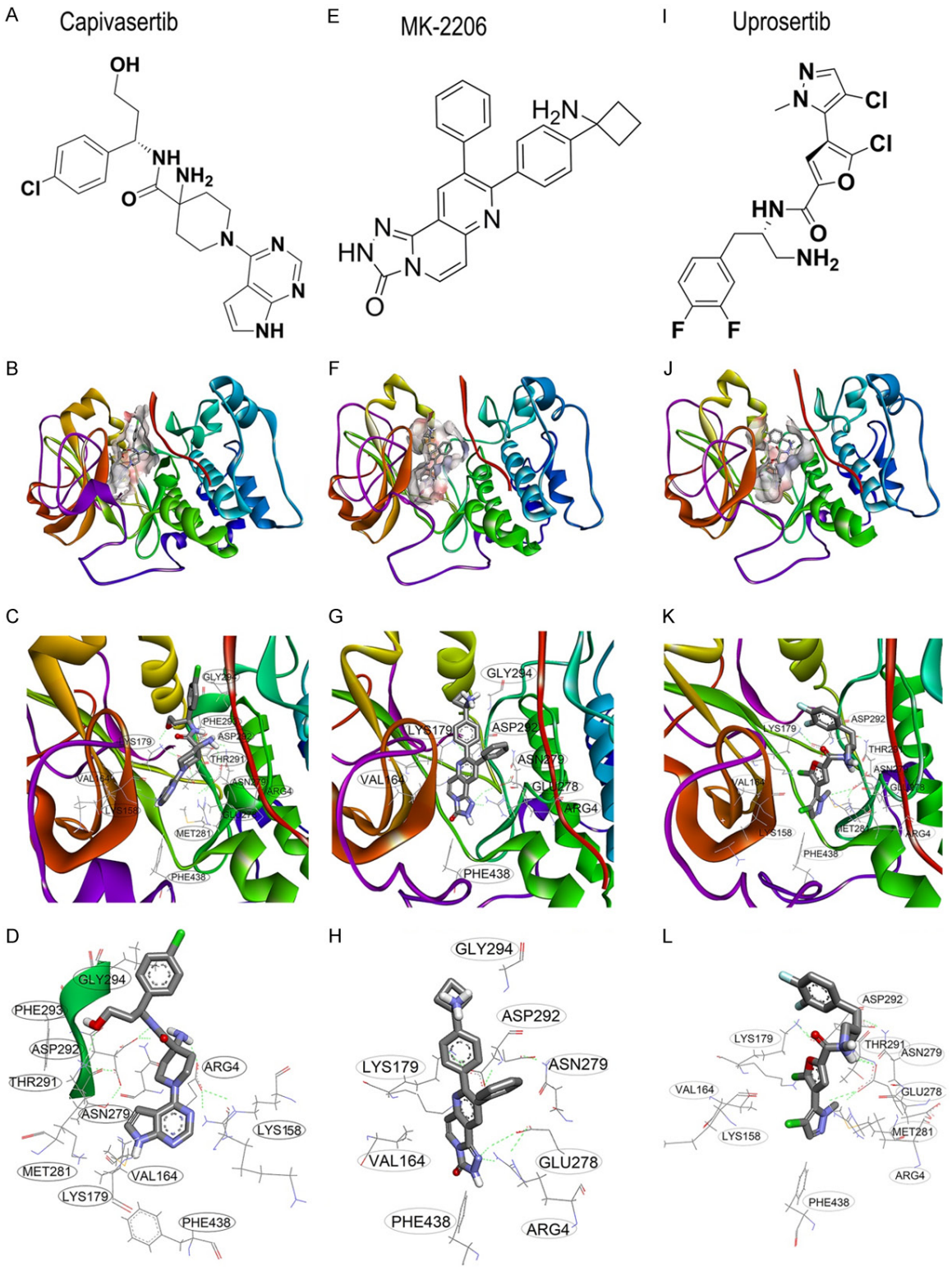

Figure 4. Interactions of Akt inhibitors with Akt kinase domain. The Akt kinase domain has a consensus $D_{292}F_{293}G_{294}$ sequence known as the DFG gate, by which Akt kinase is activated/inactivated. While the side chain of the D (Asp) residue faces toward Lys179 (DFG_{in}), the kinase is activated. In contrast, when the side chain of D is located outside of the pocket, and the F (Phe) residue faces Lys179, the enzyme is inactive [105]. To show the interactions of Akt inhibitors, molecular modelling was performed on three well-known Akt inhibitors against the crystal structure of the Akt kinase domain in the active conformation. Structures of the Akt kinase domain (PDB ID: 3OCB) were obtained from the protein databank (www.rcsb.org), and Akt inhibitors were from the PubChem database. After model-

ling the Akt structure with modeller 9.25 [106] target-template alignment, model building, and model evaluation. This unit describes how to calculate comparative models using the program MODELLER and how to use the ModBase database of such models, and discusses all four steps of comparative modeling, frequently observed errors, and some applications. Modeling lactate dehydrogenase from Trichomonas vaginalis (TvLDH, molecular docking was performed using Autodock 4.2 with 30 repeats for each compound [107, 108]. The best interactions of (A-D) capivasertib (AZD5363, CID: 25227436), (E-H) MK-2206 (CID: 24964624), and (I-L) uprosertib (GSK2141795, CID: 51042438) are shown here with binding energies of -10.98, -9.81, and -7.49 kcal/mol, respectively. Capivasertib reached phase I/II/III clinical trials in different subtypes of breast cancer; however, MK-2206 and uprosertib are in phases I and II for metastatic breast cancer and TNBC, respectively.

MK2206. Furthermore, it has been shown that resistance to MK2206 can be reversed in acquired resistant breast cancer cells via Akt3 downregulation [109]. Higher level of Akt1 and Akt3 were detected in lymph node-positive and -negative luminal B breast cancer patients, respectively [110]. In addition, a higher Akt1 level is associated with unfavorable survival, whereas higher Akt2 expression showed a lower risk of progression in $Her2^+$ breast cancer patients [111].

These results indicated Akt2 and Akt3 as attractive targets to develop new inhibitors against PI3K/Akt signaling. Pan-Akt inhibitors or Akt2/Akt3 inhibitors may effectively sensitize breast cancer cells to chemotherapy. A study showed 2, 4, 6-trisubstituted pyridine plays as a scaffold for developing pan-Akt inhibitors [112]. Therefore, developing pan-Akt inhibitors, such as LY294002, reduces the invasive activity of breast carcinoma [113]; however, clinical roles of Akt2 and Akt3 inhibitors should be taken into account in future clinical trials in order to overcome chemoresistance in breast cancer.

mTOR inhibitors

A third option for targeting PI3K/Akt signalling is mTORC1/2. Although there are currently no active clinical trials for Akt inhibitors in $Her2^+$ breast cancer patients, mTOR inhibitors are under clinical investigation for HR^+ and $Her2^+$ subtypes. PI3K/Akt is a crucial signalling pathway in resistance to endocrine therapy. The effect of the dual mTORC inhibitor everolimus against HR^+ breast cancer has been studied more than other mTOR inhibitors. In a phase II trial, everolimus was used in combination with fulvestrant to postpone resistance to fulvestrant alone in patients who had previously failed therapy with AIs [NCT00570921]. Time to progression as well as ORR and clinical benefit rate were estimated in 31 ER^+ breast cancer patients who had a history of unsuccessful treatment with AIs. In this study, combinatorial therapy with fulvestrant and everolimus was effective. The median time to progression, ORR, and clinical benefit rate were estimated as 7.4 months (95% CI, 9-12.1), 13%, and 49% respectively; however, manageable stage 1 and 2 adverse events were observed, which were reverseable [114].

Furthermore, in a phase III trial (BOLERO-2, NCT00863655), everolimus combined with exemestane dramatically improved PFS in patients with HR^+Her2^- breast cancer who did not respond to AIs perfectly. Median PFS was doubled (7.8 months) in patients treated with combination everolimus and exemestane treatment compared with placebo-controlled cases (3.2 months), and a hazard ratio of 0.45 (95% CI, 0.38-0.54) was estimated for everolimus (*N* = 485). This study also compared PIK3CA-mutant versus PIK3CA-wild type patients to identify any impact of mutated PIK3CA on everolimus treatment. This study indicated that mTOR inhibitors are also effective with the PIK3CA-wild-type phenotype, and PI3K inhibitors, such as alpelisib, are recommended for PIK3CA-mutant breast cancer patients [115].

To date, rapamycin is being investigated for $Her2^+$ breast cancer patients, and everolimus and deforolimus are being studied against $ER^+/Her2^+$ and $ER^+/Her2^-$ breast cancer subtypes. As mentioned earlier, mTORC2 activates Akt through a positive feedback loop. Therefore, dual mTOR inhibitors (e.g., everolimus and deforolimus), which target both mTORC1 and mTORC2, are greatly effective against Akt signalling; otherwise, mTORC1 inhibitors (e.g., rapamycin) might not be beneficial for the suppression of overall Akt pathway activity. As mTORC1 inhibitors cannot completely inhibit PI3K/Akt signalling, partial activation of Akt may cause the recurrence of breast cancer

through the development of BCSC and hypoxia in the TME.

Simultaneous pharmacological targeting of androgen receptor (AR) and PIK3CA^{H1047R} decreases the viability of AR^{+} TNBC cells and would benefit from the current standard-of-care chemotherapy regimens for TNBC [116, 117]. A recent study showed that PIK3CA$^{H1047R/H1047L}$-mutated metaplastic breast cancers respond better to a combination of PIK3CA and MEK inhibitors BYL-719 and selumetinib [118]. Furthermore, *in vivo* combination therapy of PI3K and MAPK inhibitors displayed marked antitumour activity in metastatic breast cancers with genomic alterations of PIK3CA, Akt1, BRAF, and PTEN [118, 119]. Recent findings have suggested that mutation of PTEN and PIK3CA trigger the inactivation of GSK3 by phosphorylation, thereby mediating response to mTOR inhibitors and mTORC1 inhibition-induced upregulation of PD-L1 in TNBC cancer cells [117, 119, 120]. Coussy *et al*. showed that mTOR and PI3K inhibitors have marked antitumour activity *in vivo* in TNBC harbouring genomic alterations of PIK3CA and Akt1 genes. Interestingly, TNBC cells resistant to enzalutamide show that PIK3CA and Akt1 are potential therapeutic targets [119, 121].

Prospective clinical and pre-clinical directions

Constitutively activated Akt has been found in various breast tumour types. Emerging findings of Akt inhibitors suggest that Akt is a critical mediator of oncogenic signalling and participates in the formation of a chemoresistant phenotype in different subtypes of breast cancers. In the last 20 years, many preclinical trials have aimed to improve the effectiveness of antitumour therapy by inhibiting the oncogenic function of PI3K/Akt/mTOR signalling. Furthermore, resistance to CDK inhibitors has also been observed in breast cancer and more specifically, in TNBC patients with MDR proteins [122, 123]. As endocrine and anti-Her2 therapies do not have a good effect against TNBC, a combination of cell cycle inhibitors (e.g., CDK and microtubule inhibitors) and immune checkpoint inhibitors have been suggested as a novel therapeutic strategy for TNBC.

In our research over the past few years, we have focused on the role of the Akt pathway in resistance to chemotherapy. Previously, we found that MDA-MB-231 TNBC cells have over-activated Akt in response to lapatinib treatment [8, 18]. Having observed increased phosphorylation levels of Akt in lapatinib-treated MDA-MB-231 cells, we sought to observe whether MDA-MB231 cells respond to Akt inhibitors or not. Therefore, we treated TNBC cells with capivasertib, as the most frequent Akt inhibitor in clinical studies. Surprisingly, we observed that capivasertib did not inhibit Akt phosphorylation, and instead, significantly increased the level of Akt phosphorylation at S473. Accordingly, we postulated that both TKIs and Akt inhibitors might trigger Akt activation, which leads to chemoresistance of TNBC cells. These results are consistent with previous studies in which activation of the Akt pathway has been reported in TNBC cells as a response to Akt inhibitors [87]. For further elucidation, we analysed the expressions of several proteins related to Akt pathways, including Akt, PIK3CA, GSK-3β, KRAS, and c-Myc in 10 TNBC cell lines and patients with TNBC. We found that most TNBC cell lines had lower Akt levels, except HCC1143 and HCC1187, which may be related to the resistance to chemotherapy. In contrast, KRAS and c-Myc are strongly expressed in TNBC cell lines [124]. Furthermore, through comparison of data obtained from TNBC and non-TNBC patients, we found that the average expressions of EGFR and tumour suppressors p21 and RB-1 are lower in TNBC patients compared with non-TNBC patients (breast cancer, N = 113; TNBC, N = 115) (unpublished data). Lower RB-1 and p21 levels are associated with downregulation of GLUT1, lower glycolysis, and consumption of alternative compounds like glutamine as a replacement energy source when glucose availability is restricted. Of note, Akt not only affects regulation of genes in the RB-1/E2F/Myc pathway, but also reprograms the metabolism of TNBC cells to provide TNBC-specific TME, which finally leads to resistance of TNBC cells to chemotherapy [125]. Using clinical data, we also found that expression of epigenetic regulators, such as DNA methyltransferases DNMT3A, DNMT3B, and DNMT1, but not histone deacetylases (HDACs) such as HDAC3A and SIRT1, is significantly increased in TNBC compared with non-TNBC patients (unpublished data). We also recently highlighted the effects of JAK/STAT and Akt pathways on resistance to radiotherapy in exploring the possible roles of the IFN/

STAT1/Akt pathway in radioresistance. We found that IFN-related DNA damage resistance, STAT1-dependent, and Akt-related genes derived from a primary breast cancer gene expression profile were more broadly associated with resistance to X-ray-induced DNA damage and doxorubicin in breast cancer. Taken together, we suggest that STAT1 and Akt are critical mediators of oncogenic signalling, which participate in the formation of the radioresistant phenotype in breast cancer [126].

Moreover, it should be noted that TNBC patients with MDR have basal-like breast cancer with high expression of tumour-infiltrating lymphocytes and PD-L1. Hence, PD-L1 inhibitors, such as atezolizumab, in combination with routine chemotherapy, has been suggested to be a novel treatment approach for TNBC [127, 128]. In this regard, and supporting the investigation of IMpassion130 clinical trials, atezolizumab plus nab-paclitaxel prolonged PFS among patients with TNBC in both the intention-to-treat population and the PD-L1-positive subgroup. Combined PD-L1 inhibitor and nab-paclitaxel treatment in both PD-L1-positive patients and the overall population of the phase III trial significantly improved PFS [129-131]. Immunotherapy with other checkpoint inhibitors that target CTLA-4 and PD-1 is another therapeutic approach that has been successfully applied to the treatment of breast cancer. Growth factor receptors, such as Her2 and EGFR, and immune checkpoints, such as CTLA-4 and PD-1, induce signalling pathways thus leading to cell proliferation and induction of alternative PI3K/Akt signalling [132, 133]. Therefor, inhibition of Akt signalling represents an attractive targeted therapy of breast cancer in the modern era of personalized medicine [134, 135].

Apart from the Akt upstream RTK, Akt signalling can be activated by crosstalk with other signalling proteins. Therefore, mutations in other signalling proteins affect the Akt pathway and its response to Akt pathway inhibitors. As mentioned earlier, mutations of other signalling proteins, such as KRAS, PIK3CA, EGFR, and mTORC1/2, affect the efficacy of PI3K/Akt pathway inhibitors. In case KRAS is highly mutated, the chance of breast cancer cells acquiring resistance against EGFR/Her2 therapy is increased, and Akt signalling is highly activated in an EGFR-independent manner. On the other hand, according to BOLERO-2 clinical trials, PIK3CA mutations decrease the efficacy of other PI3K/Akt pathway inhibitors, such as everolimus [115]. Other clinical studies mentioned in the present article also highlighted that breast cancer cases possessing PIK3CA mutations, especially patients with ER^+ and $Her2^-$ phenotypes, have a low response to Akt and mTOR inhibitors. However, the number of studied patients needs to be higher to confirm this finding. A crucial direction for future studies on Akt pathway inhibitors should be the clinical investigation of the negative impact of other signalling proteins on the efficacy of Akt pathway inhibitors.

According to the comprehensive assumptions of the present article, the following challenges of Akt inhibitors should be solved to offer the best treatment and overcome the drug resistance in breast cancer: (*i*) High heterogeneity and mutagenesis of breast tumours are the main challenges for overcoming drug resistance, incomplete responders, and relapse in individualized treatment of breast cancer. (*ii*) Inhibition of PI3K/Akt/mTOR may be tolerable because this pathway has crosstalk with immuno-oncologic pathways, such as RAS/MAPK and NF-κB. Certainly, combinatorial approaches to target other pathways and related genes in breast cancer cells have significant benefits in cancer patients undergoing chemotherapy and/or immunotherapy. The development of dose-limiting toxicities has often prevented the use of optimal therapeutic concentrations. (*iii*) The phenotype of breast cancer individuals and their subtype, as well as the mutations of signalling proteins of other pathways crucially affect the response to Akt pathway inhibitors. (*iv*) Biotransformation, bioavailability, affinity, and selectivity of Akt pathway inhibitors and biosafety are crucial challenges, and more clinical trials are required to confirm the pharmacokinetics and safety of Akt pathway inhibitors. (*v*) Epigenetic regulation of the Akt pathway affects the response of breast cancer patients to Akt pathway inhibitors. The activities of proteins, such as DNMTs, SIRTs, and HDACs, and activities of tumour suppressor and oncogenic non-coding microRNAs, should be taken into account while the therapeutic potential of PI3K/Akt signalling is under investigation. Of note, the ratio of microRNAs

and other non-coding RNA expression profiles constitutes important mechanisms in controlling their response to Akt inhibitors. Thus, the balance of genetic and epigenetic mutations, as well as corresponding toxicity and adverse effects, should be taken into full account to achieve more stable efficacy during Akt pathway targeting. Based on recent advances reviewed in the present article, our research direction is to determine the mechanism of Akt-dependent chemoresistance and the roles of Akt modifications under the regulation of epigenetic factors and in response to different therapeutic strategies.

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Disclosure of conflict of interest

None.

Abbreviations

Ais, aromatase inhibitors; BCSCs, breast cancer stem cells; BCRP, breast cancer resistance protein; CDK, cyclin-dependent kinase; DNMT, DNA methyltransferase; EGFR, epidermal growth factor receptor; ER, oestrogen receptor; FDA, food and drug administration; 5-FU, 5-Fluorouracil; GSH, glutathione; GSK, glycogen synthase kinase; GRP78, glucose-regulated protein 78; HER2, human epidermal growth factor receptor 2; HIF-1α, hypoxia-inducible factor-1α; HDAC, histone deacetylase; IFN-γ, interferon-gamma; IL-6, Interleukin-6; JAK, janus kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; MDR, multidrug resistance; mTOR, mammalian target of rapamycin; Nrf2, nuclear factor erythroid 2-related factor 2; OCT4, octamer-binding transcription factor; ORR, overall response rate; PD-L1, programmed death-ligand 1; P-gp, p-glycoprotein; PI3K, phosphatidylinositol-3-kinase; PIK3CA, PI3K catalytic subunit α; PFS, progression-free survival; PTEN, phosphatase and tensin homolog; RTKs, receptor tyrosine kinases; ROS, reactive oxygen species; STAT, signal transducers and activators of transcription; T-DM1, ado-trastuzumab emtansine; TKI, tyrosine kinase inhibitor; TME, tumour microenvironment; TNBC, triple-negative breast cancer.

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