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Recent Development of Anticancer Therapeutics Targeting Akt

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Abstract

The serine/threonine kinase Akt has proven to be a significant signaling target, involved in various biological functions. Because of its cardinal role in numerous cellular responses, Akt has been implicated in many human diseases, particularly cancer. It has been established that Akt is a viable and feasible target for anticancer therapeutics. Analysis of all Akt kinases reveals conserved homology for an N-terminal regulatory domain, which contains a pleckstrin-homology (PH) domain for cellular translocation, a kinase domain with serine/threonine specificity, and a C-terminal extension domain. These well defined regions have been targeted, and various approaches, including *in silico* methods, have been implemented to develop Akt inhibitors. In spite of unique techniques and a prolific body of knowledge surrounding Akt, no targeted Akt therapeutics have reached the market yet. Here we will highlight successes and challenges to date on the development of anticancer agents modulating the Akt pathway in recent patents as well as discuss the methods employed for this task. Special attention will be given to patents with focus on those discoveries using computer-aided drug design approaches.

Keywords

Akt; anticancer therapeutics; computer-aided drug design; drug discovery and development; pleckstrin-homology (PH) domain; serine/threonine kinase

INTRODUCTION

The phosphatidylinositol-3-kinase (PI3K) signaling pathway and its downstream effectors have proven vital to a diverse set of growth and survival functions. Akt (also known as protein kinase B, PKB, or Related to A and C, RAC) is one of the main targets of PI3K. More than 50 proteins have been validated as putative substrates of Akt [1]. Its pivotal role in cell survival, apoptosis, transcription, and proliferation makes it an attractive target for regulatory drugs [2]. Additionally, genetic aberrations such as mutation, amplification, and

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

rearrangement of the Akt pathway are more frequent in human cancer. Genetic screens in model organisms have identified Akt as the primary downstream mediator of the effects of PI3K, and Akt is over-expressed or activated in major cancers [3].

The human Akt family isoforms: Akt1, Akt2, and Akt3 (also known as PKB α , PKB β , and PKB γ , respectively) are characterized by an amino terminal pleckstrin-homology (PH) domain (~ 110 amino acids), a kinase domain (~260 amino acids) separated by a hinge (LINK or Linker) region of 39 amino acids, and a C-terminal regulatory domain (~ 70 amino acids) Fig. (1). All three Akt kinases have 90–95% homology in the kinase domain, 60% homology in their PH domain, and 25% homology in their LINK region [4]. Structure gives clues as to how it is involved in the signaling cascade. After growth factors, insulin or cytokines recruit PI3K to the plasma membrane, PI3K is allosterically activated, leading to the increase of PtdIns(3,4,5)P₃ (PIP₃) on the plasma membrane. Akt is translocated from the cytoplasmic space to the plasma membrane by way of PH domain binding to PIP₃. Akt is a serine/threonine kinase member of the AGC family of kinases [5]. Akt activation is based on the integrity of the PH domain, which controls membrane translocation, and on phosphorylation of Thr308 in the activation loop and Ser473 in the hydrophobic domain [6]. After these two phosphorylation events, numerous intramolecular interactions are formed within Akt, including an anchoring salt bridge in the C-terminal α -helix and an anchoring hydrogen bond between the C-terminal α -helix and the phosphate of Thr308. The phosphorylation of Thr308 causes four more intramolecular interactions that brings all of the functional loops together – the catalytic, the magnesium binding and the substrate binding loops [7]. The X-ray structure reveals that Phe469 and Phe472 of the C terminus comprise the hydrophobic motif (HM) Phe-X-X-Phe-(Ser/Thr)-Trp. This motif binds in an intramolecular fashion to a hydrophobic groove (HG) on the kinase domain Fig. (1). The HG lies under the kinase domain 4- 5 loop, behind the α B and α C helices, including residues: Lys185, Arg202, Arg208, Lys216, and Gln220.

While Akt1 (referred to as Akt henceforth) is expressed ubiquitously at high levels with exception to the kidney, liver and spleen [8–10], Akt2 expression is elevated in insulin-responsive tissues such as brown fat, skeletal muscle and liver [11], and Akt3 expression is ubiquitous with low levels in liver and skeletal muscle. Specific functions have been implicated in the different isoforms of Akt; Akt2 amplification and overexpression corresponds with motility/invasion [12], and increased Akt3 activity is suggested to contribute to the aggressiveness of steroid hormone-insensitive cancers [13]. Knockout experiments have shown that the three isozymes are not functionally identical. Akt1^{-/-} mice are healthy, but show impaired fetal and postnatal growth [14] while Akt2^{-/-} knockout mice display normal growth characteristics, but with a mild to severe insulin resistance phenotype [15, 16]. Akt3^{-/-} mice have smaller and fewer brain cells, resulting in a 20% decrease in brain size [17].

Because the three isozymes of Akt are not functionally identical, inhibitors against them have been used to determine the function in different tissues [18]. However, similar to other kinases, the development of Akt specific inhibitors is still problematic due to its high sequence identity to other AGC family proteins (>90% homology among Akt isoforms and >50% with PKA α - and PKC α -) Fig. (2). Moreover, there is a 100% homology between the ATP binding site of Akt1 and Akt2 (96% homology with Akt2/3 and >70% with PKA α - / PKC α -), as indicated in Table 1. This implies that the development of Akt isoform-specific inhibitors is even more challenging and unselective inhibition of these isoforms will lead to various off-target toxicities due to their involvement in different pathways. Nevertheless, the fact that some kinase domain inhibitors display certain selectivity among the isoforms hints towards structural differences in binding. When comparing the kinase domain crystal structures of Akt1 (3CQU) with Akt2 (2UW9), which have similar bound inhibitors, we

found that most of the known critical residues interact with the inhibitors in similar manners; but there are several residues binding differently to the inhibitors, suggesting the subtle structural difference of these isoforms. For instance, we found that in Akt1 (3CQU), the Phe161 interacts tightly with the triazole moiety of the ligand, but in Akt2 (2UW9) the residue Phe163 seems not involved in the inhibitor binding. Similar pattern have been observed for Leu156, Thr211, Glu278 and Asn279.

Taken collectively, these findings demonstrate that Akt is a promising target, and there have been numerous and intense efforts toward the development of Akt inhibitors as anticancer agents. These attempts can be organized based upon the regions and functions of Akt to which they target. In this review, we will summarize the approaches to develop Akt inhibitors for potential cancer treatments by targeting different regions of Akt, including: the kinase domain, PH domain, LINK domain, and C-terminal extension domain. Then we will discuss peptide based pseudo-substrates/pseudo-regulators to block protein-protein interactions, followed by combined strategies. Some of these representative inhibitors are summarized in Table 2.

TARGETING THE KINASE DOMAIN FOR AKT INHIBITOR DEVELOPMENT

While the central kinase domain of Akt is the most obvious therapeutic target in the Akt pathway, it has proved to be a difficult one. Each kinase domain of Akt, which stretches from amino acid 148 to 411, terminates in a regulatory hydrophobic motif. Because of the high homology of the kinase domain, the majority of Akt inhibitors occupy the ATP binding site as pan-Akt inhibitors [22]. These compounds display promiscuity towards the general AGC kinase family (related to AMP/GMP kinase and protein kinase C). This kinase family is involved in a diverse range of critical cellular functions; the broad targeting of which leads to off-target effects and toxicity. In spite of the daunting challenge presented by targeting the Akt kinase domain, the significance and potential payoff of this has proved too seductive to ignore. Successes from similar kinase domain inhibition of other noteworthy kinases, such as inhibitors against c-Kit and Bcr/Abl, are anticipated to be repeated with Akt.

There have been numerous reports of Akt kinase inhibitors with reasonable selectivities and promising efficacy results. Luo *et al.* [23], based on structure-based computational approaches, generated indazole-pyridine based pan-Akt inhibitors, the most potent of which is A-443654 from Abbott Laboratories [24] Fig. (3A). A-443654 targets the ATP-binding pocket and has equal potency against Akt1, Akt2, or Akt3 within cells (Akt1 binding $K_i=160\text{pM}$), but is more selective against Akt than other kinases in the AGC family including PKA and PKC. Figure 3B illustrates that there are multiple hydrogen bonds as well as hydrophobic interactions involved in the binding of this inhibitor to the Akt kinase domain, explaining the high binding affinity. A-443654 was also found to induce Akt Ser473 phosphorylation in PTEN- and TSC2-deficient cancer cell lines [25], which possibly lead to its severe hypotension in rats and dogs [26].

Another pan-Akt inhibitor, GSK690693 from GlaxoSmithKline, Fig. (4) [27, 28] (Akt1: $IC_{50} = 2\text{nM}$, Akt2: $IC_{50} = 13\text{nM}$ and Akt3: $IC_{50} = 9\text{nM}$) is aminofurazan-derived, ATP-competitive inhibitor, and has been reported to be selective against 13 kinases ($IC_{50} < 100\text{nM}$), including AGC kinase family members: PKA, PrkX and PKC isoforms [29]. GSK690693 showed selective antiproliferative effects towards malignant cells, and downstream Akt phosphorylation events were reduced. In 55% of 112 cell lines tested, acute lymphoblastic leukemia (ALL), non-Hodgkin lymphoma, and Burkitt lymphoma showed 89%, 73%, and 67% proliferation sensitivity, respectively, to GSK690693. It was withdrawn prior to entering clinical trials [30], possibly because treatment resulted in increased

circulating blood glucose and insulin levels [31] due to its interactions with Akt2, which is an integral part of the insulin signaling pathway [11].

Via high-throughput screening, Wyeth reported lacto-quinomycin (WAY-178210-A-1), Fig. (5A) from the fermentation broth of a microbial strain *Streptomyces* sp. LL-AF101. It is a potent and selective inhibitor by targeting Akt (Akt1 IC_{50} = 0.149 μ M) through a novel allosteric mechanism that involves two critical catalytic activation loop (T-loop) cysteines (Cys296 and Cys310) neighboring the activating residue Thr308 [32]. Lactoquinomycin was shown to be selective against a panel of 45 kinases, but did not discriminate appreciably between Akt1 and Akt2 isozymes. The T-loops of AGC kinases, in contrast to the ATP-binding sites, show a high level of polymorphism [33]. However therapy by lactoquinomycin itself is not feasible because of its redox properties conferring nonspecific cytotoxicity [34]. Development of the proven Akt inactivating pyranonaphthoquinone (PNQ) framework might lead to less toxic compounds. Further PNQ framework exploration by Wyeth has resulted in modified compounds with increased Akt inhibition Fig. (5B) [35].

LIMITATIONS OF TARGETING KINASE DOMAIN FOR AKT INHIBITOR DEVELOPMENT

Despite these preceding results, progress in the development of Akt inhibitors targeting the kinase domain has been slow and disappointing, primarily because of high homology of ATP binding pocket in various kinases as well as the fact that Akt inhibition is often associated with promiscuous inhibition within the AGC kinase family (protein A, G and C kinases) [29]. To date, there have been no reports of specific Akt kinase inhibitors that have been shown to be both active against tumors *in vivo* and devoid of major toxicities to normal tissues [36]. Extensive conservation of the ATP-binding domain within the AGC kinase family has proved to be a major hurdle in developing small molecule inhibitors against Akt. There have been reports of libraries of small molecule inhibitors of Akt, resulting from screening of larger libraries of compounds. In future, more potent and selective Akt inhibitors may be identified by virtual screening of these libraries using structure-guided approaches based on the 3D structures of Akt as well as the chemical-physical properties of chemical compounds [37–40].

TARGETING THE PH DOMAIN FOR AKT INHIBITOR DEVELOPMENT

Although the majority of small molecule inhibitors are ATP-competitive inhibitors targeting the kinase domain of Akt [41, 42], they provide little specificity because of the high degree of homology in the ATP-binding pocket among different serine/threonine kinases [43] as discussed above. In order to overcome this drawback, alternative therapeutic modes have been pursued by targeting the PH domain of Akt to interfere with its binding to PIP3 and membrane translocation. The PH domain is a region containing 100 to 120 amino acids found in over 250 human proteins. Although the amino acid sequence of PH domains is not universally conserved, the tertiary structure is remarkably conserved [44]. The fact that the sequence identity of different PH domains is usually less than 30%, which offers the possibility to develop selective agents for different PH domain targets [45].

Akt can be activated through binding to 3'-OH phosphorylated phosphatidylinositols via the PH domain. In order to block this interaction, computer-based molecular modeling has been successfully used in drug discovery and development targeting Akt [46–55]. Based on computational modeling of Akt PH domain, a series of structurally modified phosphatidylinositol ether lipid analogues (PIAs) were synthesized to inhibit the interaction [56–58]. Five PIAs with modification at two sites on the inositol ring were found to inhibit Akt with IC_{50} less than 5 μ M Fig. (6C). These compounds inhibited Akt activation and

phosphorylation of several downstream substrates of Akt in tumor cells without affecting the activities of upstream kinases (such as PI3-K and 3-phosphoinositide-dependent protein kinase 1 (PDK1, also known as PDK1), or members of other signaling pathways such as ERK2. Moreover, PIAs increased apoptosis 20–30-fold in cancer cell lines with high levels of endogenous Akt activity, but only 4–5-fold in cancer cell lines with low levels of Akt activity. However, whether PIAs are effective *in vivo* and whether PIAs affect other PH-domain containing proteins is currently unknown.

Perifosine structurally resembles naturally occurring phospholipids, and appears to block the activation and phosphorylation of Akt in cellular settings [59–61]. Perifosine belongs to a class of antitumor alkylphospholipids with a piperidine head group Fig. (6A). It was originally based on the structure of lysophosphatidylcholine. *In vivo*, perifosine induced myelopoiesis in murine marrow and spleen, whereas it caused apoptosis in myeloma xenografts [62]. *In vitro*, perifosine inhibits Akt at low micromolar concentrations [60, 63–65], and inhibition has been measured in many xenograft models *in vivo* [63, 66, 67]. Perifosine is currently being tested in phase 2 clinical trials. So far, the clinical results with perifosine have been modest and have not lived up to the hopes for an orally available Akt inhibitor, because no modulation of Akt has been assessed [59]. In order to measure the binding affinity of perifosine to the Akt PH domain, surface plasmon resonance spectroscopy binding assay was executed, which uses PIP3 biotin-labeled liposomes to measure the displacement [68]. Although perifosine has been reported as an Akt PH domain inhibitor, our recent experimental studies showed that perifosine does not bind to the PH domain of Akt with binding affinity (K_i) $> 50\mu\text{M}$. [69]. Furthermore, another report demonstrated that the low binding affinity of perifosine to the PH domain was undetectable by titration calorimetry. Ultimately, it remains unclear if the potential effectiveness of perifosine is due to disrupting microdomains crucial to Akt activation or *via* direct PH domain displacement of PIP₂ and PIP₃ ligands [59].

Ceramides are molecules also belonging to the lipid family. A ceramide is composed of a sphingosine and a fatty acid Fig. (6B). Ceramides are found in high concentrations within the cell membrane. Experimental findings obtained with separate domains suggest that ceramide blocks the effects of insulin on both the PH domain and the catalytic/regulatory domain of Akt [70]. Major issues with the lipid-based molecules are: limited solubility, moderate potency against Akt kinases, aggregation, and poor pharmacokinetics. Hence, small molecule design was undertaken for inhibitors targeting the PH domain of Akt, but with better ADMET (Absorption, Distribution, Metabolism, Excretion and Toxicity) properties.

In order to design small molecule inhibitors of the Akt PH domain, *in silico* structure-based drug design was performed to guide the chemical synthesis. Using *in silico* library screening and interactive molecular docking, as described next, a novel class of non-lipid-based compounds were identified Fig. (6D), with binding affinity in the range 0.4 to 3.6 $\mu\text{mol/L}$ [71]. Some of them exhibited PH domain-binding selectivity for Akt compared with insulin receptor substrate-1 and PDK1. These compounds inhibited Akt in cells, induced apoptosis, and inhibited cancer cell proliferation. Unfortunately, *in vivo*, the lead compound failed to achieve the blood concentrations required to inhibit Akt in cells, most likely due to rapid metabolism and elimination, and did not show antitumor activity.

Recently, a small inhibitor with good antitumor activity was discovered by our group [68, 72]. The discovery workflow was summarized in Fig. (7A). Briefly, the Akt1 PH domain crystal structure 1UNQ [73] was retrieved from the protein database (PDB). SYBYL was employed to fix the missing residues and atoms of the protein. Hydrogen atoms were added, and crystal waters and the ligand were removed from the complex structure. For the

definition of the binding pocket, no cavity detection was executed and a cavity file was instead generated, which included all residues within 6.5 Å around the residues Lys14, Glu17, Arg23 and Arg86 which were reported as essential for the protein-ligand interactions [73]. Via high-throughput virtual screening with our in-house tool HiPCDock [73], top 10⁴ hits were initially selected for further rescoring and diversity analysis. At this stage, GOLD docking was executed using default parameters unless otherwise noted [73]. The internal hydrogen bonds were taken into account to restrict the flexibility of the ligand. No early termination was allowed for the fitness search. For each ligand, the received docking poses were evaluated via virtualization and selected according to the protein pharmacophore. About 1000 compounds were selected at this step for *in silico* ADMET evaluation, followed with experimental validation of 100 chemical candidates. After this process 22 potential PH domain inhibitors were identified and were experimentally confirmed to be active using surface plasmon resonance spectroscopy. All of these evaluations have been focused on Akt1. Due to the relatively low sequence identity of PH domains (< 20%), compared to kinase domains (> 95%), we expect that optimization of Akt isoform-specificity of our PH domain inhibitors will be not as challenging as ATP competitors. In order to improve the binding affinity and cellular permeability, the structures of these compounds were further optimized using both structure-based docking approaches and ligand-based Caco-2 QSAR models. Figure 7B shows the interaction between one of our hits Fig. (6D) and the Akt PH domain. Another hit, 4-dodecyl-N-(1,3,4-thiadiazol-2-yl) benzenesulfonamide Fig. (6E), was subsequently synthesized and measured to have a binding affinity $K_D = 40.8 \pm 2.5 \mu\text{M}$ and Akt inhibition $IC_{50} = 6.3 \pm 0.9 \mu\text{M}$ [68]. This compound inhibited Akt and its downstream targets in cells as well as in pancreatic cancer cell xenografts in immunocompromised mice, exhibiting promising antitumor activity [68].

TARGETING THE LINK DOMAIN FOR AKT INHIBITOR DEVELOPMENT

The LINK domain is the region connecting the PH domain with the catalytic domain of Akt. This domain consists of only about 50 residues, and it is poorly conserved among the Akt isoforms (17–46% identical) and has no significant homology to any other human protein [45]. Because there are no published crystal structures of the LINK region, and the low sequence homology between this domain and other known structures makes the homology modeling difficult, it presents a high challenge for the drug design targeting the LINK domain.

Two LINK domain inhibitors were developed, as claimed by Merck, using high-throughput screening techniques [74–83]. Both inhibitors are capable of inhibiting full-length Akt, and a detailed kinetic analysis indicated that these inhibitors were not competitive with ATP or peptide substrate and could not inhibit Akt lacking the PH domain [84].

Because of the sequence diversity of the LINK region, as mentioned above [45], the selectivity of the LINK domain-targeted inhibitors is presumably high. For instance, Merck Akt-I-1 Fig. (8A) inhibits Akt1 with $IC_{50} = 4.6 \mu\text{M}$, while $IC_{50} > 250 \mu\text{M}$ for the Akt2 inhibition. Merck-Akt-I-2 Fig. (8B) is also isoform selective against the full length Akt paralogs (Akt1: $IC_{50} = 3.4 \mu\text{M}$, Akt2: $IC_{50} = 23 \mu\text{M}$ and Akt3: $IC_{50} > 50 \mu\text{M}$) [79]. It is inactive against PH domain-truncated Akt [84]. Merck-Akt-I-2 was shown to reversibly inhibit the activation of Akt1 and Akt2, but not Akt3 [84–86]. Furthermore, optimization of the quinoxaline template and extension at the isopropylamine group results in higher potency Fig. (8C) [79, 87]. A series of other analogs were derived from the Merck-Akt-I-2 to improve the potency and selectivity. One of the analogs can selectively inhibit Akt1 at $IC_{50} = 760 \text{ nM}$, while another one is Akt2 selective with $IC_{50} = 325 \text{ nM}$ [88].

TARGETING THE C-TERMINAL HYDROPHOBIC MOTIF

Akt terminates with a C-terminal hydrophobic domain, in which the HM Phe-X-X-Phe-(Ser/Thr)-Trp is found to be conserved among AGC kinase family [89–91]. These hydrophobic residues play a significant role in triggering allosteric effects and fully activating Akt for substrate phosphorylation. Recent X-ray crystallography studies showed that phosphorylation of Akt2 Ser474 in the HM by a putative PDK2 stabilized the hydrophobic groove (HG) residues located in the N-lobe of the kinase domain, in which B and C-helices are highly disordered in unphosphorylated and monophosphorylated Akt2 [33]. This is achieved by the hydrophobic interaction with B and C-helices as well as a putative hydrogen bond with residues phospho-Ser474 to Gln219 of the α 4 strand [33, 92]. The conformational changes resulting from the interactions between HM and HG augment Akt kinase activity by 7–10 fold [93]. It offers the biological basis of developing novel inhibitors of Akt by hampering intramolecular hydrophobic interactions between the HM and HG structures. Furthermore, Yang *et al.* have provided insights to this novel target utilizing a mutagenesis study of chimeric protein of Akt and PIFTide [92]. PIFTide, a 23-residue peptide from PRK2 HM, is a potent activator of Akt which mimics the phosphorylated HM of Akt [89]. They found that mutations of conserved phenylalanine or aspartate residues in PIFTide might eliminate their potential to activate Akt [92]. These findings indicated an innovative drug design strategy against Akt; that is, to exploit pseudo-unphosphorylated-HM or its small-molecule derivatives that are capable of constitutively binding to HG and making Akt unresponsive to the phosphorylation on Ser473. Although no efficacious inhibitors or drugs have been disclosed so far, the therapeutic potential of C-terminal hydrophobic domain has aroused profound interest from some pharmaceutical companies including Novartis.

TARGETING TRAF6 TO INHIBIT AKT PATHWAY SIGNALING

It has been recently discovered that the E3 ligase TRAF6 forms a complex with Akt, and it is responsible for Akt ubiquitination at a specific residue (Lys8) in the PH domain. This ubiquitination promotes translocation, instead of protein degradation, to the plasma membrane for subsequent Akt activation [94]. This latest study has provided TRAF6 as a new potential target in developing novel Akt pathway inhibitors for human cancer therapy [95]. Although there are no TRAF6 inhibitors currently available, as of this publication, blocking E3 ligase functions indeed has shown great promise in clinical trials. TRAF6 is an especially attractive target due to its roles in the PI3K/Akt/PTEN pathways [96]. Combined with TRAF6 inhibition, rapamycin analogs (rapalogs) that inhibit the mammalian target of rapamycin complex 1 (mTORC1) can shift their stabilization and cytostatic effects to cytotoxic effects, and thus increase their therapeutic potential [95].

INHIBITING AKT WITH PEPTIDE-BASED AKT PSEUDOSUBSTRATES OR PSEUDOREGULATORS

The majority of inhibitors, which target the Akt ATP binding pocket or PH domain, are capable of affecting other ATP or PtdIns-utilizing proteins. Therefore, selective inhibition of Akt activity still remains challenging due to a variety of homologous proteins sharing similarity in structures and functions. In contrast to ATP (or PtdIns) competitive inhibitors, substrate-mimetic inhibitors that target the Akt substrate binding domain hold a great potential in becoming new anticancer therapeutics with high specificity and low toxicity. Akt modulates its downstream substrates, such as BAD, GSK3, FKRHL1 etc, via phosphorylation at specific serine/threonine sites in which the Akt-specific binding motif plays an important role. Recent development of peptide and non-peptidic inhibitors has proven to be selective and potent in inhibiting Akt activity *in vitro*. A series of hybrid peptides composed of 9aa from human forkhead transcription factors (hFKRHL1) and 11aa

Akt-binding consensus motif [97] have been designed to induce apoptosis and sensitize tumor cells [98–100]. With mutations of putative phosphorylation serine/threonine sites with Alanine, these pseudosubstrates are able to prevent the substrates from binding to Akt1, Akt2 and Akt3. For instance, a peptide with two Alanine mutations inhibits *in vitro* phosphorylation of biotinylated BAD peptide by Akt1 with K_i 110nM, whereas four out of five peptides exhibit at least 30-fold selectivity against Akt compared with other kinases including p70S6K, PKA, Cdc2, Src, PKC, etc. Moreover, these peptide-based inhibitors can also block the phosphorylation of GSK3 and Hela cell growth *in vitro*.

In another patent, a peptide named Akt-*in* was explored based on A strand of TCL1 or its homology MTCL1 [101]. TCL1 is a proto-oncogene and acts as the co-activator of Akt through two functional motifs, which are responsible for Akt association and homodimerization, respectively [102]. Hiromura *et al.* were the first to hypothesize and studied the inhibitory use of A peptide from TCL1 (10–24aa), which spans the crucial binding site for Akt association. NMR structure and molecular docking showed that Akt-*in* was bound to the Akt PH domain and inhibited membrane translocation by inducing conformational change on the PIP3 binding pocket in a dose-dependent manner. Akt-*in* also exhibited strong specificity against Akt over PDK1, PKC, PKA, and MAPK in both *in vitro* and cell-based experiments [103]. However, like other peptidomimetic inhibitors, Akt-*in* still retains poor bioavailability; it needs further optimization for future clinical use.

Although peptide-based Akt inhibitors have shown potent efficacy and selectivity against Akt activity, their large size and high flexibility limit the satisfactory pharmacokinetic properties and cell-permeability. Recently, Kayser-Bricker *et al.* reported a group of non-peptidomimetic small-molecule inhibitors based on truncated GSK3 binding motif Fig. (9) [104, 105]. Using structure-based modification of the N-terminal, C-terminal, and the core chemical scaffold, followed by ligand-based structure-activity relationship (SAR) optimization, small molecule inhibitors showed improved PK/PD profile compared to its original lead compound [106], with merely slight potency decreases (from 14μM to 17μM).

COMBINED STRATEGIES COMPLEMENTARY TO UNI-TARGET ANTICANCER THERAPEUTICS FOR AKT INHIBITION

Anticancer chemotherapy is more than the inhibition of hyperactivation of Akt. As far as we know, dozens of upstream or downstream regulators or effectors in the Akt signaling pathway are thought to mediate the survival, evasion of apoptosis, proliferation and angiogenesis of cancer cells [107]. Thus, personalized chemotherapy targeting multiple nodes in the Akt pathway may be effective to treat specific cancers which are resistant to a single agent or therapy. Although the combination of other anticancer inhibitors with Akt inhibitors has been extensively studied under preclinical background, only a few patents of combined strategies have been published to date.

It is known that three Akt isoforms are hyperactivated in various types of cancers, and can be phosphorylated and activated by multiple upstream kinases, such as PI3K, HER2, EGFR etc [108, 109]. In addition, irregularities of Akt activity can be achieved by Akt-independent upstream signaling, like PTEN loss and PI3KCA mutation [110]. Also Akt may be constitutively activated due to overexpression of Akt isoforms. Therefore, the rational combination of proximal upstream inhibition with Akt inhibition may solve the single drug resistance in some cases. Barnett *et al.* first reported a significant increase of caspase-3 activation by treating at least two selective Akt1 inhibitors, an Akt1 and Akt2 inhibitor, a PI3K inhibitor (LY294002), and a HER2 inhibitor (Herceptin) over one test compound alone [78]. Another patent comprising the combination of Bevacizumab, Tarceva and Gleevec (as inhibitors of VEGF mediated angiogenesis, HER1-Akt pathway, and Bcr-abl

tyrosine kinase, respectively) has been tested in Phase II clinical trials [111]. This composition was verified as sufficient to treat proliferative diseases with minimized toxicity to the host. Similar results have been observed in gefitinib (tyrosine kinase inhibitor)-resistant cancer cell lines when using a combined approach of LY294002 with EGFR inhibition [112].

Several substrates of Akt, such as mTOR, BAD, and hsp90 mediate Akt cellular functions in protein synthesis, inhibition of apoptosis, and NO signaling. On the other hand, the deregulation of other pathways such as MAPK, and the NO pathway, also contribute to carcinogenesis and metastasis [113, 114]. Hence, the combination of inhibitors of distal targets of Akt with other cancer signaling pathways has been indicated as an alternative methodology to improve and augment their therapeutic effects. For example, the synergistic effect of rapamycin-induced autophagy by a PI3K/Akt inhibitor has been reported previously in malignant glioma cells [115]. A newly-invented drug combination comprising diaryl urea compounds, PI3K inhibitors, Akt kinase inhibitors and mTOR inhibitors was recently published for treating cancer [116]. This composition is targeted to the MAP kinase signaling pathway [117] as well as the PI3K/Akt/mTOR pathway [118] and may enhance the inhibition effect of metastasis and cell proliferation of treated cancer tissue. However, it is still a challenge to effectively inhibit the PI3K pathway by targeting mTORC1 because the blockage of the negative feedback loop can eventually lead to Akt activation [119]. Therefore it is important to inhibit Akt for anticancer therapy. More recently, Akt pathway inhibitors such as LY294002 may coordinate with drugs that target hsp90 or caveolin-1 to treat NO-dependent tumor angiogenesis [120]. Another patent invention concentrates on the synergistic induction of cancer cell apoptosis by inhibiting cyclin-dependent kinases (CDKs) and XIAP [121]. Roscovitine or flavopiridol (CDK9 and XIAP inhibitors) combined with API-2 (Akt inhibitor) or LY294002 (PI3K inhibitor) elicited apoptosis on roscovitine-resistant prostate cancer PC3 cell line, while normal epidermal cells remained viable.

CURRENT & FUTURE DEVELOPMENTS

There have been over five thousand publications on Akt in the last 15 years (based on PubMed search), and as of late a marked increase has occurred in the number of Akt inhibitors. However, there are still unresolved issues in regard to Akt target specificity and isozyme selectivity that must be addressed before these inhibitors can be employed in anti-cancer therapies. The complexity of the Akt pathway provides many signaling events that can be molecular targets for potential therapeutics, both up and downstream of Akt itself. Elucidation of the structure, systemic biology, and complete molecular network of Akt and its isoforms will result in advanced therapies with higher therapeutic index and lower toxicities. In addition, Akt inhibitors can be used in combination with existing chemotherapeutic agents. Moreover, computer-aided *in silico* and rational design approaches will further help us to accelerate the discovery and development of Akt targeted therapies in a more cost-effective manner.

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Fig. 1. Schematic representation of Akt1. PH: Pleckstrin homology domain; LINK: Linker domain; HG: Hydrophobic groove; P: Phosphate; CTD: C-terminal domain.

Fig. 2.

Sequence alignment of the kinase domain of all three Akt isoforms and some other AGC proteins.

Fig. (3A).

Fig. (3B).

Fig. 3.

Fig. (3A). A-443654 from Abbott Laboratories.

Fig. (3B). Analysis of crystal structure demonstrates that Glu230, Ala232 Asn280, Asp293 and several other residues are involved in strong interactions between Akt kinase domain and the inhibitor A-443654.

Fig. 4.
Structure of GSK690693.

Fig. (5A).

Fig. (5B).

Fig. 5.

Fig. (5A). Wyeth Akt inhibitor Lactoquinomycin (WAY-178210-A-1).

Fig. (5B). The pyranonaphthoquinone (PNQ) framework of Wyeth compounds derived from structure Fig. (5A).

Fig. 6. Akt PH domain inhibitors. **A.** Perifosine. **B.** Ceramides. **C.** PIAs. **D.** The derivatives of 4-(1-benzoyl-3-methyl-5-oxo-4,5-dihydro-1H-pyrazol-4-ylazo)-N-pyrimidin-2-yl-benzenesulfonamide. **E.** 4-Dodecyl-N-(1,3,4-thiadiazol-2-yl)benzenesulfonamide.

Fig. (7A).

Fig. (7B).

Fig. 7.

Fig. (7A) The workflow employed for PH domain inhibitor identification with high-throughput virtual screening and other *in silico* modeling approaches. The gray numbers represent the reduction of numbers of compounds from the initial large chemical database collection to the final experimentally measured active hits for future studies.

Fig. (7B). One of the hits we identified through our *in silico* library screening. It has been confirmed active in our experimental testing followed with further optimization. Molecular docking demonstrates that this compound mimics the 4 and 5 phosphate groups in PIP3 to interact with the positive Akt PH domain binding pocket involved in Glu17, Arg23, Arg25, Asn53, Asn54, Arg86 and several others.

Fig. 8.
LINK domain inhibitors. **A.** Merck-Akt-I-1. **B.** Merck-Akt-I-2. **C.** Merck Akt-I-3.

Fig. 9.

Rational design of Akt pseudosubstrates and lead optimization of non-peptidomimetic small-molecule inhibitors based on human GSK3 binding motif. A series of modifications on chemical moieties (in dashed box), including the deletion of N-terminal Arginine and Proline, substitution of internal scaffold to p-aminobenzoic acid, increase of C-terminal hydrophobicity, etc. It afforded the peptidomimetic inhibitor with IC₅₀ 14 μ M. Lead optimization based on chemical scaffold and molecular docking resulted in several drug-like and synthesizable small-molecular inhibitors which were validated to inhibit Akt kinase activity at low micromolar level.

Table 1

Homology of the Kinase Binding Site.

Kinase	% Homology	Variable Residues in Binding Site																	
		L158	K160	T162	F163	K165	E193	H196	T197	T213	F227	M229	E230	A232	E236	E279	M282	T292	L296
Akt1	100																		
Akt2	100	L	K	T	F	K	E	H	T	T	F	M	E	A	E	E	M	T	L
Akt3	96	L	K	T	F	K	E	H	T	T	F	M	E	V	E	E	M	T	L
PKC-	74	L	K	S	F	K	D	C	T	T	F	M	E	V	D	D	M	A	M
PKA-	70	L49	T51	S53	F54	R56	Q84	H87	T88	V104	M118	M120	E121	V123	E127	E170	L173	T183	F187

Table 2

Summary of Some Representative Akt Inhibitors.

Inhibitor Name	Company	Target	Status	References
A-443654	Abbott	ATP binding site	Lead optimization	[23–26]
GSK690693	GlaxoSmithKline	ATP binding site	Withdrawn	[27–31]
XL418	Exelixis	ATP binding site	Phase I clinical trials	[19, 20]
Truncated GSK3 pseudosubstrate	Yale/University of South Florida	Substrate binding site on Kinase domain	Lead optimization	[104–106]
WAY-178210-A-1	Wyeth	T-loop of kinase domain	Lead optimization	[32–35]
Phosphatidylinositol ether lipid analogues (PIAs)	MD Anderson/NIH	PH domain	Preclinical development	[56–58]
Perifosine	Keryx	PH domain	Phase II clinical trials	[60, 63–67]
PHT-427	PHusis Therapeutics	PH domain	Preclinical development	[69]
PH-6	MD Anderson	PH domain	Lead optimization	[71]
DPIEL/PX-316	Prolx	PH domain	Preclinical development	[21]
Merck Akt-I-(1,2,3)	Merck	LINK domain	Preclinical development	[74–83]