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Review Article
Therapeutic implications of targeting the PI3Kinase/AKT/mTOR signaling module in melanoma therapy

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Abstract: The PI3Kinase/AKT/mTOR signaling module is implicated in various cellular functions including cell survival, growth and proliferation, glucose metabolism, apoptosis, migration, and angiogenesis. Increased expression of AKT and its up- and downstream regulators is linked to several types of cancer. Aberrant expression of AKT is observed in nearly 60% of melanomas culminating in apoptosis resistance via deactivation of apoptotic molecules Bad and Caspase-9. Through cross-talk with NF-κB, ERK1/2, JNK and p38MAPK signaling pathways, AKT induces a plethora of cellular effects often leading to tumor development and progression. Due to frequently observed resistance to other common cancer treatments such as chemotherapy, immunotherapy, and radiation, and the detrimental consequences of constitutive activation of the PI3Kinase/AKT/mTOR signaling module, targeted inhibition of the effectors and substrates involved in this module has become a viable and attractive option for molecular targeted therapy in melanoma. Pharmacological inhibitors of various components of this module, either alone or in combination with other agents, have shown significant decrease in proliferation, tumorigenesis, cell growth and survival of various tumors in phases I and II clinical trials. Some inhibitors have even received their Food and Drug Administration (FDA) approval. This review summarizes the current knowledge on this module, its cross-talk with other major cell survival pathways and its targeted inhibition for therapeutic purposes in melanoma.

Keywords: PI3Kinase, AKT, mTOR, PTEN, melanoma, targeted therapy, signal transduction, apoptosis, resistance

Introduction
The serine/threonine protein kinase B (AKT) belongs to the AGC family of protein kinases. The AGC group consists of cyclic AMP, GMP and protein kinase C. The AGC family of kinases includes several important anticancer targets (e.g. PKB), which are at the heart of intense drug discovery endeavors. Kinases from this family have many similarities within their active site as well as their means of activation; deregulation of these highly regulated networks is often observed in human diseases such as melanoma and other cancers [1]. AKT consists of three homologous members known as PKβα (AKT1), PKββ (AKT2) and PKβγ (AKT3). AKT is a growth factor regulated protein kinase which contains three functionally different sites: a pleckstrin homology (PH) domain, a central catalytic domain, and a C-terminal hydrophobic motif (HM) [2]. Binding of phosphoinositide 3-OH kinase (PI3K) products to the pleckstrin homology domain results in AKT translocation to the plasma membrane where it is activated via phosphorylation by upstream kinases such as the phosphoinoside-dependent kinase 1 (PDK1). Since its discovery over a decade ago, researchers have identified some of the key roles of AKT. Among its myriad of cellular responsibilities, AKT is implicated in cellular processes such as cell survival, proliferation and growth, glucose metabolism, apoptosis, angiogenesis, transcription and migration [1].

AKT activation and its downstream effects in a nutshell
AKT is activated by phosphorylation at multiple sites. Initially the Thr308 residue is phosphorylated, causing a charge-induced binding site conformational change. Experiments with mouse embryonic stem cells and knock out mutants have shown that PDK1 is the key kinase responsible for this initial phosphorylation step
Although phosphorylation at Thr\textsuperscript{308} partially activates AKT, full activation of AKT requires phosphorylation on a second site, the Ser\textsuperscript{473} residue, which greatly amplifies the rate of catalysis and downstream consequences of AKT activation. Interest in the understanding of the dynamics of activation of the AKT pathway has been incited by its subsequent role in promoting cell survival, resulting in inactivation of a series of major pro-apoptotic proteins [4]. AKT activation requires the recruitment of PI3K to the cell membrane, followed by its phosphorylation. AKT is thus activated by PI3K; PI3K itself is activated by a receptor tyrosine kinase (RTK) and G-protein-coupled receptors (GPCR). After its translocation to the cytoplasmic domain of receptors, activated PI3K catalyzes the production of the second messenger phosphatidylinositol-3,4,5-triphosphate (PIP3) by phosphorylation of phosphatidylinositol-4,5-diphosphate (PIP2). PIP3 levels are tightly controlled by the actions of phosphatases such as PTEN [2].

Phosphatase and tensin homologue (PTEN)

The tumor suppressor phosphatase and tensin homologue, PTEN, converts PIP3 to PIP2 in the cytoplasm, thus, antagonizing the PI3K signaling [5]. PTEN is found in both the cytoplasm and the nucleus and shuttles between each by a variety of mechanisms. PTEN, typically in the cytoplasm, is a major participant in down-regulating AKT activity, which subsequently leads to higher p27 levels. Studies on PTEN have revealed that in normal, non-dividing cells, it resides largely in the nucleus while in cells undergoing active division (e.g., cancerous cells) PTEN is mostly found in the cytoplasm. Studies on the role of nuclear PTEN are still in their preliminary stages, which warrant further scrutiny [6]. Deletions or mutations within the region of the genome encoding PTEN leads to diminished or absent expression of this vital tumor suppressor. This phenomenon is often observed in the overwhelming majority of sporadic melanomas. As PTEN expression becomes impaired, it will lead to an unhindered increase in AKT activity, which subsequently initiates downstream pro-survival effectors leading to reduced apoptosis and increased cell proliferation [7].

AKT signaling cascade and activation of gene transcription

AKT is a master-switch allowing aggressive growth in melanoma. Some of the consequences of constitutive AKT activation in melanoma are as follows: conferring resistance to several pro-apoptotic effectors (e.g., Fas ligand and tumor necrosis factor related apoptosis-inducing ligand (TRAIL)), and inhibition of tumor suppressors such as FoxO Forkhead and other transcription factors [8]. Binding of growth factors (often through the endocrine or the autocrine system) to membrane receptors, such as receptor tyrosine kinases (RTK) activates PI3K. Activated PI3K phosphorylates PIP2 to produce PIP3. PIP3 recruits PDK1 to the plasma membrane. PDK1 phosphorylates and activates AKT (at the plasma membrane) at Thr\textsuperscript{308}, PI3K antagonist, PTEN, is a lipid phosphatase which dephosphorylates PIP3 in the cytoplasm, therefore reversing the actions of PI3K, ultimately leading to decreased AKT activity. The mammalian target of rapamycin (mTOR) is a downstream target of the PI3K pathway and is activated in response to similar stimuli that activate the PI3K pathway. mTOR complex 2 (mTORC2) inhibitors may have a great potential in inhibiting major pathways involved in tumorigenesis [9]. Receptor tyrosine kinases also activate mTORC2, which phosphorylates and consequently activates AKT. AKT indirectly activates mTORC1 via the phosphorylation of TSC2, this keeps TSC2 from activating Rheb; resulting in accumulation of Rheb-GTP complex. Rheb-GTP then activates mTORC1, which phosphorylates other downstream targets. AKT substrates include Bad, Caspase 9, IKK\protect\Large\textalpha{}, NOS, TSC2, PRAS40, p27, MDM2, and GSK3 [10]. AKT-mediated phosphorylation of these proteins leads to their activation or inhibition. Regulation of these substrates by AKT contributes to activation of various cellular processes.

Role of AKT in cancer

Recent studies support the notion that one of the major functions of AKT is to promote growth factor-mediated cell survival and to block apoptosis, as observed in most types of cancers. Apoptosis in mammalian cells is a highly regulated cellular process. It is currently believed that an early apoptotic event is the breakdown of mitochondrial structure and the release of cytochrome c. The released cytochrome c then binds to the apoptosis protease-activating factor (Apaf-1) and pro-caspase 9 to form the apoptosome complex. This initiates the activation of caspase cascades. Major players in the process of apoptosis include Bcl-2, Bcl-w, Mcl-1, Bcl-1/ A1, Bcl-xL, Bim, Bad, Bid (the Bcl-2 homology...
domain 3, BH3 only subfamily), Bax and Bak. Bad is a member of the Bcl-2 family of proteins that binds to Bcl-xL or Bcl-2 and inhibits their anti-apoptotic potential. However, when Bad is phosphorylated by AKT, it fails to carry out its pro-apoptotic tasks. It is thought that caspase-9, Bad, and Bim (pro-apoptotic), and Mcl-1 (anti-apoptotic) are direct targets of AKT in determining cell fate in response to apoptotic stimuli [11].

Recent studies have demonstrated that AKT regulates cell survival through transcription factors that regulate the expression of pro- as well as anti-apoptotic genes. The NF-κB family of transcription factors has central functions in a variety of cellular mechanisms: cellular proliferation and apoptosis, inflammation, and the initiation and propagation of innate and adaptive immune responses. The most important NF-κB family members are p65 (RelA), p52 (p100), p50 (p105), cRel, and RelB. These dimers are normally kept in the cytoplasm via an inhibitor of κB (IκB). IκB prevents the NF-κB complex from nuclear localization and subsequent transcriptional activation. Upon activation of the IKK complex, IκBo or IκBβ is phosphorylated which marks IκB for ubiquitin-dependent degradation by 26S proteasome and nuclear translocation of NF-κB [12]. Nuclear translocation and activation of NF-κB leads to the transcription of NF-κB-dependent pro-survival genes, including Bcl-2, Mcl-1, Bcl-xL, caspase inhibitors (cellular inhibitors of apoptosis; cIAP) and c-Myb. Other substrates of AKT involved in transcriptional regulation and cell survival consist of FoxO Forkhead, Mdm2, CREB, and YAP.

AKT signaling regulates physiological functions such as stimulating glucose uptake in response to insulin, nutrient uptake, and metabolism. In the presence of high levels of insulin, glycogen synthase kinase 3 (GSK3) is inhibited upon phosphorylation by AKT, which promotes glucose storage as glycogen. Inhibition of GSK3 has also been shown to be anti-apoptotic. Low levels of growth factors secreted from cells leads to a reduction in their ability to utilize nutrients, thus, leading to an exhaustion of ATP and glucose-based metabolites. AKT activation promotes cellular uptake of glucose, and in this way prevents the activation of Bax and cell apoptosis.

As mentioned above AKT protein kinase family consists of three members, AKT1/PKBα, AKT2/ PKBβ and AKT3/PKBγ. Even though all three isoforms may be expressed in a particular cell type, only certain isoforms may be active [13]. Research has shown that each isoform of AKT is responsible for specific functions in the cell. Based on knockout mice experiments, AKT1 is responsible for growth and regulation of spontaneous apoptosis in the testis and thymus, AKT2 lowers blood glucose levels by the regulation of insulin. Current studies have linked increased AKT3 expression, and decreased PTEN activity to 43-60% of sporadic melanomas. This possible relationship was reinforced by the results from experiments in which AKT3 activity was specifically lowered using siRNA or increased PTEN protein expression, which led to increased apoptosis, reduced cell survival and blockage of melanoma tumorigenesis [14].

Networking and cross-talk among signaling pathways

Almost all research done on various signal transduction pathways has revealed that these pathways are not only immensely intricate and complex but they are each part of a more complex and interconnected network; the AKT pathway is no exception. AKT, directly or indirectly, communicates with RAF-MEK1/2-ERK1/2, NF-κB, JNK and p38 pathways. Through various feedback mechanisms the activity of one of these pathways can often have very strong effects on the activity of the others. Communication between all of the signaling pathways is what makes controlling a single cascade nearly impossible (Figure 1).

Overview of the NF-κB signaling pathway

The transcription factor NF-κB controls very complex gene regulatory mechanisms, which in turn control a wide array of cellular processes such as immune responses, differentiation, proliferation, survival and apoptosis [15]. There are two recognized NF-κB signaling pathways. The classical (canonical) pathway elicits a more immediate response which can develop responses to infections, proinflammatory cytokines and stress within minutes. The most relevant aspect of the classical pathway is its ability to inhibit apoptosis [15]. Once certain cell surface receptors (such as antigen-specific T- and B-cell receptor complexes; BCR, TCR) are triggered, the IκB kinase (IKK) complex will phosphorylate the
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Figure 1. The PI3K/AKT signaling module and cross-talk with other pathway. Upon binding of growth factors to receptor tyrosine kinases (RTK) a cascade of events are triggered resulting in the activation of PI3Kinase and subsequent AKT activation (module I). This cascade leads to inhibition of pro-apoptotic signaling molecules (BAD, BIM, BAX, Caspase 9) or induction of anti-apoptotic Bcl-2, Mcl-1, Bcl-xL, Bcl-w conferring a growth advantage to the tumor cells. One of the major targets of AKT is the mTOR (module II) which is also implicated in cell growth and metabolism. The intricacy of the AKT pathway is further complicated by its cross-talk, via various positive and negative regulatory feedback loops, with multiple major cell survival pathways including: NF-κB (module II), RAS/RAF/MEK/ERK MAPK (module III), p38MAPK (module IV), JNK MAPK (module V). This elaborate network of signaling pathways will amplify the anti-apoptotic signaling and confer a resistant phenotype to the malignant melanoma cells.

two conserved serine residues of IkB, the NF-κB inhibitor which keeps NF-κB in an inactive state in the cytoplasm. Subsequent phosphorylation of IkB will label it for ubiquitin-dependent degradation by the 26S proteasome. Having been freed from the IkB complex, various NF-κB dimers translocate to the nucleus, and through a series of posttranslational modifications activate transcription [16]. The non-canonical (alternate) pathway is characterized by a slower and longer lasting response mainly involved in adaptive immunity, development of secondary lymphoid organs, and proliferation. This pathway differs from the classical pathway in that in order to activate NF-κB, IkB degradation is not a necessity. NF- inducing kinase (NIK) and IKKα dimers induce phosphorylation-dependent proteolytic removal of p100 C-terminal. Now the NF -κB dimers, mainly consisting of p52/RelB, can move to the nucleus and upon binding to a unique κB site, activate transcription [17].

Cross-talk between the NF-κB and AKT signaling pathways

NF-κB activity can also be influenced by cross talk with the AKT signaling pathway through IKK activation and subsequent RelA/p65 phosphorylation at Ser529. Studies have shown that specific AKT inhibition reduces IKK activity [18].
Inhibition of AKT affects both IKK kinase activity and IKKβ/NEMO ubiquitination. This suggests that the AKT pathway can enhance IKK and IKKβ/NEMO kinase activity leading to phosphorylation, ubiquitination of IkBα and its eventual degradation. This will lead to a more constitutively active NF-κB. Higher NF-κB activity will increase self-sufficient growth signals, down regulation of growth inhibitory signals, protection from apoptosis and higher proliferation rate (Figure 1) [19].

Mitogen-activated protein kinase (MAPK) signaling pathways

Mitogen activated protein kinases (MAPKs) regulate several different cellular functions such as proliferation, differentiation and survival. These functions are activated by various environmental stimuli. There are three major groups of MAPKs: the extracellular signal-regulated kinase (ERK), the c-Jun N-terminal kinase (JNK) and the p38 MAPKs. All of these pathways are often constitutively activated in cancers. They also have a high degree of interaction and cross-talk with the PI3K/AKT pathway [20]. Each MAPK pathway follows a similar activation cascade: sequential phosphorylation and activation of a MAPK Kinase Kinase (MAPKKK), a MAPK Kinase (MAPKK) and a MAPK. Below is a brief description of each pathway, their cross-talk with the AKT pathway, and the consequences of such cross-talks in cell fate.

Overview of the RAF-MEK1/2-ERK1/2 signaling pathway

The RAS family of GTPases is a group of proteins that play a major role in cellular processes including proliferation, differentiation and apoptosis. The RAS proteins have active and inactive conformations, when the bound GDP is phosphorylated to GTP the protein is in its active conformation [21]. There are several pathways in which RAS family proteins are involved, such as the RafGEF pathway. RafGEF proteins can induce RAS proteins to activate RaIA and RaIB GTPases, playing a possible role in tumorigenesis and transformation. One of the most important pathways involving the RAS family proteins is the RAF-MEK1/2-ERK1/2 pathway. This pathway uses serine/theronine kinases, which bind to RAS-GTP, allowing it to be translocated to the plasma membrane. Once at the membrane, active RAS-GTP proceeds to phosphorylate and activate one of several RAF proteins (B-Raf, C-Raf, A-Raf), which are the MAPK kinase kinase (MAPKKK) of this pathway. Activated RAF proteins will phosphorylate and activate MEK1/2 (MAPK kinase) which in turn phosphorylates and activates ERK1/2 (MAPK) [19]. Activation of ERK1/2 can phosphorylate proapoptotic Bcl-2 family members Bad and Bim, triggering their cytosolic sequestration and proteasome-dependent degradation, respectively [20]. This cascade of events leads to cell cycle progression and proliferation (Figure 1).

Cross-talk between RAF-MEK1/2-ERK1/2 MAPK and AKT signaling pathway

The RAF pathway also has significant interaction with other pathways including the PI3K/AKT pathway. In fact, there is a direct interaction between PI3K (precursor in the AKT pathway) and RAS; consequently making PI3K a RAS effector [21]. There are several modes of cross-talk between the two, interacting with different isoforms of class I PI3Ks. In some instances active RAS (RAS-GTP) can bind to and activate p110α resulting in a 20-fold increase in PI3Ky activity. RAS can also, through direct interaction, increase the activity of p110α (catalytic subunit of PI3Kα). However, the exact mechanism is not fully understood. It may include conformational changes in substrate binding site or aiding in interactions at the membrane. Activation of p110α occurs independently of p85, through ligand binding at various RTKs, which can dimerize and autophosphorylate allowing it to interact with GRB2 and SOS, thereby RAS becomes activated [22]. There is also data supporting that direct interaction between RAS and p85 regulatory unit is a necessity for p110 activation, however, the exact mechanism is not yet deciphered [23]. In any case, in order to activate PI3K (through direct activation of p110) RTK must be phosphorylated as well as activation of RAS small GTPases. The consequence of increased PI3K activity by RAS is a higher rate of PIP2 phosphorylation, hence, AKT activation. This leads to uncontrolled cell cycle progression, proliferation, antiapoptotic phenotype, cell survival and tumorigenesis. Depending on the cell type and signal intensity, activation of the RAF-MEK1/2-ERK1/2 pathway may confer opposing outcomes such as proliferation, apoptosis and cell cycle arrest. AKT can also phosphorylate RAF in its regulatory domain at Ser259 in order to inactivate it and switch responses from cell cy-
cle arrest to proliferation [24]. This response is highly dependent on relative activity of both pathways; high PI3K/AKT activity will inhibit RAF kinase activity whereas high RAF activity will lower PI3K/AKT activity [25]. Lastly, AKT can also indirectly affect the RAF-MEK1/2-ERK1/2 pathway; AKT can activate GSK-3, which in turn can induce cyclin D1 (CD1), a downstream product of the ERK1/2 pathway. AKT can also directly inhibit p27Kip1, a downstream negative regulator of the ERK1/2 pathway (Figure 1) [26].

**Overview of the c-Jun N-terminal kinase (JNK) signaling pathway**

The second MAPK signaling transduction pathway is the JNK pathway, which is involved in cellular stress, apoptosis, survival, transformation and differentiation. MAPK kinase kinases for the JNK pathway are stimulated by a variety of factors including growth factors, tumor promoters, hormones and proinflammatory cytokines. There are several different possibilities for the MAPK kinase kinase in the beginning of the JNK pathway which include MEKK family members, ASK1, MLK, TAK1 and TPL-2. Upon stimulation, one of several MAPK kinase kinases will activate either one of JNK pathway’s MAPK kinases (MKK4 or MKK7). There is little known about the details of JNK activation, however, similar to the ERK1/2 pathway, RAS can activate the JNK pathway [27]. JNK is assumed to be constitutively activated as a result of a common deletion mutation of tumor suppressor p16(INK4a), which can bind to and inhibit JNK [28]. Following JNK activation, several substrates are prospects for phosphorylation (e.g., ATF2, c-Myc, p53, Paxilin), however, the most well studied and arguably significant one is c-Jun. This leads to a cascade of interactions ending with activation of target genes controlling cell cycle, proliferation, differentiation as well as death. There are also occasions where aside from acting as a kinase, JNK may label substrates for degradation [20]. This functional difference highlights the diverse roles of individual JNK family members (JNK1, 2, 3). Since the affinity of c-Jun is much higher for JNK2 than JNK1, it is thought that they serve different purposes. Studies conducted on knockout mice for either gene suggest that JNK2 is involved dominantly in degradation of target genes while JNK1 stabilizes and activates substrates (such as c-Jun) for transcription [29]. Shared with various other pathways, the apoptotic responses of the JNK pathway are controlled by a delicate balance of the expression levels of proteins such as Bcl-2, Bcl-xL, Bad, Bim and Bax.

**Cross-talk between the JNK1/2 MAPK and AKT signaling pathway**

The mysteries of the JNK pathway become more complex when investigating the possibility of the existence of a feedback loop between the AKT and JNK pathways. In response to various proapoptotic stimuli, many pathways including AKT can inhibit JNK to promote cell survival [30]. Apoptosis signal-regulating kinase 1 (ASK1) contains a sequence very similar to the sequences found in most common AKT substrates that can act as a phosphorylation site for AKT. ASK is an upstream regulator of the JNK pathway. This promotes apoptosis and activates the next component in the JNK pathway. One study has shown that AKT can phosphorylate ASK1 at Ser83 residue, thereby, inhibiting and making ASK1 unresponsive to normal stress and oxidative stimuli leading to blockade of apoptosis and promoting cell survival [31]. However, future studies are warranted to more definitely establish a cross-talk between AKT and JNK pathways (Figure 1).

**Overview of the p38 MAPK signaling pathway**

The last pathway of the MAPK family is the p38 signaling pathway, most commonly known for tumor suppression, negative regulation of cell survival and proliferation, differentiation and apoptosis. Activation of the p38 pathway is triggered by a variety of stimuli and stresses including proinflammatory cytokines, heat shock, UV light, hypoxia, ischemia, etc [26]. TNF receptor associated factor (TRAF) proteins are recruited via TNF-α and IL-1 to activate various MAPK kinase kinases, which include, but not limited to, MEKK1-3, MLK2/3, ASK1, Tpi2, TAK1 and TA01/2. Thereafter, MKK3 and MKK6 are phosphorylated and act as the MAPK kinases which activate p38 [32]. However, p38 can also activate itself via autophosphorylation completely independent of MAPKKs [33]. The p38 pathway is also easily down regulated by MKP-1, -4 or -5 as well as other phosphatases. There are four p38 isoforms, p38α, p38β, p38γ and p38δ. All isoforms generally remain inactive in the cytoplasm anchored by MK2, MK3 or MK5. Upon activation and translocation to the nuclei, they
phosphorylate various substrates such as ATF1/2/6, MEF2, Elk-1, GADD153, Ets1, p53 and MITF. p38 can also phosphorylate several cytoplasmic proteins [32].

**Cross-talk between the p38 MAPK and AKT signaling pathway**

The p38 pathway cross-talks with the AKT signaling pathway, both as a regulator and as a substrate. Inhibition of p38 using SB203580 effectively reduces the phosphorylation and activation of AKT, leading to increased cell death via apoptosis and decreased cell survival [34]. However, p38 activity can also be reduced by PI3K inhibition. PI3K inhibitors (wortmanin and LY294002) reduce p38 phosphorylation confirming a reciprocal relationship between the two pathways.

**Role of the AKT pathway in melanoma progression and resistance to therapy**

Among the major forms of skin cancer, malignant melanoma carries the highest risk of mortality from metastasis. The chances of survival of patients in the advanced stages of this disease are still very poor. At the moment, there are no effective long-term treatments for patients suffering from the disease, despite numerous clinical trials testing the efficacy of several therapeutics ranging from surgery to immuno-, radio-, and chemotherapy [13]. The lack of effective therapies is partially due to our insufficient information about genetic abnormalities during melanoma development and lack of effective therapies specifically targeting those defects [35].

There are many components that regulate signaling transduction pathways; however, the two most important ones are kinases and phosphatases. The intricate regulation of expression and activity of these enzymes is essential for proper cell growth and development. Constitutive activity of phosphatases and kinases involved in cell survival, proliferation, apoptosis and differentiation is often a hallmark of cancer development including melanoma. Specifically in melanoma, melanocytes can be transformed through genetic mutations (as a result of DNA damage) over several years to exhibit mutant phenotypes with constitutive activation of enzymes leading to malignancy [36]. Recent endeavors have linked AKT deregulation and melanoma tumorigenesis [37]. The AKT/PI3K pathway controls several cellular functions ranging from cell survival, proliferation, differentiation, and cell motility to tumor suppression and apoptosis. Several studies have shown constitutive activation of the AKT/PI3K pathway in a plethora of cancers including melanoma [17]. AKT is overexpressed in nearly 60% of all melanomas [13]. Point mutations or over expression of the gene causes aberrant expression of AKT. Although the three isoforms of AKT (AKT1, AKT2, AKT3) share a high degree of homology, it is the function of the specific isoform, AKT1, which has the largest effect on cell survival and cancer development. AKT knockout mice have a significant increase in cell death by apoptosis [38, 39]. The list of cellular processes by which AKT, directly or indirectly, facilitates melanoma progression is extensive [13, 40-44]. The major route of AKT activation is mediated by PI3K, an upstream lipid kinase. PI3K is activated by receptor tyrosine kinases; G-protein coupled receptors, or integrins. Upon binding to the cell surface they initiate the recruitment of the p85 and p110α subunits (PI3K) to the membrane where they mediate activation and phosphorylation of PIP2 to PIP3. PIP3 induces AKT phosphorylation at two sites (Thr308 and Ser473). In cancer cells, AKT translocates to the plasma membrane, while in normal cells AKT remains inactive in the cytoplasm [45]. There is also a receptor independent path in which RAS can activate PI3K. RAS is constitutively expressed in 10-20% of melanomas leading to a majority of PIP2 molecules being phosphorylated to PIP3, thereby activating AKT [46-48]. Although recent developments have shown that mTOR indeed has PDK2 activity, its role in AKT phosphorylation in melanoma has yet to be determined. Of course, PI3K itself can be over expressed causing higher AKT activity [49, 50]. Complex parallel paths which, directly or indirectly, induce AKT activation are also often over expressed in melanomas. The RAS-RAF-ERK pathway is constitutively activated in more than 70% of melanomas largely due to mutations in B-RAF or N-RAS [51]. Along with deregulated expression and activation of specific AKT members, several components involved in cross-talk and/or indirect interaction with the AKT pathway are altered in melanomas as well. The phosphatase and tensin homolog (PTEN) acts as an inhibitor of AKT through hydrolyzing PIP3, thereby, preventing the recruitment of PDK1 and subsequent phosphorylation and activation of AKT.
Recent studies have shown that a significant decrease in PTEN expression occurs in 43% of melanoma cell lines [52]. These results were developed from a range of experiments including the observed sensitization of melanoma cells post induction of PTEN [53, 54], increased phosphorylation of AKT3 after inhibition of PTEN in wild type cells [13], increased cell death and decreased AKT3 activity following PTEN induction [53, 13, 40]. These studies suggest that decreased expression of PTEN aids in the onset and further development of melanoma by its inability to inhibit AKT.

There is an elaborate list of the ways in which AKT can aid in the progression and continued development of melanoma. One of the proapoptotic substrates of the PI3K/AKT pathway is BAD (Bcl-2/Bcl-xL associated death promoter). In normal cells BAD inhibits Bcl-2, which is implicated in resistance to various thereapeutics. BAD can be inactivated through phosphorylation by AKT, which can confer cell survival advantage [55]. The consequence of BAD phosphorylation by AKT is the inability of BAD to form a heterodimer with Bcl-xL and Bcl-2, which would normally lead to cytochrome c release and subsequent activation of the caspase cascade (Casp9 to Casp-3,-6, and -7) [56]. Reduction of telomerase activity by inhibition of human telomerase reverse transcriptase (hTERT) as well as reduced hTERT phosphorylation through interaction with AKT can also induce higher proliferation rates [57]. B-RafV600E is one of the most commonly mutated genes in melanomas. It helps in the regulation of MAPK pathways, however, despite its common mutation in melanomas its role in early cancer development is not fully understood. This may suggest that other signaling pathways and events are involved in the disease onset. B-Raf can also be phosphorylated on either of its serine residues (Ser364, Ser428), thereby, reducing its activity as well as the activity of downstream MAPK pathways, which generally inhibit melanoma development. These actions often lead to early onset and premature development of melanoma through promoting proliferation and early melanocyte transformation [58]. A strong indicator of melanoma development is skin hypoxia. Transformation of melanocytes by AKT can only occur under hypoxic conditions. Notch1 plays a significant role in the preservation of a undifferentiated state of melanocytes in hypoxic conditions. AKT can upregulate Notch1 via the NF-κB pathway promoting the transformation of melanocytes because even in the extreme hypoxic conditions it appears that AKT protects tumors from low oxygen pressures and other chemotherapeutic agents [59]. Foxp3 (Forkhead box p3) is downregulated in a variety of cancers including melanoma. Proliferation rate of regulatory T-cells will increase with the activity of Foxp3 [60]. AKT can directly inhibit Foxp3 by phosphorylation. The consequence of this inhibition is downregulation of the tumor suppressor gene p27 and Fas ligand which upon binding to its receptor induce apoptosis [61, 62]. Other substrates include activation of Cyclin D1 (which activates CDK to induce cell cycle progression and proliferation) and inactivation of Cyclin D1 inhibitor GSK-3 [63]. Furthermore, focusing on the regulatory roles of PI3K/AKT and AKT pathways on signal transducers and activators of transcription (STAT) in resistant melanoma cell lines, it was demonstrated that targeting MAPK alone in therapy has only a temporary effect. However, melanoma cell lines exhibited extra sensitivity to treatment upon inactivation of both the AKT and the ERK signaling pathways. In order to fully inhibit tyrosine phosphorylation and subsequent activation of STAT, PI3K and MAPK work synergistically. Inhibition of STAT activation will lead to eventual decrease in apoptotic activity [64]. These results reinforce the need for a multi-pathway targeting approach in melanoma treatment (Figures 1, 2).

**Targeting the AKT pathway for therapeutic purposes in melanoma**

Considering the major role and significant implications of AKT hyperactivation in cancer development, it is no wonder why this pathway has become a hotspot target for therapeutic endeavors. There are several direct or indirect AKT inhibitors that are currently being evaluated. However, through countless pre-clinical studies and trials, and given the fact that cross-talk between signaling pathways is extensive and complicated; combination therapies are evidently becoming a necessity (Figure 2).

PI3K is the first component of the AKT pathway whose inhibition has been under investigation to not only effectively block a particular pathway (AKT) but also to circumvent compensatory mechanisms. Wortmannin, an irreversible inhibitor of PI3K can covalently bind to Lys802 residue of p110 (the catalytic subunit of PI3K). This...
Targeting PI3K/AKT pathway in melanoma

A compound shows proapoptotic activity in tumor cells [65, 66]. However, wortmannin has been used for nearly a decade to uncover new information about the PI3K signaling pathway. More recently newer compounds have been developed using wortmannin as a model. These compounds such as PWT-458 and PX-866 have less pharmaceutical limitations. PWT-458 is a derivative of wortmannin with much stronger therapeutic index in pre-clinical animal models. PWT-458 has shown in vivo proapoptotic activity. PX-866 is another derivative of wortmannin that is more stable and has more specificity towards PI3Kα, γ, and δ as opposed to PI3Kβ. PX-866 inhibits the growth of human tumor cells with a longer lasting effect. However, due to toxic side effects such as hyperglycemia and decreased glucose tolerance, these drugs have yet to enter human clinical trials [66]. Another well studied PI3K inhibitor is LY294002. LY294002 works through ATP competitive inhibition, preventing the phosphorylation of PI3K. Similar to wortmannin, LY294002 has been used as a base to develop improved compounds. One example is SF1126, a prodrug of LY294002 which inhibits all paralogs of PI3K, mTOR and even the phosphorylation and activation of AKT [66]. This drug has shown significant results both in vitro and in vivo and is currently being tested in phase I trials [66]. One final compound aimed at inhibiting PI3K is ZSTK474. Administration of escalating doses of 100, 200 or 400 mg/kg body weight once a day over the course of 13 days showed a dramatic and direct correlation between drug concentration and PI3K inhibition in mice. There was a direct correlation between percent of inhibition of tumor growth and concentration used [67].

Continuing down the AKT signaling pathway, the next attractive target is PDK1. There is only one isoform for PDK1, and its ability to phosphorylate and activate all three AKT isoforms makes it a valuable target [68]. In order for AKT to be activated it must be phosphorylated at two sites within the activation loop as well as the C-terminus. PDK1 phosphorylates the activation loop [69, 70]. UCN-01 is a nonselective inhibitor of PDK1. Unfortunately, when tested on advanced cancer patients in phase I/II clinical trials there have yet to be significant anti-tumor results. Along with the lack of impressive results come various side effects including nausea and vomiting, lactic acidosis and transaminitis. Due to the non-selective mode of UCN-01 action, it is difficult to eliminate these toxic effects [71].

Of course AKT inhibitors have also been developed. Perifosine functionally inhibits the activation of AKT by preventing phosphorylation at both sites within the activation loop, as well as impeding AKT membrane translocation. Although this drug decreases the levels of activated AKT, the effects seen on cell viability were modest at best. A study was done on melanoma patients where 900mg was administered on day 1 followed by a maintenance dose of 150mg/day for the following 20 days. Little to no change in the viability of cancer cells was seen; an evident lack of objective response [72]. However, other studies have shown an increase in apoptosis upon perifosine administration. This may be, however, due to the effects of the drug on various other pathways such as the p38 and

Figure 2. Therapeutic implications of targeting the PI3K/AKT pathway. Schematic depiction of major components of the AKT signaling module and various pharmacological inhibitors (in various sages of clinical testing) that could potentially interfere with the activity of the AKT pathway. These compounds, alone or combined with various other modalities, may improve treatment efficacy.
ERK1/2 pathways [73].

The mTOR complex is a kinase that regulates cell growth and metabolism in response to environmental stimuli. mTORC1 is a direct downstream target of AKT, [74]. Rapamycin greatly reduces the growth of human tumor cell lines (including B16 melanoma) both in vivo and in vitro at a concentration dependent manner via direct targeting of the mTORC1 complex [75]. Testing agents in combination has provided significantly better results than any single agent alone. A combination of AKT3 and B-RAS siRNAs can significantly inhibit melanoma growth. PLX-4720 specifically inhibits B-RafV600E. However, continuous exposure to the drug induces resistance to apoptosis through subsequent activation of AKT, hinting to possible success with a combination of PLX-4720 with AKT3 and B-RAS siRNAs [58, 76-78]. For example, pharmacological compounds inhibiting MAPK (U0126, PD98059, PD325901) and mTOR (rapamycin) synergistically reduced melanoma cell growth [79-81]. Although combination therapies are extremely important, new hybrid compounds are being developed that can simultaneously inhibit multiple pathways. Hexamethylene bis-acetamide (HMBA) can simultaneously inhibit AKT and MAPK pathways while also down regulating NF-κB. Although this drug has yet to be tested in melanoma, it has shown great success in breast cancer trials [82].

Conclusions

Over the past ten years, our knowledge of AKT/ PKB activation and inhibition and its consequences in cell fate has significantly increased. Ample experimental evidence supports the hypothesis that PI3K/PTEN/AKT/mTOR signal transduction pathway plays a prominent role in the promotion of cell growth and prevention of apoptosis ultimately leading to various forms of tumorigenesis and metastasis. Suppression of this and other related pathways may inhibit key survival networks important in solid tumors and hematopoietic disorders. Although our knowledge of the mechanisms for AKT/PKB activation has improved, a number of major issues still remain unclear. For instance, there may be as of yet other undiscovered substrates of AKT/ PKB. Also our understanding of the exact mechanisms of some of the key players in this signaling module remains largely hypothetical. Further research in this area could shed more light on the intricacies of these signaling molecules, which could essentially unlock the mysteries of uncontrolled cellular division and invasion; the hallmarks of cancer [83]. Recently, great strides have been taken in expanding our understanding of melanoma invasion and metastasis. The PI3K/AKT pathway is linked to several other distinct signaling cascades involved in melanoma tumorigenesis (e.g. NF-κB, ERK, JNK). This interconnected and complex network system yields a highly intricate mechanism of cell fate determination, which goes awry in melanoma. As of yet there is no “cure” for malignant melanoma. However, new treatment strategies including simultaneous targeting of single (Vemurafenib; selective BRAFV600E inhibitor) or multiple kinases (Sorafenib, Nexavar) are being devised at a promising pace. It is argued that combination of inhibitors of survival with apoptosis inducers will prove the best choice. Suitable examples may be the combination of a BH3 mimic with a MAPK inhibitor or the combination of chemotherapy with either proteasome or Raf kinase inhibitor. The scientific community is looking more into specific targeting of apoptotic signaling pathways, or even targeting multiple pathways rather than just a protein in a cascade [84]. Even combinations of three or more signaling effectors may be envisioned. The efficient development of these therapies should be accompanied by the development of customized markers specific for each patient [85]. Given the countless number of oncogenic mutations and anomalies in metastatic melanomas, it is highly unlikely that we will be able to treat all patients with the same combination therapy. In summary, great steps have been taken in improving melanoma therapy by the discovery of how certain signal transduction pathways affect cell fate in normal versus cancerous tissue. Targeted and customized therapy, aiming at multiple pathways regulating apoptosis seems to have taken center stage among melanoma investigators as the most logical and promising treatment option. Clearly more investigations and clinical trials are needed to improve melanoma therapy, but promising advances have been made by insights from understanding the dynamics of signal transduction pathways, their mode of activation and their downstream apoptosis-related targets.

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