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Repositioning HIV Protease Inhibitors as Cancer Therapeutics

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Abstract

Purpose of review—Though designed to target only the HIV protease, HIV protease inhibitors (PIs) induce toxicities in patients such as insulin resistance and lipodystrophy that suggest that PIs have other targets in mammalian cells. Akt controls insulin signaling and is an important target in cancer, but no Akt inhibitors are approved as cancer therapeutics. These observations have prompted study of HIV protease inhibitors as inhibitors of Akt and possible cancer therapeutics. This review will highlight the latest advances in repositioning HIV PIs as cancer therapeutics.

Recent findings—Although PIs can inhibit Akt activation and inhibit the proliferation of over 60 cancer cell lines, as well as improve sensitivity to radiation or chemotherapy, these effects do not always correlate with Akt inhibition. Other important processes such as the induction of endoplasmic reticulum stress appear critical to the biological activity of PIs. These impressive and surprising preclinical data have prompted clinical testing of nelfinavir as a lead HIV PI in cancer patients.

Summary—While mechanism of actions for the anti-tumor effects of HIV PIs are complex, their broad spectrum of activity, minimal toxicity, and wide availability make PIs ideal candidates for repositioning as cancer therapeutics.

Keywords

protease inhibitors; Akt; apoptosis; ER stress; autophagy

Introduction

The development of cancer drugs is slow and costly. The repositioning of HIV PIs as cancer therapeutics has been based on two facts. First, Akt is an important target in cancer, yet no Akt inhibitors are clinically available. Second, HIV PIs inhibit Akt activation, which likely explains the clinical toxicities of insulin resistance and lipodystrophy that are associated with their use. Recent studies show that HIV PIs are established broad-spectrum anti-cancer agents that work through pleiotropic mechanisms in cancer cells. The clinical efficacy of PIs is now being evaluated in cancer patients.

Protease inhibitors target more than HIV protease

The protease inhibitors were rationally designed to block HIV aspartyl protease, an enzyme that cleaves the gag and gag-pol precursor polyproteins, arresting maturation and preventing the generation of infectious virions [1,2]. Because this class of drugs is only weakly active

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against human aspartyl proteases [2,3], minimal toxicity was anticipated. Shortly after their introduction, however, off-target effects began to be described.

Some of the earliest data came from Kempf et al. [3] who found that treating mice with ritonavir could prevent the expansion of cytotoxic T cells against lymphocytic choriomeningitis virus (LCMV) epitopes. This was not because of inhibition of LCMV replication, but rather because of decreased presentation of LCMV antigen. The mechanism proposed by Schmidtke et al. [4] was inhibition of antigen processing via ritonavir-induced inhibition of the 20S proteasome chymotrypsin activity. Consistent with this, early HIV trials reported on the ability of saquinavir and ritonavir to affect immune reconstitution before virus replication was suppressed [5] and to maintain immune reconstitution in face of persistent viremia [6–10]. In 1999, a report of a patient with regression of Kaposi's sarcoma (KS) following therapy with a PI-containing regimen was published [11], and improvements in response rates and relapsed free survival in many AIDS associated malignancies followed [12]. While these findings were initially attributed to immune reconstitution and better control of oncogenic viral infections, the number of reports in solid tumors, KS [13], lymphoma, fibrosarcoma [14], multiple myeloma [15], and prostate cancer [16,17] suggested other mechanisms for the anti-neoplastic activity of PIs.

Lipodystrophy and insulin resistance

Another indication that PIs target more than the HIV protease was based on reports of lipodystrophy and insulin resistance in PI-treated HIV patients [18–21]. Explanations for lipodystrophy centered on adipocyte transcription factors such as peroxisome proliferators activated receptor γ (PPAR-γ) and sterol regulatory element binding protein-1 (SREBP-1). Carr et al. [22] proposed that through one of several mechanisms, adipocyte differentiation was blocked, ultimately resulting in apoptosis. While controversy exists regarding adipocyte differentiation [23–27], a consensus that PIs increase SREBP-1 expression developed [26, 28]. SREBP-1 expression has been shown to be increased 2.6 fold in adipocytes of HIV infected persons after treatment with ritonavir [28]. Increased expression of SREBP-1, especially SREBP-1c, is a feature of the congenital lipoatrophy syndrome, an autosomal recessive disorder, characterized by unregulated expression of SREBP-1c, loss of subcutaneous fat, insulin resistance, and dyslipidemia [29], phenotypic features of patients with PI-induced lipodystrophy.

HIV PIs may also induce insulin resistance through multiple mechanisms. PIs inhibit release of insulin by pancreatic beta cells [30], and inhibit the response of skeletal muscle cells, adipocytes, and hepatocytes to insulin. This occurs through diminished signaling through Akt and isoforms of protein kinase C [31,32], as well as through direct binding to glucose transporters such as Glut1 and Glut4 [32,33]. The role of Akt in mediating the effects of insulin was confirmed in studies of mice that lack specific isoforms of Akt. Mice lacking Akt2 are insulin resistant and have higher fasting and post-prandial glucose levels than heterozygous or wild type mice, and show compensatory but inadequate hyperinsulinemia [34]. Inhibition of Akt signaling by PIs not only provided a mechanism for a commonly observed toxicity, but also provided strong rationale to test PIs as cancer therapeutics.

The Akt pathway in cancer

The Akt pathway is the prototypic survival pathway and is constitutively activated in a number of malignancies (Figure 1) [35]. In preclinical studies, Akt promotes cellular transformation, cellular proliferation, and drives tumor formation in mice. In addition, Akt activation promotes resistance to chemotherapy as well as radiation therapy, and portends a poor prognosis for patients with many types of cancer [36]. The Akt signaling cascade is initiated with the activation of phosphatidylinositol-3-kinase (PI3K) following cross-linking of a growth factor

with its cell surface receptor. Activated PI3K phosphorylates membrane bound phosphoinositides. Phosphorylated phosphoinositides bind to Akt, leading to its translocation to the inner cell surface where it can be phosphorylated by many mechanisms [37]. Following phosphorylation, activated Akt moves to the cytosol and nucleus to activate its many substrates, which control important cellular processes such as cell cycle progression, apoptosis, transcription and translation [37,38]. Regulation of the PI3K/Akt pathway is complex, and an important negative regulator is the tumor suppressor PTEN, a lipid phosphatase that de-phosphorylates the products of PI3K.

Because Akt promotes the formation, maintenance, and therapeutic resistance of cancer, the development of Akt inhibitors has become a major effort within industry and academia. Given the inherent delay of developing Akt inhibitors de novo, the immediate availability of the PIs made them logical candidates to study in cancer.

The efficacy of PIs as anti-cancer agents

The results of several studies that assessed the efficacy of PIs in cancer cells are summarized in Table 1. HIV PIs have a very broad spectrum of activity, and can inhibit the proliferation and/or cause the death of virtually every cancer cell line tested in a dose dependent manner. Many investigators have confirmed the *in vitro* efficacy of PIs by demonstrating inhibition of growth of human tumors in mice when transplanted as xenografts. Cytotoxic chemotherapies such as docetaxel and targeted therapies such as imatinib have been successfully combined with PIs [16,39,40], suggesting that HIV PIs have properties that are shared with traditional cancer therapeutics.

Despite this wide spectrum of activity, PIs are not very potent. Most studies require ≥10 μM for cellular activity. This is relevant because pharmacokinetic studies performed in HIV patients revealed that the maximum concentrations achieved for nelfinavir were 7–9 μM. Of HIV PIs tested, nelfinavir appears to be most potent, which has led to its consideration as a lead HIV PI for cancer therapy. The recognition of PIs as broadly cytotoxic agents to cancer cells has intensified efforts to understand how PIs work, given the absence of HIV protease.

Is activated Akt the critical molecular target for protease inhibitors?

Constitutive activation of Akt protects cancer cells from apoptosis, making them resistant to the effects of ionizing radiation and/or chemotherapy [41–44]. Several groups [40,45–47] have suggested that inhibition of PI3K-induced activation of Akt by HIV PIs is an important mechanism by which the class exerts anti-tumor effect. These investigators showed that decreased phosphorylation of Akt correlated with increased sensitivity to ionizing radiation [40,45–47] and chemotherapy [40]. However, this has not been observed in all studies, and in many cases inhibition of Akt activation lags behind other effects such as induction of ER stress and inhibition of cell cycle progression. Moreover, other groups hypothesize that the anti-tumor effects of PIs are related to other mechanisms such inhibition of VEGF and HIF1α expression [48] (which may be secondary to Akt inactivation), direct inhibition of the chaperone function of Hsp90 [49], or inhibition of proteasome function [50] (Figure 2).

Administration of a PI to a cancer cell may not only result in direct Akt inhibition, but also indirect inhibition. For example, Akt inhibition has been observed in some studies after cell cycle arrest with apoptosis [14,15,40,49,51–53] cell cycle arrest without apoptosis [46,48, 52], or only apoptosis [39,54]. More recently, studies that identified cell cycle arrest as a consequence of administration of PIs also showed a correlation with the accumulation of proteins that control cell cycle progression [14,40,49,53]. This led to the important observation that protein degradation, and more generally, protein homeostasis is altered by PIs.

Beyond the Akt pathway: proteasome inhibition and ER stress

The 20S proteasome was one of the first “off target” activities of the HIV PIs [3]. Several investigators [14,50,54–58] have proposed direct inhibition of the proteasome as the mechanism for anti-neoplastic activity. In these studies, proteasome inhibition resulted in the accumulation of cell cycle inhibitors, cell cycle arrest and apoptosis [14,] or decrease in NF-κB activity [55]. Hampson et al. [58] showed that proteasome inhibition by lopinavir prevented E6 induced clearance of p53 in an HPV-16 cervical cancer cell line. Other investigators [50, 52,54,59] have extended these studies and observed that PIs induce ER stress.

The endoplasmic reticulum (ER), responsible for protein folding and maturation, is also capable of signal transduction for cell homeostasis. Any condition that alters function of the ER is called ER stress. Such conditions include accumulation of misfolded proteins resulting from proteasome inhibition, lipid or glycolipid imbalances, and changes in the ionic balance of the ER lumen [60,61]. ER stress as a result of abnormal glucose transport and proteasome inhibition has been shown to be part of PI-induced lipodystrophy [62]. The accumulation of unfolded protein aggregates prompts signaling through an evolutionarily conserved pathway called the unfolded protein response, in which misfolded proteins directly activate specific ER kinases such as PERK that cause a decrease in protein synthesis and thereby give the cell a chance to clear the denatured proteins. As part of a feedback loop, protein phosphatase 1 (PP1) can be expressed, which can de-phosphorylate and inactivate Akt, further decreasing protein synthesis. If a cell cannot recover from ER stress, it can directly undergo apoptosis via a caspase dependent pathway or can attempt survival through autophagy. Invariably, prolonged autophagy leads to cell death.

The studies of Gupta et al. [50], Gills et al. [52], and Pyrko et al. [54] have provided the most thorough analysis of how PIs induce ER stress in cancer cell lines. Gupta described induction of ER stress as a means by which Akt was de-phosphorylated by PP1. Gills et al. [52] suspected ER stress when vacuolization and cellular detachment in nelfinavir treated cell lines was observed. Transmission electron microscopy revealed marked dilatation of ER, and two markers of ER stress, phosphorylation of eIF2α and increased expression of ATF3, were increased within hours after treatment with nelfinavir, suggesting ER stress preceded other cellular responses. Similar results were observed by Pyrko et al. [54], who treated glioma cell lines with nelfinavir and atazanavir and showed that expression of ER stress markers and dilatation of ER was critical to the response to PIs.

Autophagy

Autophagy is a tightly regulated catabolic process in which a cell degrades long-lived proteins or organelles as part of normal homeostasis or as a means to survive a period of nutrient depletion. Autophagy enables the cell to transfer nutrients from less essential locations to those vital for survival [63]. Autophagy can be induced by starvation, mTOR inhibition (secondary to Akt inhibition), ER stress and the unfolded protein response, as well as inhibition of growth factor receptor signaling [64]. Several investigators [30,31,40] reported that pre-treating cells with nelfinavir blocks growth factor receptor signaling, ultimately blocking Akt phosphorylation and activation of downstream substrates such as mTOR. However, autophagy and ER stress were not evaluated in these early papers. More recently, Gills et al. [52] demonstrated evidence of autophagy in nelfinavir treated cells. Because the effects of nelfinavir on Akt inhibition were transient and cell line specific in their studies, mTOR inhibition was an unlikely mechanism for the induction of autophagy. ER stress and the UPR were more likely based upon rapid upregulation of eIF2α and ATF3 (markers of ER stress) by nelfinavir, and EM and immunofluorescence data showed that nelfinavir caused dilatation of ER and accumulation of an ER-specific fluorescent marker in vesicles, respectively. Though a pathway

leading to the induction of autophagy was identified, the expression of autophagy markers was atypical. Expression of LC3-II, a prototypic marker of autophagy, was increased and autophagosomes were observed using EM, but induction of beclin-1 was not observed. Nonetheless, an inhibitor of autophagy increased the cytotoxicity of nelfinavir in cancer cell lines. Thus, a practical concern is whether the induction of autophagy could be a means by which cancer cells could survive nelfinavir treatment.

From the bench to the bedside

There are currently two clinical trials evaluating nelfinavir in solid tumors and two as a radiation/chemotherapy sensitizer (Clinicaltrials.gov). Results are available from a phase I trial using nelfinavir as a radiation sensitizer in locally advanced pancreatic cancer [65]. Investigators treated 12 subjects with advanced pancreatic carcinoma with nelfinavir 1250 mg orally twice daily starting 3 days before radiation therapy. Subjects received cisplatin and one of two dose levels of gemcitabine concurrently with 59.4 Gy radiation over 6 weeks. Tumor response was determined using Response Evaluation Criteria in Solid Tumors (RECIST). None of the observed toxicities were attributed to nelfinavir. Partial responses were seen in five of 10 subjects. Negative resection margins were obtained six of the 10 responders who underwent surgical resection. These data compare favorably with historical controls, as tumor responses rates following combined modality therapy for pancreatic cancer are approximately 30%. Interestingly, inhibition of Akt phosphorylation did not correlate with clinical responses, although this was a preliminary analysis.

Two other Phase I dose escalation studies using nelfinavir as a single agent in solid tumor patients are underway. In each, the objectives are to establish the maximum tolerated dose and define dose-limiting toxicities of nelfinavir. One study at the National Cancer Institute and National Naval Medical Center is open to patients with any solid tumor, and one study at City of Hope is open to patients with liposarcomas. These dose escalation studies are important because when nelfinavir was originally developed, a maximum tolerated dose was never established. Given the dose dependent cytotoxic effects of nelfinavir in preclinical studies, it is possible that higher doses could yield greater clinical responses. Currently, each trial is at a dose level where over 3,000 mg are being administered bid without significant toxicities. In addition to clinical endpoints, each study is assessing biomarkers for nelfinavir administration. The NCI study is focusing on assessment of Akt activation, and expression of markers of apoptosis, ER stress, and autophagy. The City of Hope trial is focusing on SREBP-1 expression, which explains the restriction to patients with liposarcomas, and is based on preclinical data from these investigators [51].

Conclusion

Are the data convincing enough to conclude that HIV PIs could be repositioned as anti-cancer agents? Clearly, PIs inhibit a variety of malignant cell lines and xenografts, with nelfinavir consistently being the most potent and effective at clinically achievable concentrations. Although lipodystrophy and insulin resistance in PI-treated HIV patients originally linked these agents to the Akt signaling pathway, induction of other molecular processes such as proteasome inhibition [50], ER stress, the unfolded protein response, and autophagy [52,59] must now also be considered as being critical to the effects of PI in cancer cells. Despite the uncertainty of a unifying mechanism of action for PIs, their track record of minimal toxicity, FDA approved status, and readily availability makes them excellent candidates for further evaluation as cancer therapeutics.

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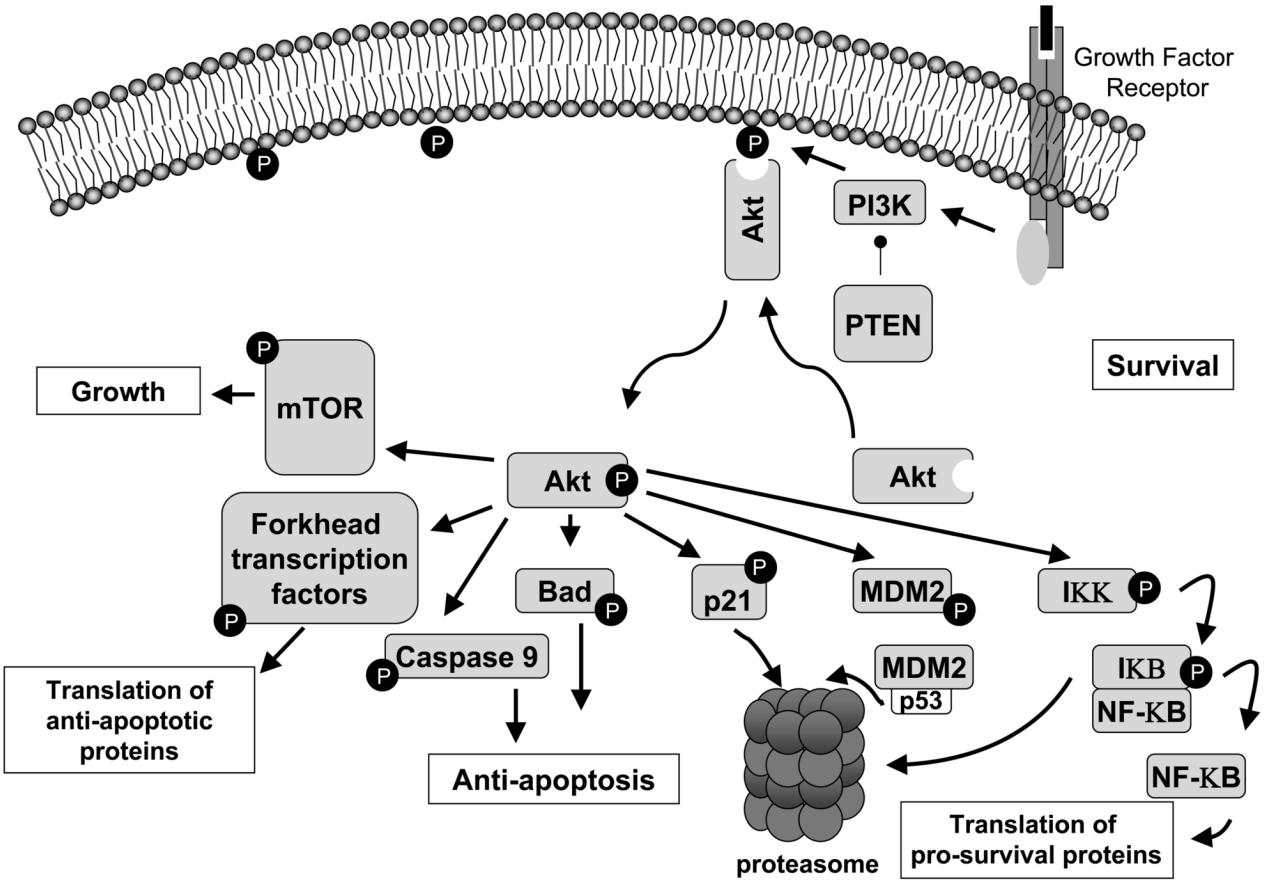

Figure 1. Activation of Akt promotes cellular survival through multiple mechanisms

Akt inhibits apoptosis by phosphorylation of many substrates, including 1. the FoxO subfamily of forkhead family transcription factors that inhibits the transcription of pro-apoptotic genes, 2. pro-apoptotic proteins such as BAD and caspase 9, which inactivates them, and 3. IKK, which indirectly increases the activity of NF-κB and stimulates the transcription of pro-survival genes. Cell cycle progression is promoted by phosphorylation of the cdk inhibitor p21, which is subsequently cleared via the proteasome. Phosphorylation of MDM2 by Akt leads to increased ubiquitinylation of p53 and increased cleared by the proteasome.

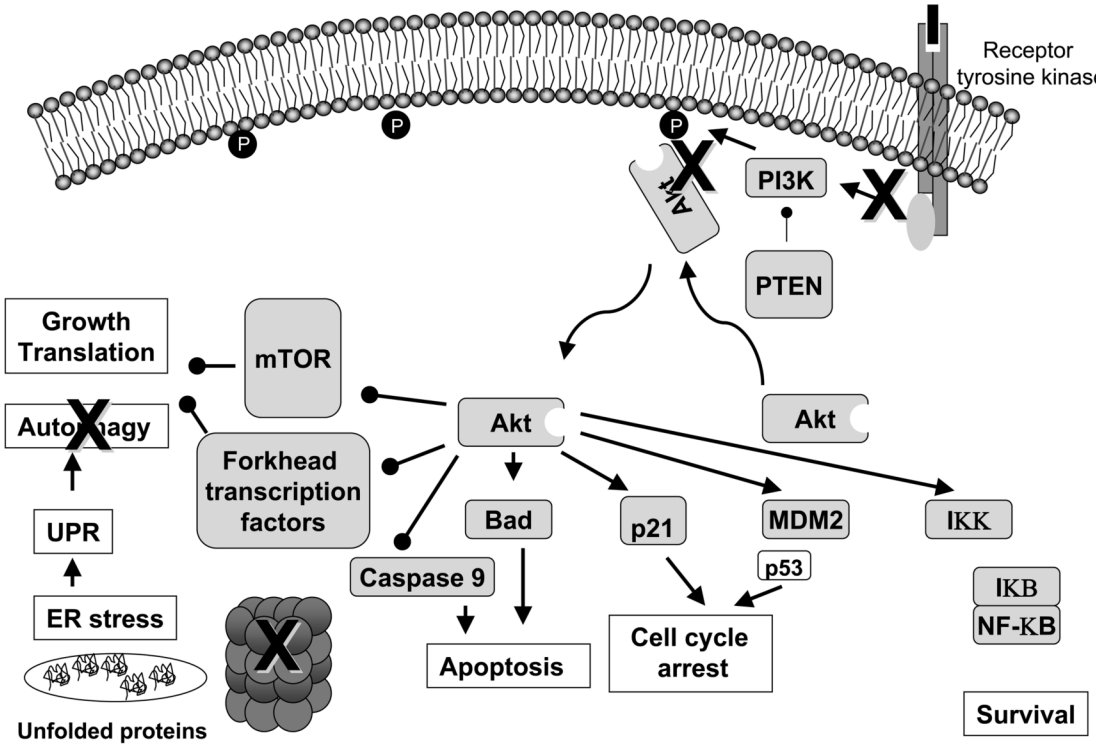

Figure 2. Potential sites of action of HIV PIs

The black Xs represent possible sites of action of protease inhibitors. The inhibition of Akt phosphorylation results in a loss translation of the pro-survival genes and a loss in the inhibitory phosphorylation of the pro-apoptotic proteins caspase 9 and BAD. p21 and p53 may induce cell cycle arrest. Inhibition of the proteasome results in the accumulation of unfolded proteins and the unfolded protein response. To permit cell recovery, a global decrease in protein synthesis or autophagy may follow.

Table 1

Cell Lines	Cell Type	Mechanism	p-Akt Inhibition	Other Observations	Xenograft data	Increased efficacy of chemoradiation therapy?	Ref
EL4-T, Jurkat, T1 Meth-A, P815, 3T3	Thymoma, leukemia, T/B lymphoblastoid hybird, Murine Fibrosarcoma, Murine Mastocytoma, Non transformed fibroblasts	Inhibition of 20S proteasome	Not studied	Apoptosis, accumulation of cell cycle inhibitors	RIT induced growth inhibition in C57BL/6 with ELT-4 tumors	Not studied	14
LnCaP, DU-145, PC-3, U373, K562, Jurkat	Prostate (LnCaP, DU-145, PC-3) Glioblastoma (U373) Leukemia (K562, Jurkat)	Inhibition of 20S and 26S proteasome, inhibition of NF-κB activity	Not studied	Apoptosis	None	Radiation	55
U266, RPMI8226, ARH77	Multiple myeloma	Inactivation of STAT3 and ERK1/2	Not studied	Growth arrest, apoptosis, and anti- angiogenesis	None	Not studied	15
PC-3, DU-145	Androgen independent prostate	Inhibition of NF-κB binding activity, inhibition of CYP 3A4 by RIT	Not studied	Apoptosis	RIT induced growth inhibition in BNX *nu/nu mice* with DU145 tumors	Docetaxel	16
SQ20B, T24, MIAPACA2, A549	SCCa head and neck, Bladder, Pancreatic, Lung	Inhibition of Akt activation	Decreased p-Akt		AMP, NEL induced growth inhibition via decr p-Akt in Ncr nude mice with SQ20B and T24 tumors	Radiation	45
HL-60	Leukemia	Inhibition of 20S proteasome	Not studied		None	Not studied	57
SW872, LiSa-2, HT1080	Liposarcoma	Up regulation SREBP-1 expression	Not studied	G1 cell cycle arrest, apoptosis	None	Not studied	51
E6 transfected C33A, SiHa	SCCa Cervix	Inhibition of 20S proteasome preventing E6-induced clearance of p53	Not studied	Apoptosis	None	Not studied	58
SQ20B, A549	SCCa head and neck, Lung	Decreased VEGF expression, decreased hypoxic induction of HIF1α via inactivated Akt pathway	Decreased p-Akt	Increased tumor oxygenation	NEL induced decreased VEGF expression in nude mice with SQ20 or A549 tumors	Radiation	48
MCF7, T47D, MDA-MB-436, MDA- MB-231	Breast cancer	Inhibition of chaparone function of Hsp90	Decreased p-Akt	G1 arrest, depletion of cyclin dependent kinases 2,4,6 and Cyclin D1, and p-Rb	RIT induced growth inhibition in nude mice with	Not studied	49

Cell Lines	Cell Type	Mechanism	p-Akt Inhibition	Other Observations	Xenograft data	Increased efficacy of chemoradiation therapy?	Ref
					MDA-MB- 231 tumors		
H460, H520, A549, EBC-1, ABC-1	Non small cell lung cancer	Inhibition of Akt activation	Decreased p-Akt	Apoptosis, accumulation of cell cycle inhibitors	NEL induced growth inhibition and increased apoptosis in BALB/c nude mice with H460 tumors	Docetaxel	40
NA	Human umbilical vein endotheial cells (HUVEC)	Inhibition of Akt activation	Decreased p-Akt	Apoptosis	NEL induced increased sensitivity to radiation in vascular window model	Radiation	46
NCI-60, H157, A549	NCI-60 (leukemia, lung, colon, CNS, melanoma, ovarian, renal, prostate, breast); H157, A549 Lung	ER stress	Varies among cell lines	G1 cell cycle arrest, apoptosis, autophagy	NEL induced growth inhibtion in BAL/c AnNCr nude mice with H157 tumors	Not studied	52
SQ20B	SCCa	Proteasome inhibition, ER stress, and unfolded protein response causing dephosphorylation of p-Akt by PP1	Decreased p-Akt		None	Radiation	50
1205 LU, A375, C8161, NIH1286, WM35, WM115	Melanoma	Cell cycle arrest via inhibition of CDK2, dephosphorylation of Rb; proteasome induced degradation of Cdc25A phosphatase	No change in p- Akt	Growth inhibition and apoptosis	None	Not studied	53
U251MG, U87MG	PTEN deficient glioblastoma	Inhibition of Akt activation	Decreased p-Akt, p-S6		Radiation sensitization in nude mice with U87 tumors	Radiation and temozolomide	47
HUVEC, KSIIM	Normal endothelial cells, KS	Anti-angiogenesis, inhibition of NF-κ B activity	Not studied	Inhibiton of proliferation of HUVEC, inhibition of KS promoting inflammatory proteins	RIT induced growth inhibition in BNX mice with KSIMM tumors	Not studied	13
T98G, LN229, U251, U87	Glioblastoma	Proteasome inhibition, ER stress, and	Not studied	Apoptosis	NEL induced growth inhibition in	Not studied	54

Curr Opin HIV AIDS. Author manuscript; available in PMC 2008 November 1.

Cell Lines	Cell Type	Mechanism	p-Akt Inhibition	Other Observations	Xenograft data	Increased efficacy of chemoradiation therapy?	Ref
		unfolded protein response			nude mice with U87 tumors		
Raj, Daudi	Lymphoma	Proteasome inhibition for both, no data presented	Not studied	Additive effect on growth inhibition	No	Not studied	56
IOMM - Lee	Meningioma cell line and Fresh meningioma tissue	Increased Bax:Bcl- 2 and decreased survivin leading to apoptosis	Not studied	Additive effect on growth inhibition	NEL + Imatinib induced growth inhibition in athymic nude mice and IOMM-Lee tumors	Imatinib	39

d Akt; Hsp90 is heat shock protein 90

ost active agent

saquinavir, RIT ritonavir, IND indinavir, AMP amprenavir, TAZ tazanavir, LOP lopinavir

l carcinoma