MECHANISM OF ANTI-CANCER ACTIVITY
OF 9-AMINOACRIDINE BASED DRUGS

by
CANHUI GUO

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Thesis Advisor: Dr. Andrei V. Gudkov

Department of Biochemistry
CASE WESTERN RESERVE UNIVERSITY

August, 2008
We hereby approve the thesis/dissertation of

Canhui Guo

candidate for the PhD degree*.

(signed) Hung-Ying Kao

(Chair of the committee)

David Samols

Genes Sen

Andrei Gudkov

George Stark

(date) July 1st, 2008

*We also certify that written approval has been obtained for any proprietary material contained therein.
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In the tumors that maintain the wild type p53 tumor suppressor gene, p53 activity is frequently inactivated by a variety of mechanisms, such as, overexpression of Mdm2 or MdmX, loss of Arf, or expression of human papilloma virus protein E6. None of these mechanisms, however, explains the suppressed p53 function in the majority of renal cell carcinomas (RCC), tumors that largely have wild type p53. In our previous study, we found that a small molecule, 9-aminoacrididine (9AA) can restore p53 function in RCC and strongly stimulate activity of wild type p53 in other types of cancer cells. In addition, this compound was found capable of inhibiting NF-kB signaling, the pathway that is constitutively active in RCC as well as in the majority of other tumor types. To determine the mechanism of simultaneous modulation of p53 and NF-kB by 9AA we compared global gene and protein expression profiles of two 9AA-treated and untreated RCC cell lines. The set of genes that changed their expression in response to 9AA treatment contained many known p53- and NF-kB targets and therefore was consistent with p53 activation and NF-kB suppression by this compound. Proteomics analysis revealed P110gamma, one of the catalytic subunits of PI3K, to be one of
a few proteins that were strongly down-regulated by 9AA treatment. We further found that the observed decrease in P110gamma protein is mediated through the 9AA-induced inhibition of the PI3K/AKT/mTOR signaling pathway. Consistent with the known role of PI3K in regulating p53 and NF-kB functions, knocking down of P110gamma expression by RNA interference in RCC cells inhibited NF-kB and activated p53. Moreover, ectopic overexpression of P110gamma in 293T cells resulted in constitutive activation of NF-kB. Furthermore, overexpression of P110gamma in RCC45 cells made them less sensitive to 9AA treatment. Together with all the above-mentioned observations indicate that p110gamma is one of the targets of 9AA that mediate its modulation of the p53 and NF-kB pathways in tumor cells.
1 Literature Review

1.1 p53 and its function

1.1.1 A Historical Overview of p53

P53 was first identified in 1979 as a cellular protein that bound to SV40 DNA tumor virus large T antigen (LT) (Lane and Crawford, 1979; Linzer and Levine, 1979). Consistent with its association with LT, early studies have showed that p53 cooperated with other oncogene products in in vitro transformation assays and p53 was overexpressed in mouse and human tumor cells (Dippold et al., 1981). Therefore, p53 at first seemed to be a bona-fide oncogene. In the late 1980s, however, researchers discovered that they were studying TP53 with missense mutation instead of wild-type gene and the oncogenic properties of p53 were due to this mutation (Baker et al., 1989; Finlay et al., 1989; Hinds et al., 1989). In fact, wild type p53 was not an oncogene but a tumor suppressor gene. It negatively regulates the cell cycle and requires loss-of-function mutations for tumor formation (Vogelstein et al., 1989; Lane and Bencimol, 1990). The generation and characterization of p53/- mice conclusively demonstrated that p53 is a tumor suppressor, because these animals develop malignant tumors within 4-6 months after birth (Donehower, et al., 1992). After this paradigm shift of the late 1980s, TP53 was widely recognized as a tumor suppressor and referred to as “the guardian of genome” (Lane, 1992).
1.1.2 Structure of p53 protein

The 393-aa p53 protein has a complex domain structure. The N-terminal region contains the transactivation domain (residues 1-62), followed by a proline-rich region (residues 63-94). The transactivation domain interacts with a number of regulatory proteins, such as the negative regulator transformed 3T3 cell double minute 2, p53 binding protein (mouse)(MDM2), components of the transcription initiation complex and the acetyltransferases p300 and CBP, which act as co-activators and regulate p53 via acetylation of its C-terminus. The proline-rich region contains SH2-domain binding motifs (PXXP) and is thought to have a regulatory role. The central (core) domain (residues 94-292) binds specifically to double-stranded target DNA that contains two decameric ‘half-site’ motifs 5’Pu.Pu.Pu.C.(A.T).(T.A/A).G.Py.Py.Py-3’(Pu=A/G, Py=T/C) separated by up to 13 base pairs. Four core domains bind these DNA response elements in a cooperative manner to give a 4:1 complex. The C-terminal region includes the tetramerization domain (residue 325-356), which regulates the oligomerization state of p53, and the negative auto-regulatory domain at the extreme C-terminus, which contains acetylation sites and binds DNA nonspecifically (Joerger and Fersht, 2007).

1.1.3 Modulation and stabilization of p53

P53 protein is kept at a very low level as a result of constitutive degradation in normal cells. In response to oncogenic and other stresses, p53 protein is stabilized by protein modification. These protein modifications appear to alter the
p53 protein in two ways: first, the half-life of the protein in a cell increases from 6-20 min to hours, and this results in a 3-10 fold increased concentration of the p53 protein in a cell. Second, as a transcription factor, the ability of the p53 protein to bind to specific DNA sequences is enhanced and causes up- or down-regulation of a variety of target genes. (Harris and Levine, 2004)

**Mdm2** The p53-mdm2 relationship is vital in the regulation of cell growth and death. The mdm2 protein regulates the activity of the p53 protein with more than one mechanism. It binds to the N-terminus of p53 (within the transactivation domain) and blocks the transcriptional activity of the p53 protein. Through binding to p53, mdm2 shuttles p53 out of the nucleus to the cytoplasm for degradation (Freeman & Levine, 1998). The mdm2 protein also functions as a ubiquitin ligase and can ubiquitinate p53 (Ashcroft and Vousden, 1999).

**E3 ligases** In addition to mdm2, other E3 ligases have been shown to be involved in proteasome-mediated degradation of p53. Human papilloma virus E6-associated cellular protein E6AP was first shown to be able to degrade p53 through ubiquitination (Scheffner et al., 1993). Some other E3 ubiquitin ligases (PirH2, COP1, ARF-BP) (Leng et al., 2003; Dorna et al. 2004; chen et al., 2005) were recently identified as E3 ligases that can ubiquitinate and degrade p53.

Phosphorylation 10% of N-terminus and C-terminus amino acids are being phosphorylated after stimulation (Lavin and Gueven, 2006). These phosphorylations are brought about by a number of protein kinases that respond to different stress stimuli including ATM (mutated in ataxia-telangiectasia); ATR(A-T and Rad3-related); the checkpoint kinases, Ck1 and Chk2; Jun NH2-terminal
kinase (JNK), p38 and others (www.biology.bnl.gov/cellbiol/anderson.html) The most frequently described phosphorylation is on Ser15 of p53 and occurs in response to different stress signals. The sites most commonly phosphorylated are Ser46, Ser15, Ser20 and Ser33.

Acetylation P300/CBP serves as a co-activator for numerous transcription factors, including p53 by mediating histone acetylation (Chan and La Thangue, 2001). Phosphorylations at Ser15, Thr18 and Ser20 stimulate the recruitment of other factors including p300 and CBP that promote C-terminal acetylation and the acetylations of p53 may prevent ubiquitination and degradation (Li et al., 2002)

Sumoylation Small ubiquitin-like protein, SUMO-1, can be attached to many transcription factors, including p53 (Gostisssa et al., 1999; Rodriguez et al., 1999). This process is termed sumoylation which can stimulate p53 transcriptional activity.

Deacetylation Histone deacetylase (HDAC)-1, -2, and -3 are all capable of down regulating p53 transcriptional activity. Coexpression of HDAC1 greatly reduced the in vivo acetylation level of p53 (Juan et al., 2000)

1.1.4 Function of p53

The tumor suppressor p53 has a major role in the cellular response to a wide range of stress signals. Activation of p53 induces a range of responses, including cell cycle arrest or DNA repair to apoptosis or senescence. Under mild or transient stress conditions, p53 induces cell cycle arrest and DNA repair. In response to severe, irreparable stress, p53 induces a permanent inhibition of cell
proliferation through apoptosis or senescence (Bensaad, 2005)


**Apoptosis** Apoptosis is a pattern of molecular and morphological changes that result in the packaging and removal of the dying cells. Central to our understanding of the mechanism of apoptosis is the induction of caspase activity. There are two general signaling pathways that trigger apoptosis. The intrinsic pathway is engaged by the transcriptional or post-translational regulation of Bcl-2 proteins that directly impact mitochondrial outer-membrane permeabilization (MOMP)(spierings et al., 2005). The extrinsic pathway is activated by ligand binding to the “death receptor”. "Death receptors" are specialized cell-surface receptors including Fas/CD95, tumor necrosis factor-alpha (TNF-alpha) receptor 1, and two receptors, DR4 and DR5 (Ashkenazi, 2002). The binding of death receptor with ligand causes the recruitment of an adaptor molecule. Procaspase-8 molecule can be activated by binding of the adapter molecular, and then procaspase-8 can activate executioner caspases.

P53 can regulate apoptosis through both transcriptional dependent and independent mechanisms. Nuclear p53 regulates the expression of numerous pro-apoptotic genes (e.g. Bax, NOXA, PUMA, BID, CD95, APAF-1, DR5,
p53AIP1) (Miyashita et al., 1995; Oda et al., 2000; Nakano et al., 2001; Sax et al., 2002; Muller et al., 1998; Moroni et al., 2001). Recent studies have started to define the mechanisms of non-transcriptional pro-apoptotic p53 activity. So far, two different mechanisms have been described, each of which is assigned to a specific localization of the p53 protein, either in the cytosol or directly at the mitochondria. Although mechanistically different, both transcription-independent modes of apoptosis induction initiate permeabilization of the outer mitochondrial membrane via activation of the pro-apoptotic Bcl-2 family members Bax or Bak (Moll et al., 2005). In response to a broad spectrum of apoptotic stimuli, fractions of wild type p53 translocate to mitochondria and localize to the outer membrane and induce apoptosis (Mihara et al., 2003). Several studies suggest that cytosolic p53 can directly activate apoptosis (Speidel et al., 2006; Chipuk et al., 2004). Both transcriptional and nontranscriptional pro-apoptotic activities of p53 cooperate to ultimately cause cell death.

**Senescence** Senescence is an almost irreversible stage of permanent G1 cell-cycle arrest that is linked to morphological changes (flattening of the cells), metabolic changes and changes in gene expression (for example beta-galactosidase) (Lowe et al., 2004). It is an important mechanism of suppressing the development of malignant tumors in vivo (Yeager et al., 1998). There are many stimuli that contribute to cellular senescence. Among them, telomere shortening, DNA damage and oxidative stress are the best described (Campisi et al., 2007). Both damage and telomere-initiated senescence depend strongly on p53 and are usually accompanied by expression of p21 (Dimri et al., 2005). Since
p21 also mediates a transient DNA-damage-induced growth arrest, what determines whether cells senesces or arrests transiently is still a question needing to be answered (Campisi et al., 2007)

**DNA repair** In eukaryotes, the five main DNA-repair processes are nucleotide-excision repair (NER), base-excision repair (BER), mismatch repair (MMR), non-homologous end-joining (NHEJ) and homologous recombination (HR). The DNA damage signal from DNA double-strand breaks or replication stress is primarily recognized by ataxia telangienctasia mutated protein (ATM) or ATM- and Rad3-related (ATR) complex. As a major transducer protein, p53 is activated by ATM, ATR as well as their target kinases-The checkpoint kinase(chk1 and chk2) (Saito et al, 2003; Vaziri et al, 1997; Wang et al, 1998). P53 modulates DNA-repair process by both transactivation-dependent and independent pathways. During the transactivation independent process, p53 interacts with proteins that are involved in all the repair process. As a transcriptional factor, it also functions by binding to p53-response elements (p53RE) to upregulate the expression of genes involved in NHEJ, MMR, BER, NER (Sengupta et al., 2005).

**P53 and metabolism** Most cancer cells show the characteristic increase in aerobic glycolysis instead of mitochondria respiration known as the Warburg effect (Gsyrynbey et al, 2004). Recently one of the transcriptional targets of p53, Synthesis of cytochrome c oxidase 2 (SCO2), was identified (Matoba et al., 2006). It is a key regulator of the cytochrome c oxidase complex that is essential for mitochondria respiration. In cells that lack functional p53, the cytocrome c oxidase complex is disrupted. This leads to a shift to glycolysis for the production
of energy. P53 also downregulates the expression of phosphoglycerate mutase (PGM), an enzyme that enhances glycolysis. In this case, losses of p53 functions enhance PGM expression and glycolysis (Kondoh et al, 2005). Thus these new discoveries shed some light on the role of p53 in tumor suppression from the metabolic point of view.

1.1.5 Mutation of p53

The strongest and most undisputed fact about p53 is the high frequency of p53 alterations in human cancer. There is mutation of p53 in almost half of all human tumors.

The central domain of the p53 protein, the target of 80% of p53 mutations, is associated with the DNA-binding activity (Soussi, 2007). In contrast to other tumor suppressor genes that are mainly altered by truncating mutations, the majority of TP53 mutations are missense substitutions (75%). Other alterations include frameshift insertions and deletions (9%), nosense mutations (7%), silent mutations (5%) and other infrequent alterations (Olivier et al, 2002).

The p53 pathway is almost certainly dysfunctional in a majority of wild-type TP53-carrying tumors. This could occur through overexpression of the p53 antagonist Mdm2, loss of the Mdm2 inhibitor p14ARF via homozygous deletion of the INK4a locus, or expression of the human papilloma virus E6 protein that triggers p53 degradation (Asker et al., 2005).
1.1.6 Therapeutic strategies for mutant TP53-carrying tumors

Wild-type TP53 reconstitution in mutant TP53-carrying tumors can be accomplished by gene therapy, that is, introduction of an intact cDNA copy of TP53 gene using a viral vector, typically one based on adenovirus (Adp53). Such studies have demonstrated a significant clinical effect with stabilized tumor growth or even tumor regression (Bykov and Wiman, 2003). However, the progress is far below expectation due to the limited efficacy of gene delivery to tumor cells and other problems associated with virus delivery such as the immune response (Wiman et al., 2006).

Some synthetic peptides corresponding to certain fragments of the p53 sequence or antibodies recognizing p53 epitopes have proven to be effective in stabilizing p53 protein or restoring mutant p53. A significant proportion of mutant p53 can be activated for DNA binding by C-terminal manipulation, such as binding of p53 antibody Pab421 (Hupp et al., 1993; Halazonetis and kandil, 1993). In addition, several monoclonal antibodies that recognize the N-terminal epitope can partially rescue the DNA binding of p53 mutants (Cohen et al., 1999). A synthetic peptide derived from the p53 C-terminal domain (peptide 46) has been shown to restore the specific DNA binding and transcriptional transactivation of several mutant p53 proteins (Selivanova et al., 1997; Kim et al., 1999).

Screening for mutant p53 reactivating compounds has been carried out. By screening a chemical library using Saos-2-His-273, a p53 null human osteosarcoma cell line has been engineered to stably express a His-273 mutant of p53 under the control of a tetracycline-regulatory promoter, PRIMA-1
reaktivation and induction of massive apoptosis) was identified as having the capability of rescuing the wild type conformation of mutant p53 protein. This compound rescues wild type conformation of mutant p53 proteins in vitro and induces apoptosis selectively in human tumor cells expressing exogenous mutant p53 (Bykov et al., 2005).

1.1.7 Therapeutic strategies for wild-type TP53-carrying tumors

The p53 pathway is disrupted in a large fraction of wild-type p53-carrying tumors. Mdm2 is a critical regulator of p53 that is frequently overexpressed in wild type TP53-carrying tumors.

In 2004, a group of imidazoline compounds named Nutlins were identified from a diverse chemical library that could interfere with p53-mdm2 binding. Through x-ray crystallography, the MDM2-p53 complex was solved and showed a well-defined hydrophobic cleft which represented the binding site for p53. In addition, the structure revealed that this cleft was filled by only three side chains of the helical region of p53: Phe19, Leu26 and Trp23. This revelation leads to the possibility that a small molecular inhibitor could mimic these three amino acids and their orientation. The inhibitor could disrupt the mdm2-p53 interaction by binding specifically in this cleft, liberating functional p53. (Vassilev et al, 2004). They seem to have great promise in B-cell chronic lymphocytic leukemia (CLL) treatment (Kojima et al., 2006).

Using a cell-proliferation assay, RITA was identified as a compound that suppresses tumor cells with wild type p53 by binding to the p53 N-terminus and
causing a conformational change that prevents MDM2 binding, resulting in p53 accumulation and upregulation of p53 target genes. It also reduces tumor growth in xenograft animal models (Issaeva et al., 2004). But it is still in only preclinical trial stage.

Other investigators have screened for compounds that inhibit the E3 ligase activity of Mdm2. This approach led to the identification of a family of compounds designated HL198 (Yang et al., 2005).

1.2 NF-kB signaling pathway

1.2.1 NF-kB pathway

Nuclear factor –kB (NF-kB) was first described in 1986 as a protein binding to an enhancer element of the immunoglobulin k light chain gene in B lymphocytes (Sen et al., 1986). However, subsequent studies revealed that NF-kB is present in virtually every cell type (Sen et al., 1986). The NF-kB family consists of five members, p50, p52, p65 (RelA), c-Rel, and RelB, encoded by NFKB1, NFKB2, RELA, REL, and RELB respectively. They share an N-terminal Rel Homology domain (RHD) responsible for DNA binding and homo- and heterodimerization. These NF-kB dimers bind to kB sites in the promoters/enhancers of target genes and regulate transcription through the recruitment of coactivators and corepressors (Hayden et al, 2008).

NF-kB dimers are initially expressed in inactive form in the cytoplasm by associating with IкB protein IкBalpha (NFKB1A), IкaBbeta (NFKB1B), or IкBε
(NFKBIE), or the precursor protein p100 (NFKB2) and p105 (NFKB1). To date seven IkBs have been identified: IkB-alpha, IkB-beta, IkB-gamma, IkB-epsilon, Bcl-3, p100 and p105. All known IkBs contain multiple copies of a 30-33 aa sequence, called ankyrin repeats which mediate the association between IkB and NF-kB dimers. The ankyrin repeats interact with a region in the RHD of the NF-kB proteins and mask their NLS preventing nuclear translocation.

Degradation of IkB is a rapidly induced signaling event that is initiated upon specific phosphorylation of these molecules by activated IKK (inhibitors of NF-kb kinase). The IKK complex contains highly homologous kinase subunits, IKKalpha/IKKK1 and IKKbeta/IKK2, and a regulatory subunit NEMO.

Two pathways lead to the activation of NF-kB. The classical pathway is triggered by pro-inflammatory stimuli and genotoxic stress, including cytokines such as tumor-necrosis factor (TNF) and interleukin-1 (IL-1); bacterial cell-wall components, such as lipopolysaccharide; viruses, and DNA-damaging agents. This leads to the IKKbeta-dependent and IKK-gamma-dependent phosphorylation of IkBs, which results in their proteasomal degradation, and to the subsequent liberation of NF-kB dimers (which are mostly p50-p65). The alternative pathway is triggered by LTalpha/beta, CD40L and Blys/BAFF, but not by TNF-alpha, IL-1 or LPS. This leads to an IKK-beta-independent and IKK-gamma-independent manner, to the phosphorylation of p100 by IKK-alpha and the degradation of its carboxy-terminal half by the proteasome. This results in nuclear translocation of p52-REL-B dimers (Karin, 2005).

The classical pathway induces the transcription of a variety of genes encoding
cell adhesion molecules, inflammatory and chemotactic cytokines, cytokine receptors, and enzymes that produce inflammatory mediators. The alternative pathway plays a central role in the expression of genes involved in development and maintenance of secondary lymphoid organs (Karin, 2005).

1.2.2 Inflammation and cancer
Epidemiological studies suggested that chronic infections and inflammation can be major risk factors for various types of cancer. Collectively, underlying infections and inflammation are linked to 15-20% of all cancer death (Kuper, 2000). For instance, chronic infections with hepatitis B (HBV) and hepatitis C virus (HCV) are major risk of hepatocellular carcinomas (HCC), whereas infections with Helicobacter pylori are associated with most gastric cancers (Roder et al, 2002). Chronic inflammatory bowel diseases (IBDs), such as ulcerative colitis (UC), are thought to increase the risk of colorectal cancer by approximately 1% per year (Ekbom, 1998), and chronic airway irritation and inflammation caused by airborne particles and tobacco smoke are likely to be important promoters of lung carcinogenesis (Dhala et al., 2004).

1.2.3 NF-kB and cancer
NF-kB is a central coordinator of innate and adaptive immune response. Recently, it has become clear that NF-kB signaling provides a mechanistic link between inflammation and cancer (Karin, 2005). There is now considerable
evidence that sustained or constitutive activation of NF-kB is prevalent in cell lines and tumor tissue specimens and contributes to malignant progression and therapeutic resistance of the major forms of human cancer. NF-kB/Rels are constitutively activated in human lymphomas (Bargou et al., 1997), carcinomas of breast (Sovak et al., 1997), prostate (Suh et al., 2002), lung (Mukhopadhyay et al., 1995), colon (Kojima et al., 2004), pancreas (Wang et al., 2004), head and neck (Ondrey et al., 1999), esophagus (Abdel et al., 2004), and cervix (Nair et al., 2003).

A number of oncoproteins have been shown to induce NF-kB activation (Baldwin et al., 2001). For example, oncogenic Ras and Her-2/Neu (ErbB2) have been demonstrated to activate NF-kB (Karin, 2002). Bcr-Abl, the fusion protein associated with chronic myelogenous leukemia, was shown to activate an NF-kB through activation of the kinase MEKK1 (Nawata et al., 2003). Also HTVL-1Tax oncoprotein activates NF-kB through direct binding and activates the IKK complex (Jeong et al., 2001). Several studies indicate that PI3K/Akt-dependent signaling activates NF-kB activity (Gustin et al., 2003).

Carcinogenesis is the process by which normal cells are transformed into cancer cells. It can be divided into three mechanistic phases: initiation (which involves stable genomic alterations), promotion (which involves the proliferation of genetically altered cells) and progression (which involves an increase in the size of the tumor, the spreading of the tumor and the acquisition of additional genetic changes (Hanahan et al., 2000). NF-kB activation has been shown to be involved in every stage of carcinogenesis. Mouse studies of inflammation-
associated liver and colon cancer support the hypothesis that sustained NF-kB activation is necessary for tumor promotion and progression. Another mouse model of chemically induced liver cancer suggests that NF-kB mediated inflammatory processes in nonmalignant cells is an important determinant of tumor development (Karin, 2005).

As an inducible transcription factor, NF-kB controls a number of genes involved in immuno-inflammatory responses, cell cycle progression, inhibition of apoptosis and cell adhesion, thus promoting carcinogenesis and cancer progression. NF-kB suppresses both the death receptor (DR) (extrinsic) pathway and the mitochondrial (intrinsic) pathway of apoptosis. NF-kB exerts its pro-survival activity through several anti-apoptotic proteins, including FLIP, Bcl-XI, A1/Bfl-1, cellular inhibitor of apoptosis (c-IAP), X chromosome-linked inhibitor of apoptosis (XIAP), TRAF1, and TRAF2. NF-kB also induces several bcl-2 family members most notably Bcl-XI and A1/Bfl-1 which prevent apoptosis by inhibiting permeability transition and depolarization of mitochondria, and cytochrome c release (Kucharczak et al, 2003).

### 1.2.4 NF-kB inhibitors

Since many studies have demonstrated that NF-kB could protect transformed cells from apoptosis and therefore participate in the onset or progression of many
human cancers, the number of NF-κB inhibitors has been increasing at a staggering rate.

**Anti-inflammatory drugs:** Several nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin (sodium salicylate) have been shown to inhibit NF-κB. At higher concentrations, aspirin blocks NF-κB activity by directly binding to and inhibiting the kinase activity of IKKbeta by reducing its ability to bind ATP (Yin et al., 1998); more recently, aspirin has also been reported to inhibit proteasome activity and consequently to interfere with degradation of IkB (Dikshit et al., 2006).

**Novel anti-NF-κB agents:** Inhibition of NF-κB can occur on several levels: upstream of IKK (e.g. at a receptor or adaptor level); directly at the IKK complex or IkB phosphorylation; ubiquitination or proteasomal degradation of IkB; nuclear translocation of NF-κB; NF-κB DNA binding; and NF-κB-directed gene transactivation (Gilmore et al., 2006).

**IkB super-repressors:** Effective blockade of NF-κB DNA binding can be accomplished by using mutant forms of IkBs, called super-repressors (SRs), which cannot be phosphorylated or degraded; thus, these mutant IkBs stably bind to NF-κB complexes. In general, the use of IkBalpha-SR has been taken as strong evidence for the involvement of NF-κB.

**Proteasome inhibitors:** PS-341 or bortezomib is a proteasome inhibitor that has entered clinical development. It inhibits the proliferation and induces apoptosis in cells of a variety of tumors. The anti-proliferative and pro-apoptotic actions of PS-341 largely correlate with its ability to block the degradation of the NF-κB inhibitor IkBalpha.
**IKK inhibitors:** Much of the effort to develop an IKK inhibitor entails the screening of compound libraries to identify inhibitors of IKK-alpha specific and/or IKK-beta catalytic activities. No potent IKK-alpha-specific inhibitors have been described to date. By comparison, the development of specific IKK-beta inhibitors has progressed rather rapidly. For example, one of beta-carboline derivative, a small molecule named PS-1145, was shown to inhibit IKK complex selectively (Lam et al, 2005)

### 1.2.5 Cross talk between p53 and NF-kB

Depending on the cell type and the nature of the inducing stimulus, p53 and NF-kB can either cooperate or antagonize each other's function. P53 and NF-kB transcription factor families are important, multifunctional regulators of cellular response to stress. P53 induction can activate an apoptotic program, and resistance to chemotherapy correlates with the loss of a functional p53 pathway. By contrast, NF-kB prevents apoptosis in response to chemotherapeutic agents. In response to inflammation stimuli, NF-kB induces anti-apoptotic genes that antagonizes the pro-apoptotic function of p53. Moreover, NF-kB negatively regulates p53 stability by controlling the levels of the p53 E3 ubiquitin ligase Mdm2. For example, a member of ankyrin-repeat-containing IκB family of NF-kB inhibitors, Bcl-3, is overexpressed in human cancers and can suppress DNA damage-induced p53 activation. It also inhibits p53-induced apoptosis through a mechanism that is partly dependent on the up-regulation of Hdm2 (Kashatus et al., 2006). Also, IKKbeta mediated NF-kB
activation has been shown to modulate basal levels of Mdm2 which results in the blockage of chemotherapy induced p53 and cell death (Rocha et al., 2005).

Phosphoinositie 3-kinase (PtdIns 3-kinase)-Akt signaling involves regulation of both p53 and NF-kB pathways. It promotes the phosphorylation and movement of the Mdm2 oncoprotein into the nucleus, where it downregulates the p53 tumor suppressor protein. In contrast, the PTEN tumor suppressor protein inhibits activation of AKT and restricts Mdm2 to the cytoplasm. While it activates the phosphorylation of NF-kB through AKT mediated phosphorylation of IKK (Jeong, 2005).

Furthermore, p53 and NF-kB have been reported to antagonize each other's function by competing for the limited pool of transcriptional co-activator p300 and CREB-binding protein (CBP)(Webster et al., 1999). Huang et al recently revealed that upon TNF-alpha and LTbeta stimulation, CBP becomes phosphorylated at serines 1382 and 1386, resulting in changing its binding preference from p53 to NF-kB regulated promoters (Huang et al., 2007).

Under certain circumstances, p53 and NF-kB cooperate with each other. For example, p53 can induce ribosomal S6 kinase 1 activity, which phosphorylates RelA at Ser536, resulting in the nuclear localization of NF-kB (Bohuslav et al., 2004).
1.3 PI3K/AKT/mTOR pathway

1.3.1 PI3Ks signaling

Phosphoinositide 3-kinases (PI3Ks) belong to a family of lipid signaling kinases that phosphorylate phosphoinositides at the 3'-OH position of the inositol ring after exposure of cells to various biological stimuli, creating 3'-phosphorulated inositol lipids (Cantley, 2002). The phosphorylated phosphoinositides (PIP3) have an important role as second messengers by working as a docking platform for lipid-binding domains, such as the pleckstrin homology (PH) domains of various cellular proteins. These include kinases such as 3-phosphoinositide-dependent protein kinase 1 (PDK1) and protein kinase B (PKB/Akt) that trigger downstream kinase cascades, and guanine-nucleotide exchange factors (such as Vav and P-Rex) that control the activity of small GTPases (Donahue et al., 2004).

1.3.2 Family members of PI3Ks

PI3Ks are subdivided onto four classes (IA, IB, II and III) according to their primary and secondary structures, mode of regulation and substrate specificity. The class IA and IB PI3K isoforms are the only ones that are able to produce PIP3 (Fruman et al., 1998). Class I PI3Ks have been the most extensively studied so far, and are heterodimeric proteins that consist of a catalytic and a regulatory adaptor subunit, the nature of which determines a further subdivision into class IA and IB PI3Ks. The class IA members- PI3Kalpha, beta and gamma- consist of an SH2-domain-containing regulatory subunit p85 that forms a complex with one of three catalytic subunits, p110alpha, p110beta or p110theta.
(Cantley, 2002). Class IA PI3Ks are activated by growth factor receptor tyrosine kinases (RTKs). Class IB PI3Ks is heterodimer consisting of a p101 regulatory subunit and a p110gamma catalytic subunit. It is activated by G-protein-coupled receptors (GPCRs).

Complete genetic inactivation of PI3Kalpha or PI3Kbeta results in embryonic lethality, which indicates that PI3Kalpha and PI3Kbeta have nonredundant and essential roles (Rommel et al, 2007). Amplification and point mutations of the gene encoding PI3Kalpha that increase the enzymatic activity of the protein have been frequently found in human cancers (Engelman et al, 2006).

1.3.3 PI3K-AKT signaling

After the activation of PI3K by any of several many growth factors, it generates membrane-bound phosphoinositides, PIP3, which act as secondary messengers and serve to recruit proteins, which contain a pleckstrin homology (PH) domain. The protein serine/threonine kinase AKT (also known as PKB) is a principle target of PIP3 (Testa et al, 2001). Aberrant loss or gain of Akt activation underlies the pathophysiological properties of a variety of complex disease, including type 2 diabetes and cancer (manning et al, 2007).

PKB/AKT is the cellular homologue of the transforming oncogene of AKT8 oncovirus (v-AKT) (Bellacosa et al., 1991). In humans, there are three cellular homologues of AKT designated AKT1, AKT2 and AKT3. All three gene products are similar in structure and size and contain an amino-terminal PH domain (Murthy et al., 2000). AKT can be inactivated by the action of protein
phosphatase 2A (PP2A) (Andjelkovic et al, 1996). The Akt-activating ability of PI3-K is opposed by the lipid phosphatase PTEN (Stambolic et al, 1998).

1.3.4 AKT activation

AKT activation is a multistep process involving both membrane translocation and phosphorylation. Phosphorylation of PIP2 to PIP3 by PI3Ks provides the docking sites for AKT and triggers the translocation of AKT to the plasma membrane. Upon membrane localization, Akt molecules are phosphorylated at two residues: a threonine (residue 308 in PKBalpha/AKT1) within the protein kinase 'T-loop' and a serine buried in a hydrophobic motif proximal to the carboxyl terminus (serine 473 in AKT1). In unstimulated cells, a threonine residue at position 308 in the activation loop is largely unphosphorylated and PKB/AKT is therefore inactive. Following agonist dependent activation of PI3K, this residue undergoes rapid phosphorylation and the activity of PKB/AKT increases markedly. It is now clear that the T-loop of PKB/AKT is the target of PDK1 (Scheid et al, 2001).

The second important phosphorylation event is associated with PKB/AKT activation at Ser473, which is situated in a hydrophobic region near the carboxyl terminus. Ser473 phosphorylation always parallels the activation of PI3K and is required for full activation of AKT. The identity of the kinase which is responsible for phosphorylation of the second critical site in AKT, Ser473, in the hydrophobic motif has long been sought and remains controversial. Many different candidate kinases have been reported to mediate this event in vitro, with few convincing genetic or physiologically relevant data in vivo (Shaw et al., 2006)
Up until now, there are more than 100 Akt substrates have been found. AKT mediated phosphorylation of these substrates leads to their activation or inhibition. Regulation of these proteins by AKT contributes to activation of the various cellular processes such as cell survival, growth, proliferation, glucose uptake, metabolism and angiogenesis (Manning et al., 2007).

1.3.5 mTOR Complex (mammalian target of rapamycin)

The target of rapamycin protein (TOR) was first identified in Saccharomyces cerevisiae through mutants that confer resistance to growth inhibition induced by an immunosuppressive macrolide, rapamycin (Kunz et al., 1993). Genetic studies have demonstrated that TOR is essential for cell growth and development in Drosophila, C.elegans, and mammals (Gingras et al, 2001). TOR orthologs have been identified in mammals (mTOR, also known as FRAP, RAFT1, or RAPT) (Sabatini et al, 1994). mTOR is a highly conserved kinase belonging to PI3K-related family of serine/threonine kinases (PIKKs). MTOR is found in two complexes, mTORC1 and mTORC2 (Kim et al, 2002). MTORC1 contains of mTOR, raptor (regulatory associated protein of mTOR), mLST8 (also known as GbetaL) and PRAS40 (praline-rich AKT substrate 40kDa). mTORC2, like mTORC1, also includes the mTOR, mLST8 protein, but instead of RAPTOR, mTORC2 contains the rictor (rapamycin-insensitive companion of mTOR) and mSIN1 protein (Guertin et al, 2007).
1.3.6 Upstream of mTOR

The mTOR pathway responds to growth factors via the PI3K pathway. Binding of insulin or insulin-like growth factors (IGFs) to their receptors leads to recruitment and phosphorylation of the insulin receptor substrate (IRS), and subsequent recruitment of PI3K. PI3K bound to IRS converts PIP2 in the cell membrane to PIP3. PIP3 recruits Akt to the membrane, resulting in the phosphorylation and activation of Akt. mTOR is wired to the PI3K pathway through the tuberous sclerosis proteins TSC1 (harmartin) and TSC2 (tuberin), TSC2 is phosphorylated and functionally inactivated by Akt in response to insulin. TSC1 and TSC2 act as a heterodimer that negatively regulates mTOR signaling by converting GTPase Rheb from an active GTP-bound state into an inactive GDP-bound state. Rheb binds directly to the kinase domain in mTOR and activates mTOR in a GTP-dependent manner. Expression of Rheb in cells leads to growth factor dependent activation of the mTOR pathway.

1.3.7 Function of mTORC1

mTOR regulates a large number of cellular processes, including transcription, translation, mRNA turnover, protein stability, autophagy, and cytoskeleton organization, but the best characterized function is regulation of translation (Gingras et al, 2001).

MTORC1 phosphorylates two known substrates, eiF4E binding protein 1 and ribosomal protein S6 kinase 1. It is currently believed that TORC1 mediates its
progrowth effects through the activation of S6 kinase 1 (S6K1) and suppression of 4E-BP1, an inhibitor of cap-dependent translation (Hay et al, 2004).

All nuclear transcribed eukaryotic mRNAs contain a ‘cap’ structure, m7GpppN (where ‘N’ is any nucleotide and ‘m’ is a methyl group) at the 5’ terminus. The cap is specifically bound by the initiation factor eIF4E that associates with two additional initiation factors, eIF4G (a large scaffolding protein) and eIF4A (a helicase that is believed to unwind mRNA 5’ secondary structures), to form the eIF4F complex, which facilitates the recruitment of ribosome to the mRNA. Activation of mTORC1 results in increased translation initiation by stimulating eIF4F complex assembly eIF4E levels are limiting for cap-dependent translation in most system (Averous et al, 2006).

The mammalian 4E-BPs constitute a three-member family of translational inhibitors that bind to eIF4E, inhibit formation of the eIF4F complex and thus cap-dependent translation. Binding of 4EBP1 (the best studied 4e-BP family member) to eIF4E is regulated by its phosphorylation status: the hypophosphorylated form (residue Thr37 and 46) of 4E-BP1 binds very tightly to eIF4E and inhibits cap-dependent translation. In response to stimuli such as growth factors, hormones, nutrients or increased energy levels, mTOR phosphorylates 4E-BP1 primarily at residue Ser64 and Thr73. As a result, eIF4E is released from 4E-BP1 with the subsequent increase in cap-dependent translation (Richter et al., 2005).

The ribosomal protein S6 kinase is also an important substrate of mTOR. S6K1 is implicated in positive regulation of cell growth and proliferation. S6K1 activation correlates with enhanced translation of a subset of mRNAs that contain
a terminal 5’ oligopyrimidine tract (TOP mRNAs). These mRNAs encode ribosomal proteins, elongation factors, the poly-A binding protein and other components of the translational machinery that become electively translated in response to growth factors (Wullschleger et al, 2006).

1.3.8 Function of mTORC2

Study of mTORC2 is in its infancy, but the finding that mTORC2 directly phosphorylates AKT adds a new twist in thinking about the role of mTOR in cancer. Phosphorylation of S473 in a C-terminal hydrophobic motif is necessary for the full activation of AKT. MTOR has been added to the list of kinase that can phosphorylate AKT on Ser473 (Sarbassov et al, 2005).
2 Materials and Methods

2.1 Cell Lines
Primary RCC cell lines (RCC 45, RCC 54, and RCC26) were provided by James Finke (The Cleveland Clinic Foundation); normal kidney epithelial cells (NKE) was provided by Joe Didanato (The Cleveland Clinic Foundation); Other cell lines used HT1080 (lung fibrosarcoma), 293 (embryonic kidney epithelial cells transformed with DNA of adenovirus type 5), HCT116 (colorectal carcinomas), ACHN( kidney adenocarcinoma), H1299 (lung carcinoma) were purchased from American Type Culture Collection. Primary RCC cell lines, NKE, ACHN cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 10 mM HEPES buffer, 55 nM beta-mercaptoethanol, and antibiotics. The other cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplement with 10% fetal bovine serum and antibiotics (30mg/l penicillin, 50ng/l streptomycin sulfate).

2.2 Chemicals
9-aminoacridine (9-AA), Doxorubicin (Dox), translation inhibitor cycloheximide (chx) were purchased from Sigma, PI3K inhibitor LY294002 was purchased from Alexis, recombinant rat 4EBP1 was purchased from Calbiochem, TNF-alpha was purchased from Fisher.
2.3 Plasmids

pNF-kB-Luc plasmid was provided by N. Neznanov (Cleveland Clinic Foundation). pCDNA3.1 vector expressing ssIkB (a stable IkB mutant lacking both phosphorylation sites) was provided by Inder Verma (Salk Institute for Biological Sciences, La Jolla, CA). pCBP plasmid was provided by Tony Kong (Rutgers, The State University of New Jersey, Piscataway, NJ). Plasmid encoding the full length p110gamma was purchased from ATCC. Plasmid encoding the full length PTEN is a gift from LD Mayo (Case Western Reserve University). Plasmid encoding AKT2 and kinase dead AKT (E299K) is a gift from Eugene Kandle (Cleveland Clinic Foundation). p53, Arf expression vectors, pBabeH1-siHdm2, and p21-ConALuc reporter plasmid are described in (Gurova et al., 2004). Human P110gamma cDNA were inserted into pLA-CMV lentivirus vector using SnaBI/Xhol restriction sites. For design and cloning of shRNA directed against human p110gamma, a pair of 70-nucleotide oligonucleotides encoding a 21-nucleotide P110gamma shRNA (5’-GATCCCGTGTTTGAGCATCATTTAAGTTGATATCCGCTTAAATGATGCTTCCAACATTTTTTCCAAG-3’ and 5’-GGCACAAACCTTGCAGTTAAATTCAACTATAGGCGAATTTTACAAGGTTTGTAAAAAGGTTC-3’) were designed, which contained extra BamHI and EcoRI restriction sites to facilitate cloning. The position of the core 21-nucleotide sequence (underlined) targeted nucleotide 4061 to 4082 of the human p10gamma. The 70 nucleotide ologonucleotides were annealed and cloned into the BamHI and EcoRI sites of the pLPLw vector and the resulting vector, designated pLPLw-shp110gamma, was subsequently sequenced to
confirm identity. shRNA against human papillomavirus oncoproteins E6, a gene that is not expressed in RCC45 or HT1080 cells, was used as control. The sequence against E6 gene was as follows: 5’-CTAACACTGGTTATACAA-3’.

2.4 Western Blot

Whole-cell extracts were prepared in RIPA buffer (25 mM Tris-HCl, pH 7.2/125 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate/1 mM EDTA) containing 1 mM PMSF (Sigma), 10 mg/ml of aprotinin (Sigma), and 10 mg/ml of leupeptin (Sigma). Protein concentrations were determined with Bio-Rad DC protein assay kit. Following heat denaturation for 3 min, proteins were separated by 4-12% NuPAGE precast Bis-Tris polyacrylamide gel electrophoresis system with MOP or MES running buffer depending on protein size. Proteins were transferred to PVDF members. Proteins were blocked with 5% non-fat milk in PBS with 0.1% Tween, incubate overnight at 4°C with primary antibody and analyzed by Enhanced Chemiluminescence (Amersham). The following antibodies were used: anti-p53, monoclonal mouse DO1 (Santa Cruz Biotechnology); anti-p21, monoclonal mouse F-5 (Santa Cruz Biotechnology); and anti-mdm2, monoclonal mouse SMP14 (Santa Cruz Biotechnology); anti-p65, (C20, Santa Cruz Biotechnology); anti-phospho-p65, (Ser-536, Cell Signaling Technology); anti-FRAP, (Santa Cruz Biotechnology), anti-4EBP1, (Santa Cruz Biotechnology); anti-P110gamma, (Santa Cruz Biotechnology), anti-p110alpha, (Cell Signaling Technology); anti-PTEN, (Cell Signaling Technology); mouse anti-GAPDH(Santa Cruz Biotechnology); anti-AKT(ser473), (Cell Signaling Technology), rabbit anti-
4EBP1(Ser65), (Santa Cruz Biotechnology) total AKT, (Cell Signaling Technology), anti-p70S6K1(Thr389) (Cell Signaling Technology). Horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology.

2.5 mTOR in vitro kinase assay
RCC45 cells were treated with or without 9aa for overnight. Then the cell extracts were prepared using RIPA buffer. Crude cell extracts were precleared with 40ul of protein A/G sepharose beads for 30-60min at 4°C, after brief centrifugation, the supernatant was collected and used for incubation with primary anti-FRAP antibodies. After complex formation at 4 °C for overnight, immunocomplexes were precipitated with protein A/G agarose beads. Immunoprecipitate was washed twice with lysis buffer and once with 1X kinase buffer (50mM Tris, 10mM MgSO4, 0.02%BSA).

In vitro kinase assay solution (substrate rabbit 4EBP1 2ug, 1Xkinase assay buffer, ATP 5mM) incubated with immunocomplexes at 30°C for 30min. The kinase reaction was stopped by adding protein loading dye and heating at 100°C for 4min.

2.6 Semiquantitative Reverse Transcription-PCR
RNA was isolated from cell lines using Trizol reagent (Life Technologies,Inc.) according to the manufacturer’s instructions. Single-stranded cDNAs were synthesized using SuperScript II reverse transcriptase (Life Technologies, Inc.)
and random hexamers as primers. The cDNAs were amplified using Advantage polymerase mix (Clontech) in a Peltier thermocycler (DYAD). Primers for PCR were as follows: P110gamma specific primers: 5’-GGAGCAGATGAAGGCCCAGGTGT-3’ and 5’-GGCGCCGCGGGGTGTCGTC-3’; MDM2 specific primers: 5’-ATCTACAGGGACGCCATCGA-3’ and 5’-TGCCTGATACACGTAACTTGAT ATACCT; GAPDH specific primers: 5’-CAGACACCAACTTTCGCAT-3’ and 5’-TGTTCCGGGTGGTTCTGCAG-3’.

2.7 Transient transfection and reporter Assay

Transient transfection Cells (2x10^5) were plated into six-well plates and, after overnight incubation, transfected with Lipofectamin Plus reagent with 0.5ug of reporter plasmids p21-ConALuc or pNF-kBluc) in combination with p53, Arf, ss-IkB, siHDM2, pCBP, p110gamma expression plasmids. Corresponding empty vectors were added into all transfections up to 2ug of total DNA amount. Normalization of transfection efficiency was done by adding 0.2ug of pCMV-LacZ plasmid. Luciferase activity and beta-galactosidase activity was measured in lysates prepared 48h after transfection. Luminometric and colorimetric reactions were read on the Wallack 1420 plate reader (PerkinElmer).

Stable transduction of reporters Cells (2X10^4) or (2X10^5) with integrated reporter were plated in 96-well plate or 6-well plate. After overnight incubation with chemical compounds or media from lentivirus producing cells, cell lysates were prepared. Luciferase activity and protein concentration were measured in
aliquots of cell lysates by using standard kits (Promega, luciferase and beta-galactosidase assay systems; Bio-Rad Protein Assay Kit).

2.8 Cell survival assay

Clonogenic survival Cells (5X10^3) were plated in six-well plates and treated with different concentration of drugs (48h) or media from lentivirus producing cell. Then fresh media was added and number of colonies was estimated 7 days later.

Growth inhibition assay Cells were plated (5X10^3) per well on 96-well plates, and the next day, different concentrations of 9aa, doxorubicin were added for 24h, cell survival was estimated after an additional 48h by using standard methylene blue staining followed by extraction and colorimetrical detection of the dye.

2.9 Lentivirus transduction

293T cells were plated subconfluently on a 10cm tissue culture plate. The next day, the cells were transfected with 3.3ug of lentiviral vector DNA, 3.3ug of pVSV-G expression plasmid and 3.3ug of packaging vector pCMVdeltaR8.2 using lipofectamine plus reagent (Invitrogen) according to the manufacture’s recommendation. The medium was changed 16h after transfection and the virus-containing medium was collected 48h and 72h post transfection.

2.10 Microarray hybridization

RCC45 and RCC54 cells were treated with 2uM and 10uM of 9aa. Total RNA was prepared by using the TRIzol method (invitrogen), followed by clean-up
utilizing the Rneasy Mini kit (Qiagen, CA, SA). cDNA preparation, hybridization to human gene expression Homo sapiens 1-Plex Array, scanning, data extraction and analysis were done according to the manufacture’s instructions (NimbleGene, Madison, WC).

2.11 Proteomic analysis
RCC45 and RCC54 cells were treated with 9aa at the concentration of 10uM for 24h. The following procedure was performed by our collaborator Zhuang Zhengping (National institute of Health). 2-dimensonal electrophoresis (2-D) was used to separate the protein. By comparing the spot density of control cells and 9aa-treated cells, specific spots whose densities have been changed most dramatically were selected. Then the information regarding the isolated protein was obtained by excising individual spots, digesting protein, and identification of the resulting fragments by mass spectrometry.

2.12 Analysis of Cell Cycle Distribution
Approximately 10^5 cells were removed from culture dishes using trypsin washed with PBS and resuspended in 300 ul 3%BSA in PBS. Five milliliters of 70% ethanol was then added dropwise. Cells were kept at -20 °C for several hours and then stained with 10 mg/ml propidiumiodide in the presence of 30 mg/ml RNaseA at 37 °C for 2 hr. Cell cycle distribution based upon propidium iodide staining of DNA content was analyzed using a FACS Calibur instrument (Becton Dickinson) and CellQuest software.
2.13 Taqman One-step real time RT-PCR

RCC45 cells were treated with different concentration of 9AA 24h. RNA was isolated by TRIzol reagent. Taqman one step real time RT-PCR was performed according to the manufacture’s protocol using with p53 specific primer (Applied Biosystem). The signal was detected by ABI7700 machine (Applied Biosystems).

2.14 Electromobility Shift Assay (EMSA)

Nuclear extracts were prepared as published in ref. 7. Annealed oligonucleotide, corresponding to NF-kB binding site (Santa Cruz Biotechnology), was radiolabeled by [α-32P]dCTP by Klenow polymerase and then by [γ-32P]dATP by T4 polynucleotide kinase. Labeled oligonucleotide (107 cpm) was affinity-purified on Probe Quant columns (Amersham Pharmacia). Radiolabeled oligonucleotide was added to 10 mg of protein nuclear extract together with 1 mg of polydIdC (Amersham Pharmacia) to prevent nonspecific binding and incubated for 30 min at room temperature. For supershift, 200 ng of anti-p65, anti-p50, or anti-antibodies were added to the reaction (all antibodies are from Santa Cruz Biotechnology). After 30 min of incubation, the entire reaction mixtures were loaded into 4% polyacrilamide gel in 0.5´ 90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3 buffer and run for 2 h at 200V. Dried gels were exposed to x-ray films for 30 min–1·h.

2.15 Metabolic labeling of cells and monitoring of general protein synthesis

To measure total protein synthesis, HT1080 cells were incubated in methionine-free DMEM medium with dialysis serum for 1h before labeling. In vivo metabolic
labeling was performed by adding Trans[35S]label (MP Biomedicals) at a final concentration of 100 μCi/mL containing labeled L-methionine. The cells were washed with ice-cold PBS and incubated with 1:1 1× PBS and 1 M NaOH for 10–15 min at 37°C to stop the labeling at different time point. Ice-cold TCA was then added to the aliquots of cell lysates to a final concentration of 10%, mixed well, placed on ice for 1 h, and filtered through GF/C filter discs (Millipore). The GF/C discs were washed three times with 5% TCA and once with 10 mL of EtOH (ice cold). Air-dried filter discs were placed in scintillation vials with scintillation fluid and counted using a liquid scintillation counter. Total protein content was measured using the Bradford assay to determine the counts per minute per microgram of protein.
3. Identification of 9AA as a new type of p53 activator that acts through inhibition of NF-kB

3.1 Introduction

The protein p53 controls genetic stability and reduces the risk of cancer through induction of growth arrest or apoptosis in response to DNA damage or deregulation of proto-oncogenes (Prives et al., 1999). The efficacy of p53 as a tumor-preventing factor is reflected by the high frequency of p53 loss, in at least 50% of human tumors, due to inactivating mutations (Olivier et al., 2004). Understanding the mechanisms of functional inactivation of wild-type p53 in human tumors, for example, by overexpression the natural antagonist of p53, Mdm2, or the viral protein E6, helps to define prospective targets for treating cancer by restoring p53 function (Gudkov et al., 2005).

Renal cell carcinomas (RCC) the most frequent and least curable type of kidney cancer, maintain wild-type but functionally inactive p53 (Gurova et al., 2004). The mechanism of p53 repression in RCC is dominant, and therefore presumably “druggable”, and different from that of all reported cases of p53 repression in tumors, suggesting the existence of an as-yet-unknown molecular target for restoring p53 function in cancer (Gurova et al., 2004). An important feature of the p53 pathway in RCC cells is that neither of several DNA damaging agents tested was able to induce p53-dependent reporter activity, while the same reporter in normal kidney epithelial cells or tumor cells others than RCC was activated by DNA damage as expected (Gurova et al, 2004). At the same moment, if p53 was overexpressed in these cells, delivered by lentivirus vector, p53-Luc reporter was
activated and cells died in 48-96 hours after transduction (Gurova et al, 2005). This demonstrated that p53 activity can be restored in RCC cells and this activity is toxic for tumor cells. Thus p53-Luc reporter in RCC45 cells was used as a readout for screening a small molecule library (DiverSet, Chembridge Corporation, Inc) for the agents capable of p53 activation in RCC cells. Among the most effective compounds isolated from this screening were several derivatives of 9-aminoacridine (9AA), including an old-known antimalaria drug quinacrine (QC) (Gurova et al., 2005).

3.2 Results

3.2.1 9AA is a potent activator of p53 in a variety of cell types

In addition to RCC45, we tested the effect of 9AA on p53 transactivation in several RCC and non-RCC-derived cancer cell lines with wild type p53. In the majority of cell lines, 9AA stimulated p53-induced transactivation stronger than doxorubicin or other DNA-damaging drugs used (Fig 3.1). This observation suggests that a common mechanism may be involved in negative control of wild type p53 in different tumor cell types. The strongest induction of p53 by 9AA was observed in human fibrosarcoma HT1080 cells, which were further used in many assays in parallel with RCC cells. The effect of 9AA on the reporter was indeed p53 dependent since the compound could not activate reporter activity in p53-null H1299 cells.
Figure 3.1 9AA activates p53 stronger than doxorubicin in the majority of tumor cells tested. Indicated cells with integrated p53 responsive reporter were treated with different concentrations of 9AA (1-10μM) and doxorubicin (dox, 0.2-2μM) for 24 hours and then luciferase activity was measured and normalized by protein content. Data presented as a fold of reporter induction by the most effective dose of 9AA over effect of dox.
3.2.2 9AA is much more toxic to tumor cells with than to normal cells and isogenic tumor cells without p53

9AA as a potent p53 activator in a variety of tumor cell lines and should be toxic for tumor cells. To test this assumption, we treated several RCC cell lines in parallel with normal kidney epithelial cells (NKE) with different concentrations of 9AA for 72h. The cell survival experiment suggests that RCC-derived cell lines were more sensitive to 9AA than normal kidney epithelial cells, probably due to the constant present of the p53 activation stimuli in tumor cells in contrast to normal cells (Fig 3.2).

Then we tested p53 dependence of 9AA toxicity. We did this by comparing growth of colonies of synergetic cell pairs differing in p53 status, HT1080 with shRNA construct against p53 and GFP as a control. The results demonstrated that cells without functional p53 are more resistant to 9AA toxicity, although they were still sensitive but to higher concentrations of 9AA than cells with functional p53 (Fig 3.3).

Looking for the mechanism of cell loss upon 9AA treatment we assess DNA content in HT1080 cells treated with 9AA by FACS analysis. We have found that at low concentrations of 9AA (up to 3uM), cells undergo growth arrest, as judged by increased G1 DNA contented cells (Fig 3.4). Higher concentrations of 9AA caused apoptosis, since a number of cells with subG1 content of DNA prevailed after this treatment (Fig 3.4). All these traits were more significant in p53 wild type HT1080 cells than in cells with p53 suppressed by shRNA expression (Fig
3.4). Thus 9AA is toxic for cells and this toxicity is more specific for tumor cells with wild type p53.
Figure 3.2 9AA is more toxic for RCC than normal kidney epithelium (NKE) cells. Cell numbers were estimated 72h after treatment with the indicated concentrations of 9AA. The box shows western analysis of the indicated proteins in lysates of NKE cells treated with dox (1uM) or 9AA (5uM) for 16h.
Figure 3.3 9AA is more toxic to HT1080 cells with normal p53 levels. HT1080 cells expressing either siGFP or sip53 were plated at 2X10³ cells/well in 96-well plates. The next day the cells were treated with the indicated concentrations of 9AA for 24h. After 24h, the cells were passaged into 6 well plates and allowed to form colonies for 5 days. Cells were stained with methylene blue and photographed. Results were quantitated by measuring absorbance of methylene blue.
Figure 3.4 Effect of 9AA on the cell cycle. HT1080siGFP cells or HT1080siP53 cells were treated with 9AA at the indicated concentration and time or dox (2uM, 24h), then the cells were subjected to FACS analysis.
3.2.3 Inhibition of NF-κB signaling pathway by 9AA

From prior mechanistic study, we know that 9AA did not cause an accumulating p53 by causing DNA damage or by functioning as a proteasome inhibitor (Gurova et al., 2005). Surprisingly, we found that 9AA decreased the protein expression of IκB, a target of proteasomal degradation (Gurova et al., 2005). This unexpected observation directed our attention to the analysis of the effects of 9AA on the NF-κB pathway.

We used p53-null H1299 cells with an integrated NF-κB-responsive reporter to test the effect of 9AA on the transcriptional activity of NF-κB. We found that 9AA inhibited basal and TNF-induced activity of the reporter (Fig 3.5a). DNA binding assay suggested that simultaneously with the inhibition of TNF-stimulated NF-κB dependent transcription, 9AA significantly increased the binding of NF-κB to DNA as judged by the nuclear accumulation of p65-containing NF-κB complexes (Fig 3.5a). 9aa treatment also reduced the phosphorylation of Ser-536 in the p65 subunit of NF-κB (Fig 3.5b). In conclusion, 9AA acts by converting NF-κB to a transcriptionally inactive state that becomes trapped in the nucleus. Therefore it represents an inhibitor of a previously undescribed type that convert NF-κB from a transactivator into a transrepressor.
Figure 3.5 Effect of 9AA on NF-kB pathway. (A) 9AA causes nuclear accumulation of inactive NF-kB. H1299-NF-kBLuc cells were treated with different concentrations of 9AA and TNF (10ng/ml). After 6h, cytoplasmic and nuclear extracts were isolated and used for luciferase or gel-shift assays, respectively. (b) Western analysis with antibodies against phosphor-Ser-536 in complexes immunoprecipitated from HT1080 or RCC45 cells by anti-p65 antibodies.
3.2.4 Inhibition of NF-κB by ss-IκB activates p53

We were interested on knowing whether p53 activation and NF-κB repression by 9AA were related or distinct activities of the drug, and, if interrelated, which one is the primary event. We could readily exclude the possibility of p53 activation being the driver of NF-κB repression since all the effects of 9AA on the NF-κB pathway were seen in p53-deficient (H1299, PC3) as well as in p53 wild type (HT1080, MCF7) cells (Gurova et al., 2005). Moreover, inhibition of NF-κB by 9AA is detectable within one hour after application of the compound, while its effects on p53 were unusually slow and required 12-16 hours of treatment before an effect was detectable. To explore the opposite model (NF-κB repression by 9AA drives p53 activation) we tested what happens with p53 activity when NF-κB is inhibited. To suppress NF-κB activity in RCC cells we used a stable IκB mutant lacking both phosphorylation sites, IκB super suppressor (ss-IκB). Transduction of this mutant into RCC ACHN cells resulted in a 3 fold inhibition of NF-κB reporter activity, that is consistent with our observations that NF-κB is constitutively active in all RCC cell lines tested. Importantly, the activity of the p53- responsive reporter in the same cells was increased up to 5 times upon transduction of ss-IκB. A similar effect was observed in another RCC line, RCC54, as well as in non-RCC cells HT1080 (data not shown), both responded to 9AA by p53 activation. Remarkably, ss-IκB activated p53 in RCC more strongly than transduction of “direct” regulators of the p53 pathway such as Arf, siRNA to Hdm2 or p53 itself. Thus, we have demonstrated that blocking NF-κB activity can restore p53 transactivation in RCC cells and that 9AA likely to acts through this mechanism (Fig 3.6).
Figure 3.6 sr-IkB inhibits NF-κB transcriptional activity (Left) and activates p53 (Right) in RCC cells. Luciferase activity in ACHN cells cotransfected with p21-ConALuc and the indicated plasmids. Normalization in both assays was done by cotransfection of the pCMV-LacZ plasmid.
3.2.5 Effect of 9AA on gene and protein expression profiles of RCC cells: potential link to the mechanism of action

The current literature does not provide clear leads to cellular factors that might serve as 9AA targets and explain its biological effects (simultaneous targeting of p53 and NF-kB pathways). We therefore chose to use “unbiased” approaches that allowed us to analyze changes in global gene and protein expression in response to treatment of cells with 9AA. We hypothesized that by characterizing genes and proteins, the expression of which is changed in response to 9AA, we may identify those which are responsible for 9AA-mediated activation of p53 and suppression of NF-kB.

To look for genes that are up and down regulated by 9AA, we did a microarray analysis. In order to increase the reliability of the experiment, two of the RCC cell lines, RCC45 and RCC54 (both have wild type but non-functional p53 (Gurova et al., 2004) were used for microarray hybridization. The cells were treated for 24h with 9AA at the concentration of 2uM and 10uM (before appearance of visible cytotoxicity). We analyzed gene expression profiles using Nimblegen NimbleChip array gene expression array that contains 36,847 genes with each gene represented by 8 probes. After data normalization, using Nimblegen and GeneSpring software, those genes whose expression is reliably changed in both RCC cells have been chosen. The following criteria for the selection was applied: (i) expression of candidate gene should be at least 2 times different from the expression in control, untreated cells (ii) expression should be changed in both cell lines, (iii)
expression should be changed dose-dependently. We have generated two lists of genes: (i) up-regulated and (ii) down-regulated as a result of 9AA treatment (Table I, II).

Microarray analysis identified around 200 genes whose expression has been up- and down-regulated by 9AA. Among the up-regulated genes, there are 10% of p53 targets such as p21, Mdm2, PUMA (Table I); among the down-regulated genes, there are 15% were NF-kB targets such as IL-8, IkBalpha and chemokines CXCL1, CXCL6, CCL20 etc. (Table II), confirming that 9AA does affect the p53 and NF-kB pathways. Thus this analysis confirmed, in general, the already observed affect of 9AA on p53 and NF-kB dependent genes. In parallel, we have compared protein expression change before and after 9AA treatment in the same RCC cells by proteomics analysis under the same conditions (only 10uM of 9AA was used). RCC cells were treated with or without 9AA and two-dimensional (2-D) electrophoresis was used to separate the proteins in the control cells and 9AA treated cells (this technical step was carried out by our collaborator Zhuang Zhengping at NIH; Figure 3.8). By comparing the spot densities of control cells and 9AA treated cells, specific spots, whose densities have been changed most dramatically were selected and picked. Then the nature of the protein present in the picked spots was identified by mass spectrometry. Nine proteins whose expression was most significantly suppressed by 9AA treatment and seven proteins whose expressions have been increased after 9AA treatment (Table III, IV) were analyzed. The results identified totally
different candidates regulated by 9AA when microarray and proteomic data was compared.

3.2.6 Selection of candidates for the role of p53/NF-κB modulator in response to 9AA treatment

A wealth of information has been obtained from both the microarray and proteomic analysis. In order to prioritize these candidates, certain criterion have been applied: (i) the degree of deregulation by 9AA and (ii) literature links to p53 and/or NF-κB regulation. Based on these criterions, we selected several candidates: Aurora B Kinase (from microarray analysis), Activating Transcription Factor 3 (ATF3, from microarray analysis) and p110gamma (from proteomic analysis). Our plan was to independently confirm changes in their expression level after 9AA treatment on the protein and RNA level and, if confirmed, then to modulate their expression by genetic means (shRNA or cDNA overexpression) and assess their effect on NF-κB and p53. For Aurora B kinase a decrease of mRNA accumulation was not translated into the similar changes at the protein level and suppression of shRNA expression does not cause any changes in NF-κB, p53 activity in RCC cells. Thus this candidate was excluded from further analysis (data not shown). ATF3 expression was changed at the RNA and protein levels in the same direction, but again, suppression of ATF3 expression by siRNA approach did not affect activity of either NF-κB, or p53. Finally we were left with p110gamma. We chose it as our primary candidate not only because it was identified as the most down-regulated protein in quantity in both
RCC45 and RCC54 cell lines after 9AA treatment, but also because its tightly involved in p53 and NF-kB regulation.

P110gamma is a catalytic subunit of phosphoinositide 3-kinases (PI3Ks) family member. PI3Ks are members of a unique and conserved family of intracellular lipid kinases that phosphorylate the 3'-hydroxyl group of phosphatidylinositol and phosphoinositides. This reaction leads to the activation of many intracellular signalling pathways that regulate functions as diverse as cell metabolism, survival and polarity, and vesicle trafficking (Engelman et al., 2006).

PI3K/AKT can promote the activation of NF-kB by phosphorylation of IKK, which in turn, augments the transcriptional activity of NF-kB (Ozes et al., 1999). This pathway can also lead to phosphorylation of Mdm2, induction of translocation of Mdm2 into the nucleus and targeting of p53 for proteasomal degradation (Mayo et al., 2002). A recent report also links PI3K/AKT to NF-kB activation, p53 inhibition and cell survival (Jeong et al., 2005). So we decided to choose P110gamma as our candidate.
Table I. List of gene up-regulation more than 2 times by 9AA treatment in both RCC45 and RCC54 cells

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Relative expression compared To untreated cells</th>
<th>2uM</th>
<th>10uM</th>
<th>2uM</th>
<th>10uM</th>
<th>p53 target or not</th>
</tr>
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<td>Pregnancy specific beta-1-glycoprotein</td>
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<td>4.3</td>
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<td>7.72</td>
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<td>2.68</td>
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<td>Ratio 3</td>
<td>Ratio 4</td>
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<td>p-value</td>
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<td>1.02</td>
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<td>Disabled homolog 2, mitogen-responsive phosphoprotein (Drosophila)</td>
<td>1.18</td>
<td>2.71</td>
<td>0.99</td>
<td>2.25</td>
<td>No</td>
<td></td>
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<tr>
<td>RAD51 homolog C (S. cerevisiae)</td>
<td>0.98</td>
<td>2.33</td>
<td>1.03</td>
<td>3.05</td>
<td>No</td>
<td></td>
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<tr>
<td>Cyclin-dependent kinase inhibitor 1A (p21, Cip1)</td>
<td>1.25</td>
<td>3.80</td>
<td>1.33</td>
<td>4.62</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Mdm2, transformed 3T3 cell double minute 2, p53 binding protein (mouse)</td>
<td>0.96</td>
<td>2.86</td>
<td>0.91</td>
<td>2.30</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Growth arrest and DNA-damage-inducible,</td>
<td>1.22</td>
<td>2.78</td>
<td>0.92</td>
<td>2.66</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Ribonucleotide reductase M2 B (TP53 inducible)</td>
<td>1.11</td>
<td>3.53</td>
<td>1.08</td>
<td>3.33</td>
<td>Yes</td>
<td></td>
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<tr>
<td>Protein Name</td>
<td>Fold Change 1</td>
<td>Fold Change 2</td>
<td>Fold Change 3</td>
<td>Fold Change 4</td>
<td>Yes/No</td>
<td></td>
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<tr>
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<td>---------------</td>
<td>---------------</td>
<td>---------------</td>
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<td></td>
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<tr>
<td>Annexin A1</td>
<td>1.01</td>
<td>3.04</td>
<td>1.13</td>
<td>2.02</td>
<td>Yes</td>
<td></td>
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<tr>
<td>Leucine-rich repeats and death domain containing</td>
<td>1.12</td>
<td>2.12</td>
<td>1.10</td>
<td>1.64</td>
<td>Yes</td>
<td></td>
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<tr>
<td>Tumor protein p53 inducible nuclear protein 1</td>
<td>1.03</td>
<td>1.61</td>
<td>1.09</td>
<td>1.86</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Apoptotic protease activating factor</td>
<td>0.78</td>
<td>1.27</td>
<td>0.85</td>
<td>1.72</td>
<td>Yes</td>
<td></td>
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<tr>
<td>Heat shock 27kDa protein 1</td>
<td>0.82</td>
<td>1.30</td>
<td>1.13</td>
<td>1.71</td>
<td>Yes</td>
<td></td>
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<tr>
<td>KILLER/DR5</td>
<td>1.1</td>
<td>1.9</td>
<td>1.1</td>
<td>2.4</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>BCL2 binding component 3 (PUMA)</td>
<td>1.03</td>
<td>1.57</td>
<td>0.78</td>
<td>1.79</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Damage-specific DNA binding protein 2, 48kDa</td>
<td>0.89</td>
<td>1.38</td>
<td>1.00</td>
<td>1.79</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Carboxylesterase 2 (intestine, liver)</td>
<td>0.80</td>
<td>1.75</td>
<td>1.04</td>
<td>3.17</td>
<td>Yes</td>
<td></td>
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<tr>
<td>Thiosulfate sulfurtransferase (rhodanese)</td>
<td>1.18</td>
<td>3.59</td>
<td>1.13</td>
<td>2.79</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Amyloid (A4) precursor-like protein 2</td>
<td>1.19</td>
<td>1.68</td>
<td>0.97</td>
<td>1.90</td>
<td>Yes</td>
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</tr>
<tr>
<td>Activating transcription factor 3</td>
<td>1.07</td>
<td>3.56</td>
<td>0.91</td>
<td>2.99</td>
<td>Yes</td>
<td></td>
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<tr>
<td>BTG family member 2</td>
<td>1.44</td>
<td>1.88</td>
<td>0.9</td>
<td>2.4</td>
<td>Yes</td>
<td></td>
</tr>
</tbody>
</table>
Table II. List of gene down-regulated more than 50% by 9AA treatment in both RCC45 and RCC54 cells

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Relative expression compared to untreated cells</th>
<th>NF-kB target or not</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RCC54 2uM 10uM RCC45 2uM 10uM</td>
<td></td>
</tr>
<tr>
<td>Similar to ribosomal protein L22</td>
<td>0.28 0.62 0.09 1.68</td>
<td>No</td>
</tr>
<tr>
<td>Kinetochore protein Spc24</td>
<td>0.41 0.31 0.57 0.34</td>
<td>No</td>
</tr>
<tr>
<td>Hypothetical protein FLJ12442</td>
<td>0.49 0.34 0.55 0.38</td>
<td>No</td>
</tr>
<tr>
<td>Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)</td>
<td>0.40 0.08 0.93 0.15</td>
<td>Yes</td>
</tr>
<tr>
<td>Ribonucleotide reductase M2 polypeptide</td>
<td>0.38 0.14 0.91 0.19</td>
<td>No</td>
</tr>
<tr>
<td>Full-length cDNA clone CS0DC016YK18 of Neuroblastoma Cot 25-normalized of Homo sapiens (human)</td>
<td>0.51 0.33 0.80 0.35</td>
<td>No</td>
</tr>
<tr>
<td>Ubiquitin-like, containing PHD and RING finger domains, 1</td>
<td>0.65 0.28 0.85 0.23</td>
<td>No</td>
</tr>
<tr>
<td>Tubulin, beta polypeptide</td>
<td>0.67 0.36 0.73 0.41</td>
<td>No</td>
</tr>
<tr>
<td>Gene Name</td>
<td>Value1</td>
<td>Value2</td>
</tr>
<tr>
<td>--------------------------------------------------------------------------</td>
<td>--------</td>
<td>--------</td>
</tr>
<tr>
<td>Ubiquitin-conjugating enzyme E2C</td>
<td>0.47</td>
<td>0.32</td>
</tr>
<tr>
<td>Kinesin family member 2C</td>
<td>0.55</td>
<td>0.37</td>
</tr>
<tr>
<td>Ubiquitin-conjugating enzyme E2S</td>
<td>0.61</td>
<td>0.37</td>
</tr>
<tr>
<td>Actin, gamma 1</td>
<td>0.46</td>
<td>0.52</td>
</tr>
<tr>
<td>Aurora kinase B</td>
<td>0.62</td>
<td>0.32</td>
</tr>
<tr>
<td>Phosphoglycerate dehydrogenase</td>
<td>0.54</td>
<td>0.33</td>
</tr>
<tr>
<td>Major histocompatibility complex, class II, DP beta 1</td>
<td>0.53</td>
<td>0.38</td>
</tr>
<tr>
<td>Ubiquitin-like, containing PHD and RING finger domains, 1</td>
<td>0.58</td>
<td>0.28</td>
</tr>
<tr>
<td>High-mobility group box 2</td>
<td>0.67</td>
<td>0.35</td>
</tr>
<tr>
<td>Solute carrier family 25 (mitochondrial carrier; dicarboxylate transporter), member 10</td>
<td>0.69</td>
<td>0.43</td>
</tr>
<tr>
<td>H2A histone family, member X</td>
<td>0.85</td>
<td>0.32</td>
</tr>
<tr>
<td>Hypothetical protein FLJ34922</td>
<td>0.64</td>
<td>0.47</td>
</tr>
<tr>
<td>Proteolipid protein 2 (colonic epithelium-enriched)</td>
<td>0.74</td>
<td>0.38</td>
</tr>
<tr>
<td>Protein Name</td>
<td>Column 1</td>
<td>Column 2</td>
</tr>
<tr>
<td>------------------------------------------------------------------------------</td>
<td>----------</td>
<td>----------</td>
</tr>
<tr>
<td>Hypothetical protein LOC149603</td>
<td>0.80</td>
<td>0.5</td>
</tr>
<tr>
<td>Ribonucleotide reductase M2 polypeptide</td>
<td>0.65</td>
<td>0.33</td>
</tr>
<tr>
<td>MCM3 minichromosome maintenance deficient 3 (S. cerevisiae)</td>
<td>0.70</td>
<td>0.39</td>
</tr>
<tr>
<td>Stimulated by retinoic acid 13</td>
<td>0.65</td>
<td>0.47</td>
</tr>
<tr>
<td>Exportin, tRNA (nuclear export receptor for tRNAs)</td>
<td>0.95</td>
<td>0.49</td>
</tr>
<tr>
<td>Mitogen-activated protein kinase-activated protein kinase 3</td>
<td>0.91</td>
<td>0.49</td>
</tr>
<tr>
<td>Tumor necrosis factor, alpha-induced protein 6</td>
<td>1.26</td>
<td>0.48</td>
</tr>
<tr>
<td>Chemokine (C-C motif) ligand 20</td>
<td>0.64</td>
<td>0.26</td>
</tr>
<tr>
<td>Chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)</td>
<td>1.21</td>
<td>0.11</td>
</tr>
<tr>
<td>HSPC150 protein similar to ubiquitin-conjugating enzyme</td>
<td>0.73</td>
<td>0.43</td>
</tr>
<tr>
<td>Interleukin 8</td>
<td>0.62</td>
<td>0.09</td>
</tr>
<tr>
<td>Chemokine (C-C motif) ligand 20</td>
<td>0.64</td>
<td>0.26</td>
</tr>
<tr>
<td>Tenascin C (hexabrachion)</td>
<td>0.70</td>
<td>0.59</td>
</tr>
<tr>
<td>Nuclear factor of klight polypeptide gene enhancer in B-cells inhibitor, k</td>
<td>0.73</td>
<td>0.54</td>
</tr>
<tr>
<td>Protein Name and Description</td>
<td>EXP</td>
<td>FPKM</td>
</tr>
<tr>
<td>------------------------------------------------------------------------------------------------</td>
<td>-----</td>
<td>------</td>
</tr>
<tr>
<td>Cyclin D1 (PRAD1: parathyroid adenomatosis 1)</td>
<td>0.77</td>
<td>0.29</td>
</tr>
<tr>
<td>V-myc myelocytomatosis viral oncogene homolog (avian)</td>
<td>1.19</td>
<td>0.40</td>
</tr>
<tr>
<td>Chemokine-like factor super family 3</td>
<td>0.87</td>
<td>0.56</td>
</tr>
<tr>
<td>Tumor necrosis factor, alpha-induced protein 6</td>
<td>1.26</td>
<td>0.48</td>
</tr>
<tr>
<td>Tumor necrosis factor receptor superfamily, member 9</td>
<td>0.63</td>
<td>0.85</td>
</tr>
<tr>
<td>Vascular cell adhesion molecule 1</td>
<td>0.88</td>
<td>0.53</td>
</tr>
<tr>
<td>Chemokine (C-C motif) ligand 2</td>
<td>0.64</td>
<td>0.26</td>
</tr>
<tr>
<td>CD44 antigen (homing function and Indian blood group system)</td>
<td>0.88</td>
<td>0.65</td>
</tr>
</tbody>
</table>
Figure 3.7 2-D Gel of RCC45 and RCC54 cells treated with 9AA. RCC45 and RCC54 cells were treated with 9AA (10uM) for 24h. The cells were lysed and the proteins were separated by 2-D electrophoresis.
Table III. A list of proteins most up-regulated by 9AA treatment in RCC45 and RCC54 cells

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Fold of change in RCC45(10uM)/control</th>
<th>Fold of change in RCC54(10uM)/control</th>
<th>Literature link to NFkB or p53</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteasome activator complex subunit 2</td>
<td>2.9</td>
<td>1.6</td>
<td>No</td>
</tr>
<tr>
<td>Cathepsin B (CTSB)</td>
<td>3.1</td>
<td>3.1</td>
<td>No</td>
</tr>
<tr>
<td>Annexin A1</td>
<td></td>
<td></td>
<td>Yes (Rahman-Roblick et al., 2008)</td>
</tr>
<tr>
<td>Adeninephosphonbosyltransferase (APRT)</td>
<td>2.6</td>
<td>1.7</td>
<td>No</td>
</tr>
<tr>
<td>Ubiquitin-conjugating enzyme E2N (UBE2N)</td>
<td>2.2</td>
<td>1.8</td>
<td>No</td>
</tr>
<tr>
<td>Proteasome subunit beta type 6 (PSMB6)</td>
<td>1.6</td>
<td>2.4</td>
<td>No</td>
</tr>
<tr>
<td>Ubiquitin carboxyl-terminal hydrolase isozyme L1</td>
<td>1.7</td>
<td>2.2</td>
<td>No</td>
</tr>
<tr>
<td>ATP synthase D chain, mitochondrial (ATP5H)</td>
<td>1.4</td>
<td>2.5</td>
<td>No</td>
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</table>
Table IV. A list of proteins most down-regulated by 9AA treatment in RCC45 and RCC54 cells

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Fold of change in RCC45(10uM)/control</th>
<th>Fold of change in RCC54(10uM)/control</th>
<th>Literature link to NFkB or p53</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vascular endothelia growth factor receptor 3(FLT4)</td>
<td>3.8</td>
<td>1.8</td>
<td>No</td>
</tr>
<tr>
<td>Fucose-1-phosphate guanylytransferase (GFPP)</td>
<td>1.3</td>
<td>2.9</td>
<td>No</td>
</tr>
<tr>
<td>Lysyl-oxidase-like 4(LOXC)</td>
<td>2.2</td>
<td>2.2</td>
<td>No</td>
</tr>
<tr>
<td>Calcipression 3</td>
<td>1.6</td>
<td>4.2</td>
<td>No</td>
</tr>
<tr>
<td>Tropomysin 1 alpha chain (TPM1)</td>
<td>2.0</td>
<td>3.7</td>
<td>No</td>
</tr>
<tr>
<td>Isophentenyl-diphosphate delta isomerase 1(IDI1)</td>
<td>2.6</td>
<td>3.2</td>
<td>No</td>
</tr>
<tr>
<td>Cargo selection protein TIP47(M6PRBP1)</td>
<td>1.4</td>
<td>3.6</td>
<td>No</td>
</tr>
<tr>
<td>Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit, gamma isoform (PIK3CG)</td>
<td>10</td>
<td>4.2</td>
<td>Yes (Jeong et al., 2006; Mayo et al., 2002)</td>
</tr>
</tbody>
</table>
3.2.7 Confirmation of decreased protein expression of p110gamma by 9AA treatment

Phosphatidylinositol 4, 5-bisphosphate 3-kinase catalytic subunit, gamma isoform (p110gamma) was the most down regulated protein of the seven examined on the proteomic analysis of 9AA treated both cell lines. To assess the validity of this analysis, western blotting was performed using an antibody specific against p110gamma (Fig 3.8). RCC45 cells were treated with 9AA at a concentration of 10uM from 0 to 24 hr, cell lysates were prepared at different time point. The western blot results showed that p110 gamma expression was decreased by 9AA treatment starting from 8 hours. Expression of GAPDH served as a control. This result confirms the accuracy of proteomic analysis that p110gamma protein was decreased by 9AA treatment.

In conclusion, we have found that as a strong activator of p53, 9AA is more toxic to tumor cells than normal cells. It is toxic to cancer cells partially through p53 dependent mechanism. Besides activation of p53, 9AA inhibits NF-kB signaling pathway. Inhibition of NF-kB is an earlier event compared to activation of p53, and inhibition of NF-kB by ss-IkB could activate p53. Global gene expression profile confirmed the activation of p53 and inhibition of NF-kB by 9AA. Through proteomic analysis, we chose p110gamma as the primary candidate to study its effect on NF-kB inhibition and p53 activation.
Figure 3.8 Confirmation of the decreased p110 gamma protein expression after 9AA treatment by western blot. RCC45 cells were treated with 9AA (10uM) for different time (0-24h), p110gamma protein expression was examined by western blot using p110gamma antibody. The expression of GAPDH was used as control.
4 Effect of P110gamma on p53, NF-kB activation and cell survival

4.1 Introduction

The PI3K pathway is an important driver of cell proliferation and cell survival, most notably in cells that are responding to growth-factor-receptor engagement. AKT kinase is one of the key survival kinases of PI3K (Cantley, 2002). It exerts its function through activation or inhibition of variety of targets. AKT enhances the cell survival by blocking the function of proapoptotic proteins and processes (Manning et al., 2007). AKT phosphorylates MDM2 on S166 and S186, and this promotes translocation of MDM2 to the nucleus (Mayo et al., 2002). As an E3 ubiquitin ligase, MDM2 triggers degradation of p53. AKT also promotes the survival of the cell by activation of antiapoptotic genes such as NF-kB. AKT activates NF-kB through direct activation of the canonical IKK complex or IKKe (Ozes et al., 1999; Boehm et al., 2007).

9AA is a small molecule with anti-cancer activity that can simultaneously activate p53 and NF-kB. In chapter 3, we described that 9AA inhibits the protein expression of p110gamma, a catalytic subunit of PI3K. In endothelial cells, p110gamma has been demonstrated to be crucial for NF-kB activation, targeted deletion of p110gamma in mice blocks NF-kB activation (Randall et al., 2006). Here we tested whether inhibition of p110gamma protein by 9AA is the reason for the inhibition of NF-kB in RCC cells; we also tested whether inhibition of this protein could activate p53. In order to test this, we planed to use pharmacological and genetic tools to inhibit or activate p110gamma.
4.2 Results

4.2.1 Effect of inhibition of p110gamma expression by shRNA on p53, NF-kB transactivation and cell survival

To study the effect of P110gamma on p53 and NF-kB activity, a shRNA approach was applied to inhibit accumulation of P110gamma. The design and cloning of the shRNA directed against human P110gamma was described in Materials and Methods. To determine the silencing efficiency of the shRNA against P110gamma, RCC45 cells were infected with lentivector expression shRNA against p110gamma or against E6 as control. 48 hours post-infection, RNA was extracted and the p110gamma RNA level was detected by reverse-transcriptase PCR using P110gamma specific primers. RT-PCR result confirmed that shRNA effectively inhibited P110gamma mRNA expression (Fig 4.1).

Then we tested the effect of inhibition of P110gamma by shRNA on p53 and NF-kB transactivation by reporter assay. RCC45 and HT1080 cells containing an integrated p53 responsive luciferase reporter (RCC45ConAluc and HT1080ConAluc) were used to study the effect of inhibition of p110gamma by shRNA on p53 transactivation. We infected RCC45ConAluc and HT1080ConAluc cells with lentivirus containing shP110gamma or shE6 as a control for 24 h. The reporter activity was measured 48 h post-infection. The results revealed that inhibition of p110gamma by shRNA activated p53 reporter activity 2-3 fold in both cell lines (Fig 4.2).

To examine if shp110gamma could affect NF-kB transactivation, we used RCC45 cells containing an integrated NF-kB responsive reporter kBluc (RCC45kBluc)
cells. Inhibition of P110gamma expression by shRNA significantly inhibited basal NF-κB transactivation compared to control shRNA infected RCC45kBluc cells (Fig 4.3). These results suggest the inhibition of p110gamma by shRNA activates p53 and inhibits NF-κB.

PI3K/AKT is an evolutionarily conserved cell survival pathway that can transmit an anti-apoptotic signal by either inactivation of p53 or activation of NF-κB. In the absence of this signal, we expected programmed cell death to ensue. We examined the effect of shp110gamma on cell survival by Methylene blue assays. RCC45 cells or HT1080 cells were infected with shp110gamma or control shRNA for 24h, and allowed to grow for another 6 days. Surviving cells were stained with methylene blue solution. We found that inhibition of P110gamma by shRNA compromised the viability of RCC45 and HT1080 cells (Fig 4.4).

Since 9AA drug is toxic to a variety of cancer cells with or without wild type p53, we wanted to test whether inhibition of p110gamma by shRNA killed cancer cells through a p53-dependent mechanism. RCC45 and HT1080 isogenic cell lines with knock-down of p53 gene (RCC45 shp53 and HT1080 shp53) were used to test this. shp110gamma infection killed RCC45 and HT1080 cells indifferent to their p53 status, suggesting this is a p53 independent process (Fig 4.4).
Figure 4.1 Inhibition of P110gamma gene expression by shRNA. RCC45 cells were infected with shRNA against p110gamma for 24h. 48 h post infection, RNA isolated was reverse transcribed to cDNA and subjected to PCR using p110gamma and GAPDH specific probe.
Figure 4.2 Inhibition of p110gamma by shRNA on p53 transactivation. HT1080ConAluc(A) and RCC45ConAluc(B) cells were plated onto 12-well plate. The next day, cells were infected with shRNA against p110gamma for 24h. 48h post infection, p53 transactivation was measured by luciferase assay. The cell lysates were normalized by protein concentration.
Figure 4.3 Inhibition of p110gamma by shRNA on NF-kB transactivation. RCC45ConA and HT1080ConA cells were plated onto 12-well plate. The next day, cells were infected with shRNA against p110gamma for 24h. 48h post infection, NF-kB transactivation was measured by luciferase assay. The cell lysates were normalized by protein concentration.
Figure 4.4 Inhibition of p110gamma by shRNA on cell survival. RCC45 and its isogenic RCC45shp110gamma (A), HT1080 and its isogenic HT1080shp110gamma (B) cells were plated into 12 well plate at the number of 104 cells per well. The next day, cells were infected with shRNA against p110gamma or control shRNA for 24h. The cells were allowed to grow on the fresh medium for one more week. Cell survival was examined by methylene blue.
4.3.2 Effect of PI3K inhibitor on p53 activation

To confirm the importance of inhibition of PI3K/AKT in NF-kb inhibition and p53 activation, we used the PI3K inhibitor, LY294002, to block the PI3K/AKT pathway. We expected that LY294002 treatment would have the same effect on p53 activation as inhibition of P110gamma by shRNA. RT-PCR suggests that 12h of LY294002 treatment in RCC45 cells increased the transcription level of the p53 target gene mdm2 (Fig 4.5B). In parallel, western blotting showed that there is increased expression of mdm2 and p21 protein in RCC45 cells after LY294002 treatment (Fig 4.5A). All together, these results suggest that inhibition of p110gamma by either a genetic or pharmacological approach leads to the activation of p53 and caner cell death.

4.3.3 Effect of overexpression of p110gamma on NF-kB transactivation

To further confirm the role of p110gamma on NF-kB transactivation and cell survival, we examined the effect of overexpression of P110gamma in 293T cells. Western blotting showed that p110gamma protein expression was dramatically increased after the transient transfection (Fig 4.6B). Then the NF-kB transactivation was examined by reporter assay (Fig 4.6A). The results demonstrated that overexpression of P110gamma cDNA elevated the basal level of NF-kB transactivation approximately 2 fold.
4.4.4 Effect of overexpression of p110gamma on the cell sensitivity to 9AA treatment

If p110gamma is one of the targets of 9AA, we hypothesize that by overexpressing this target, cells will be resistant or be less sensitive to 9AA treatment. Since RCC45 is very difficult to transfect, we first cloned p110gamma into a lentivirus vector, then infected RCC45 cells p110gamma containing virus. After that, both RCC45 cells and RCC45 cells overexpressing p110gamma protein were treated with 9AA at different concentrations (4uM, 5uM, 6uM) for 48h. Cell survival assay revealed that 48h treatment effectively killed the RCC45 cell. But when p110gamma protein was overexpressed in those cells, they became much less sensitive to 9AA treatment, suggesting p110gamma may be one of the targets of 9AA (Fig 4.7).
Figure 4.5 Effect of LY294002 on p53 activation. RCC45 cells were treated with or without LY294002 at different concentration for 12h (A) Cell lysates from LY294002 treated or untreated cells were subjected to western blot using mdm2, p53, p21 and GAPDH antibodies. (B) RNA from LY294002 treated or untreated cells were reverse transcribed to cDNA and subjected to semi-quantitative PCR.
Figure 4.6 Effect of overexpression of p110gamma on NF-kB transactivation. 293T cells were transiently transfected with p110gamma cDNA or corresponding amount of control empty vector, NF-kB responsive reporter kBluc together with pCMV-LacZ as control. 48h post transfection, p110gamma protein expression was examined by western blot using p110gamma and GAPDH specific antibody. NF-kB activity was measured by NF-kB luciferase. The transfection efficiency was normalized by LacZ reporter.
Figure 4.7 Sensitivity of RCC45 cells to 9AA treatment after p110gamma overexpression. RCC45 cells were infected with p110gamma containing virus or control virus. 24h post infection, cells were replated onto 24-well plate at the number of 10X10^4 per well. Both cells were treated with 9AA at the concentration of 4uM, 5uM and 6uM for 48h, the cell survival was measured by methylene blue.
5 Mechanism of down-regulation of P110gamma by 9AA

5.1 Introduction

Class I PI3Ks are dual-specificity lipid and protein kinases involved in numerous intracellular signaling pathways. Class IA includes three catalytic subunits, p110alpha (encoded by PIK3CA), p110 beta (encoded by PIK3CB) and p110theta (encoded by PIK3CD), which form a complex with the SH2-containing regulatory p85 subunits, and are activated through tyrosine kinase signaling. Class IB p110gamma is mainly activated by seven transmembrane G-protein-coupled receptors (GPCRs), through its regulatory subunit p101 and G-protein betagamma subunits (Vanhaesebroeck et al., 2001). Expression of PI3Kalpha and beta is ubiquitous, PI3Kgamma and theta are mainly restricted to the hematopoietic system, but the expression pattern is broader than originally reported (Camps et al., 2005; Rommel et al., 2007).

Class IA PI3K isoforms (p110alpha, p110beta and p110theta) bind directly or indirectly to receptors through the interaction of their regulatory subunits (p85) with tyrosine-phosphorylated recognition motifs on the receptor cytoplasmic domains or soluble adaptor proteins such as GAB2 (GRB2-associated binding protein 2). The only class IB PI3K isoform, PI3Kgamma, is recruited to G-protein-couple receptors (GPCRs) by direct interaction with G-protein beta-gamma subunits, through both the catalytic p110 and the regulatory subunit. RAS, in its active GTP-bound state, also binds directly to the p110gamma subunit of PI3kgamma further regulating its catalytic activity (Vanhaesebroeck et al., 2001).
Knockout experiments show that the alpha and beta isoforms are essential for early embryonic development, whereas genetic inactivation of the gamma or theta isoform allows normal development to adulthood but causes defects in the immune system (Ruckel et al., 2007). Recent reports suggest involvement of the gamma isoform in tumor angiogenesis and drug resistance of chronic myeloid leukemia cells (Hamada et al., 2005; Hickey et al., 2006).

Although only p110alpha has been well documented for its oncogenic potential, recent cell culture experiments suggest that all forms of p110 can induce oncogenic transformation (Kang et al., 2006).

In chapter 4, we have demonstrated p110 gamma protein expression in RCC cell. This observation broadens the expression of this protein to cells other than the hematopoietic system. We also identified p110gamma as the protein that was most down-regulated after 9AA treatment among the seven we examined. Inhibition of p110gamma inhibits NF-κB and activates p53. This brings us to the question of why there is decreased protein expression of p110 gamma by 9AA treatment?

PI3K signaling plays a pivotal role in translating signals into a variety of cellular functions. The activation of PI3K by cell surface receptor mediates the phosphorylation of membrane phospholipids PIP2 to PIP3 and proteins PDK1 and PDK2. Protein kinase B or AKT, major downstream target of PI3K, binds through PH-(pleckstirn homology)-domain to PIP3 on the plasma membrane (Cully, 2006). Membrane associated AKT/PKB is phosphorylated and activated by PDkinases. Thus, the major outcome of PI3K activation is phosphorylation
and activation of AKT. Activated AKT in turn phosphorylates several different targets, among which the most consistent are FOXO transcriptional repressors, which are inactivated by phosphorylation and mammalian target of rapamycin, mTOR, which is activated by AKT. Importantly, full activation of PKB/AKT requires the phosphorylation Thr308 by PDKinases and Ser473 by PDkinases and mTOR (Manning, 2007). Thus, there is a signal-amplifying circuit between AKT and mTOR.

mTOR regulates a large number of cellular processes, but the best characterized function is regulation of translation (Gingras et al, 2001). mTOR enhances translation initiation in part by phosphorylating two major targets, the eIF4E binding proteins (4E-BPs) and the ribosomal protein S6 kinases (S6K1 and S6K2) that cooperate to regulate translation initiation rates (Hay et al, 2004). 4E-binding proteins (4E-BPs) are eIF4E binding inhibitory proteins which bind and sequester translation initiation factor 4E (eIF4E) preventing the formation of the translation initiation complex. eIF4E levels are the rate limiting step for cap-dependent translation in most systems (Averous et al., 2006). The kinase mTOR phosphorylates 4E-BP on several sites (Mamane et al., 2006), this causes the liberation of eIF4E from 4E-BP1, and the association of eIF4E with capped mRNA to initiate the translation.
5.2 Results

5.2.1 Effect of 9AA on the transcription of p110gamma and its family members

In order to determine why there is decreased p110gamma protein expression, we first performed reverse transcription-polymerase chain reaction (RT-PCR) to check whether the decrease of p110gamma protein is at the level of mRNA accumulation. Both RCC45 and RCC54 cells were treated with 9AA at the concentration of 2uM and 10uM for 24h. After the isolation of RNA, RT-PCR was performed as described in the Materials and Methods. The results showed that p110gamma mRNA remained constant during the 24h of 9AA treatment in both RCC45 and RCC54 cells (Figure 5.1). Taking advantage of the microarray analysis data we generated (Chapter 3), we compared mRNA expression levels of p110gamma and other catalytic PI3Ks with or without 9AA treatment. Consistent with the RT-PCR results, microarray data did not show any significant gene expression change of p110gamma during 9AA treatment (Table V). In addition, 9AA treatment did not affect the gene expression of other members of catalytic subunits of PI3K (Table V).

We also checked whether expression of p110alpha protein, a catalytic subunit of class IA PI3K which is very frequently deregulated in cancer, is changed after 9AA treatment. We have found that amount of p110alpha protein expression did not change during the treatment. (Fig 5.2) As expected, 9AA treatment inhibited the protein expression of p110gamma at 24h. The above results suggested that
the decrease of p110gamma occurs at the protein level and is specific for the gamma subunit versus other PI3K catalytic proteins.

5.2.2 Effect of 9AA on PI3K pathway and activity

We examined the effect of 9AA treatment on AKT phosphorylation. There were no consistent changes of AKT Thr308 phosphorylation, although AKT Ser473 phosphorylation was significantly inhibited after 9AA treatment. This site is essential for the full activation of AKT (Shaw et al., 2006). 10μM of 9AA treatment decreased the phosphorylation of AKT on Ser473 (Figure 5.2), suggesting the activation of AKT was impaired by 9AA treatment with the total AKT level remained constant during the treatment. Surprisingly, in some cells decreased phosphorylation of AKT is an earlier event than decreased level of p110gamma protein level after 9AA treatment (Fig 5.2).

Combined with the fact that the decrease in p110gamma is at the protein level and not the transcriptional level and that the literature links AKT/mTOR signaling to translation regulation, we hypothesize that the decrease of p110gamma is through an AKT mediated translational regulation pathway.
Figure 5.1 Down-regulation of P110gamma is not on RNA level. RNA isolated from RCC45, RCC54 or 9AA treated cells was reverse transcribed to cDNA and subjected to RT-PCR, as described in Materials and methods. PCR products were separated by 1.5% agarose gel electrophoresis and visualized. The relative expression levels of GAPDH were used as control.
Table V mRNA expression of p110gamma (PIK3CG) and other PI3K family member with 9AA treatment.

RCC45 and RCC54 cells were treated with 9AA at the concentration of 2uM and 10uM for 24h. Microarray analysis was performed. The mRNA expression level of PIK3CG (p110gamma) and other catalytic subunit of PI3K with or without 9aa treatment was compared.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>RCC45 2uM/control</th>
<th>RCC45 10uM/control</th>
<th>RCC45 2uM/control</th>
<th>RCC45 10uM/control</th>
</tr>
</thead>
<tbody>
<tr>
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<td>1.04</td>
<td>1.07</td>
<td>1.03</td>
<td></td>
</tr>
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<td>1.18</td>
<td>0.97</td>
<td>1.04</td>
</tr>
<tr>
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<td>1.06</td>
<td>1.01</td>
<td>0.96</td>
</tr>
<tr>
<td>PIK3CD</td>
<td>0.92</td>
<td>0.96</td>
<td>0.88</td>
<td>1.20</td>
</tr>
</tbody>
</table>
Figure 5.2 Effect of 9AA treatment on the protein expression of catalytic subunit of PI3K and AKT phosphorylation. RCC45 cells were treated with 9AA (10uM) for different time (0-24h). Cell lysates were subjected to western blot using p110gamma, p110alpha, pAKT$^{Ser473}$, AKT, and GAPDH antibodies.
5.2.3 Inhibition of AKT/mTOR signaling by 9AA treatment

To test this hypothesis, we first investigated if there is impaired mTOR signaling downstream of AKT. RCC45 cells were treated with or without 9AA at the concentration of 10μM for 24h, cell lysates were immunoprecipitated with mTOR antibody, then the mTOR in vitro kinase activity was performed by using mTOR substrate Rat 4EBP1. The results showed that mTOR immunoprecipitated in control cells effectively phosphorylated 4EBP1 on Ser65, but that phosphorylation of 4EBP1 on Ser65 was almost completely abolished in 9AA treated RCC45 cells. We conclude that mTOR kinase activity has been inhibited by 9AA treatment in RCC cells (Fig 5.3).

To exclude the possibility that the 9AA effect on mTOR was cell-specific and limited to RCC45 cells, we also examined the effect of 9AA treatment to p110gamma protein expression and mTOR activity in human colon cancer carcinoma cell line HCT116 and lung fibrosarcoma cell line HT1080 cell lines, which retain wild type p53 and are very responsive to 9AA treatment (Gurova et al., 2005). HCT116 cells were treated with 9AA at different concentration (0-10μM) for 24h, phosphorylation of mTOR target 4EBP1, S6K and expression of p110gamma were analyzed by western blotting. We detected decreased phosphorylation of 4EBP1, S6K, and the decreased protein expression of p110gamma in HCT116 after 9AA treatment. This decrease of p110gamma correlated with the decreased mTOR kinase activity in HCT116 cells in a dose-dependent manner (Fig 5.4). In HT1080 cells, time dependent (0-24h) 9AA
treatment (10uM) also decreased mTOR kinase activity which was examined by the phosphorylation of ser65 of 4EBP1, and this decrease also correlated with the decrease of p110gamma protein accumulation (Fig 5.5).

5.2.4 Inhibition of general translation by 9AA

We have shown that 9AA treatment decreased mTOR kinase activity. Since one of the best characterized functions of mTOR is its regulation of protein translation, we wondered whether 9AA will also affect the general protein translation. To test this, we performed a pulse labeling experiment. HT1080 cells first incubated with dialyzed serum-containing medium without methionine for 1h, and then pulsed with S$^{35}$methionine for different times. General protein synthesis was measured by the incorporation of methionine S$^{35}$. The results demonstrated that 9AA treatment reduced the amount of S$^{35}$methionine incorporation into the cells in a dose-dependent manner (Fig 5,6). However, the inhibition of translation by 9AA treatment was not as dramatic as cycloheximide, a known inhibitor of translation (Fig 5,6).

5.2.5 Effect of inhibition of AKT/mTOR pathway on p110gamma protein expression

The correlation between the decrease of p110gamma protein expression and mTOR kinase activity strongly favors our hypothesis that the decrease of p110gamma is through an mTOR-mediated translational regulatory pathway. To prove this, we used different ways to inhibit AKT/mTOR signaling pathways.
In order to inhibit AKT/mTOR signaling, first we introduced wild type AKT2 or kinase dead AKT2 (E299K) mutant into 293T cells by transient transfection and detected p110gamma by western blotting. The results revealed that P110gamma protein accumulation was significantly decreased in kinase dead AKT2 (E299K) mutant cells compared to the control cells. Cells transfected with wild type AKT2 (Fig 5.7) showed little effect. These results suggest that the kinase activity of AKT is necessary for the expression of p110gamma.

Phosphatase and tensin homologue deleted from chromosome 10 (PTEN) is a lipid phosphatase that negatively regulates PI3K signaling pathway (Cantley, 2002). As a tumor suppressor, PTEN opposes the effects of PI3K activation. When PTEN is inactivated, activation of PI3K effectors particularly the activation of the key survival kinase AKT, will occur. Genetic studies have linked the PTEN deficiency to stimulation of mTOR activity (Tee et al, 2005). So we examined if inhibition of PI3K/AKT pathway by overexpression of PTEN would affect the protein expression of p110gamma levels. RCC45 cells were infected with PTEN containing lentivirus or control GFP virus for 48h (before the appearance of any obvious cell death), then the cell lysates were collected for the examination by western blotting. The results showed that with the overexpression of PTEN, p110gamma accumulation was significantly decreased (Fig 5.7).

LY294002 is a known inhibitor of the PI3K/AKT pathway by inhibiting the catalytic subunit of PI3K. 10uM of LY294002 inhibits the lipid kinase activity of all isoforms of p110-type PI3K as well as mTOR (Brunn et al., 1996). We therefore examined the effect of LY294002 treatment on p110 gamma protein expression. RCC45
cells were treated with LY294002 at different concentrations (0-15uM) for 12h. Western blotting results showed that LY294002 treatment decreased p110gamma protein accumulation in a dose-dependent manner starting from 10uM in RCC45 cells (Fig 5.9). All these results suggest that p110gamma protein is through a AKT-mediated translational pathway.
Figure 5.3 Inhibition of mTOR kinase activity by 9AA treatment. RCC45 cells were treated with 9AA at the concentration of 10μM for 24h. The cell lysates were immunoprecipitated by mTOR antibody or control antibody, then mTOR in vitro kinases activity of the immunoprecipitants was measured using rat 4EBP1 as substrate.
Figure 5.4 Effect of 9AA treatment on AKT/mTOR signaling in HCT116 cells. HCT116 cells were treated with different concentration of 9AA for 24h, cell lysates were subjected to western blot by p110gamma, pAKT, and mTOR target p4EBP1 and pP70S6K. Western blot of GAPDH served as control.
Figure 5.5 Effect of 9AA treatment on AKT/mTOR signaling in HT1080 cells. HT1080 cells were treated 9AA(10uM) for different time, cell lysates were subjected to western blot by p110gamma, and mTOR target p4EBP1 Western blot of GAPDH served as control.
Figure 5.6 Effect of 9AA on general protein synthesis. HT1080 cells were incubated in media without methionine in medium with dialyzed serum for 1h. Than s35 methionine was added for indicated periods of time. After that proteins were collected by trichloric acid precipitation and S35 incorporation was counted on scintillation counter.
Figure 5.7 Effect of inhibition of AKT pathway on p110gamma expression. 293 cells were transiently transfected with control, AKT2 and AKT2 mutant (E299K) constructs. 48h post transfection, western blot was performed using p110gamma. Western blot of GAPDH served as control.
Figure 5.8 Effect of inhibition of PI3K/AKT pathway by PTEN on p110gamma protein expression. RCC45 cells were infected with control GFP or PTEN virus. 48h post infection, western blot was performed using p110gamma, PTEN antibody. Western blot of GAPDH served as control.
Figure 5.9 Effect of PI3K inhibitor on the expression of p110gamma protein. RCC45 cells were treated with PI3K inhibitor (LY294002) at different concentration (0-20uM) for 12h, and then the cell lysates were immunoblotted with p110gamma. The expression of GAPDH was served as control.
6 Discussion

6.1 9AA and QC as anti-cancer agents

P53 is one of the most potent tumor suppressor genes known. It is activated in response to DNA damage, oncogene activation, reactive oxygen species formation, and hypoxia, all conditions present in transformed and tumor cells. The outcome of p53 activation is growth arrest or suicidal death of cells undergoing these kinds of stress. This is incompatible with tumor growth and that is why p53 is mutated in half of human tumors. We hypothesized that in those tumors in which p53 is wild type, p53 functions may be inactivated by other mechanisms. There are many examples already known such as overexpression of the natural negative regulator of p53 mdm2 (Oren et al., 2002), loss of positive regulator Arf (Sherr et al., 2001) or by viral protein E6 of human papilloma virus (Thomas et al., 1999). RCC is a type of tumor with wild type p53, but in RCC cells, p53 function has been inhibited by an unknown mechanism (Gurova et al., 2004). Recently, in an attempt to look for the small molecules that can activate p53 in RCC cells, 9AA was identified as the core structure that can activate p53 better than DNA damaging agents. 9AA is a highly fluorescent small molecule that has been used as a topical antiseptic agent since 1942 (Rubbo et al., 1942). On studying the mechanism of 9AA induced p53 activation we found that besides activation of p53, 9AA also inhibited NF-kB activity, either constitutive in RCC cells or induced by TNF or other cytokines (Fig 3.5). NF-kB is a central coordinator of innate and adaptive immune responses. As an inducible
transcription factor, NF-kB controls a number of genes involved in the immuno-inflammatory response, cell cycle progression, inhibition of apoptosis and cell adhesion, thus promoting carcinogenesis and cancer progression (Karin, 2005). There is now considerable evidence that sustained or constitutive activation of NF-kB is prevalent in cell lines and tumor tissue specimens and contributes to malignant progression and therapeutic resistance of the major forms of human cancer (Luo et al., 2005). Importantly 9AA inhibits NF-kB even in p53 deficient cells, which means that NF-kB inhibition by 9AA is p53 independent (Gurova et al., 2005). Manipulation with NF-kB activity in RCC and other tumor cells by using a degradation resistant mutant of IkB revealed that p53 activity in many tumor cells is attenuated by high basal activity of NF-kB (Fig 3.6). But the exact mechanism of how NF-kB affects p53 and how 9AA inhibits NF-kB is still unknown. Understanding of the mechanism of 9AA effects on NF-kB and p53 may open a new avenue for therapeutic intervention in cancer since both of these factors are well recognized targets for anti-cancer therapy. Simultaneous modulation of their activities in a desired way may present a very prospective and unique up-to-date approach for cancer treatment. Quinacrine, an analogue of 9AA, which is an old anti-malaria drug, has the same effect on the activation of p53 and inhibition of NF-kB, and it is now in the phase II clinical trial for hormone refractory prostate cancer. Thus in this work, we were interested in elucidating a mechanism of the 9AA effect on NF-kB and p53.
6.2 Biochemical modification of p53 and NF-κB in 9AA treated cells.

The mechanisms of NF-KB inhibition and p53 activation are rather well studied. Most of the known inhibitors of NF-κB act through inhibition of the enzymatic activity of the IKK kinases leading to stabilization of IkB and anchoring of NF-κB in cytoplasm of cells. As opposed to NF-κB inhibitors that lock transcription complexes in the cytoplasm by inhibiting the phosphorylation and degradation of IkB, 9AA and QC not only allow the TNF induced nuclear translocation and accumulation of NF-κB, but greatly prolong the time of its presence in nuclei (Fig 3.5). Meanwhile, the complex present in nuclei is transcriptionally inactive. Since there is no NF-κB-dependent transcription, including transcription of IkB, and the existing one is destroyed upon TNF induction (Fig 3.5), this explains NF-κB trapping in the nuclei of cells. Thus, 9AA and QC act by converting NF-κB to a transcriptionally inactive state that becomes trapped in the nucleus. So it represents inhibitors of a previously undescribed type that convert NF-κB from a transactivator into a transrepressor.

6.3 Proposed mechanisms of NF-κB mediated inhibition of p53

One of the known mechanisms of cross talk between p53 and NF-κB is their competition for transcriptional coactivators such as the histone acetylase, CBP/p300 (Webster, 2002). But based on our results, saturation of tumor cells with exogenous CBP, which is normally limited in cells, did not lead to p53 activation. So 9AA mediated activation of p53 and inhibition of NF-κB can’t be explained by their competition for CBP/p300 (Gurova et al., 2005)
Since there was no literature enabling us to build a hypothesis, we used a fishing expedition to look for those genes or proteins that may be involved in 9AA mediated inhibition of NF-kB and activation of p53. A regulator of NF-kB activity may be among up- and down- regulated candidates by 9AA treatment. We propose also that a p53 inhibitor may be among genes regulated by NF-kB. Expression of this gene should be significantly suppressed by 9AA treatment. By analysis of gene expression profiles through microarray or protein expression profiles we generated a long list of candidates. Assessing these candidates by their role in regulation of NF-kB and p53, we chose p110gamma, the catalytic subunit of PI3K as our primary candidate. First, it is one of the most down-regulated proteins in the proteomics analysis. Second, the PI3K family is a signaling pathway that is crucial to many aspects of cell growth and survival (Cantley, 2002). Genomic aberrations of PI3K signaling including mutation, amplification and rearrangement are more frequent than any other pathway in human cancer (Cantley 2002). This pathway is very possibly the target of anti-cancer drugs. Finally, PI3K/AKT has been shown to play a crucial role in the transactivation of NF-kB by phosphorylating its p65 subunit (Sizemore et al., 2001; Jeong et al, 2005), There is a strong literature linking p65 phosphorylation and p53 inhibition (Jeong et al, 2005). AKT can also phosphorylate Mdm2, inducing translocation of Mdm2 into the nucleus, targeting p53 for proteasome degradation (Mayo et al., 2002).
6.4 P110gamma as the catalytic subunit of PI3K family

PI3Kgamma is the only member of class IB- phosphoinositol kinases. It is composed of the regulatory subunits p101 or p84, as well as the catalytic subunit p110gamma (Stephen et al., 1994). Whereas the classes IA PI3Ks are involved in receptor tyrosine kinase signaling, PI3Kgamma is mostly activated by seven-transmembrane-spanning G-protein-coupled receptors (GPCRs), through its regulatory subunits interacting with the beta gamma-subunits of G proteins (Krugmann et al., 1999). In addition, PI3Kgamma is directly activated by Ras (Pacold et al., 2000). AKT is the downstream target of all of the class I PI3K.

PI3Kgamma expression is mainly restricted to the haematopoietic system, although PI3Kgamma can also be detected in endothelium, heart and brain (Camps et al., 2005). Genetic inactivation of the gamma isoform allows normal development to adulthood but causes defects in the immune system (Ruckle et al., 2007). Class IB phosphatidylinositol 3-kinase p110gamma (PI3Kgamma) has gained increasing attention as a promising drug target for the treatment of inflammatory disease (Ruckle et al., 2007). Here we described that this protein is expressed in human cancer cell lines (RCC, HCT116, HT1080 etc). Treatment of these cells with the anti-cancer compound 9AA leads to a decrease in expression of p110gamma. Independently to the role of PI3Kgamma in regulation of NF-kB and p53 we made two observations: (i) PI3Kgamma is expressed in different tumor cells; (ii) candidate anti-cancer compound 9AA down-regulates the level of PI3K gamma in tumor cells. Two important questions arose with these
observations: (i) does PI3Kgamma play any role in the regulation of NF-kB and p53 and (ii) what is the mechanism of its down-regulation by 9AA.

6.5 The role of inhibition of p110gamma in p53 activation and NF-kB inhibition

We have found that inhibition of p110gamma by either knock-down or pharmacological inhibitors activated p53 and inhibited NF-kB, and induced cell death (Fig 4.2; Fig 4.3; Fig 4.4; Fig 4.5). This is a p53 independent mechanism because isogenic cell lines with p53 expression suppressed by shRNA were equally sensitive to inhibition of p110gamma (Fig 4.4). This may indicate that p110gamma is an independent survival factor for tumor cells and in this situation; p53 activation is not a cause of cell death. The effect of inhibition of p110 gamma on cell death may explain why 9AA can kill cancer cells with wild type p53 and with no p53 expression.

Although cancer-specific mutations have been identified only in p110alpha, overexpression of either p110beta, p110gamma, or p110theta is sufficient to transform chicken embryo fibroblasts, suggesting an inherent oncogenic potential of these proteins (Kang et al., 2006). Overexpression of p110gamma has the ability to elevate NF-kB transactivation in cells with low basal NF-kB (Fig 4.7). In our experiment, overexpression of p110 gamma also activated NF-kB in human kidney cells. Most importantly, overexpression of this protein in RCC45 resulted in the cells becoming resistant to 9AA treatment (Fig 4.8). We speculate that the introduction of this protein induces survival signaling through AKT pathways, which make the cancer cells resistant to 9AA.
6.6 Inhibition of AKT/mTOR signaling by 9AA treatment

The decrease in p110gamma protein accumulation after 9AA treatment detected by proteomics analysis and western blotting (Fig 3.8; Table IV) was not paralleled by changing in mRNA expression. Two reasons for down-regulation of p110gamma after 9AA treatment could be: (i) inhibition of p110gamma translation or (ii) increased degradation of the protein.

Looking carefully at the effect of 9AA on other members of PI3K signaling pathways, we paid attention to several important facts: (i) decrease of the protein level was specific for p110gamma subunit and not for p110alpha (ii) phosphorylation of AKT on Ser 473 was inhibited by 9AA. Importantly, in some cells, the AKT phosphorylation occurs earlier than the decrease in p110gamma level. Thus, we propose that decreases Ser473 phosphorylation of AKT after 9AA treatment is not the result of decreased p110gamma catalytic activity in cells (Fig 5.2). As an integrator of growth factor and nutrient signals, mTOR is a downstream target of PI3K/AKT whose function is exclusively involved in cell growth. Hyperactive AKT and mTOR signaling is involved in many cancers and mTOR inhibitors are promising anti-cancer drugs (Guertin et al., 2005). We decided to assess catalytic activity of the mTOR complex in 9AA treated cells and have found that it is inhibited (Fig 5.3).

Downstream effectors of the mTOR complexes include 4EBP1 and the p70 ribosomal S6 kinase (S6K), its two well-characterized substrates. MTOR exerts its effects by phosphorylating eukaryotic initiation factor 4E binding protein 1
(4EBP1), which inhibits 5’-cap-dependent mRNA translation (the majority of cellular translation) by binding and inactivating eIF4E. Phosphorylation of 4EBP1 releases eIF4E, allowing initiation of translation (Gingras et al., 2004). In addition to 4EBP1, mTOR also regulates translation via S6 kinase (S6K, formerly known as p70S6K).

6.7 Decrease of p110gamma protein expression through suppression of AKT/mTOR pathway

We hypothesized that the decrease of p110gamma is through mTOR mediated translational pathway because we found (i) the decreased phosphorylation of mTOR substrates correlates with the decrease of p110gamma protein expression (ii) decrease of p110gamma is at the protein level but not at mRNA level and (iii) decreased phosphorylation of AKT is not the consequence for the decreased protein expression of p110gamma. All these phenomena suggest that decrease of p110gamma may be through the AKT/mTOR signaling pathway. Genetic or pharmacological inhibition of AKT/mTOR pathway proved that inhibition of AKT/mTOR inhibits the p110gamma protein expression (Fig 5.7; Fig 5.8; Fig 5.9).

One of the questions raised by our results is how mTOR inhibits p110gamma protein expression but not p110alpha. Our possible explanation is that mTOR mediated cap dependent translation initiation is a rate-limiting step in many systems because eIF4E is limited in the cell (Duncan et al., 1987). EIF4E overexpression in cells enables efficient translation of mRNAs with (1) relatively
long and structured 5'UTRs, and (2) most importantly, protein products that function in controlling cell growth and proliferation. Among these mRNAs include ODC (ornithine decarboxylase), FGF (fibroblast growth factor), and VEGF (vascular endothelial growth factor) (Hay, 2004). In contrast, most housekeeping genes contain relatively short and unstructured 5'UTR (Wagner, 2007). Activation of mTOR phosphorylates 4E-BP and the phosphorylated of 4EBP releases eIF4E allowing to form the translation initiation complex. So mTOR activation induces the expression of those proteins with long 5'UTRs that are usually involved in cell growth and proliferation. P110gamma mRNA (NM_002649) has a relatively long 5'UTR (around 300nt), which may explain why mTOR mediates p110gamma protein expression but not p110alpha. The latter has a short 5'UTR around 100nt.
7. Summary and working model

7.1 Summary

9aa is a small molecule that affects two anti-cancer targets p53 and NF-kB in a desired way. Through the mechanistic studies, we have made the following discoveries:

1. 9aa affects two anti-cancer targets in a desired way—activation of p53 and inhibition of NF-kB. It activates p53 in RCC45 and many other cell lines. 9aa inhibits both basal and TNF-induced NF-kB transactivation by converting it from a transactivator into a transrepressor.

2. Microarray data confirms the activation of p53 and the inhibition of NF-kB by 9aa treatment. Among the up-regulated genes >10% are p53 targets. Among the down-regulated genes >15% are NF-kB target genes.

3. Proteomics analysis identified p110gamma, one of the catalytic subunits of PI3K, as the most down-regulated protein after 9aa treatment.

4. Down-regulation of p110gamma occurs at the protein level and not at the transcriptional level.

5. Inhibition of p110gamma by genomic knock-down activates p53 and inhibits NF-kB. Overexpression of p110gamma elevates NF-kB transactivation in normal kidney cells, and overexpression of p110gamma in cancer cells make them less sensitive to 9aa treatment.

6. 9aa suppresses the phosphorylation of AKT and its downstream target mTOR.
7. Down-regulation of p110gamma by 9aa treatment is mediated by AKT/mTOR mediated translational pathway

7.2 Working Model

We have shown that 9AA, a small molecule with anti-cancer activity, inhibits the AKT/mTOR signaling pathway which is usually hyperactive in human cancer. But who is the direct target of 9aa is still unknown. Quinacrine (QC), an analogue of 9AA is known to be very lipophilic and targets phospholipase A2 (PLA2) by direct binding to the enzyme and inhibition of its enzymatic activity (Mustonen et al., 1998). The PLA2 family represents a diverse group of enzymes that hydrolyze sn-2 fatty acid from the cell membrane. PLA2 has been implicated in a wide range of cellular responses including signal transduction. AKT has recently been reported to be activated by PLA2 (Park et al., 2003). We can not exclude the possibility that 9AA targets the PLA2 enzyme family.

We propose that most probably 9AA amplifies the signal between AKT and mTOR. This results in a decrease in Ser473 phosphorylation and inhibition of AKT activity. This is the first event we observe after 9AA treatment. Decreased activity of AKT leads to additional inhibition of mTOR, in TORC2 as well as TORC1 complexes and inhibition of translation. The consequence of this event is reduction in p110 gamma level as well as reduced translation of other proteins (Fig 5.6). Lowered p110gamma expression in turn additionally inhibits AKT activity.
AKT is a known positive regulator of NF-kB through phosphorylation of p65 (Ozen et al., 1999) and an inhibitor of p53 through phosphorylation of mdm2 (Mayo et al., 2002). We have shown that inhibition of p110gamma by shRNA inhibits NF-kB and activates p53 transcription. We proposed that, similar to other members of class IA PI3Ks, inhibition of p110gamma could inhibit AKT kinase activity. AKT inhibition could affect NF-kB transactivation through inhibition the phosphorylation of p65 on Ser536 (Jeong et al., 2005). Decreased phosphorylation of p65 could activate p53 through some unknown mechanism (Jeong et al., 2005). On the other hand, inhibition of AKT could prevent the nuclear translocation of mdm2 which could activate p53 through protein stabilization (Mayo et al., 2002).

Importantly, the effects of 9AA on NF-kB and especially p53 activity were stronger than the effect of shRNA to p110gamma. This may be explained by the fact that inhibition of p110gamma expression leads to only partial inhibition of AKT since there are additional PI3K proteins in cells, while 9AA acts more directly on the AKT/mTOR amplifying circuit and thus provides stronger inhibition of AKT, than suppression of p110gamma expression by shRNA approach. In this scheme, a decreased level of p110 expression is the consequence of AKT inhibition, but not the reason. Our scheme is based on the known role of AKT in the regulation of p53 and NF-kB, although we cannot exclude the existence of additional signals going directly from p110gamma to either of p53 or NF-kB pathways.
An important feature of all these signaling pathways in cells, also confirmed in this study, is the presence of intercrossing signals between pathways. By modulating the activity of one of the members, we affect the activity of many other members which are also regulating each other. As an example, we first observed that NF-κB inhibition in tumor cells leads to p53 activation. Then we have found that also PI3K/AKT pathway inhibition activates p53 and inhibits NF-κB. Thus p53 suppression in tumor cells may be the sum of signals coming from active AKT and NF-κB, and 9AA through inhibition of both pathways induces p53 activity stronger than just DNA damaging signals in tumor cells.
Figure 7.1 Current model of signaling events within AKT/mTOR/p53/NF-kB (left) and proposed mechanism of 9AA anti-cancer activity (right). 9AA effects are shown in red. Detailed explanations can be found in the text.

Reference List


Cohen PA, Hupp TR, Lane DP, Daniels DA (1999) Biochemical characterization of different conformational states of the Sf9 cell-purified p53His175 mutant protein. FEBS Lett. 463, 179-84


Donahue AC and Fruman DA (2004) PI3K signaling controls cell fate at many points in B lymphocyte development and activation. Seminar in cell and developmental biology 15 183-197


Gingras AC, Raught B, Sonenberg N (2001) Regulation of translation initiation by FRAP/mTOR. Genes Dev. 15, 807-26


Gingras AC, Raught B, Sonenberg N (2001) Regulation of translation initiation by FRAP/mTOR. Genes Dev. 15, 807-26


Halazonetis TD, Kandil AN (1993) Conformational shifts propagate from the oligomerization domain of p53 to its tetrameric DNA binding domain and restore DNA binding to select p53 mutants. EMBO J. 12, 5057-64


Huang WC, JU TK, Hung MC and Chen CC (2007) Phosphorylation of CBP by IKKalpha promotes cell growth by switching the binding preference of CBP from p53 to NF-kappaB. Molecular Cell 26:75-87

Hupp TR, Meek DW, Midgley CA, Lane DP (1993) Activation of the cryptic DNA binding function of mutant forms of p53. Nucleic Acids Res. 21, 3167-74


Karin M and Greten FR. (2005) NF-kB: linking inflammation and immunity to cancer development and progression. Nature Reviews Immunology. 5:749-759


Krugmann S, Hawkins PT, Pryer N, Braselmann S (1999) Characterizing the interactions between the two subunits of the p101/p110gamma phosphoinositide 3-kinase and their role in the activation of this enzyme by G beta gamma subunits. J Biol Chem. 274, 17152-8


Lane DP, Crawford LV (1979) T antigen is bound to a host protein in SV40-transformed cells. Nature. 278,261-3


Mayo LD, Donner DB (2001) phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus. Proc Natl Acad Sci U S A. 98, 11598-603

Miyashita T, Reed JC (1995) Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell. 80, 293-9


Rocha S, Garrett MD, Campbell KJ, Schumm K, Perkins ND(2005)Regulation of NF-kappaB and p53 through activation of ATR and Chk1 by the ARF tumour suppressor. EMBO J. 24, 1157-69


Sizemore N, Lerner N, Dombrowski N, Sakurai H, and Stark GR (2001) Distinct roles of the IkB kinase alpha and beta subunits in liberating nuclear factor kB (NF0kB) from IkB and in phosphorylating the p53 subunit of NF-kB


molecule inhibitors of HDM2 ubiquitin ligase activity stabilize and activate p53 in cells. Cancer Cell. 7, 547-59
