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ORIGINAL ARTICLE

9-Aminoacridine-based anticancer drugs target the PI3K/AKT/mTOR, NF-κB and p53 pathways

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Acquisition of a transformed phenotype involves deregulation of several signal transduction pathways contributing to unconstrained cell growth. Understanding the interplay of different cancer-related signaling pathways is important for development of efficacious multitargeted anticancer drugs. The small molecule 9-aminoacridine (9AA) and its derivative, the antimalaria drug quinacrine, have selective toxicity for tumor cells and can simultaneously suppress nuclear factor-κB (NF-κB) and activate p53 signaling. To investigate the mechanism underlying these drug activities, we used a combination of two-dimensional protein separation by gel electrophoresis and mass spectrometry to identify proteins whose expression is altered in tumor cells by 9AA treatment. We found that 9AA treatment results in selective downregulation of a specific catalytic subunit of the phosphoinositide 3-kinase (PI3K) family, p110γ. Further exploration of this observation demonstrated that the mechanism of action of 9AA involves inhibition of the prosurvival AKT/mammalian target of rapamycin (mTOR) pathway that lies downstream of PI3K. p110γ translation appears to be regulated by mTOR and feeds back to further modulate mTOR and AKT, thereby impacting the p53 and NF-κB pathways as well. These results reveal functional interplay among the PI3K/AKT/mTOR, p53 and NF-κB pathways that are frequently deregulated in cancer and suggest that their simultaneous targeting by a single small molecule such as 9AA could result in efficacious and selective killing of transformed cells.

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Introduction

Tumor cells frequently acquire prosurvival mechanisms leading to deregulation of major stress response pathways. Suppression of p53 and constitutive activation of nuclear factor-κB (NF-κB) are among the most common properties of tumor cells and are, therefore, considered potential targets for therapeutic intervention aimed at selective elimination of such cells. In addition, cell transformation often involves activation of prosurvival pathways contributing to unconstrained cell growth under normally restrictive conditions, including AKT/mammalian target of rapamycin (mTOR) and phosphoinositide 3-kinase (PI3K) pathways. There are numerous indications of interaction between these pathways, which suggests that their simultaneous pharmacological modulation would provide enhanced anticancer efficacy (Mayo and Donner, 2001; Sizemore *et al*., 2002; Levine *et al*., 2006; Budanov and Karin, 2008).

p53 is a key tumor suppressor that plays an undisputed role in cancer prevention. p53 tumor suppressive activity stems from its ability to respond to a variety of stresses and trigger cell cycle arrest, apoptosis or senescence, thereby protecting against malignant transformation (Bensaad and Vousden, 2005). Understanding the mechanisms responsible for functional inactivation of wild-type p53 in human tumors (for example, overexpression of natural antagonists of p53 such as Mdm2 or the viral E6 protein) helps to define prospective targets for treating cancer by restoring p53 function (Gudkov, 2005).

Like p53, NF-κB is a major sensor of cell stress. Activation of the NF-κB signaling pathway results in inhibition of apoptosis by induction of antiapoptotic genes and/or suppression of proapoptotic genes (Kucharczak *et al*., 2003). Consistent with its antiapoptotic activity, NF-κB has been reported to be constitutively active in numerous tumors, reducing their sensitivity to proapoptotic stresses, including those associated with cancer treatment (Karin and Gretin, 2005). p53 and NF-κB antagonize each other and the balance in activity of the two pathways determines whether a cell proliferates or undergoes apoptosis in stress conditions.

We have identified a group of structurally related compounds, derivatives of 9-aminoacridine (9AA), which can simultaneously activate p53 and inhibit NF-κB in tumor cells and have anticancer effects *in vitro* and *in vivo* (Gurova *et al*., 2005). The antimalarial drug quinacrine belongs to this group and is currently in clinical trials for the novel application of cancer treatment. We have shown that NF-κB activation can attenuate p53 activity in tumor cells with wild-type p53. However, the details of the molecular mechanisms underlying 9AA activity and the NF-κB-p53 interrelationship remained undefined.

In this study, we investigated these mechanisms by comparing protein expression in tumor cells before and after 9AA treatment, hypothesizing that differentially expressed proteins would be candidate mediators of the effect of 9AA on p53 or NF-κB signaling. Interestingly, we found that expression of a catalytic subunit of PI3Ks, p110γ, was downregulated in two different tumor cell lines following 9AA treatment. By following this lead, we have identified events occurring upstream and downstream of p110γ downregulation. Reduction in p110γ appeared to be a part of suppression of AKT/mTOR pathway by 9AA. Thus, inhibition of mTOR seems to be the most upstream in the chain of events triggered by 9AA that results in simultaneous deregulation of several interplaying pathways such as p53 and NF-κB that are frequently deregulated in cancer. The impact of 9AA on these three interwoven pathways provides strong support for the potential of this compound and its derivatives as anticancer therapeutics.

Results

P110γ expression is decreased in tumor cells treated with 9AA

9AA is a potent inhibitor of NF-κB and inducer of p53 activity in tumor cells (Gurova *et al*., 2005). 9AA and the related drug quinacrine are toxic to many different tumor cell types *in vitro* and *in vivo* (Gurova *et al*., 2005). To identify potential mechanisms responsible for the antitumor activity of 9AA as well as its ability to induce p53 and inhibit NF-κB, we compared global protein expression profiles in tumor cells before and after treatment with 9AA. We used two RCC cell lines, RCC45 and RCC54, in which 9AA treatment causes p53 activation and NF-κB inhibition (Gurova *et al*., 2005). Cells were left untreated or treated with 10 μM 9AA for 24 h. Importantly, cell lysates for proteomic analysis were collected before any signs of 9AA-induced toxicity appeared (after 48 h) to eliminate effects related to the process of cell death. Proteins in the lysates were separated by 2D gel electrophoresis and the pattern of protein spots was compared between 9AA-treated and untreated samples. Protein spots that displayed consistent differential expression between the treated and untreated groups with statistically significant values by *t*-test ($P<0.05$) were identified using 2D gel software (Proteomweaver; Definiens, Munich, Germany). Fifteen proteins that were most significantly affected by 9AA treatment in both the RCC45 and RCC54 cell lines (seven upregulated and eight downregulated) were isolated from gels and identified by MS (Tables 1 and 2). The protein showing the greatest fold change upon 9AA treatment in both cell lines was p110γ, one of the catalytic subunits of PI3K. Notably, among the signaling pathways regulated by members of the PI3K family are important prosurvival pathways, including those controlled by the AKT/PKB and FRAP/mTOR kinases. AKT/PKB is involved in a complex crosstalk with the NF-κB and p53 pathways (Ozes *et al*., 1999; Mayo *et al*., 2002; Jeong *et al*., 2005). Therefore, we chose to investigate p110γ further as a promising candidate for mediating the effects of 9AA on tumor cells, in particular its effects on the p53 and NF-κB pathways.

First, to confirm the results of the proteomics analysis, we analysed the effect of 9AA on p110γ protein levels in independent experiments on several other tumor cell lines (HT1080, HCT116) by western blotting (Figure 1a and data not shown). The results confirmed that 9AA treatment results in downregulation of p110γ levels. In addition, whereas p110γ expression

Table 1 A list of up-regulated proteins in RCC45 and RCC54 cells treated with 9AA

Gene name	*Fold of change in RCC45 (9AA/control)*	*Fold of change in RCC54 (9AA/control)*
Proteasome activaotor complex subunit 2	2.9	1.6
Cathepsin B(CTSB) Annexin A1	3.1	3.1
Adeninephosphonbosyltranferse (APRT)	2.6	1.7
Ubiquitin-conjugating enzyme E2N (UBE2N)	2.2	1.8
Proteasome subunit beta type 6(PSMB6)	1.6	2.4
Ubiquitin carboxyl-terminal hydrolase isozyme L1	1.7	2.2
ATP synthase D chain, mitochondrial (ATP5 H)	1.4	2.5

Table 2 A list of down-regulated proteins in RCC45 and RCC54 cells treated with 9AA

Gene name	*Fold of change in RCC45 (control/ 9AA)*	*Fold of change in RCC54 (control/ 9AA)*
Vascular endothelia growth factor receptor 3(FLT4)	3.8	1.8
Fucose-1-phosphate guanyly-transferase (GFPP)	1.3	2.9
Lysyl-oxidase-like 4(LOXC)	2.2	2.2
Calcipression 3	1.6	4.2
Tropomysin 1 alpha chain (TPM1)	2.0	3.7
Isophentenyl-diphosphate delta isomerase 1(IDI1)	2.6	3.2
Cargo selection protein TIP47(M6PRBP1)	1.4	3.6
Phosphatidylinositol 4,5-bispho-sphate 3-kinase catalytic subunit, gamma isoform (PIK3CG)	10	4.2

was previously shown to be limited to cells of hematological origin (Camps *et al*., 2005; Rommel *et al*., 2007), we found that p110γ is expressed in tumor cell lines of various origins, including RCC, fibrosarcoma (HT1080), colon carcinoma (HCT116). We tested different 9AA doses and lengths of treatment and observed downregulation of p110γ expression as early as 4–8 h after treatment with at least 5 μM 9AA (Figure 1 and data not shown).

Next, we sought to determine whether 9AA affects p110γ at the mRNA or protein level and whether this effect is specific for p110γ versus other PI3K catalytic subunits. We used reverse transcriptase (RT)–PCR and microarray hybridization to quantify mRNA transcript levels for p110γ and other catalytic subunits in RCC45 and RCC54 cells before and after 9AA treatment. We found no changes in mRNA levels for any of the tested subunits, including p110γ (Figure 1b; Table 3). Moreover, there was no change in the protein level of one of the most abundant PI3K catalytic subunits, p110α, upon treatment of RCC45 cells with 9AA (Figure 2a). Taken together, these data indicate that 9AA treatment specifically affects p110γ expression at the protein level.

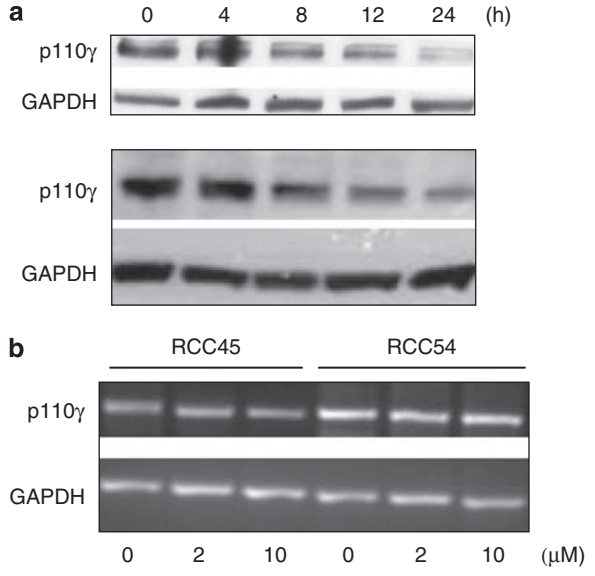

Figure 1 P110γ protein expression is suppressed in tumor cells treated with 9-aminoacridine (9AA). (**a**) Western blotting of renal cell carcinoma 45 (RCC45; top) and HT1080 (bottom) cells treated with 10 μM 9AA for the indicated periods of time. (**b**) Reverse transcriptase (RT)–PCR analysis of p110γ mRNA expression in RCC45 and RCC54 cells treated for 24 h with the indicated concentrations of 9AA.

9AA inhibits AKT/mTOR activity

To determine whether 9AA treatment affects phosphorylation-dependent activation of AKT, we treated RCC45 cells with 10 μM 9AA, a dose shown to reduce p110γ levels. After various times of treatment (30 min to 24 h), we assessed the level of phospho-AKT and total AKT protein by immunoblotting with specific antibodies (Figure 2a). As might be expected because activation of AKT is known to require PI3K activity, we observed a decrease in AKT phosphorylation upon 9AA treatment, whereas the total level of AKT protein remained unchanged. However, two aspects of the data argue against the possibility of 9AA-mediated downregulation of p110γ leading to reduced AKT activation. First, the decrease in AKT phosphorylation was observed earlier than the decrease in p110γ levels. Second, 9AA treatment affected phosphorylation of AKT at Ser^{473} but not at Thr^{308}. These results suggest that inhibition of AKT activation by 9AA is not because of reduced p110γ levels. This unexpected finding led us to consider other potential explanations for the observed 9AA-dependent inhibition of AKT Ser^{473} phosphorylation. Although phosphoinositide-dependent kinase kinases have been reported to phosphorylate AKT at Thr^{308}, it was recently shown that the kinase critical for Ser^{473} phosphorylation is actually mTOR (Sarbassov *et al*., 2005). At the same time, mTOR is a target for AKT kinase activity, creating a positive feedback loop that serves as a signal amplifying circuit.

As 9AA treatment specifically affected phosphorylation of AKT Ser^{473}, we investigated whether the kinase activity of mTOR is inhibited by 9AA. We immunoprecipitated mTOR from untreated and 9AA-treated RCC45 cells and used the precipitated protein for *in vitro* kinase reactions with recombinant 4EBP1 as a substrate. *In vitro* phosphorylated 4EBP1 was detected by immunoblotting with a phospho-specific antibody. As shown in Figure 2b, 9AA treatment resulted in reduced phosphorylation of 4EBP1, indicating reduced mTOR kinase activity. To confirm this finding, we looked at the phosphorylation status of two endogenous proteins that are substrates of mTOR kinase activity, 4EBP and p70S6K. Through western blotting with phospho-specific antibodies, we demonstrated that phosphorylation of both of these substrates is reduced in 9AA-treated cells as compared to untreated cells (Figure 2c). These data demonstrate that 9AA treatment of tumor cells leads to a decrease in mTOR kinase activity and suggests that this then leads to reduced AKT activation. Downregulation of p110γ does not

Table 3 Relative mRNA expression levels for p110γ (PIK3CG) and other PI3 K catalytic subunit family members in RCC45 and RCC54 cells left untreated (control) or treated with either 2 μM or 10 μM 9AA for 24 h

Symbol	*RCC54 2 μM/control*	*RCC54 10 μM/control*	*RCC45 2 μM/control*	*RCC45 10 μM/control*
PIK3CG	1.04	1.00	1.07	1.03
PIK3CA	1.00	1.18	0.97	1.04
PIK3CB	1.04	1.06	1.01	0.96
PIK3CD	0.92	0.96	0.88	1.20

Transcript levels were quantified by the average signal of hybridization the whole array (Details in Gurova *et al*., 2005).

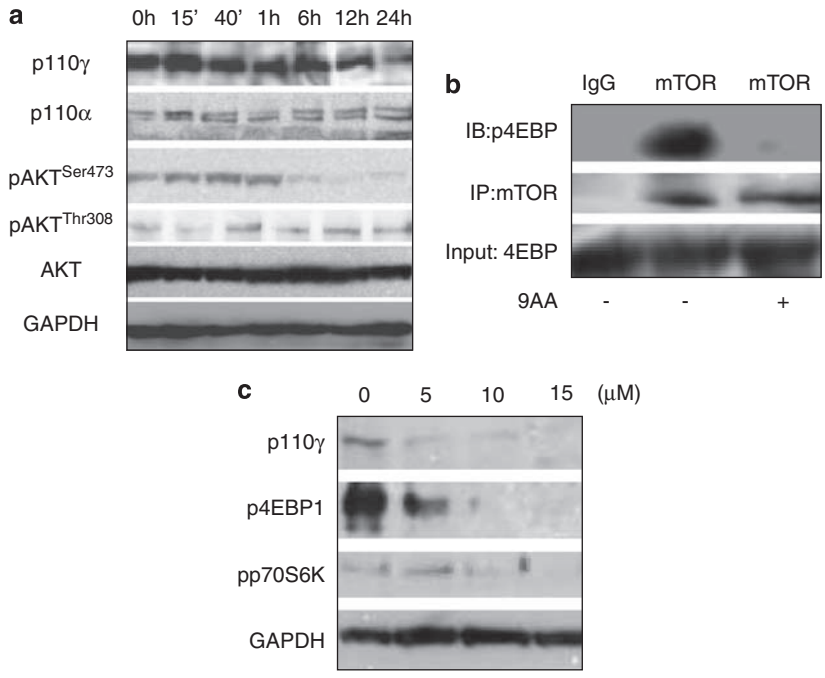

Figure 2 9-Aminoacridine (9AA) treatment suppresses the activity of AKT and mammalian target of rapamycin (mTOR) in tumor cells. (**a**) Western blotting of lysates from renal cell carcinoma 45 (RCC45) cells treated with 10 μM 9AA for the indicated periods of time. (**b**) *In vitro* kinase assay testing the activity of mTOR protein. RCC45 cells left untreated or treated with 10 μM 9AA. The cell lysates were immuoprecipitated using mTOR-specific antibody. Immunoprecipitated products were used for *in vitro* kinase assay using recombinant eIF4E-binding protein (4EBP) as a substrate. Phosphorylation of 4EBP1 was examined by western blotting using phosphor-specific antibody. The antibodies used for immunoprecipitation (IP) and immunoblotting (IB) are indicated. The bottom panel is a western blotting showing total 4EBP levels in the input lysates before *in vitro* kinase assay. (**c**) Western blotting of lysates from HCT116 cells treated with different concentrations of 9AA for 24 h. Phospho-specific antibodies were used to assess phosphorylation of endogenous 4EBP1 and p70 ribosomal S6 kinase (p70S6K).

appear to be involved in modulation of the AKT/mTOR pathway by 9AA because it occurs at later time points (Figure 2a).

p110γ downregulation is a consequence of mTOR inhibition

mTOR regulates a large number of cellular processes, but its best characterized function is regulation of translation (Gingras *et al*., 2004). Our finding that 9AA inhibits mTOR activity led us to hypothesize that the reduction in p110γ levels observed in cells treated with 9AA might be because of inefficient mTOR-dependent translation of p110γ. To test this possibility, we looked at whether p110γ protein levels were affected by inhibition of mTOR by means other than 9AA treatment. Rapamycin is a specific chemical inhibitor of mTOR complex 1, which is involved in the regulation of translation (Huang *et al*., 2003). Treatment of HT1080 cells with rapamycin reduced p110γ levels, indicating that p110γ is translated through an mTOR-dependent mechanism (Figure 3a). In contrast, p110α levels were not affected by rapamycin treatment. Phosphorylation of endogenous 4EBP1 was monitored to confirm that rapamycin indeed blocked mTOR activity in this experiment. Our finding that the effect of rapamycin treatment on p110γ and p110α levels mirrored that of 9AA treatment supports our hypothesis that inhibition of mTOR leads to downregulation of p110γ in 9AA-treated cells. As mTOR is a substrate for phosphorylation by AKT, we used several strategies of AKT inhibition to indirectly inhibit mTOR and assess its role in p110γ regulation. First, we tested the effect of a kinase deficient AKT mutant (E299K) that acts as a dominant inhibitor of AKT by titrating away AKT targets. Overexpression of this mutant in 293T cells led to reduction of p110γ levels, similar to 9AA treatment (Figure 3b). PTEN is a lipid phosphatase whose activity opposes that of PI3K. By converting PIP3 to PIP2, PTEN inhibits AKT activity, which is translated then into inhibition of mTOR activity. Overexpression of the PTEN cDNA in RCC45 cells also led to decreased p110γ protein levels (Figure 3c). Use of a chemical inhibitor of the PI3K pathway, Ly294002, had similar consequences—downregulation of p110γ protein expression (Figure 3d). Taken together, these data indicate that p110γ levels are reduced in cells treated with 9AA because of 9AA-mediated inhibition of AKT/mTOR activity.

To test whether 9AA inhibits only mTOR-dependent translation or translation in general, we compared the effect of 9AA on protein synthesis with that of cycloheximide, a general inhibitor of translation that acts by interrupting translation elongation within the ribosome. Incorporation of $^{[35]}$S-methionine/cysteine was compared between control, cycloheximide-treated and 9AA-treated HT1080 cells (Figure 4). In 9AA-treated

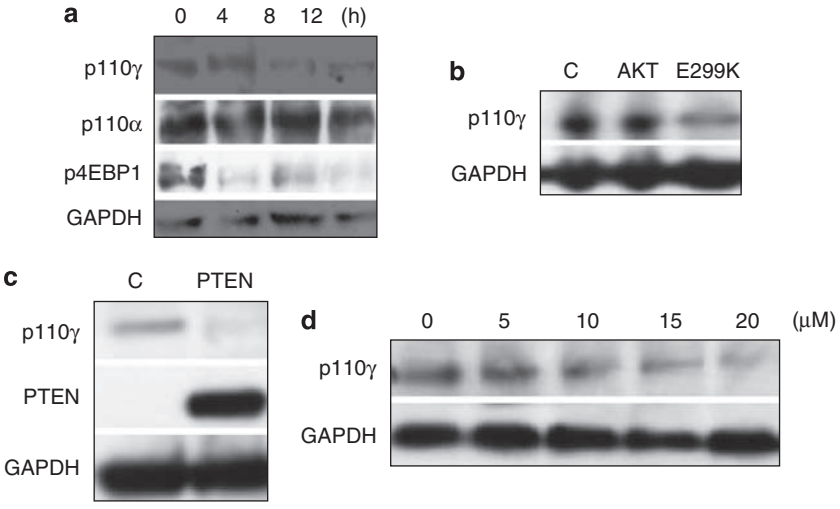

Figure 3 Inhibition of different members of the phosphoinositide 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway leads to the decreased expression of p110γ. (**a**) Western blotting of lysates from HT1080 cells treated with 1 μM of the mTOR inhibitor rapamycin for the indicated periods of time. (**b**) Western blotting of lysates from 293 cells expressing SV-40 T-antigen (293T) transfected with empty vector (C), wild-type AKT or dominant-negative kinase dead AKT mutant (E299K). (**c**) Western blotting of lysates from renal cell carcinoma 45 (RCC45) cells transduced with empty vector (C) or a phosphatase and tensin homolog (PTEN) expression construct. (**d**) Western blotting of lysates from RCC45 cells treated with different concentrations of the PI3K inhibitor Ly294002.

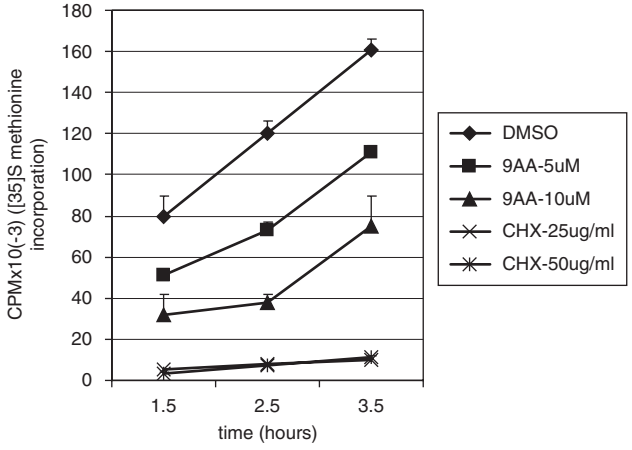

Figure 4 9-Aminoacridine (9AA) inhibits translation in HT1080 cells, but to a lesser extent than cycloheximide (CHX). HT1080 cells were incubated in cysteine/methionine-free medium for 0.5 h. $^{[35]}$S-labeled methionine and cysteine were then added and cells were treated with 9AA or CHX for the indicated amounts of time. Incorporation of $^{[35]}$S-labeled amino acids into cellular proteins was assessed by trichloro-acetic acid (TCA) precipitation of proteins and scintillation counting.

cells, $^{[35]}$S-Met incorporation was reduced 40–50% as compared to control cells. Under the same conditions, cycloheximide treatment resulted in more than 90% inhibition of protein synthesis. Even the highest dose of 9AA, 20 μM, which caused complete death of all cells tested after longer times of incubation, did not completely inhibit translation, but only reduced it to 54% of control, an effect comparable to that of the mTOR inhibitor rapamycin (Terada *et al*., 1994). This experiment demonstrated that 9AA affects translation to a similar extent as a known inhibitor of mTOR.

On the basis of these data, we conclude that 9AA is an inhibitor of AKT/mTOR activity. The effect of 9AA on mTOR leads to reduced translation of mTOR-dependent proteins and reduction of p110γ level.

Interplay between the p53, NF-κB and PI3K/AKT/mTOR pathways in tumor cells treated with 9AA

We have demonstrated that 9AA modulates the activity of several key signaling pathways in tumor cells, the p53, NF-κB and PI3K/AKT/mTOR pathways. From both the literature and our own studies, it is clear that these signaling pathways influence each other's activity and that the outcome of their activation depends upon the cell type and the stimulus used (Levine *et al*., 2006). As activation of p53 has been shown to inhibit mTOR (Feng *et al*., 2005), we compared the effect of 9AA on the PI3K/AKT/mTOR pathway in the presence or absence of wild-type p53. shRNA was used to knock-down expression of endogenous wild-type p53 in RCC45 cells. There was no difference in the reduction in p110γ protein levels and AKT Ser^{473} phosphorylation in 9AA-treated RCC45 cells expressing either shRNA against p53 or shRNA against green fluorescence protein (GFP) as a control (Figure 5). Therefore, p53 does not mediate inhibition of AKT or downregulation of p110γ in tumor cells treated with 9AA.

AKT is a known regulator of both p53 and NF-κB activity. AKT phosphorylates Ser^{536} of the p65 subunit of NF-κB, thereby stimulating NF-κB transcriptional activity (Sizemore *et al*., 2002). In addition, AKT phosphorylates Hdm2 on Ser^{166} (Mayo *et al*., 2002). Phosphorylated Hdm2 shuttles p53 from the nucleus to the cytoplasm and induces p53 degradation. In this way, AKT acts to suppress p53 activity. We tested whether 9AA treatment affected these AKT-dependent

phosphorylation events in tumor cells and confirmed our previous finding (Gurova *et al*., 2005) that 9AA inhibits phosphorylation of Ser^{536} of p65 (Figure 6a). Moreover, although 9AA treatment results in an initial increase in total Hdm2 protein because of transcriptional activation through p53, this newly synthesized Hdm2 does not become phosphorylated at Ser^{166} as shown by the slight decrease, rather than an increase, in the level of phosphorylated Hdm2 over time (Figure 6b). Thus, the effects of 9AA on p65 and Hdm2 are similar to the effects caused by AKT inhibition, which suggests that 9AA affects p53 and NF-κB activity at least partially through inhibition of AKT.

We also tested whether modulation of p110γ levels in RCC cells had any added effect on modulation of p53 and NF-κB mediated by AKT. We designed and cloned an shRNA against p110γ into a lentiviral vector (Figure 7a). This vector and a control vector were used to transduce RCC45 cells carrying p53- and NF-κB-dependent reporter constructs. Reporter activity was measured 48 h posttransduction and showed that the effect of downregulation of p110γ expression on p53 and NF-κB activity mirrors that of AKT inhibition. As compared to shE6 transduced control cells, cells with reduced p110γ displayed decreased NF-κB reporter activity and elevated p53 reporter activity (Figure 7c and d). Moreover, in contrast to control cells, RCC45 cells transduced with shRNA to p110γ did not form colonies in a colony assay (Figure 7b). This effect was independent of p53 status (tested with shRNA against p53), demonstrating that p110γ is an independent survival factor for these cells. Finally, to determine whether overexpression of p110γ can confer resistance to the toxic effect of 9AA on tumor cells, we ectopically expressed the p110γ cDNA in RCC45 cells (Figure 8a) and compared survival of cells with normal and elevated levels of p110γ following 9AA treatment. As shown in Figure 8, overexpression of p110γ protects cells from the toxic effect of 9AA. Moreover, we observed elevated NF-κB transcriptional activity in the reporter assay after transduction of p110γ (Figure 8c). Therefore, modulation of p110γ in tumor cells can, on its own, affect the activity of the p53 and NF-κB pathways.

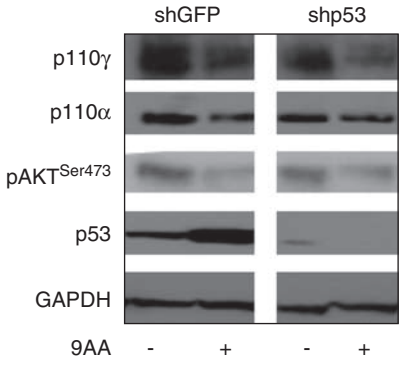

Figure 5 9-Aminoacridine (9AA) treatment downregulates p110γ protein levels in tumor cells independently of p53 status. Renal cell carcinoma 45 (RCC45) cells transduced with lentivector expressing short-hairpin RNA (shRNA) against either p53 or green fluorescence protein (GFP) were left untreated or treated with 10 μM 9AA for 24 h. Cell lysates were then prepared and used for western blotting with the indicated antibodies.

Discussion

In this study, we set out to clarify the mechanism of action of 9AA, a compound that we previously identified as toxic to tumor cells displaying simultaneous inhibition of NF-κB and induction of p53. Through proteomic analysis, we found that p110γ protein levels are specifically reduced in tumor cells treated with 9AA. p110γ is the catalytic subunit of the single member of class IB of the PI3K family (Stephens *et al*., 1994). Class I PI3K activity is required for phosphorylation-dependent activation of the prosurvival AKT signaling pathway. Therefore, the importance of PI3K for tumorigenesis is likely due, at least in part, to its regulation of AKT. AKT, in turn, has been shown to be involved in transactivation of NF-κB by phosphorylating its p65 subunit (Sizemore *et al*., 2002; Jeong *et al*., 2005). Moreover, a correlation was shown between AKT kinase activity and p53 inhibition (Jeong *et al*., 2005). AKT can also phosphorylate Mdm2, which induces translocation of Mdm2 into the nucleus and targets p53 for proteasomal degradation (Mayo *et al*., 2002).

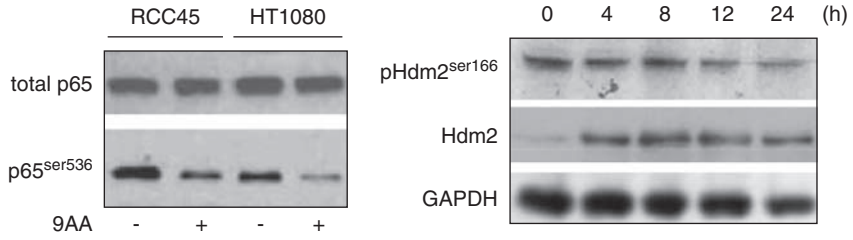

Figure 6 9-Aminoacridine (9AA) treatment of tumor cells suppresses phosphorylation of p65 and Hdm2, mimicking the effect of AKT inhibition. (**a**) Renal cell carcinoma 45 (RCC45) and HT1080 cells were treated with 10 μM 9AA overnight. Lysates were subjected to immunoprecipitation with anti-p65 antibody and then analysed by western blotting with antibodies recognizing total p65 or the phopsho-Ser^{536} form of p65. (**b**) RCC45 cells were treated with 5 μM 9AA for the indicated periods of time. Cell lysates were used for immunoblotting with antibodies specific for Hdm2 phosphorylated at Ser^{166}, total Hdm2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

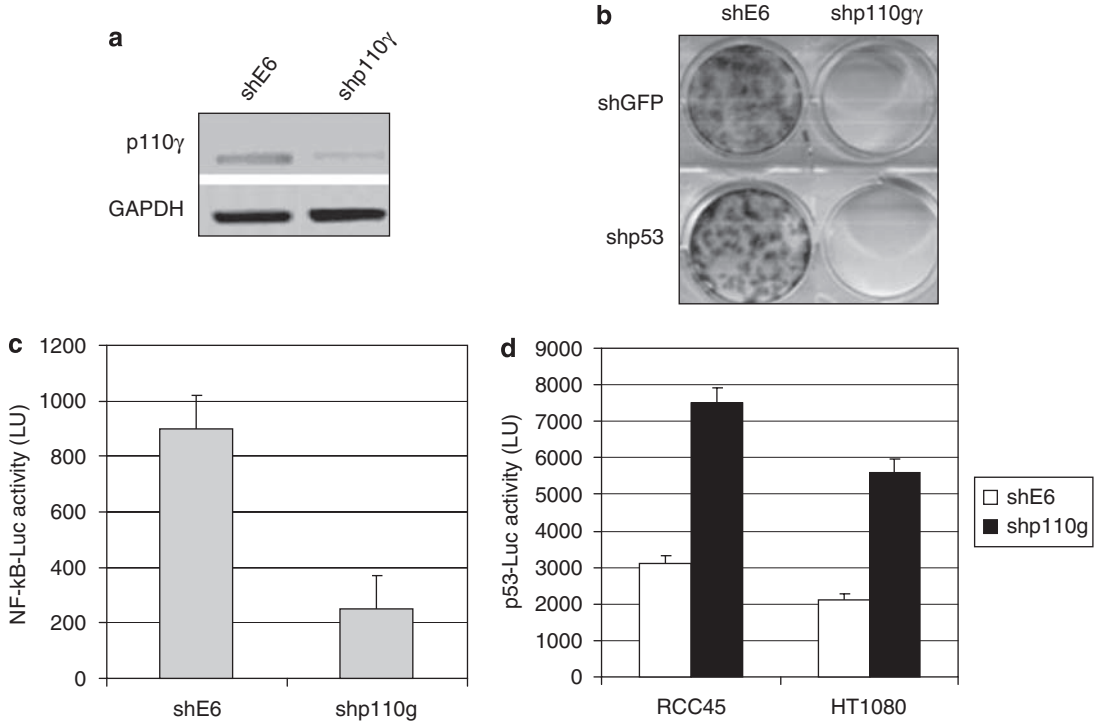

Figure 7 Modulation of p110γ expression affects cell survival and changes nuclear factor-κB (NF-κB) and p53 transcriptional activity in tumor cells. (**a**) Reverse transcriptase (RT)–PCR analysis of p110γ expression in renal cell carcinoma 45 (RCC45) cells transduced with short-hairpin RNA (shRNA) vectors against p110γ or E6. (**b**) RCC45 cells were cotransduced with lentiviral vectors expressing the indicated shRNA constructs. Cells were stained with methylene blue 7 days after transduction. (**c**, **d**) NF-κB (**c**) and p53-dependent (**d**) reporter luciferase activity in RCC45 and HT1080 cells transduced with indicated lentiviral constructs (measured 48 h after transduction).

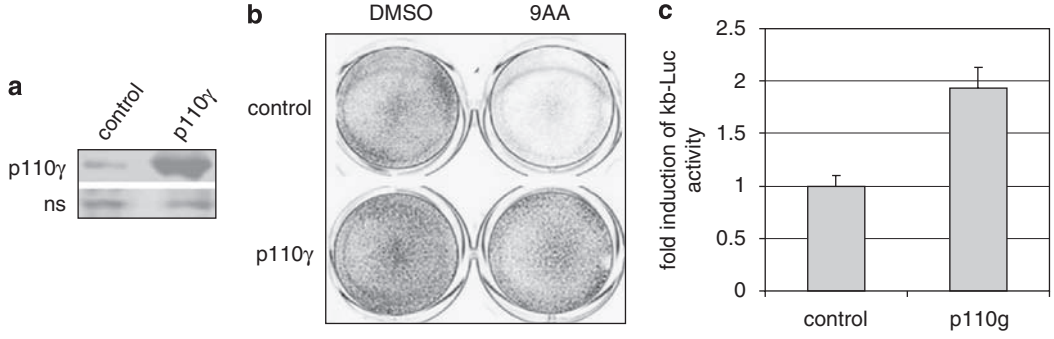

Figure 8 Effect of overexpression of p110γ on sensitivity of tumor cells to 9-aminoacridine (9AA) treatment and on nuclear factor-κB (NF-κB) transactivation. (**a**) p110γ protein expression was examined by western blot using p110γ specific antibody. (**b**) Renal cell carcinoma 45 (RCC45) cells were infected with p110γ containing virus or control virus. Then equal numbers of cells were plated into 24-well plate. The next day, cells were treated with 9AA (μM) for 48 h, the cell survival was measured by methylene blue. (**c**) 293T (293 cells expressing SV-40 T-antigen) cells were transiently transfected with p110γ cDNA or corresponding amount of control empty vector, NF-κB-responsive reporter κBluc together with pCMV-LacZ as control. 48 h posttransfection, NF-κB activity was measured by NF-κB luciferase. The transfection efficiency was normalized by LacZ reporter.

Under normal conditions p110γ expression is largely restricted to the hematopoietic system, although it can also be detected in endothelium, heart and brain (Camps *et al*., 2005). Genetic inactivation of p110γ allows normal development to adulthood, but causes defects in the immune system (Rückle *et al*., 2006). p110γ is gaining attention as a promising drug target for the treatment of inflammatory disease (Rückle *et al*., 2006). Here we showed that the p110γ protein is expressed in human cancer cell lines of various origins (RCC, HCT116, HT1080, etc.) and investigated the roles it might play in tumor cells.

Suppression of p110γ levels by either 9AA treatment or shRNA-mediated gene knockdown resulted in

activation of p53 and inhibition of NF-κB and induced tumor cell death (Figure 7). The effect of shRNA-mediated p110γ suppression on cell survival did not depend upon p53 (Figure 7b). This suggests that p110γ is an independent survival factor for tumor cells and that, in this situation, p53 activation is not the cause of cell death. Our finding that death of tumor cells resulting from suppressed expression of p110γ is p53 independent is consistent with the ability of 9AA to not only kill cancer cells with wild-type p53, but also those with no p53 expression, albeit to a lesser extent (Gurova *et al*., 2005).

Cancer-specific mutations have been identified only in p110α, but ectopic expression of other PI3K family members, including p110β, p110γ and p110δ, is sufficient to transform chicken embryo fibroblasts, suggesting that these proteins have inherent oncogenic potential (Kang *et al*., 2006). This is consistent with, and may even be explained by, our finding that overexpression of p110γ induces NF-κB-dependent transcription in cells with low basal levels of NF-κB activity (293 cells, Figure 8c). p110γ-mediated induction of NF-κB activity in precancerous cells likely creates a cellular environment conducive to cancer initiation. Overexpression of p110γ in RCC45 cells makes them resistant to cell death induced by 9AA treatment (Figure 8b), suggesting that the reduction in p110γ protein levels in 9AA-treated tumor cells be involved in the toxicity of 9AA toward tumor cells. The mechanism by which p110γ affects NF-κB and p53 probably involves AKT kinase activity, which is at least partially responsible for NF-κB activation through phosphorylation of p65 and p53 inhibition by phosphorylation of Hdm2. Modulation of both of these biochemical events was observed in tumor cells treated with 9AA (Figure 6).

In our experiments aimed at defining events downstream of 9AA-mediated p110γ downregulation, we found that AKT activity is inhibited by 9AA treatment, but that this is not likely a direct effect of reduced PI3K activity. As only phosphorylation of AKT at Ser^{473} (and not at Thr^{308}) was blocked by 9AA treatment (Figure 2a), we hypothesized that 9AA might inhibit mTOR, a kinase previously shown to target this particular site in AKT (Sarbassov *et al*., 2005). This was found to be true (Figure 2b), thereby implicating mTOR along with AKT in the mechanism of action of 9AA. Interestingly, mTOR is both the major component of the TORC2 that phosphorylates AKT on Ser^{473} and a substrate itself for phosphorylation by AKT. This creates a positive feedback circuit between AKT and mTOR.

In addition to the mTORC2 mentioned above, mTOR is also a component of the mTORC1. mTORC1 exerts its effect on 5′-cap-dependent mRNA translation (the majority of cellular translation) through phosphorylation of 4EBP1. In its unphosphorylated state, 4EBP1 binds tightly to eIF4E and inhibits the activity of eIF4E in the initiation of protein synthesis. Phosphorylation of 4EBP1 by mTOR releases eIF4E and allows initiation of translation (Gingras *et al*., 2004). These considerations led us to hypothesize that the observed decrease in p110γ protein levels upon 9AA treatment is because of inhibition of mTOR-mediated translation. Several aspects of our data support this: (i) the decreased phosphorylation of mTOR substrate correlates with the decrease in p110γ protein expression (Figure 3); (ii) the decrease of p110γ is at the protein level, not at the mRNA level; (iii) decreased phosphorylation of AKT is observed earlier than the decreased expression of p110γ protein. These observations suggest that the decrease in p110γ that results upon 9AA treatment may be because of a primary effect of 9AA on the AKT/mTOR signaling pathway. Indeed, genetic and pharmacological inhibition of various members of the AKT/mTOR pathway resulted in reduced p110γ protein expression (Figure 3).

One question raised by these data is why 9AA-mediated suppression of mTOR leads to inhibition of p110γ protein expression specifically (and not of p110α, for example). One possible explanation is that p110α expression might rely on cap-independent translation initiation mechanism involving internal ribosome entry site (IRES) element (Komar and Hatzoglou, 2005). IRESs elements are generally insensitive to mTOR inhibition by rapamycin and their activity can be even enhanced under conditions of rapamycin treatment (see for example, Shi *et al*., 2005).

Although we feel that suppression of mTOR-dependent translation of p110γ is the most likely explanation for 9AA-mediated reduction in p110γ protein levels, our data does not rule out the possibility that altered protein degradation is responsible for this effect.

In summary, we propose the following working model of 9AA action in tumor cells (Figure 9). Inhibition of mTOR activity by 9AA appears to involve a different mechanism than the known mTOR inhibitor rapamycin because rapamycin inhibits mTOR through binding to FKBP12 protein, which is a component of mTORC1, but not mTORC2.

We originally defined 9AA as a compound capable of simultaneous inhibition of NF-κB and activation of p53 (Gurova *et al*., 2005). In this study, we demonstrated that 9AA also inhibits the PI3K/AKT/mTOR signaling pathway, a property that is expected to contribute to the anticancer effect of the compound. Inhibition of AKT activity by 9AA was recently confirmed in a report from our collaborators on a model of human T-cell leukemia virus-transformed cells (Jung *et al*., 2008). The unique ability of 9AA to modulate multiple pathways that are deregulated in tumor cells in the desired direction further strengthens the prospect of using molecules similar to 9AA as anticancer agents. Quinacrine, a 9AA derivative that has been widely used in humans as an antimalaria drug, has effects on the PI3K/AKT/mTOR, NF-κB and p53 signaling pathways that are similar to what is observed with 9AA. The anticancer efficacy of quinacrine is currently being assessed in a stage II clinical trial in hormone refractory prostate cancer patients. The results of this trial will provide an indication of the feasibility of using 9AA derivatives as cancer therapeutics.

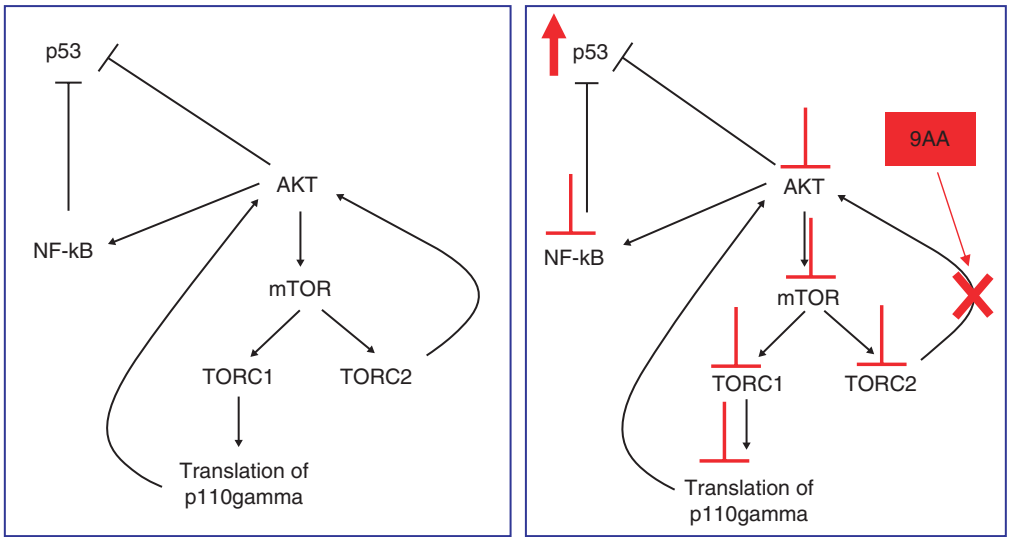

Figure 9 Working model of the interplay between the phosphoinositide 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR), p53 and nuclear factor-κB (NF-κB) B pathways in tumor cells (left) and the effect of 9-aminoacridine (9AA) treatment (right). In cancer cells, there is constitutively active AKT/mTOR signaling pathway. We propose that most probably 9AA affect amplifying signal between AKT and mTORC2. This results in the decrease of Ser^{473} of AKT and inhibition of AKT activity. This is the first event observed after 9AA treatment. Decreased activity of AKT leads to additional inhibition of mTOR (TORC2 as well as TORC1) and inhibition of translation. The consequence of this event is dropping down in p110γ level as well as translations of some other proteins. Lowered p110γ expression in turn additionally inhibits AKT activity, thereby impacting the p53 and NF-κB pathways as well.

Materials and methods

Cell lines and cultures

Renal cell carcinoma (RCC) cell lines (RCC45, RCC54) were provided by Dr James Finke (Cleveland Clinic, Cleveland, OH, USA). The HT1080 (lung fibrosarcoma), 293 cells expressing SV-40 T-antigen (293T) and HCT116 (colorectal carcinoma) cell lines were purchased from American Type Culture Collection (ATCC). Cell lines carrying p53- or NF-κB-responsive luciferase reporter constructs were described before (Gurova *et al*., 2005). The RCC45 shGFP and RCC45 shp53 cell lines were generated by infecting RCC45 cells with the corresponding short-hairpin RNA (shRNA)-encoding lenti-vectors as described (Gurova *et al*., 2005).

Reagents and antibodies

The PI3K inhibitor LY294002 and the mTOR inhibitor rapamycin were purchased from Alexis Biochemicals (AXXORA, LLC, San Diego, CA, USA). Recombinant rat eIF4E-binding protein 1 (4EBP1) was purchased from Calbiochem (EMD Chemicals, Inc., Gibbstown, NJ, USA). The anti-p53 (monoclonal mouse DO1), anti-p21 (monoclonal mouse F-5), anti-Hdm2 (monoclonal mouse SMP14), anti-p65, anti-fluorescence recovery after photobleaching (FRAP; mTOR), anti-4EBP1, anti-P110γ and mouse antiglyceraldehyde 3-phosphate dehydrogenase antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-p110α, antiphosphatase and tensin homolog (PTEN), anti-AKT and phospho-specific antibodies recognizing AKT(Ser^{473}), AKT(Thr^{308}), MDM2(Ser^{166}), 4EBP1(Ser^{65}), p70 ribosomal S6 kinase 1 (p70S6K1; Thr^{389}) and p65(Ser^{536}) were also purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Plasmids

pNF-κB-Luc plasmid was provided by N Neznanov (Cleveland Clinic Foundation). The plasmid encoding the full length PTEN cDNA was from L Mayo (Case Western Reserve University, Cleveland, OH, USA). The plasmids encoding wild-type AKT and kinase dead AKT (E299K mutant) were gifts from Dr Cheng (Lee Moffit Cancer Center, Tampa, FL, USA). The human p110γ cDNA was purchased from ATCC and inserted into the pLA CMV lentiviral vector to generate pLA-CMV-p110γ. To generate a shRNA construct directed against human p110γ, a pair of complementary 70-nucleotide (nt) oligonucleotides encoding a 21-nt p110γ-specific shRNA (5′-GATCCCGTGTTTGGAAGCACATTTAAGTTGATAT CCGCTTAAATGATGCTTCCAAACATTTTTTCCAAG-3′ and 5′-GGCACAAACCTTCGTAGTAAATTCAACTATA GGCGAATTTACTAGAAGGTTTGTAAAAAAGGTTCT TAA-3′) were designed. The core 21-nt sequence targeting human p110γ is underlined. The 70 nt oligonucleotides were annealed and cloned into the $pLPL_w$ vector and the resulting vector, designated pLPLw-shp110γ. shRNA against the human papillomavirus E6 oncoprotein, which is not expressed in RCC45 or HT1080 cells, was used as a negative control.

Immunoprecipitation

RCC45 or HT1080 cells in p100 plate were treated with or without 9AA (10 μM) for overnight. Equivalent amounts of cell extracts were precleared with 40 μl of protein A/G sepharose beads (Santa Cruz) for 30–60 min at 4 °C, after brief centrifugation, the supernatant was collected and used for incubation with p65 or mTOR antibody. After complex formation at 4 °C for overnight, immunocomplexes were precipitated with protein A/G agarose beads. Immunoprecipitates were then subjected to western blot using anti-p65(Ser536) antibody or mTOR *in vitro* kinase assay.

mTOR in vitro *kinase assay*

Immunoprecipitates was washed twice with lysis buffer and once with 1 × kinase buffer (50 mM Tris, 10 mM $MgSO_4$, 0.02% bovine serum albumin). *In vitro* kinase assay solution

20 μl (substrate rabbit 4EBP1 2 μg, 1 × kinase assay buffer, adenosine-5′-triphosphate 5 mM) incubated with immunocomplexes at 30 °C for 30 min. The kinase reaction was stopped by adding protein loading dye and heating at 100 °C for 4 min. Proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE) and subjected to western blot.

RT–PCR
Total RNA was isolated from cell lines using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. First-strand cDNA was obtained using SuperScript II reverse transcriptase according to the manufacturer's protocol (Invitrogen). PCR was performed using the cDNA as template, Platinum PCR SuperMix (Invitrogen) and primers that were described before (Yamboliev *et al*., 2000).

Lentivirus transduction
293T cells were plated subconfluently on a 10 cm tissue culture plate. The next day, cells were transfected with 3.3 μg lentiviral vector DNA, 3.3 μg pVSV-G expression plasmid and 3.3 μg packaging vector pCMVdeltaR8.2 using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's recommendations. The medium was changed 16 h after transfection and the virus-containing medium was collected 48 and 72 h posttransfection, filtered through 0.45 μM filter (Fisher Scientific, Pittsburgh, PA, USA).

Transient transfection and reporter assay
Transient transfection A total of 2×10^5 cells per well were plated in six-well plates and, after overnight incubation, transfected using Lipofectamine Plus reagent with 0.5 μg pNF-κBluc in combination with pLA-CMV-p110γ expression plasmids. Corresponding empty vectors were used as controls. Normalization of transfection efficiency was performed by cotransfection of 0.2 μg pCMV-LacZ plasmid. Measurements of luciferase activity and lacZ activity was described previously (Gurova *et al*., 2005).

Stable transduction of reporters A total of 2×10^5 cells per well with integrated reporter were plated in six-well plate. After lentiviral transduction, cell lysates were prepared 48 h later. Luciferase activity and protein concentration were measured in aliquots of cell lysates by using standard kits (Promega Corporation, Madison, WI, USA, Luciferase Assay Systems; Bio-Rad Protein Assay Kit, Bio-Rad, Hercules, CA, USA).

Colony formation assay
A total of 5×10^3 cells per well were plated in six-well plates. The next day, cells were transduced with lentiviral vectors. Medium was changed at 24 h and the cells were stained with methylene blue 6 days after infection.

Microarray hybridization
RCC45 and RCC54 cells were treated with or without 9AA (2 or 10 μM) for 24 h. Total RNA was prepared using Trizol reagent (Invitrogen) and 'cleaned-up' using the Rneasy Mini kit (Qiagen, Valencia, CA, USA). cDNA preparation, hybridization to human gene expression *Homo sapiens* 1-Plex Array, scanning, data extraction and analysis were carried out according to the manufacturer's instructions (NimbleGen, Madison, WI, USA).

Proteomic analysis
RCC45 and RCC54 cells in p100 plate were treated with or without 9AA (10 μM) for 24 h. After two-dimensional (2D) gel electrophoresis, Silver Stain kit (Bio-Rad) was used to detect protein spots according to the manufacturer's instructions. Silver-stained spots were destained as previously described (Okamoto *et al*., 2007), then subjected to reduction and alkylation, followed by *in situ* digestion with trypsin. The resulting peptide mixtures were recovered by sequential extraction as previously described (Stone and Williams, 1993). Peptides obtained from in-gel digests were analysed by nanospray liquid chromatography tandem mass spectrometry (MS/MS) as previously described (Furuta *et al*., 2004). The unprocessed data files containing MS/MS spectra were submitted to the Mascot search engine (MatrixScience Ltd., London, UK) for database searching using the Mascot daemon. The SwissProt-Trmbl database was searched using *H. sapiens* as a taxonomic restrictor. To optimize the accuracy of protein identification, we required a minimum of two unique peptides at different sites within the protein for a positive identification. Therefore, a protein sequence would be identified only after mass spectrometric analysis of two or more unique peptides, each of whose probability scores met or exceeded the threshold ($P<0.05$) for statistical significance.

Metabolic labeling of cells and monitoring of general protein synthesis
To measure total protein synthesis, HT1080 cells were incubated in Met/Cys-free Dulbecco's modified Eagle's medium (MP Biomedicals Inc., Solon, OH, USA) with 5% dialysed fetal bovine serum for 0.5 h. Cells were metabolically labeled for 1.5 or 3 h with 2 μCi of ^{35}[S]methionine and ^{35}[S]cysteine per milliliter of medium (Pro-mix L-[^{35}S] *in vitro* cell labeling mix, Amersham Biosciences, GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). The cells were incubated with 1:1 1 × phosphate buffer saline and 1 M NaOH for 10–15 min at 37 °C to stop the labeling at different time points. Ice-cold trichloro-acetic acid (TCA) was then added to the aliquots of cell lysates to a final concentration of 10% and filtered through GF/C Whatman glass filters. The GF/C filters were washed three times with 5% TCA and once with 10 ml of ethanol (ice cold). Air-dried filter disks were placed in scintillation vials with scintillation fluid and counted using a liquid scintillation counter (Beckman, LS 6500). Total protein content of the cell lysates was measured using the bicinchoninic acid assay (Pierce, Rockford, IL, USA) to allow calculation of counts per minute per microgram of protein.

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