Intrinsically lower AKT, mammalian target of rapamycin, and hypoxia-inducible factor activity correlates with increased sensitivity to 2-deoxy-D-glucose under hypoxia in lung cancer cell lines

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Abstract

Down-regulation by small interfering RNA or absence of hypoxia-inducible factor (HIF-1α) has been shown to lead to increased sensitivity to glycolytic inhibitors in hypoxic tumor cells. In surveying a number of tumor types for differences in intrinsic levels of HIF under hypoxia, we find that the reduction of the upstream pathways of HIF, AKT, and mammalian target of rapamycin (mTOR) correlates with increased toxic effects of 2-deoxy-D-glucose (2-DG) in lung cancer cell lines when treated under hypoxia. Because HIF-1α translation is regulated by mTOR, we examined the effects of blocking mTOR under hypoxia with an analogue of rapamycin (CCI-779) in those cell lines that showed increased mTOR and AKT activity and found that HIF-1α down-regulation coincided with increased 2-DG killing. CCI-779, however, was ineffective in increasing 2-DG toxicity in cell lines that did not express HIF. These results support the hypothesis that although mTOR inhibition leads to the blockage of numerous downstream targets, CCI-779 increases the toxicity of 2-DG in hypoxic cells through down-regulation of HIF-1α. Overall, our findings show that CCI-779 hypersensitizes hypoxic tumor cells to 2-DG and suggests that the intrinsic expression of AKT, mTOR, and HIF in lung cancer, as well as other tumor types, may be important in dictating the decision on how best to use 2-DG alone or in combination with CCI-799 to kill hypoxic tumor cells clinically. [Mol Cancer Ther 2008;7(6):1506–13]

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Introduction

Glucose transporters and glycolytic enzymes are among many of the genes activated by hypoxia-inducible factor-1α (HIF-1α), a key regulator of a wide range of cellular responses to lowered oxygen tension (1–4). Previously, it was reported that although hypoxic tumor cells are hypersensitive to glycolytic inhibitors such as 2-deoxy-D-glucose (2-DG), HIF-1α seems to confer a level of resistance to this treatment (5). A mechanism offered to explain these results is that HIF-1α increases the expression of hexokinase, which is the enzyme that 2-DG interacts with to block glycolysis; thus, greater amounts of this glycolytic inhibitor are required to shut it down. This hypothesis was supported by results which showed that hypoxic cells unable to activate the HIF response pathway either through small interfering RNA (siRNA) or mutation, showed decreased levels of hexokinase which correlated with increased sensitivity to 2-DG (5). Thus, although tumor cells under hypoxic conditions are compromised in their ability to undergo oxidative phosphorylation and consequently become vulnerable to inhibitors of glycolysis, blocking HIF potentiates the toxicity of this type of treatment.

2-DG is currently in phase I clinical trials, based on the rationale that by killing the hypoxic population of solid tumors, it will raise treatment efficacy when combined with standard chemotherapy. Thus, it becomes important to identify the mechanisms by which the use of this strategy can be maximized. Therefore, combining inhibitors of HIF with 2-DG may prove to be one way to increase the effectiveness of this latter agent's cytotoxic activity in hypoxic tumor cells. In this regard, CCI-779, a soluble analogue of rapamycin has been shown to down-regulate the translation of HIF-1α protein by inhibiting the mammalian target of rapamycin (mTOR), which functions as a central modulator of cell growth at the level of mRNA translation (6–9). mTOR controls the translation of HIF-1α as well as many other proteins through phosphorylation of two downstream targets, p70S6K and 4E-BP1 (10–12). Activation of both of these proteins up-regulates the translation of mRNAs that contain polypyrimidine tracts at their 5′ transcriptional (5′TOP) starting sites (13–15). Because HIF-1α mRNA contains such polypyrimidine tracts, its translation is directly regulated by mTOR (14, 16). Upstream of mTOR, the AKT pathway (when activated) positively stimulates mTOR activity. Thus, tumor cells with intrinsic activation of AKT and/or mTOR would be expected to express HIF under hypoxic conditions, whereas cell types with low or absent levels of

these activated pathways would not. In this regard, we report here that human lung cancer cell lines which show intrinsically lower phosphorylated AKT (pAKT) and phosphorylated mTOR (pmTOR) activities correlate with barely detectable levels of HIF, and consequently, increased sensitivity to the glycolytic inhibitor 2-DG. Moreover, we find that when mTOR is blocked by CCI-779 in the lung cancer cell lines which express high levels of pAKT and pmTOR, HIF-1α is down-regulated which in turn leads to the hypersensitization of hypoxic tumor cells to 2-DG.

Materials and Methods

Cell Lines

SCLC 1 and SCLC SR-2 cell lines were derived from the bone marrow, and SCLC B and SCLC BC came from the lymph nodes, as described previously (17, 18). NSCLC A and NSCLC ALC lines were established from metastatic adenocarcinomas to the brain (19). HEPA-1 and C4 cell lines (20) were purchased from American Type Culture Collection.

Compounds and Antibodies

CCI-779 was kindly provided by Wyeth-Ayrest. 2-DG was purchased from Sigma Chemicals. Hexokinase type II (HKII), AKT, pAKT (Ser^{473}), mTOR, pmTOR (Ser^{2448}), and phosphorylated P70S6K (Thr^{389}) were purchased from Cell Signaling, Inc. HIF-1α was purchased from BD Bioscience.

Cytotoxicity Assay

Cells were seeded in 24-well dishes at the following numbers per well: 8×10^4 transfected SCLC, 4×10^4 HEPA-1 or C4, 1.5×10^4 SCLC, and 4×10^4 NSCLC. Transfected SCLC cells were incubated under 5% CO_2 and 95% air at 37°C for 6 to 8 h to allow attachment and then transferred to either normoxic or hypoxic conditions. All other cell lines were cultured under 5% CO_2 and 95% air at 37°C for 24 h before the various treatment conditions. Cells were treated under hypoxia for 48 h with different combinations of 2-DG and CCI-779 as described in the figure legends. The culture mediums as well as the trypsinized cells were collected and this mixture was centrifuged at $400 \times g$ for 5 min, The supernatant was discarded, and the cells were resuspended in 1 mL of Hank's buffer and assayed for live and dead cells using a Vi-Cell cell viability analyzer (Beckman Coulter, Inc.).

Hypoxia

Cells were exposed to hypoxia (0.5% O_2) by incubation in a hypoxia glove box (Coy Laboratory Products, Inc.). After an initial exposure to low oxygen, all subsequent treatments were given within the glove box to prevent cellular damage due to reoxygenation. Additionally, if the procedure required a change of medium after hypoxic exposure, the replacement medium was equilibrated to the low-oxygen environment 24 h before use. Normoxia-equilibrated medium was used for the normoxic control cells.

siRNA

Cells (8×10^5) were seeded in a 60-mm Petri dish and incubated under normal culture conditions for 24 h. Dharmafect 1 transfection reagent was then used to transfect 100 nmol/L of HIF-directed SMARTpool siRNA or siCONTROL (Dharmacon, Co.). Cells were incubated in the presence of transfection medium for 24 h under 5% CO_2 and 95% air at 37°C. The transfection medium was then removed, cells were gently rinsed thrice with PBS, and fresh culture medium was replaced. Cells were then allowed to recover for 24 h under normal culture conditions, and then trypsinized and reseeded either in 24-well plates or in 100-mm Petri dishes depending on the proceeding experimental protocols as described in the appropriate figure legends.

ATP

Cells were seeded at 1.5×10^4 and grown under normal culture conditions for 24 h. The cells were either maintained under normoxia or transferred to hypoxia for 24 h. The medium was then replaced with 100 μL of fresh medium conditioned in either normoxic or hypoxic conditions, and the appropriate doses of 2-DG with or without the combination of CCI-779 were given. Following 6 h of treatment, the Cell Titer-Glo kit (Promega) was used to quantify ATP by luminescence as measured on a FLUOstar OPTIMA (BMG Lab Tech). Each data point was normalized to an average of three tandem cell counts.

Western Blot Analysis

Cells were seeded at 1×10^5/mL onto 100 mm dishes, and allowed to attach overnight, then treated with various doses of 2-DG, with and without CCI-779 under either normoxic or hypoxic conditions. At 48 h, cells were harvested by scraping in radioimmunoprecipitation assay buffer, which contains 10 mmol/L of Tris (pH 7.4), 100 mmol/L of NaCl, 1 mmol/L of EDTA, 20 mmol/L of $Na_4P_2O_7$, 2 mmol/L of Na_3VO_4 10%, 0.5% deoxycholate, 1 mmol/L of phenylmethylsulfonyl fluoride, and a phosphatase and protease inhibitor cocktail (purchased from Sigma). Cell lysis was completed by sonication and centrifugation. The total protein was separated on an 8% SDS-PAGE, transferred onto a nitrocellulose membrane (Amersham Biosciences) and immunoblotted with the indicated antibodies. Bands were visualized using an enhanced chemiluminescence reagent (Pierce Biotechnology, Inc.). The membrane was stripped using a stripping buffer (Pierce Biotechnology) for successive re-blotting with additional antibodies. Equal protein loading was verified using the Micro BCA Protein Assay (Pierce Biotechnology) as well as using actin as a loading control (actin antibody was purchased from Sigma). Band density (intensity × mm^2) was measured for each protein using a molecular imager Chemidoc system with Quality One software (Bio-Rad).

Results

Reduced AKTand mTOR Activity Correlates with Low Levels of HIF and Increased Sensitivity to 2-DG in Lung Cancer Cell Lines under Hypoxia

Because the down-regulation or absence of HIF renders hypoxic cells more sensitive to glycolytic inhibitors, we surveyed a number of cell lines for their intrinsic ability to

express this transcription factor as well as its upstream regulators AKT and mTOR. Under hypoxia, low pAKT and pmTOR levels (which are the active forms) were found in two cell lines (NSCLC) correlating with undetectable levels of HIF-1α (Fig. 1A and B) and with increased sensitivity to 2-DG, as compared with four cell lines (SCLC) that express higher levels of these proteins (Fig. 1C). The average of 6-fold higher mTOR activity and 10-fold higher AKT activity detected in the SCLC lines (quantification of Fig. 1A) corresponds with robust amounts of HIF-1α (Fig. 1B). Interestingly, the levels of the inactive forms of mTOR and AKT (unphosphorylated) under normoxia and hypoxia are the same for both lung cancer cell types (Fig. 1A). Overall, these results show that decreased levels of HIF-1α in cells growing under hypoxia correlate with decreased AKT and mTOR activity and increased sensitivity to 2-DG.

Blocking mTOR with CCI-779 Correlates with Down-regulation of HIF-1α and Increased 2-DG Toxicity in Hypoxic Tumor Cells

In order to determine whether blockage of mTOR results in the down-regulation of HIF and thereby increases the sensitivity of hypoxic cells to 2-DG, SCLC line B was treated with the water-soluble analogue of rapamycin, CCI-779. At a nontoxic dose, CCI-779 is found to completely block mTOR activity as measured by assaying its direct downstream target phosphorylated P70S6K. This, in turn, coincides with the lowering of HIF-1α and HKII levels by CCI-779 (Fig. 1D and E).

Cotreatment of SCLC B with 2-DG and CCI-779 results in increased toxicity as compared with cells treated with 2-DG alone (Fig. 2A). Interestingly, the percentage of dead cells elicited by this treatment in SCLC B is strikingly similar to that in NSCLC cells treated with 2-DG alone where HIF is not expressed; 78% and 88% compared with 73% and 82%, respectively. However, when NSCLC line A, which has a low mTOR activity, was treated with CCI-779 under hypoxia, 2-DG toxicity did not increase, and in fact, a slight protective effect was observed (Fig. 2B). Overall, these data show that inhibiting mTOR with CCI-779 in lung cancer cell lines that intrinsically express high levels of this protein leads to the blockage of HIF-1α translation culminating in the increased sensitivity of hypoxic tumor cells to 2-DG.

CCI-779 does not Increase 2-DG Toxicity in Hypoxic Cells When HIF-1α Is Down-regulated by siRNA or Is Nonfunctional

Inhibition of mTOR is known to affect the translation of numerous proteins. To address the question of whether the increased sensitivity to 2-DG by CCI-779 was specific to its effects on HIF under hypoxia, siRNA directed against HIF-1α or mutant HIF-1β cells that cannot express functional HIF were used. In SCLC cells in which HIF-1α is significantly decreased with siRNA (Fig. 3A), the addition of CCI-779 did not augment 2-DG cytotoxicity (Fig. 3B). As expected, in SCLC cells transfected with scrambled siRNA in which HIF is expressed under hypoxia, treatment with CCI-779 down-regulated HIF, which correlated with increased sensitivity to 2-DG (Fig. 3C). Similarly, CCI-779 did not increase the sensitivity to 2-DG in a hepatoma mutant cell line deficient in HIF-1β (c4), whereas 2-DG sensitivity was augmented with CCI-779 treatment under hypoxia in its wild-type cell counterpart (Hepa-1; Fig. 4A and B). Moreover, in the two NSCLC lines studied, CCI-779 did not increase sensitivity to 2-DG under hypoxia (where HIF-1α is barely detectable), whereas in the four SCLC lines (where HIF is expressed), it did (Fig. 4C). Interestingly, in all three models in which HIF was nonfunctional under hypoxia, CCI-779 not only failed to increase sensitivity to 2-DG but displayed a slight protective effect. Overall, the results in the HIF+ and HIF− models presented above support the interpretation that CCI-779 increases the sensitivity to 2-DG in hypoxic tumor cells by down-regulating HIF-1α.

In Cells in which HIF Is Absent, Inhibiting mTOR Results in Reduced 2-DG Toxicity

Surprisingly, in cells unable to mount a HIF response, cotreatment with CCI-779 and 2-DG resulted in decreased rather than increased toxicity (as shown above in Fig. 4C). In this figure, a 20% to 25% decrease in cell death was observed in NSCLC cell lines growing under hypoxia (in which HIF is not detectable) when CCI-779 was combined with 2-DG as compared with 2-DG alone. This phenomenon was also observed in cells in which HIF was inactive either through siRNA or a mutation in HIF-1β (C4 cell line). A possible explanation for this result could be that inhibition of mTOR with CCI-779 stimulates an energy conservation effect that somewhat decreases 2-DG–induced ATP depletion under hypoxia. Indeed, it has previously been shown that blockage of mTOR by rapamycin in Lewis lung carcinoma cells growing under prolonged conditions of hypoxia (5 days) results in the conservation of energy as measured by increased ATP (21). In order to determine whether such activity of CCI-779 could account for the reduced cytotoxic effects of 2-DG in the conditions in which HIF is inactive, ATP levels were measured. As compared with 2-DG treatment alone, CCI-779 was found to increase ATP levels under hypoxia by 26% and 18% in the NSCLC cell line A treated with 2-DG at 4 and 6 mmol/L, respectively (Fig. 5A). Similarly, CCI-779 increases ATP levels by 34% and 25% in HIF− mutant hepatoma (C4) cell lines, respectively, as compared with cells treated with 2-DG alone (Fig. 5B). Overall, these data further support the idea that CCI-779 reduces the cytotoxic effect of 2-DG in cells in which HIF is inactive by increasing ATP levels.

Discussion

Previously, it was shown that treatment with siRNA specific for HIF-1α increased sensitivity to 2-DG, which could be explained by correspondingly decreasing amounts of hexokinase, the enzyme which 2-DG interacts with to block glycolysis (5). Because the techniques for effectively applying siRNA in patients remain to be elucidated, it was not clear how this information could be used clinically. However, CCI-779, which inhibits the translation of HIF-1α

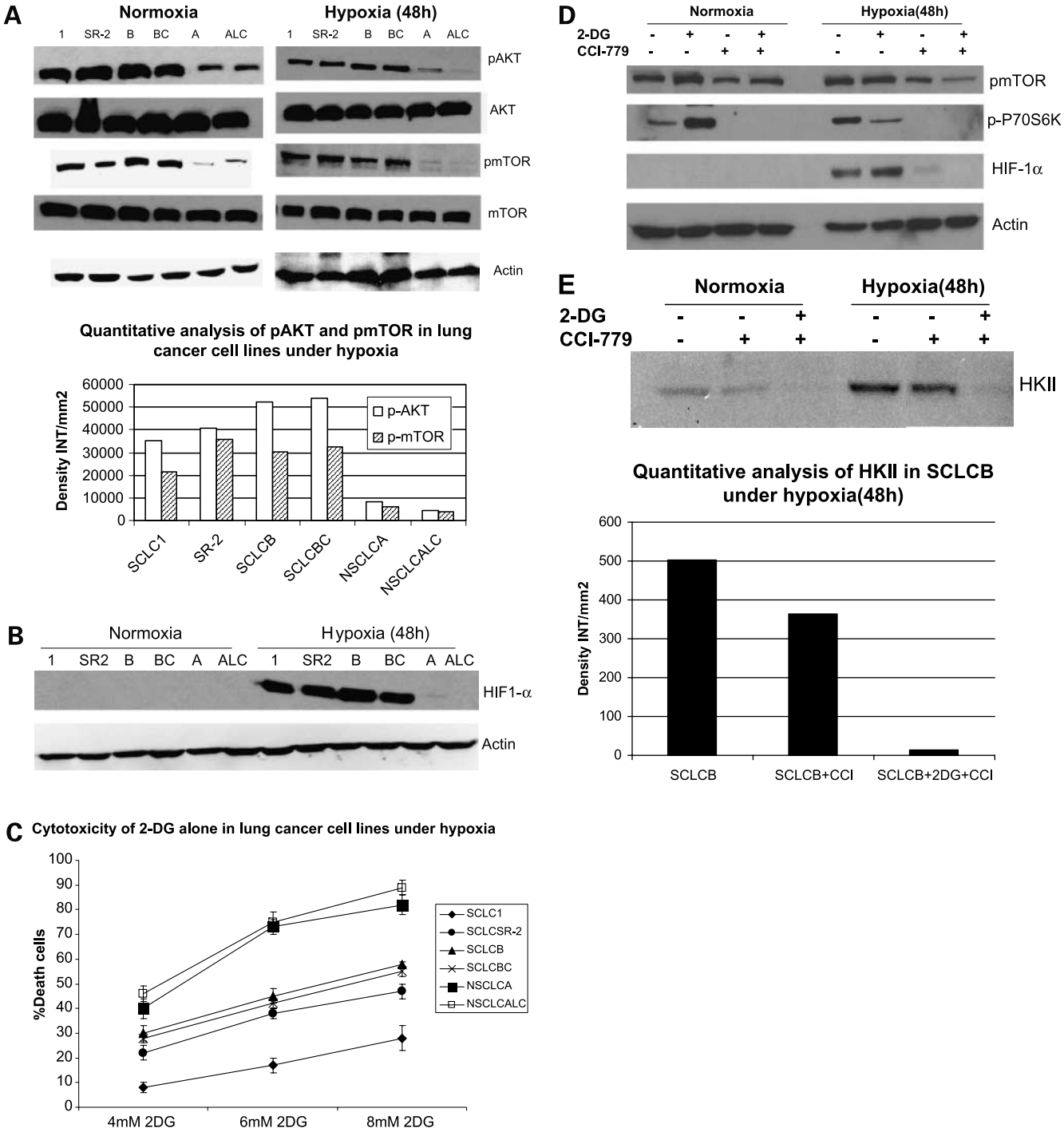

Figure 1. Decreased AKT and mTOR activity in lung cancer cell lines correlates with reduced levels of HIF-1α and increased sensitivity to 2-DG under hypoxia. **A,** Western blots and quantitative analysis of pAKT and pmTOR levels in lung cancer cell lines grown under normoxia or hypoxia for 48 h. Under normoxia as well as hypoxia, two NSCLC lines displayed intrinsically low levels of pAKT and pmTOR as compared with four SCLC lines. **B,** immunoblots of cells grown under normoxic or hypoxic conditions for 48 h showed increased HIF-1α in those cell lines expressing high levels of pAKT and pmTOR (SCLC lines), whereas in the NSCLC lines which expressed lower levels of pAKT and pmTOR, HIF-1α was barely detectable. **C,** low levels of HIF-1α in NSCLC lines correlated with increased sensitivity to 2-DG under hypoxia (48 h). *Points,* average of triplicate samples with SD ($P < 0.05$). **D,** immunoblots of pmTOR, p-P70S6K, and HIF-1α in SCLC line B treated with 4 mmol/L of 2-DG or 0.1 μg/mL of CCI-779 alone and in combination under both normoxic and hypoxic conditions for 48 h. Note that under hypoxic conditions, CCI-779 down-regulates HIF-1α and when 2-DG is added, HIF-1α is completely eliminated. **E,** immunoblot of HKII expression in SCLC line B when treated with 0.1 μg/mL of CCI-779 alone or in combination with 4 mmol/L of 2-DG under 48 h of normoxia and hypoxia. Quantitative analysis indicates that HKII is decreased by 30% when SCLC line B under hypoxia is treated with CCI-779 alone and further reduced when cotreated with 2-DG (4 mmol/L).

by blocking mTOR, has recently been approved for patient use as an anticancer agent. Therefore, this compound could have activity as a HIF inhibitor and, when applied clinically, could thereby increase tumor cell sensitivity to 2-DG under hypoxic conditions. Indeed in Fig. 1D and E, we show that under hypoxia, CCI-779 blocks mTOR activity which in turn leads to the down-regulation of HIF-1α and HKII. It should be noted that mTOR activity is measured by the phosphorylated levels of its downstream effector molecule P70S6K. Thus, loss of the phosphorylated form of this protein when cells are treated with CCI-779 indicates the blockage of mTOR activity.

Moreover, the levels of HIF-1α were further reduced when 2-DG was combined with CCI-779 in SCLC line B (Fig. 1D). Thus, although the mechanism remains unclear, it seems that by lowering ATP levels with 2-DG in hypoxic

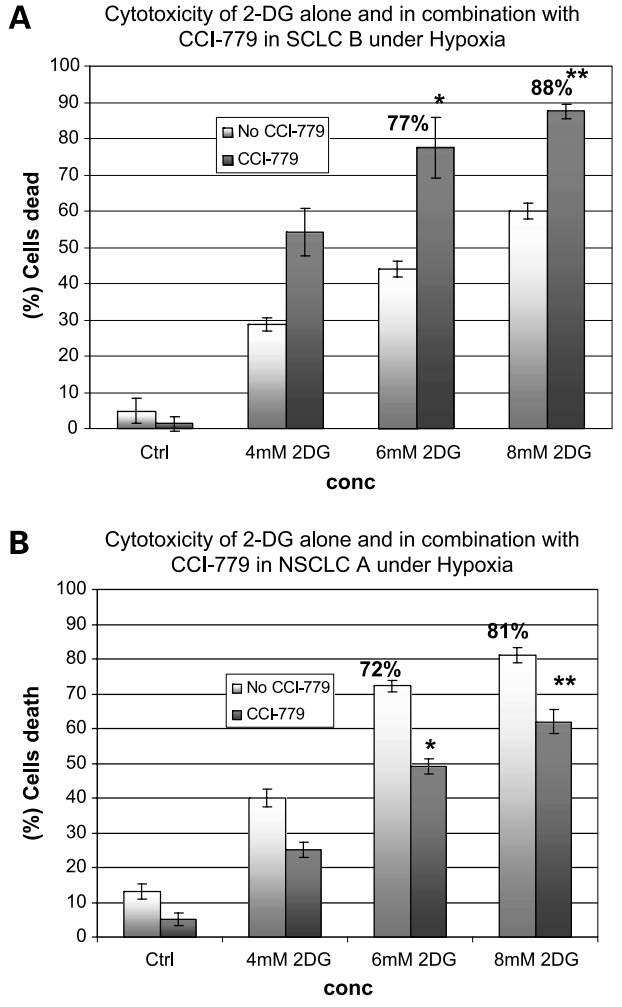

Figure 2. Blocking mTOR with CCI-779 correlates with down-regulation of HIF-1α and increased sensitivity to 2-DG. **A** and **B,** under hypoxia, CCI-779 increased the cytotoxicity of 2-DG in SCLC line B, which expresses high levels of pmTOR, but not in NSCLC line A, which expresses low levels of pmTOR. *Columns,* average of triplicate samples; *bars,* SD (*, $P = 0.002$; **, $P = 0.004$).

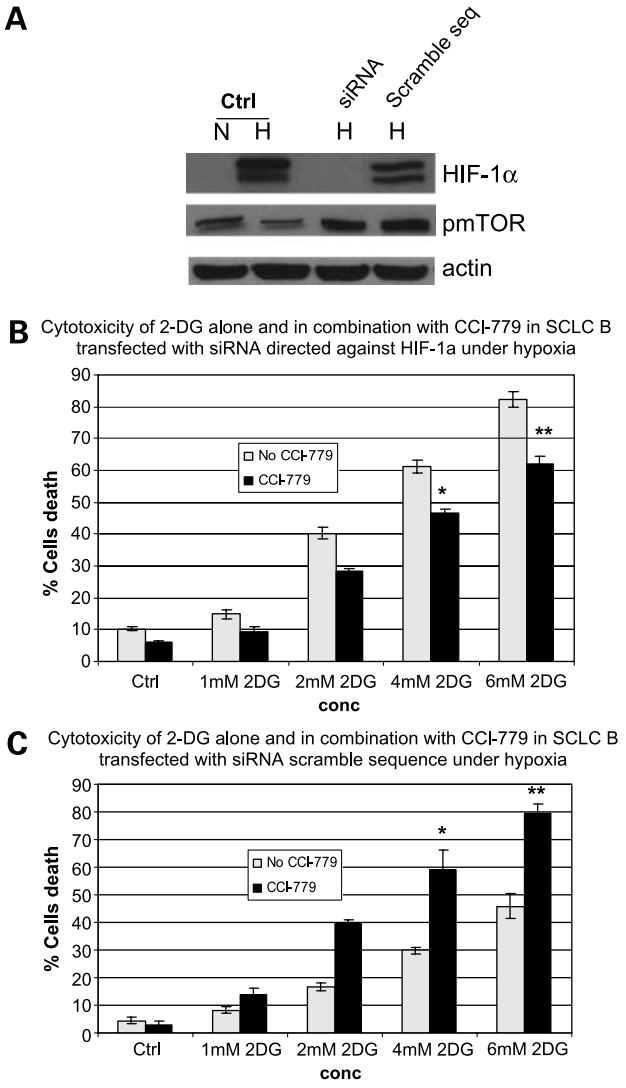

Figure 3. In cells in which HIF-1α is down-regulated by siRNA, CCI-779 does not increase the toxicity of 2-DG in hypoxic cells. **A,** Western blot analysis of SCLC line B treated under normoxic (*N*) and hypoxic (*H*) conditions confirming the effectiveness of siRNA in down-regulating HIF-1α. **B** and **C,** under hypoxia, CCI-779 does not increase 2-DG toxicity in cells transfected with siRNA directed against HIF-1α. In contrast, CCI-779 increases 2-DG toxicity in cells treated with scrambled RNA. *Columns,* average of triplicate samples; *bars,* SD (*, $P = 0.07$; **, $P = 0.016$).

cells as previously shown (5), favorably affects the effectiveness of CCI-779 in further down-regulating HIF-1α (and perhaps other mTOR targets). This idea is supported by previous results which showed that inadequate levels of ATP trigger AMP-activated protein kinase phosphorylation which in turn suppresses mTOR activity in cells under hypoxia (22–24). As can be seen in Supplemental Data S1,[3] 2-DG increases the phosphorylation levels

[3] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

of AMP-activated protein kinase which correlates with the attenuation of mTOR activity as reflected by reduced levels of p-P70S6K. Overall, the lowering of HIF-1α as a consequence of cotreatment with 2-DG and CCI-779 corresponds with increased toxicity by 2-DG when CCI-779 (at a nontoxic dose) is combined with this glycolytic inhibitor in hypoxic tumor cells (Figs. 2A, 3C, and 4A). Thus, CCI-779 may prove to be useful in the clinical setting as an anti-HIF agent for increasing the effectiveness of 2-DG in killing the hypoxic cell population found in most solid tumors.

Hexokinase, the enzyme which 2-DG interacts with to block glycolysis, has been shown to be reduced when HIF is inhibited by siRNA (5). This result was found to correlate with increased toxicity of 2-DG in cells under hypoxia (5). Here, we find similarly that when HIF is inhibited via blockage of mTOR by CCI-779, HKII is reduced by 30% (Fig. 1E). Interestingly, when 2-DG is combined with CCI-779, a further decrease in HKII is observed. A possible explanation for this result is that the binding of 2-DG to HKII leads to the dissociation of this enzyme from its normal mitochondrial localization, hence, resulting in cytosolic degradation (25). Additionally, we find that HKII is reduced in hypoxic cells treated with 2-DG alone (data not shown), which further supports the possibility that binding of HKII by 2-DG lowers its levels. Recently, it has been reported that HIF-1α increases mitochondrial respiration in cells under low oxygen tension (26). This latter result presents the possibility that HIF-1α may render hypoxic cells resistant to glycolytic inhibitors by increasing mitochondrial ATP production. The contributions of

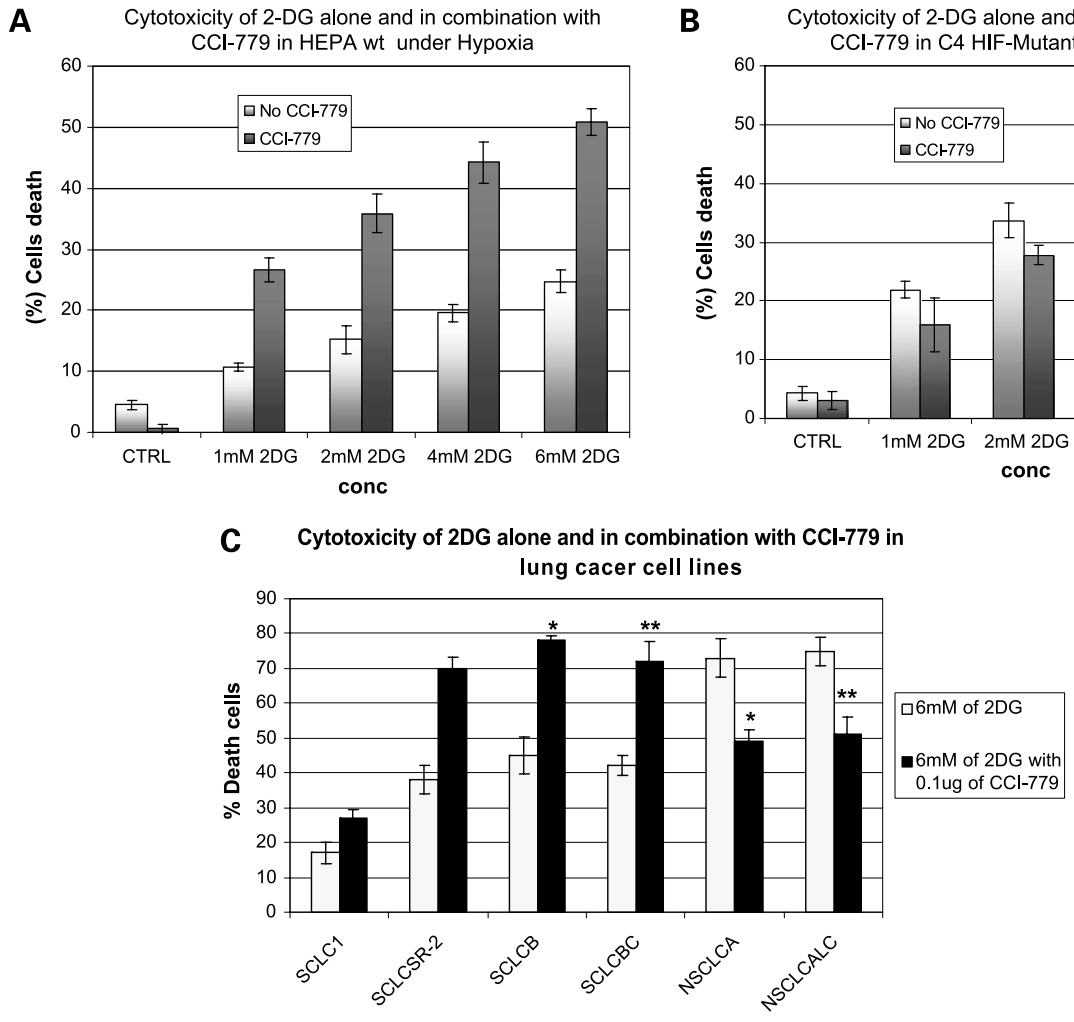

Figure 4. In mutant cells in which HIF-1α is nonfunctional, CCI-779 does not increase 2-DG toxicity under hypoxic conditions. **A** and **B,** cytotoxicity assays in HEPA (HIF+) and C4 (HIF−) cells after exposure to various doses of 2-DG and 0.1 μg/mL of CCI-779 under hypoxic conditions for 48 h. Note the increase in cell death after treatment with 2-DG in combination with CCI-779 in the HEPA ($P < 0.02$; **A**) cell line that expresses functional HIF but not in the C4 HIF-deficient ($P < 0.05$; **B**) mutant cell line. **C,** cytotoxicity assays in four SCLC vs. two NSCLC lines. Note that in NSCLC cells which do not express HIF under hypoxia, cotreatment with CCI-779 and 2-DG results in decreased toxicity. *Columns,* average of triplicate samples; *bars,* SD (*, $P = 0.001$; **, $P = 0.002$).

HIF-induced increases in hexokinase and efficiency to use oxygen in hypoxic cells for conferring resistance to glycolytic inhibitors are currently being investigated in our laboratory to determine the importance of each.

It has been shown that blocking mTOR with rapamycin or its analogues leads to suppression of the translation of numerous proteins (27–31). In order to address whether the increased sensitivity to 2-DG by treatment with CCI-779 is indeed due to the down-regulation of HIF-1α, cells transfected with siRNA directed against HIF-1α as well as a HIF− mutant cell line were cotreated with these two agents. The results, which show that CCI-779 did not increase the sensitivities of these cell lines to 2-DG when grown under hypoxia, clearly implicate the down-regulation of HIF as the primary mechanism by which CCI-779 augments 2-DG toxicity in wild-type cells.

Interestingly, mTOR activity was found to be highly expressed intrinsically in four lung cancer cell lines which corresponded with high HIF-1α levels and relative resistance to 2-DG when cells were grown under hypoxia (Fig. 1C). In contrast, mTOR activity was significantly lower in two lung cancer cell lines correlating with barely or nondetectable levels of HIF-1α (Fig. 1B) and increased sensitivity to 2-DG under hypoxic conditions (Fig. 1C). Furthermore, the level of resistance to 2-DG in the cell lines overexpressing pAKT, pmTOR, and HIF-1α could be overcome by cotreating them with CCI-779 (Fig. 4C). Similar to the experiments described above for the HIF− mutant and HIF knockdown cells, when the lung cancer cell lines expressing extremely low or nondetectable levels of HIF protein were cotreated with CCI-779, increased sensitivity to 2-DG was not observed (Figs. 2B, 3B, and 4B). Thus, the data from lung cancer cell lines support our findings with hepatoma, which indicate that down-regulation of HIF is the mechanism by which CCI-779 sensitizes hypoxic cells to glycolytic inhibitors. Additionally, the amount of 2-DG–induced cell death by CCI-779 in HIF+ cells compares favorably with the amount of cell death in HIF− mutant, HIF-1α siRNA knockdown cells, and lung cancer cell lines intrinsically deficient in HIF-1α when treated with 2-DG alone. Therefore, these results highlight the importance of HIF in attenuating tumor cell sensitivity to glycolytic inhibitors, which is in agreement with our previous findings (5).

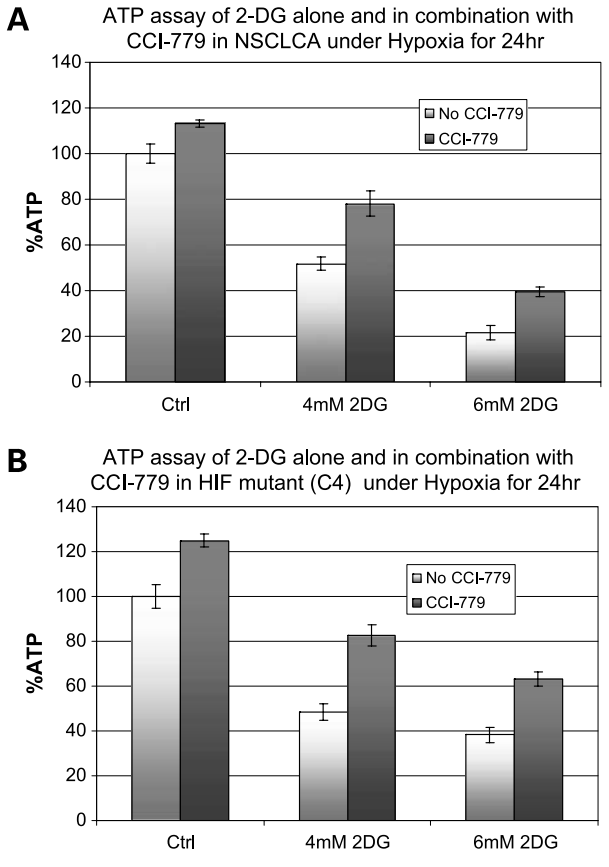

Figure 5. In cells unable to mount a HIF response, CCI-779 increases the resistance to 2-DG which correlates with increased ATP levels. **A** and **B,** ATP assays in NSCLCA and C4 cells after exposure to various doses of 2-DG and 0.1 μg/mL of CCI-779 under hypoxic conditions for 24 h. Note the increase in ATP levels in both NSCLC line A (**A**) and hepatoma (HIF−) cell line C4 (**B**), after treatment with CCI-779. *Columns,* average of triplicate samples; *bars,* SD ($P < 0.05$).

From the data presented in this article, it seems that NSCLC are fundamentally different from SCLC in that they have lower mTOR and AKT activity, as well as reduced HIF expression under hypoxia. However, in another study in which 110 NSCLC tissue samples were evaluated from patients, it was noted that pAKT levels were expressed in 51% of these patients (32). Thus, our findings of differences in pAKT between NSCLC and SCLC seem to be due to small sampling. It is clear, however, that intrinsic sensitivity to 2-DG under hypoxia correlates with lower levels of this upstream pathway of mTOR. Moreover, in the SCLC cell lines in which we find increased levels of pAKT, inhibiting mTOR with CCI-779 is more effective in increasing sensitivity to 2-DG than in cells that express very low levels of this oncogenic protein. Increases in pAKT have been implicated in many different tumors and it has been suggested that cancer cells overexpressing this signaling molecule are more sensitive to mTOR inhibitors compared with those expressing lower levels (19, 33). Taken together, intrinsic differences in pAKT, pmTOR, and HIF found in lung cancer tumors could dictate future decisions on whether to use CCI-779 in combination with 2-DG when treating patients with either of these types of cancer.

A possible disadvantage of using mTOR inhibitors as anticancer agents directed at killing aerobically proliferating cells is that rapamycin has been shown to protect Lewis lung carcinoma cells from hypoxic-induced death (21). The explanation for this effect is that under hypoxia, mTOR is not completely shutdown, and therefore, the addition of rapamycin will further block the function of this protein resulting in lowered energy consumption and thereby increased survival. If this is a general phenomenon of rapamycin, then this agent would be expected to decrease 2-DG toxicity in hypoxic cells by a similar mechanism. Because, however, we find that CCI-779 increases 2-DG

toxicity in hypoxic cells by decreasing HIF, it seems that the role of this agent in attenuating HIF supercedes its effects on the conservation of energy resulting in greater inhibition of glycolysis and overall cell death. This idea may also explain our observation that when CCI-779 is applied to cells deficient in HIF, their survival from 2-DG under hypoxia is slightly increased (Figs. 2B and 4B) via its activity as an energy conserver, which is reflected in the increased ATP levels (Fig. 5A and B). In support of this data are the results in which the level of 2-DG toxicity in SCLC treated with siRNA against HIF-1α went down by ~15% to 20% when CCI-779 was added (Fig. 3B).

Our findings that the combination of CCI-779 with 2-DG increases killing of hypoxic cells suggest that 2-DG may be an important additive when rapamycin and/or its analogues are to be used to treat patients with cancer. Moreover, our data indicate that the addition of mTOR inhibitors to clinical protocols that include 2-DG should increase the killing of the hypoxic tumor cell population by decreasing HIF.

Disclosure of Potential Conflicts of Interest

T.J. Lampidis: Threshold Pharmaceuticals consultant/scientific advisory board. The other authors reported no potential conflicts of interest.

References

1. Semenza GL. Hypoxia, clonal selection, and the role of HIF-1 in tumor progression. Crit Rev Biochem Mol Biol 2000;35:71 – 103.

2. Wang GL, Semenza GL. General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia. Proc Natl Acad Sci U S A 1993;90:4304 – 8.

3. Vaupel P. The role of hypoxia-induced factors in tumor progression. Oncologist 2004;9:10 – 7.

4. Maxwell PH, Pugh CW, Ratcliffe PJ. The pVHL-hIF-1 system. A key mediator of oxygen homeostasis. Adv Exp Med Biol 2001;502:365 – 76.

5. Maher JC, Wangpaichitr M, Savaraj N, et al. Hypoxia-inducible factor-1 confers resistance to the glycolytic inhibitor 2-deoxy-D-glucose. Mol Cancer Ther 2007;6:732 – 41.

6. Sabatini DM. mTOR and cancer: insights into a complex relationship. Nat Rev Cancer 2006;6:729 – 34.

7. Thomas GV, Tran C, Mellinghoff IK, et al. Hypoxia-inducible factor determines sensitivity to inhibitors of mTOR in kidney cancer. Nat Med 2006;12:122 – 7.

8. Land SC, Tee AR. Hypoxia-inducible factor 1α is regulated by the mammalian target of rapamycin (mTOR) via an mTOR signaling motif. J Biol Chem 2007;282:20534 – 43.

9. Mamane Y, Petroulakis E, LeBacquer O, et al. mTOR, translation initiation and cancer. Oncogene 2006;25:6416 – 22.

10. Abraham RT. mTOR as a positive regulator of tumor cell responses to hypoxia. Curr Top Microbiol Immunol 2004;279:299 – 319.

11. Gingras AC, Raught B, Gygi SP, et al. Hierarchical phosphorylation of the translation inhibitor 4E-BP1. Genes Dev 2001;15:2852 – 64.

12. Wouters BG, van den Beucken T, Magagnin MG, et al. Control of the hypoxic response through regulation of mRNA translation. Semin Cell Dev Biol 2005;16:487 – 501.

13. Tang H, Hornstein E, Stolovich M, et al. Amino acid-induced translation of TOP mRNAs is fully dependent on phosphatidylinositol 3-kinase-mediated signaling, is partially inhibited by rapamycin, and is independent of S6K1 and rpS6 phosphorylation. Mol Cell Biol 2001;21:8671 – 83.

14. Jefferies HB, Fumagalli S, Dennis PB, et al. Rapamycin suppresses 5′TOP mRNA translation through inhibition of p70s6k. EMBO J 1997;16: 3693 – 704.

15. Hay N, Sonenberg N. Upstream and downstream of mTOR. Genes Dev 2004;18:1926 – 45.

16. Pause A, Belsham GJ, Gingras AC, et al. Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5′-cap function. Nature 1994;371:762 – 7.

17. Savaraj N, Wu C, Wangpaichitr M, et al. Overexpression of mutated MRP4 in cisplatin resistant small cell lung cancer cell line: collateral sensitivity to azidothymidine. Int J Oncol 2003;23:173 – 9.

18. Xu J, Tyan T, Cedrone E, et al. Detection of 11q13 amplification as the origin of a homogeneously staining region in small cell lung cancer by chromosome microdissection. Genes Chromosomes Cancer 1996;17:172 – 8.

19. Wu C, Wangpaichitr M, Feun L, et al. Overcoming cisplatin resistance by mTOR inhibitor in lung cancer. Mol Cancer 2005;4:25.

20. Wood SM, Gleadle JM, Pugh CW, et al. The role of the aryl hydrocarbon receptor nuclear translocator (ARNT) in hypoxic induction of gene expression. Studies in ARNT-deficient cells. J Biol Chem 1996; 271:15117 – 23.

21. Hamanaka Y, Mukai M, Shimamura M, et al. Suppression of PI3K/mTOR pathway rescues LLC cells from cell death induced by hypoxia. Biochem Biophys Res Commun 2005;330:318 – 26.

22. Kimura N, Tokunaga C, Dalal S, et al. A possible linkage between AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) signalling pathway. Genes Cells 2003;8:65 – 79.

23. Sarbassov DD, Ali SM, Sabatini DM. Growing roles for the mTOR pathway. Curr Opin Cell Biol 2005;17:596 – 603.

24. Kimball SR. Interaction between the AMP-activated protein kinase and mTOR signaling pathways. Med Sci Sports Exerc 2006;38: 1958 – 64.

25. Robey RB, Hay N. Akt, hexokinase, mTOR: targeting cellular energy metabolism for cancer therapy. Drug Discov Today 2005;2:239 – 46.

26. Semenza GL. Oxygen-dependent regulation of mitochondrial respiration by hypoxia-inducible factor 1. Biochem J 2007;405:1 – 9.

27. Crespo JL, Hall MN. Elucidating TOR signaling and rapamycin action: lessons from *Saccharomyces cerevisiae*. Microbiol Mol Biol Rev 2002;66: 579 – 91, table of contents.

28. Dennis PB, Fumagalli S, Thomas G. Target of rapamycin (TOR): balancing the opposing forces of protein synthesis and degradation. Curr Opin Genet Dev 1999;9:49 – 54.

29. Easton JB, Kurmasheva RT, Houghton PJ. IRS-1: auditing the effectiveness of mTOR inhibitors. Cancer Cell 2006;9:153 – 5.

30. Rubio-Viqueira B, Hidalgo M. Targeting mTOR for cancer treatment. Adv Exp Med Biol 2006;587:309 – 27.

31. Janus A, Robak T, Smolewski P. The mammalian target of the rapamycin (mTOR) kinase pathway: its role in tumourigenesis and targeted antitumour therapy. Cell Mol Biol Lett 2005;10:479 – 98.

32. Balsara BR, Pei J, Mitsuuchi Y, et al. Frequent activation of AKT in non-small cell lung carcinomas and preneoplastic bronchial lesions. Carcinogenesis 2004;25:2053 – 9.

33. Dancey JE. Therapeutic targets: MTOR and related pathways. Cancer Biol Ther 2006;5:1065 – 73.