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Research Paper

Response and determinants of cancer cell susceptibility to PI3K inhibitors

Combined targeting of PI3K and Mek1 as an effective anticancer strategy

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Abbreviations: PI3K, phosphatidylinositide-3-kinase; PIP3, inositol 1,4,5-trisphosphate; mTOR, target of rapamycin; PTEN, phosphatase and tensin homolog; Mek1, mitogen activated protein kinase kinase 1; ERK, extracellular signal-regulated kinase; PARP, Poly (ADP-ribose) polymerase; 17-HWT, 17-hydroxywortmannin; DMSO, dimethyl sulfoxide; PBS, phosphate buffered saline; IC_{50}, inhibition concentration 50

Key words: PI3K, Ras, Mek1, kinase inhibitor, anticancer

While small molecule inhibitors of the phosphatidylinositide-3-kinase (PI3K) are expected to impact the development of new cancer therapy, the tumor types and underlying cellular pathways determining inhibitor response remain poorly defined. In this report, we have studied anti-proliferative effects of the PI3K inhibitors WAY-266176 and WAY-266175 in a panel of histologically diverse cancer cells. Inactivation of PI3K caused potent growth suppression in some cells (MDA468, BT549, MDA361, MCF7, LNCap, PC3MM2) but minimal suppression in others (MDA231, MDA435, DU145, HCT116, A549), which correlated with a differential down-regulation of cyclin D1, c-Myc, and induction of apoptosis. A heightened PI3K/AKT/mTOR signaling was linked to the sensitive phenotype but did not generally predict inhibitor response. Interestingly, the resistant cells all displayed an elevated phospho-ERK that remained elevated after serum deprivation. In HCT116 cells, activation mutations in the PI3K catalytic subunit PIK3CA and Ki-Ras correlated with a resistant phenotype, which was partially sensitized by homologous replacement with the wild-type Ki-Ras but not by deletion of cellular PTEN. Depletion of Mek1 via siRNA in resistant cells enhanced PI3K inhibitor-induced growth suppression. Moreover, a profoundly augmented growth suppression and apoptosis were achieved in resistant cells by combination treatment with WAY-266176/WAY-266175 and Mek1 kinase inhibitor CI-1040 or UO126. The combination therapy efficiently inhibited mitogenic signaling and reduced expression of cyclin D1 and c-Myc. Our results identify deregulation of the Ras/Raf/Mek/ERK pathway as a dominant determinant in cancer cell resistance to PI3K inhibitors and highlight combined targeting of PI3K and Mek1 as an effective anticancer strategy.

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Introduction

The class-1a PI3-kinases (PI3K) catalyze the production of lipid second messenger phosphatidylinositol-3,4,5-triphosphate (PIP3), which is required for activation of a wide range of cellular functions of growth and survival.[1] The serine/threonine kinase AKT and mTOR are essential mediators of the PI3K signaling pathways. AKT and mTOR collectively confer dominant survival signal and promote cell growth via respective downstream substrates that directly participate in apoptosis, cell cycle progression, and cellular translation.[2-4] Deregulated PI3K/AKT/mTOR signaling pathway is widely implicated in human cancer (reviewed in refs. 5–7). These alterations include the mutational activation of the p110α catalytic subunit of PI3K (PIK3CA),[8-11] loss of the tumor suppressor gene PTEN, a PIP3 phosphatase that inhibits the PI3K signaling (reviewed in refs. 5 and 7), as well as constitutive PI3K expression and/or AKT activation in numerous cancer types.[12-18] Together, these clinical data establish a central involvement of the PI3K pathway in tumor growth and resistance to chemotherapy.

While small molecule inhibitors of the PI3K/AKT/mTOR pathways are expected to impact the development of novel cancer therapy, the tumor types and underlying complexity of the biochemical pathways determining inhibitor response remain poorly defined. It has been previously suggested that the mTOR inhibitor rapamycin and analogs are particularly effective against a subset of tumors that harbor PTEN-deficiency and/or heightened AKT activation.[19-21] Similarly, the known PI3K inhibitors appear to kill certain cancer cells with activated PI3K/AKT status.[22-24] Wortmannin-derived PI3K inhibitors PWT-458 and PX-866 efficiently suppress PI3K signaling in vivo and elicit promising anticancer efficacy in preclinical xenograft tumors harboring the PTEN loss or constitutive PI3K activation.[25,26] Furthermore, a dual inhibitor of PI3K and mTOR elicits a greater antitumor efficacy in the PTEN-negative glioma.[27] On the other hand, many cancer cell lines are intrinsically resistant to mTOR inhibition by rapamycin analogs, and the classical PI3K inhibitors are not known as broad spectrum inhibitors of cancer cell growth. Intriguingly, a heightened AKT activation fails to sensitize ovarian tumor cells that are already resistant to mTOR inhibitors.[28] Thus, while these previous observations highlighted a

critical dependence on the PI3K/AKT/mTOR pathway in a subset of cancers, the activity status of the pathway per se may not generally predict inhibitor response in broad tumor types.

It is well known that malignant tumors typically acquire numerous genetic alterations to support tumor growth, and cooperation among various redundant biochemical pathways may differentially determine the outcome of PI3K inhibitor therapy. Hence, biochemical and molecular understanding of the cancer cell sensitivity and resistance is necessary in development of effective PI3K inhibitor therapies. In this report, we have studied the functional effects of new PI3K inhibitors in a panel of histologically diverse cancer cells. We found that while the phosphorylation of AKT was completely suppressed in all tested cell lines, potent inhibition of growth was only observed in a subset of cancer cells. A heightened PI3K/AKT activation was linked to the sensitive phenotype but did not generally predict inhibitor response. We have identified the aberrant Ras/Raf/Mek/ERK signaling as a dominant determinant of cancer cell resistance to PI3K inhibitors and highlight combined targeting of PI3K and Mek1 pathways as an effective anticancer therapy.

Results

WAY-266176 and WAY-266175 are potent and covalent inhibitors of PI3K in vitro and in vivo. As we recently reported,[32] WAY-266176 and WAY-266175 (WAY-176 and WAY-175) are furan ring-opened 17-HWT irreversible inhibitors of PI3Kα and isoforms. Both inhibitors are >20-fold selective over mTOR and are inactive against a panel of protein kinases. Importantly, the serum half-life for 17-HWT, WAY-176 and WAY-175 are 0.3 h, 2.3 h and >24 h, respectively. The sharply improved serum stability of WAY-176/WAY-175 resulted in sustained PI3K suppression and anticancer efficacy in cell culture and in tumor model in vivo.[32] In the Rat1-IGF1 stimulation model, both WAY-176 and WAY-175 potently inhibited PI3K signaling (Fig. 1A). To confirm that these inhibitors covalently target PI3K in cancer cells, we performed inhibitor wash-out assay with WAY-176, 17-HWT and a reversible inhibitor LY294002 in the PTEN-negative U87MG glioma cells. Inhibitors were added to cells for 1 h and then washed out for 1 h and 3 h. Immunoblot analysis indicated that while all three inhibitors suppressed PI3K signaling markers, the phosphorylated forms of AKT, GSK3, S6K1 and 4EBP1, at the end of 1 h drug incubation, no inhibitory effect was seen for LY294002 in the wash-out samples (Fig. 1B). In contrast, both wash-out samples of WAY-176- and 17-HWT-treated cells retained a nearly complete (1 h wash-out) and substantial (3 h wash-out) suppression of PI3K signaling markers (Fig. 1B). A similarly retained inhibitory activity in the wash-out assay was seen for WAY-175 (not shown). Thus, like 17-HWT, WAY-176/WAY-175 covalently inhibited cellular PI3K. A single bolus IV injection of WAY-176 (5 mg/kg) into nude mice bearing U87MG glioma xenografts resulted in a rapid and sustained suppression of PI3K signaling in the tumor for at least 6 h (Fig. 1C).

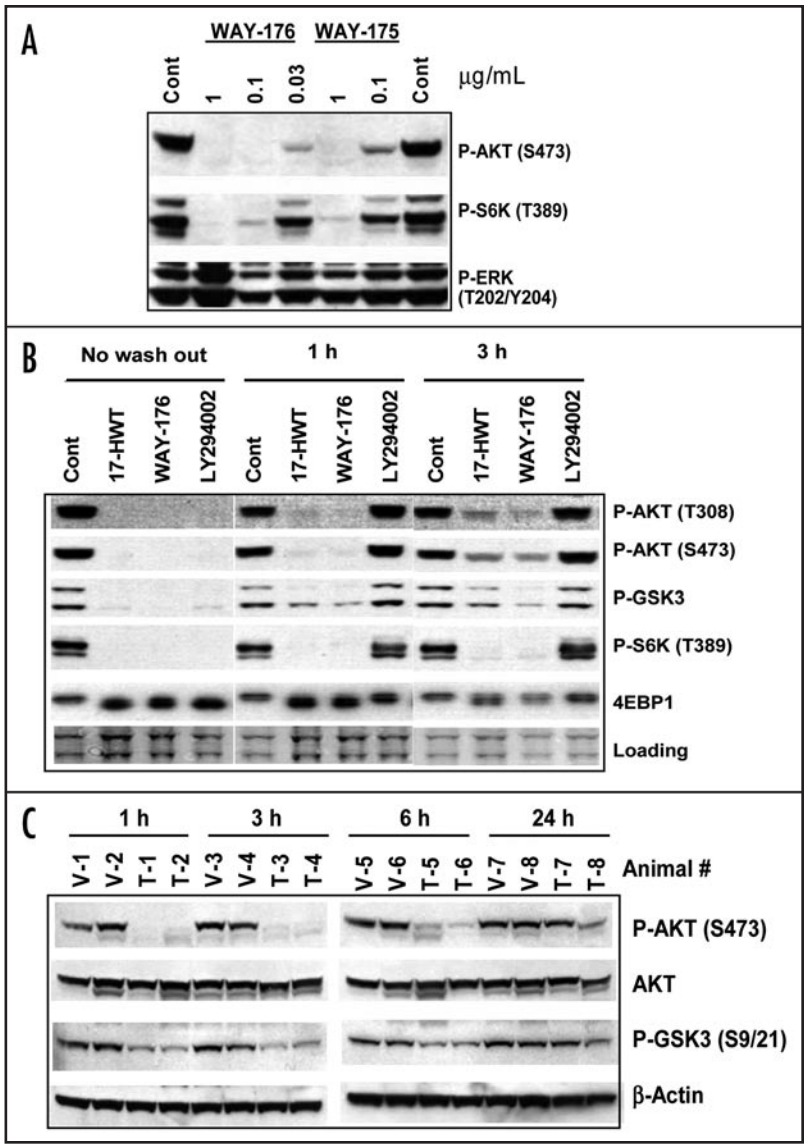

Figure 1. WAY-176 and WAY-175 are potent and covalent inhibitors of PI3K in vitro and in vivo. (A) Rat1 cells were plated in 6-well culture plates, serum starved for 24 h and stimulated with IGF1 without or with the indicated doses of WAY-176 and WAY-175. Total lysates were prepared and subjected to immunoblotting analysis with P-AKT (S473), P-S6K (T389), and P-ERK (T202/Y204) as described in Materials and Methods. (B) U87MG cells in full growth medium were treated with the indicated inhibitors for 1 h and harvested immediately (no wash out). In parallel sets, treated cells were washed thoroughly and incubated without inhibitors for additional 1 h (1 h) or 3 h (3 h). Lysates were analyzed similarly as (A) with P-AKT (S473, T308), P-GSK3 (S9/21) and 4EBP1. (C) Nude mice bearing U87MG xenograft tumors were dosed with vehicle (PBS) or 5 mg/kg WAY-176. At the indicated times tumors were dissected. Tumor lysates were prepared as described in Materials and Methods, and subjected to immunoblotting analysis with P-AKT (S473), AKT, and P-GSK3 (S9/21). V, vehicle-treated; T, WAY-176-treated.

Together, these data further establish WAY-176 and WAY-175 as potent and covalent inhibitors of PI3K in vitro and in vivo.

Activation of the PI3K/AKT signaling pathway contributes to susceptibility to PI3K inhibitors. To gain insight into the tumor types that might be particularly susceptible to PI3K inhibitors, we studied a panel of commonly used tumor cell lines. Expression of PTEN, phospho-AKT and phospho-S6K1, two prominent signaling

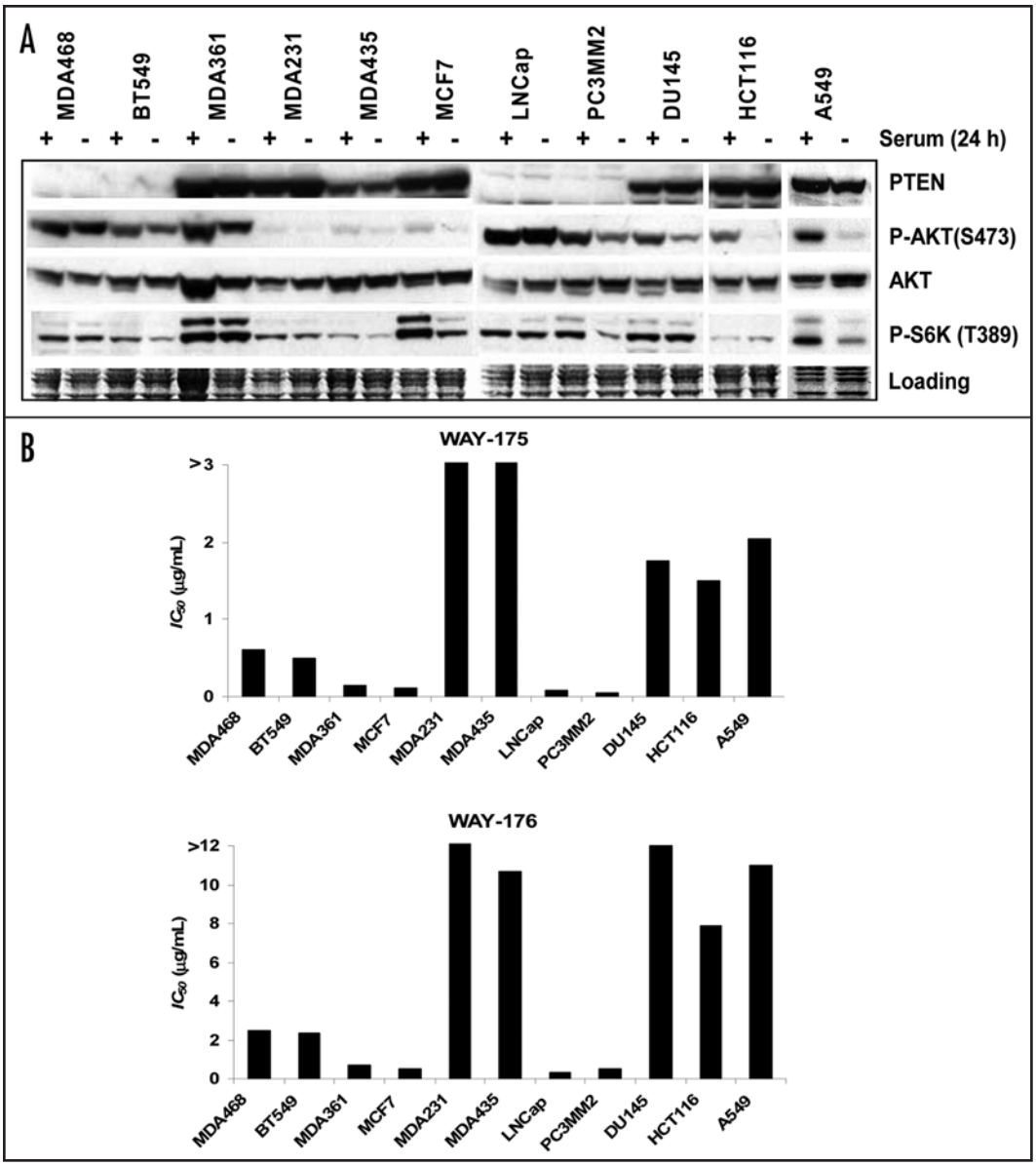

Figure 2. PI3K/AKT/mTOR pathway deregulation correlates with susceptibility to PI3K inhibitors. (A) Indicated cancer cell lines were plated in 10 cm culture plates for 24 h, then washed and refed with fresh media with or without 10% serum for 24 h. Total lysates were prepared as described in Materials and Methods. Equal amounts of total lysates were subjected immunoblotting with PTEN, P-AKT (S473), AKT, and P-S6K (T389). (B) Indicated cancer cell lines were plated in 96-well culture plates at predetermined cell density (1,000 to 10,000 cells per well) for 1 day, treated with vehicle or various doses of WAY-176 and WAY-175 (0.06-15 μg/mL) for 3 days. Growth inhibitions were determined as described in Materials and Methods. IC_{50} values were determined (mean of triplicate) and plotted.

markers of the PI3K/AKT/mTOR pathway, were analyzed by immunoblotting using total cellular lysates prepared from serum starved- and serum-stimulated cells (Fig. 2A). Growth inhibition IC_{50} values of WAY-176 and WAY-175 against each cell line were determined in a 4-day growth assay in which cells were exposed to various concentrations of the inhibitors once on day 1 (Fig. 2B). While WAY-175 was more potent than WAY-176 in growth assays due to its superior serum stability, both inhibitors displayed a similar inhibition profile with variable IC_{50} values ranging from low ng/mL to high μg/mL (Fig. 2B). Interestingly, the sensitive cells included those breast and prostate lines possessing functional loss of PTEN (MDA468, BT549, LNCap and PC3MM2) or mutational activation of the PI3K catalytic subunit PIK3CA (MDA361, MCF7).[10] The resistant cells included breast MDA231, melanoma MDA435, prostate DU145, colon HCT116 and lung A549. Notably, these cells are not known to harbor genetic alterations in the PI3K/AKT pathway except for HCT116, which contains the PIK3CA mutation.[33] It is also noteworthy that the sensitive MCF7 expressed a low level of phospho-AKT, whereas resistant DU145, HCT116 and A549 expressed moderate levels of phospho-AKT that were reduced upon serum deprivation. Thus, a heightened PI3K/AKT signaling is linked to the sensitive phenotype but does not generally predict inhibitor response.

Differential modulation of cell cycle factors and death markers in sensitive versus resistant cells. To study the cellular mechanism that may account for the differential sensitivity, we next performed immunoblot analysis on a subset of sensitive (LNCap, MDA361, PC3MM2) and resistant (DU145, MDA231, HCT116) cells after exposure to various concentrations of WAY-176, WAY-175 and LY294002 (5 μg/mL) for 6 h (Fig. 3). Phospho-AKT was downregulated in all cells indicating that PI3K was suppressed in both the sensitive and resistant lines (Fig. 3A). Significantly, we observed a rapid and nearly complete or substantial inhibition of cyclin D1 and c-Myc in the sensitive LNCap and MDA361 cells in response to WAY-176, WAY-175 and LY294002, which correlated with the inhibition of phospho-AKT (Fig. 3A; Fig. 3B), left and middle panels). In contrast, both cyclin D1 and c-Myc were poorly or partially inhibited in the resistant DU145 and MDA231 cells even at higher inhibitor concentrations, which did not correlate with the inhibition of phospho-AKT (Fig. 3A; Fig. 3B, left and middle panels). Since both cyclin D1 and c-Myc are well established positive regulators of growth and proliferation, the observed differential suppression of these proteins may be critical factors underlying the differential susceptibility of cancer cells to PI3K inhibitors. It was further shown that PI3K inhibition in LNCap and MDA361 cells resulted in a rapid

induction of apoptosis as indicated by the caspase-mediated PARP cleavage in these cells 6 h post exposure to the inhibitors (Fig. 3B, right panel). Inhibition of PI3K in the resistant DU145 and MDA231 cells did not cause PARP cleavage (Fig. 3B, right panel). We conclude from these data that although the PI3K biochemical pathway is inactivated by the inhibitors in all studied cell lines, suppression of growth in the sensitive cancer cells is accompanied by a rapid and potent inhibition of cell cycle factors and induction of apoptosis.

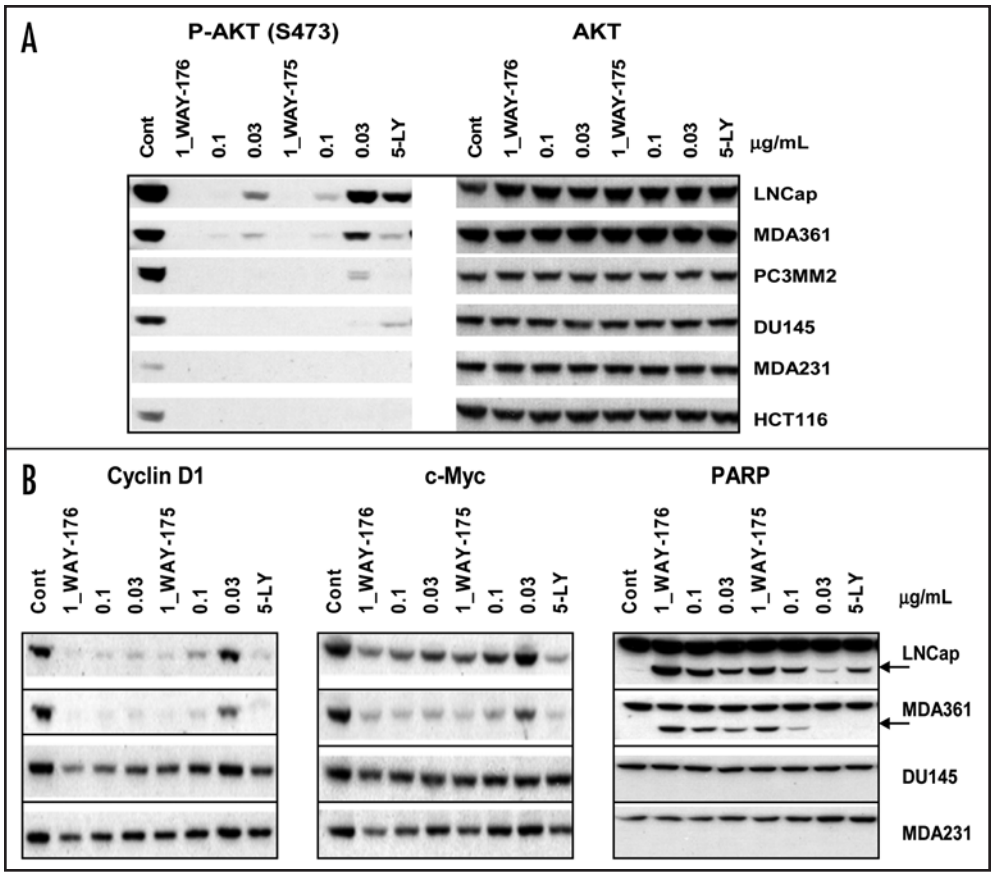

Figure 3. Differential modulation of cell cycle factors and apoptosis in the sensitive versus resistant cancer cells. (A) Indicated cell lines were plated in 6-well culture plates for 24 h, treated with the indicated doses of WAY-176, WAY-175 and LY294002 in full growth media for 6 h. Total lysates were prepared in LDS lysis buffer and subjected to immunoblotting with P-AKT (S473) and AKT. (B) Total lysates as in (A) were subjected to immunoblotting with cyclin D1, c-Myc and PARP. Arrows indicate the PARP fragment resulted from caspase-mediated cleavage.

Resistant cancer cells display constitutively phosphorylated ERK and are sensitized by homologous replacement of mutant Ki-Ras with the wild-type Ki-Ras but not by PTEN deletion. Our data thus far suggested a link between deregulated PI3K/AKT signaling and susceptibility to PI3K inhibitors in certain cancer cells. However, the relatively poor suppression of growth in the PIK3CA mutant HCT116 as well as the lack of correlation of phospho-AKT with sensitivity in several additional cell lines indicated that the activity status of the PI3K pathway per se does not predict sensitivity (Fig. 2). The Ras/Raf/Mek/ERK signaling is frequently activated in colon and other major cancers, and may influence cellular response to PI3K inhibition. To test this possibility we conducted immunoblot analysis of the expression and phosphorylation status of ERK in the same panel of cell lines (Fig. 4A). We found that the resistant MDA231, MDA435, DU145, HCT116 and A549 all exhibited an elevated phospho-ERK that remained elevated even after serum deprivation. In contrast, the sensitive MDA361, MCF7, BT549, LNCap and PC3MM2 all expressed low or barely detectable levels of phospho-ERK. Notably, the sensitive MDA468 expressed high phospho-ERK that was down-regulated upon serum deprivation. The data in Figure 4A indicate that in addition to the PI3K pathway activation, the activity status of cellular Ras/Raf/Mek/ERK is inversely correlated with sensitivity to PI3K inhibitors. While the mechanism for elevated phospho-ERK in DU145 cells is not known, MDA231, MDA435, HCT116 and A549 are known to harbor mutations in components of the Ras/Raf/Mek/ERK pathway.[33] We next compared HCT116 with its isogenic line HKH, in which the mutant Ki-Ras is replaced by the wild-type gene.[30] WAY-175 dose response data from four independent assays demonstrated a 4.7-fold reduction in IC_{50} in the HKH cells relative to HCT116 cells (Fig. 4B, left panel) indicating that mutant Ras plays a role in the cellular resistance. As expected, HKH expressed a reduced level of phospho-ERK as compared with HCT116 (Fig. 4B, right panel). Interestingly, WAY-175/WAY-176 and LY294002 all substantially inhibited c-Myc expression in the Ras-wild-type HKH cells but not in the Ras-mutant HCT116 cells even at higher inhibitor concentrations (Fig. 4B, right panel). To assess the effects of PTEN status on sensitivity in these cells, we conducted similar assays on the HCT116-derived isogenic PTEN-knockout cell pair (Fig. 4C, left panel). Surprisingly, in contrast to the observation with the restoration of wild-type Ras, WAY-175 dose response obtained with CR259 cells ($PTEN^{-/-}$) was nearly identical to that of CR208 cells ($PTEN^{+/+}$, vector control) (Fig. 4C, right panel). Thus, we conclude from the data in Figure 4 that deregulation of the Ras/Raf/Mek/ERK signaling significantly reduces susceptibility of the cancer cells to PI3K inhibitors.

Depletion of Mek1 sensitizes resistant cancer cells to PI3K inhibitors. To further define the role of Ras/Raf/Mek/ERK signaling in resistance to PI3K inhibitors, we depleted cellular Mek1, a key effector kinase that phosphorylates ERK, via siRNA in the resistant DU145, HCT116 and A549 cells. A substantial reduction (DU145 and HCT116) or a nearly complete knockdown (A549) of Mek1 expression could be confirmed (Fig. 5A). Both the control siRNA- and the Mek1 siRNA-transfected cells were analyzed for growth inhibition response after exposure to various doses of WAY-175 and WAY-176. Depletion of Mek1 significantly sensitized these cells to WAY-175 (Fig. 5B). The reduction in WAY-175 IC_{50} values in the

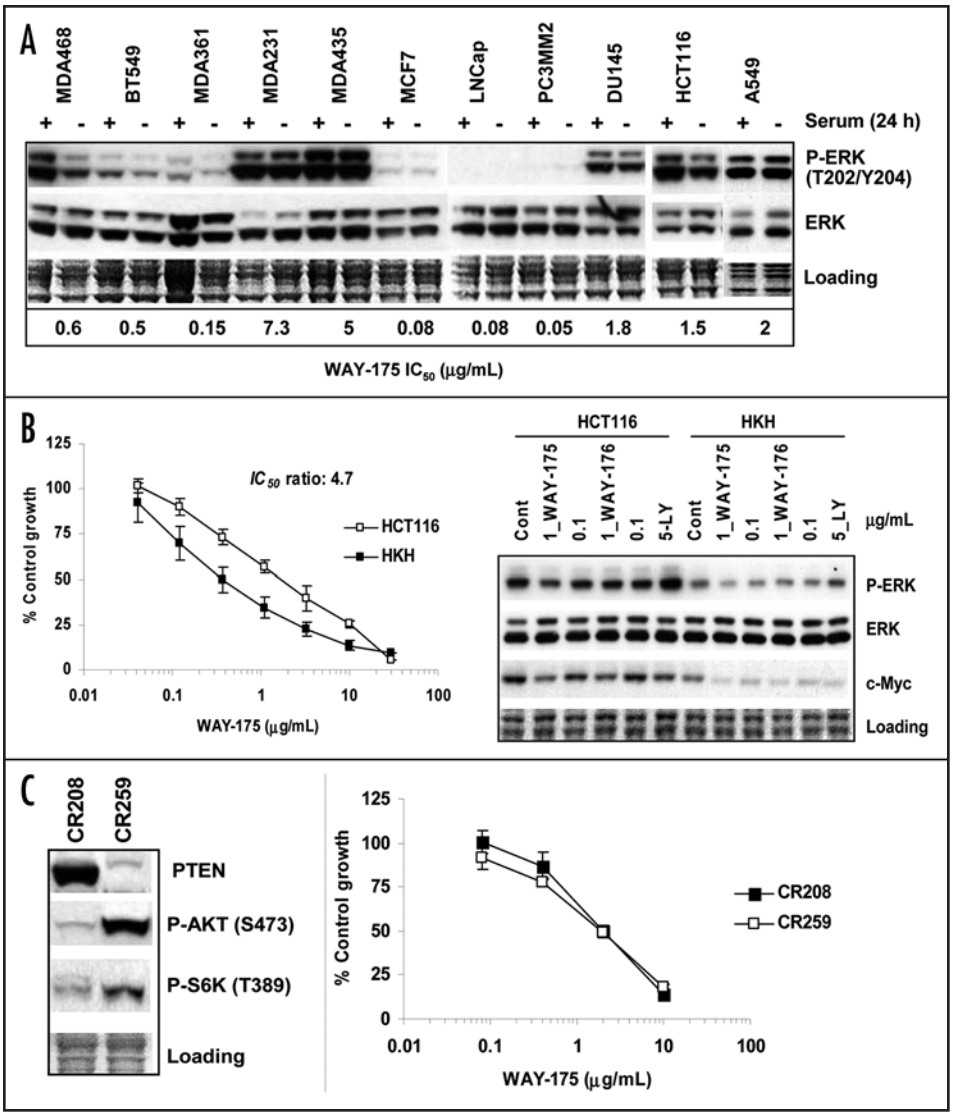

Figure 4. Deregulated Ras/Raf/Mek/ERK pathway confers cellular resistance to PI3K inhibitors. (A) Total lysates of the indicated cancer cells were subjected to immunoblotting with P-ERK (T202/Y204) and ERK. WAY-175 IC_{50} values of the corresponding cell lines are listed below the blot image. (B) Left panel, growth inhibition curves of the isogenic HCT116 (Ras mutant) versus HKH (Ras wild-type) in response to WAY-175. Cells were plated in 96-well plates for 1 day, treated with vehicle or various doses of WAY-175 for 3 days. Growth inhibitions were assayed as described in Materials and Methods. Mean of four experiments are shown. Right panel, immunoblotting of HCT116 and HKH cell lysates, harvested after 16 h treatment with PI3K inhibitors, with P-ERK, ERK and c-Myc. (C) Left panel, immunoblotting of the isogenic CR208 ($PTEN^{+/+}$) and CR259 ($PTEN^{-/-}$) cell lysates with PTEN, P-AKT (S473), and P-S6K (T389). Right panel, growth inhibition curves of CR208 versus CR259. Cells were plated, treated, and analyzed similarly as in (B).

Mek1 depleted cells relative to the control cells were 6.6 fold in DU145 (Fig. 5B, left panel), 18.3 fold in HCT116 (Fig. 5B, middle panel), and 27.2 fold in A549 (Fig. 5B, right panel). A similar sensitization was seen for WAY-176 (not shown).

A profoundly augmented growth suppression and apoptosis induced by combination treatment with kinase inhibitors of Mek1 and PI3K. Because deregulation of the Ras/Raf/Mek/ERK pathways is prevalent in major solid cancers and induces resistance to the PI3K inhibitor therapy, we next studied the combination anti-tumor activity employing kinase inhibitors of Mek1 and PI3K (Fig. 6). In HCT116 cells, cotreatment with a suboptimal dose of the Mek1 inhibitor UO126 (5 μg/mL) or CI-1040 (1 μg/mL) resulted in a dramatic sensitization to growth suppression by WAY-175 (Fig. 6A, left panel). The normalized IC_{50} values for WAY-175, WAY-175+UO126 and WAY-175+CI-1040 were 1.7 μg/mL, 0.11 μg/mL and 0.09 μg/mL, respectively. The Mek1 inhibitors similarly sensitized response to WAY-176 (Fig. 6A, right panel). The normalized IC_{50} values for WAY-176, WAY-176+UO126 and WAY-176+CI-1040 were 7.6 μg/mL, 0.53 μg/mL and 0.47 μg/mL, respectively. Similar results were obtained with additional cell lines of colon, lung, breast and prostate (not shown). Cell cycle analysis indicated that while a suboptimal dose of either WAY-175 (1 μg/mL) or WAY-176 (7 μg/mL) alone increased cells in G_0/G_1, the corresponding combinations with CI-1040 (1 μg/mL) resulted in a substantial rise in the sub-G_1 population (Fig. 6B). To determine the mode of cell death, we measured caspase 3 activity in the cells treated with the single inhibitors or various combinations (Fig. 6C). While a suboptimal dose of UO126 (5 μg/mL) or CI-1040 (1 μg/mL) alone was inactive, both Mek1 inhibitors greatly enhanced the levels of active caspase 3 when combined with WAY-175 (Fig. 6C, left panel) or WAY-176 (Fig. 6C, right panel) at 24 h post treatment. Together these data demonstrate a profoundly augmented suppression of growth and rapid induction of apoptosis resulting from a combined targeting of Mek1 and PI3K.

Inhibition of mitogenic signaling by combined targeting of PI3K and Mek1. We wished to examine the effects of combination therapy on cellular mitogenic signaling markers that may reflect the enhanced suppression of growth. We performed immunoblot analysis of HCT116 cells that were treated singly with a low dose of WAY-175 (0.1 μg/mL), CI-1040 (1 μg/mL) or combination of the two inhibitors (Fig. 7A). As expected CI-1040 alone inhibited phospho-ERK but had minimal effect on the signaling markers of the PI3K/AKT/mTOR pathway. WAY-175 alone inhibited phospho-AKT but only weakly inhibited the phosphorylation of S6K1, 4EBP1 and GSK3, and caused minimal suppression of cyclin D1 and c-Myc. Interestingly, combination treatment with CI-1040 and WAY-175 resulted in a further reduction in the phosphorylated forms of S6K1, 4EBP1 and GSK3. These biochemical changes were accompanied by substantially reduced expression of both cyclin D1 and c-Myc (Fig. 7A). We also examined the protein levels of cyclin D1 and c-Myc in the control- and Mek1-siRNA transfected cells without or with 6 h exposure to the low dose WAY-175 (Fig. 7B). It

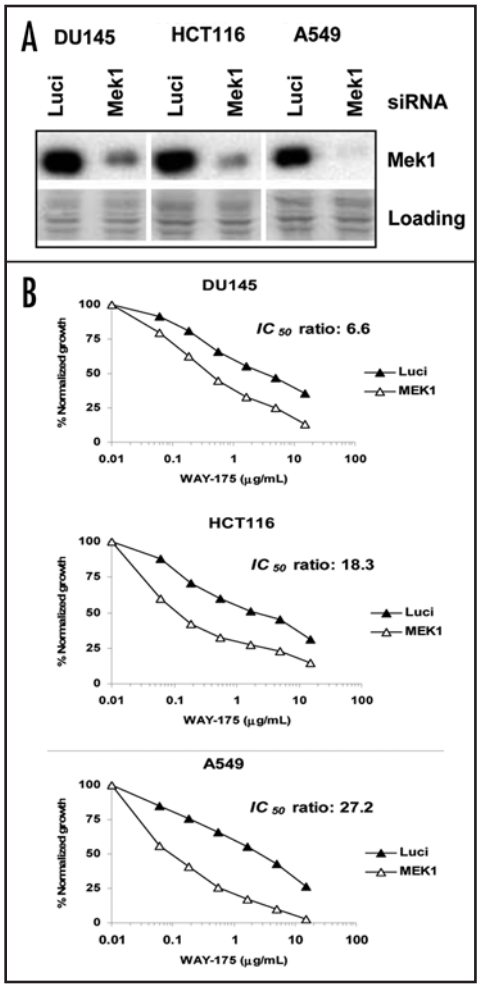

Figure 5. Depletion of Mek1 sensitizes resistant cancer cells to PI3K inhibition. (A) Cells of DU145, HCT116 and A549 were transfected with the luciferase control siRNA or Mek1 siRNA for 3 days. Total lysates were subjected to immunoblotting with Mek1. (B) One day after siRNA transfection, cells were replated in 96-well plates for 2 days, treated with various doses of WAY-175 for additional 3 days. Growth inhibitions were assayed as described in Materials and Methods.

was apparent that 0.1 μg/mL of WAY-175 caused greater suppression of both cyclin D1 and c-Myc in the Mek1-depleted DU145 (Fig. 7B, left panel), HCT116 (Fig. 7B, middle panel) and A549 (Fig. 7B, right panel) as compared with the corresponding WAY-175-treated control cells. Taken together, the results in Figures 6 and 7 strongly indicate that in the resistant cells, combined targeting of Mek1 and PI3K can efficiently inactivate mitogenic signaling pathways and suppress cell cycle factors cyclin D1 and c-Myc, which are likely critical for the broad sensitization to the combination therapy.

Discussion

Due to its prevalent deregulation in human cancer, the PI3K signaling pathway represents a rational target for cancer treatment. WAY-176 and WAY-175 are potent PI3K inhibitors with promising in vivo anticancer efficacy.[32] In this report, we have employed WAY-176 and WAY-175 to study cancer cell response to PI3K inhibition. We found that while phospho-AKT was efficiently inhibited in all tested cells, potent growth suppression only occurred in a subset of cancer cells. Consistent with the notion of "pathway addiction", several cell lines harboring mutations in components of the PI3K/AKT/mTOR pathway were highly susceptible to these inhibitors as indicated by potent suppression of growth and rapid induction of apoptosis. WAY-176, WAY-175 and LY294002 all substantially decreased expression of cyclin D1 and c-Myc in the sensitive LNCap and MDA361 cells, whereas a much weaker suppression of these proteins was observed in the resistant DU145 and MDA231 cells. These data suggest that expression of cyclin D1 and c-Myc in these cells is dependent on PI3K, and may be relevant biomarkers for growth inhibitory response to PI3K inhibitors. While majority of the sensitive cell lines displayed a heightened PI3K/AKT signaling, the phospho-AKT levels did not always correlate with susceptibility to PI3K inhibition. Interestingly, HCT116 cells that contain activation mutations in both PIK3CA and Ki-Ras displayed a relatively resistant phenotype, which was not sensitized by deletion of cellular PTEN. Thus, while these findings together further establish the dependence of PI3K signaling for cell growth in certain cancer cells, the activity status of the PI3K pathway per se will not generally predict inhibitor response.

It is apparent from the current study that deregulated Ras/Raf/Mek/ERK plays an important role in cellular resistance to PI3K inhibitors. We observed that the resistant cells all displayed an elevated and constitutively phosphorylated ERK presumably resulting from mutations and/or deregulation in components of the Ras/Raf/Mek/ERK pathway.[33] In HCT116 cells activation mutation in the Ki-Ras appeared to be dominant over the activation of PI3K in determining response. Consistent with this assessment, homologous replacement of mutant Ras in these cells with the wild-type Ras enhanced sensitivity, whereas deletion of cellular PTEN did not. The enhanced growth suppression in the Ras-wild-type HKH cells correlated with a substantial reduction in c-Myc expression. The role of Ras/Raf/Mek/ERK pathway in resistance was further supported by the knockdown studies on Mek1, a key effector kinase of the pathway. siRNA mediated depletion of Mek1 in resistant DU145, HCT116 and A549 cells uniformly sensitized these cells for growth inhibition by PI3K inhibitors, which was accompanied by a further decrease in the levels of cyclin D1 and c-Myc compared to the control cells. Previous studies on the mTOR inhibitors have linked PTEN-loss or heightened AKT activation to inhibitor sensitivity.[19-21] Interestingly, an activated AKT fails to sensitize ovarian tumor cells that are already resistant to mTOR inhibitor rapamycin.[28] Thus, these observations collectively highlight redundant biochemical pathways of PI3K and Ras/Raf/Mek/ERK in deregulation of key cell cycle machinery that may critically determine a poor growth inhibitory response to PI3K pathway inhibitors.

Oncogenic mutations in the Ras genes are highly common in human malignancies, and can often lead to deregulated signaling of both Raf/MEK/ERK and PI3K pathways.[34,35] It is well established that these biochemical pathways cooperate to promote tumor growth, survival, and resistance to chemotherapy.[36-38] In the current study, combination treatment with the kinase inhibitors of PI3K and Mek1 resulted in an augmented suppression of growth in the PI3K inhibitor-resistant HCT116 colon cells. Further studies revealed a similar sensitization in additional colon cell lines and other tumor types including breast, prostate and lung (not shown). Biochemical studies indicated that the combination therapy

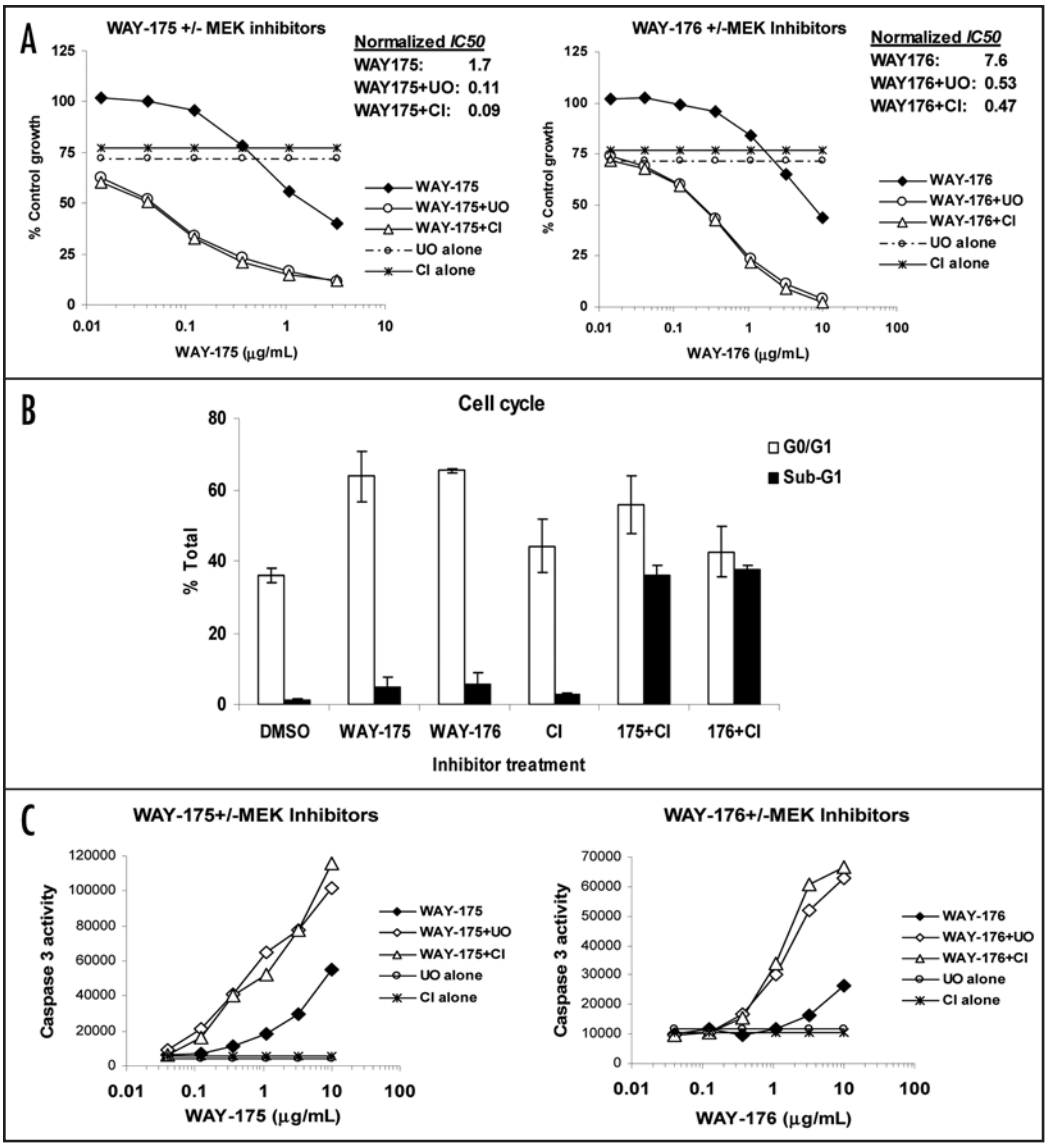

Figure 6. Augmented growth suppression and apoptosis induced by combination treatment with kinase inhibitors of Mek1 and PI3K. (A) HCT116 growth inhibition curve in response to WAY-175 (left panel) or WAY-176 (right panel) in combination with Mek1 inhibitors. Cells were plated in 96-well plates (1,000 cells per well) for 1 day, treated with vehicle, various doses of WAY-175 or WAY-176 alone, or with CI-1040 (1 μg/ml) or UO126 (5 μg/ml). A set of wells were treated with CI-1040 or UO126 alone. Cells were exposed to the inhibitors for 3 days. Growth inhibitions were assayed as described in Materials and Methods. For calculation of normalized IC_{50} values, growth inhibitions in the combination treatment groups were normalized against the value obtained with the corresponding Mek1 inhibitor alone. (B) Cell cycle analysis. HCT116 cells were treated for 24 h with vehicle, WAY-175 (1 μg/ml), WAY-176 (7 μg/ml), CI-1040 (1 μg/ml), combination of WAY-175 and CI-1040, or combination of WAY-176 and CI-1040. Treated cells were harvested and analyzed for cell cycle profile as described in Materials and Methods. Percent of cells in G_0/G_1 and sub-G_1 are plotted. (C) Caspase assays of cells treated with inhibitors. HCT116 cells were plated and treated similarly as in (B). Caspase 3/7 activities were measured 24 h later as described in Materials and Methods.

potentiated dephosphorylation of several key components of mitogenic signaling including phospho-S6K1, phospho-4EBP1 and phospho-GSK3, which together negatively impact cellular machinery of protein synthesis and gene transcription. Lastly, we found that the levels of cyclin D1 and/or c-Myc were substantially down-regulated in resistant cancer cells in response to combination therapy. Together, these observations provide cellular and biochemical evidence supporting combination treatment with inhibitors of PI3K and Mek1 in broad tumor types.

In summary, results from the current study have provided insight into the cellular and biochemical pathways that regulate cancer cell response to PI3K inhibitor therapy. These findings will be useful in future development of clinical strategies that target PI3K and Raf/Mek/ERK pathways for cancer treatment.

Materials and Methods

Chemicals. The natural product wortmannin was obtained from fermentation broths of the fungal culture ZIMV298 of the Wyeth microbial collection. 17-HWT, WAY-266175 and WAY-266176 were synthesized (as described previously in ref. 29). LY294002 and UO126 were obtained from CalBiochem. CI-1040 was synthesized by Wyeth Discovery Synthetic Chemistry. All chemicals used for buffers and assays were obtained from Sigma unless specified.

Cell culture. Cell lines of Rat1, U87MG, MDA-MB-468, BT549, MCF7, MDA-MB-361, MDA-MB-231, MDA-MB-435, LNCap, DU145, HCT116 and A549 were obtained from American Type Culture Collection (ATCC). PC3MM2 was obtained from Dr. Carolyn Discafani, Wyeth Discovery Oncology. The isogenic lines HCT116 (parent) and HKH (mutant Ki-Ras replaced with the wild-type Ki-Ras)[30] were obtained from Dr. Kim Arndt, Wyeth Discovery Oncology. The HCT116-derived isogenic PTEN knockout cell lines CR208 (vector control) and CR259 (PTEN$^{-/-}$) were kindly provided by Dr. Bert Vogelstein and Dr. Carlo Rago (Johns Hopkins University). All cells were maintained in a 37°C incubator with 5% CO_2, and were cultured using standard cell culture methods.

Mek1/2 depletion via RNA interference. DU145, HCT116 and A549 cells were plated in 10 cm culture dish at 30–50% confluence. One day after plating, cells were transfected with 80 nM siRNAs of the luciferase (control) or Mek1 (CCCGCAAUCCGGAACCAGA UCAUAA)[31] using lipofecatmine 2000 reagent (Invitrogen). Cells were replated 24 h later in the 96-well culture plates for growth assays (see below), or 6-well culture plates for immunoblotting at 72 h post transfection.

Tumor cell growth assays. Cells were plated in 96-well culture plates at 1000 to 3000 cells per well. One day following plating, cells were treated with various doses of WAY-266175 or WAY-266176 alone or with a low constant dose of CI-1040 (1 μg/mL) or UO126 (5 μg/mL). In all combination assays, the single and combination treatments were performed in the same 96-well plate along with control wells treated with vehicle (DMSO) or alone with the same dose of CI-1040 or UO126 included in combination. For assays of siRNA-transfected DU145, HCT116 and A549, cells were replated in 96-well plates 24 h post transfection. Inhibitors were added to cells 2 days after replating (3 days after transfection). Three days after exposure to inhibitors, viable cell densities were determined by MTS assay. The assays were performed using an assay kit (Promega) following the protocol supplied with the kit. The assay results were read in a 96-well plate reader by measuring absorbance at 490 nm. The effect of each treatment was calculated as percent of control growth relative to the vehicle DMSO-treated cells grown in the same culture plate.

Cell cycle analysis and apoptosis assay. For cell cycle analysis, cells were seeded in 96-well culture plates at 10,000 cells/well for 24 h. Cells were then exposed to various inhibitors for 24 h. Following treatment, cells were harvested, washed with PBS, and fixed overnight at -20°C in 70% ethanol. Cells were washed, stained with propidium iodide, and analyzed for cell cycle profile (acquired 5000 cells/well) on Guava PCA-96 instrument (Guava Technologies) according to the Guava Cell Cycle Protocol. Apoptosis was also measured 24 h post inhibitor treatment using Apo-ONE Caspase-3/7 assay kit (Cat # G7791, Promega). Assay mix was added to cells and incubated at room temperature for 3 h with gentle mixing using a plate shaker. The fluorescence of each well was read using the Victor plate reader (Wallac-PerkinElmer). Each experimental plate contains blank wells (without cells) as background fluorescence that was subtracted from all wells to obtain experimental values. Data are expressed as relative fluorescent units (RFLU) or as Apo-ONE units.

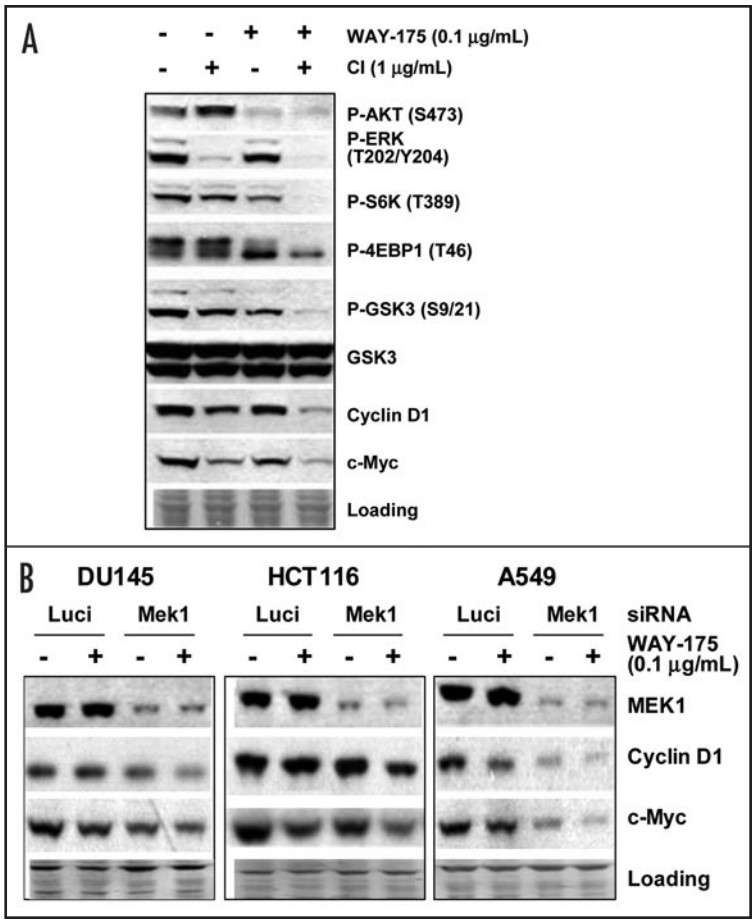

Figure 7. Combination therapy profoundly inhibits mitogenic signaling and cell cycle factors. (A) HCT116 cells were plated in 6-well plates for 24 h, treated with vehicle, the indicated doses of WAY-175, CI-1040, or combination for 16 h. Total lysates were prepared in LDS lysis buffer and subjected to immunoblotting analysis with P-AKT (S473), P-ERK (T202/Y204), P-4EBP-1 (T46), P-S6K (T389), P-GSK3 (S9/21), cyclin D1 and c-Myc. (B) Cells of DU145, HCT116 and A549 were plated and transfected with the indicated siRNAs similarly as in (Fig. 5A). After 3 days, cells were treated with WAY-175 for 6 h. Total lysates were subjected to immunoblotting with Mek1, cyclin D1, and c-Myc.

Protein lysates and immunoblotting. For total cell lysates of xenograft tumors and in vitro cultured tumor cells grown in serum-stimulated or serum-depleted conditions, cells from culture dishes or xenograft tumors (dissected and minced) were lysed in lysis buffer (25 mM Hepes, pH 7.5, 100 mM NaCl, 20 mM β-glycerophosphate, 1.5 mM $MgCl_2$, 0.5 mM EGTA, 0.25 mM EDTA 1% NP-40, 10 mM Na_3VO_4, 10 μg/ml Aprotinin, 10 μg/mL Leupeptin, 1 mM PMSF, 1 μM Microcystin LR and 0.1% 2-mercaptoethanol). Crude lysates were sonicated and clarified by centrifugation. For in vitro cell culture treatment with various inhibitors, total cellular lysates were prepared using NuPAGE-LDS sample buffer (Invitrogen). Crude lysates were sonicated and then clarified by centrifugation. Equal amounts of proteins were subjected to immunoblotting analysis using NuPAGE electrophoresis system (Invitrogen). Antibodies for PTEN, P-AKT (S473 and T308), total AKT, P-S6K (T389), P-GSK3 (S9/21), P-ERK (T202/Y204), P-4EBP1 (T46), 4EBP1, cyclin D1 and Mek1 were obtained from Cell Signaling Technology. Antibodies for c-Myc and PARP were obtained from Santa Cruz Biotechnology. β-actin antibody was from Chemicon International. Immunoblots were probed with primary and secondary antibodies following manufacturer's instructions and detected using enhanced chemiluminescence (Amersham Pharmacia).

PI3K inhibition in xenograft tumors. U87MG glioma cells were implanted subcutaneously in female nude mice. When tumor size reached 400 mm^3, they were randomized into treatment groups. The tumor bearing mice were dosed once with either PBS or WAY-266176 (formulated in PBS) intravenously (IV). At the indicated times post dosing, tumors were dissected. Total protein lysates were prepared, quantified, and subjected to immunoblotting (as described previously in ref. 25).

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