

# Rapamycin synergizes with the epidermal growth factor receptor inhibitor erlotinib in non-small-cell lung, pancreatic, colon, and breast tumors

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## Abstract

The receptor for epidermal growth factor (EGFR) is overexpressed in many cancers. One important signaling pathway regulated by EGFR is the phosphatidylinositol 3'-kinase (PI3K)-phosphoinositide-dependent kinase 1-Akt pathway. Activation of Akt leads to the stimulation of antiapoptotic pathways, promoting cell survival. Akt also regulates the mammalian target of rapamycin (mTOR)-S6K-S6 pathway to control cell growth in response to growth factors and nutrients. Recent reports have shown that the sensitivity of non-small-cell lung cancer cell lines to EGFR inhibitors such as erlotinib (Tarceva, OSI Pharmaceuticals) is dependent on inhibition of the phosphatidylinositol 3'-kinase-phosphoinositide-dependent kinase 1-Akt-mTOR pathway. There can be multiple inputs to this pathway as activity can be regulated by other receptors or upstream mutations. Therefore, inhibiting EGFR alone may not be sufficient for substantial inhibition of all tumor cells, highlighting the need for multipoint intervention. Herein, we sought to determine if rapamycin, an inhibitor of mTOR, could enhance erlotinib sensitivity for cell lines derived from a variety of tissue types (non-small-cell lung, pancreatic, colon, and breast). Erlotinib could inhibit extracellular signal-regulated kinase, Akt, and S6 only in cell lines that were the most sensitive. Rapamycin could fully inhibit S6 in all cell lines, but this was accompanied by activation of Akt phosphorylation. However, combination with erlotinib could down-modulate rapamycin-stimulated Akt activity. Therefore, in select cell lines, inhibition of both S6 and Akt was

achieved only with the combination of erlotinib and rapamycin. This produced a synergistic effect on cell growth inhibition, observations that extended *in vivo* using xenograft models. These results suggest that combining rapamycin with erlotinib might be clinically useful to enhance response to erlotinib. [Mol Cancer Ther 2006;5(11):2676–84]

## Introduction

The epidermal growth factor receptor (EGFR) is a member of the HER family of receptor tyrosine kinases that are overexpressed in a wide range of tumor types including non-small-cell lung cancer (NSCLC) and pancreatic, head and neck, and breast cancers (1–4). Erlotinib is an orally bioavailable low molecular weight inhibitor of the EGFR that has been approved for both NSCLC and pancreatic cancer (5, 6). Recent reports have shown that the sensitivity of cell lines to growth inhibition by EGFR inhibitors is dependent on the down-regulation of activity in the phosphatidylinositol 3'-kinase (PI3K)-Akt pathway. Akt activates both cell survival signals (through regulation of apoptosis signaling proteins including BAD and procaspase-9) and cell proliferative signals [through activation of the mammalian target of rapamycin (mTOR)-S6K-S6 pathway; ref. 7]. For NSCLC cell lines, inhibition of Akt activity correlated with sensitivity to EGFR inhibition, and Akt activity seemed to be mediated through EGFR trans-activation of HER3 in sensitive cell lines (8). We have recently found that Akt activity is also inhibited by erlotinib only in erlotinib-sensitive pancreatic tumor cell lines, where Akt activity is mediated through HER3 trans-activation by EGFR (9).

There can be multiple inputs to the Akt pathway such as other receptor tyrosine kinases including insulin-like growth factor-I receptor (IGF-IR; ref. 7). In addition to redundancies in growth factor receptor signaling, specific mutations or changes in the expression of regulatory proteins that affect signaling downstream of receptors can contribute to the lack of sensitivity to EGFR inhibitors (10–13). Therefore, inhibiting EGFR alone may not be sufficient for growth inhibition of all tumor cells, and this highlights the usefulness of multipoint intervention (14, 15).

Rapamycin is a high molecular weight polyketide that inhibits signaling downstream of Akt by blocking select functions of mTOR. However, there are two regulatory loops that may limit the efficacy of rapamycin as a single agent: (a) there is the potential for feedback inhibition whereby down-regulation of mTOR-S6K signaling by rapamycin activates the IRS-PI3K pathway to promote Akt signaling; (b) mTOR can form two different complexes within the cell, a mTOR-GβL-raptor complex and a mTOR-GβL-rictor complex. Only

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the mTOR-GβL-raptor complex is sensitive to inhibition by rapamycin (16–20). Blockade of mTOR interactions with raptor by rapamycin could shift the equilibrium toward the rictor-bound complex, promoting an increase in Akt phosphorylation. Therefore, rapamycin can be thought of as possessing two counteractive activities toward tumor cell growth, one that inhibits cell proliferation and one that activates prosurvival signals.

The synergistic behavior of rapamycin combined with EGFR inhibitors to block tumor cell growth has previously been described for two tumor types harboring specific classes of mutations: renal cell carcinoma and glioblastoma multiforme (21–23). Both renal cell carcinoma and glioblastoma multiforme are unique in that they are characterized by a high frequency of specific mutations that result in activation of Akt in a growth factor-independent manner, tuberous sclerosis complex mutations and EGFR-vIII mutations, respectively. Such studies highlight the potential for combining mTOR and EGFR inhibitors for these specific classes of tumors; however, the success of this combination across a broad set of solid tumors has not been established. Herein, we investigated whether the combination of rapamycin with the EGFR inhibitor erlotinib would show synergy for other solid tumors that do not fit into the same mutation class as renal cell carcinoma or glioblastoma multiforme. We also explored the signaling mechanism by which mTOR and EGFR inhibitors cooperate to inhibit cell growth. We determined the correlation between the ability of erlotinib to regulate the Ras-Raf-mitogen-activated protein kinase/extracellular signal-regulated kinase (Erk) kinase-Erk and PI3K-Akt-mTOR pathways and sensitivity to growth inhibition in a group of 22 cell lines derived from four tumor types (NSCLC, colon, pancreatic, and breast). We find that erlotinib could inhibit the phosphorylation of Akt, S6, and Erk only in erlotinib-sensitive cells. Rapamycin was effective in blocking S6 phosphorylation in all cell lines tested; however, this was accompanied by an increase in Akt phosphorylation, and rapamycin by itself substantially inhibited growth in only three cell lines. In erlotinib-insensitive cell lines, although erlotinib could not down-regulate basal Akt activity, it could inhibit rapamycin-stimulated Akt activity. Moreover, only when erlotinib was combined with rapamycin was there substantial induction of apoptosis, which corresponded to a synergistic effect on overall cell growth inhibition.

Our observations of synergy in cell culture could be extended *in vivo* to a xenograft model. These results suggest that combining rapamycin or rapamycin analogues with erlotinib might be clinically useful across a range of solid tumors in patients to enhance the sensitivity to erlotinib.

## Materials and Methods

### Cell Lines

The cell lines H460, Calu6, SW1573, H1703, H292, H358, HCT-116, HT-29, FET, GEO, HCT-15, Colo201, Colo205, CBS, SW480, BxPC3, HPAC, CFPAC, MiaPaca-2, Panc1, and

MDA-MB-468 were grown in media containing 10% FCS containing 10% FCS as prescribed by the American Type Culture Collection.

### Measurement of Cell Proliferation

Cell proliferation was determined using the Cell Titer Glo assay (Promega, Madison, WI). Cell lines were seeded at a density of 3,000 cells per well in a 96-well plate. Twenty-four hours after plating, cells were dosed with varying concentrations of drug, either as a single agent or in combination. The signal for Cell Titer Glo was determined 72 hours after dosing.

### Analysis of Additivity and Synergy

The Bliss additivity model was used to classify the effect of combining rapamycin and erlotinib as additive, synergistic, or antagonistic. A theoretical curve was calculated for combined inhibition using the equation  $E_{\text{bliss}} = E_A + E_B - E_A \times E_B$ , where  $E_A$  and  $E_B$  are the fractional inhibitions obtained by drug A alone and drug B alone at specific concentrations. Here,  $E_{\text{bliss}}$  is the fractional inhibition that would be expected if the combination of the two drugs was exactly additive. If the experimentally measured fractional inhibition is less than  $E_{\text{bliss}}$ , the combination was said to be synergistic. If the experimentally measured fractional inhibition is greater than  $E_{\text{bliss}}$ , the combination was said to be antagonistic. For dose-response curves, the Bliss additivity value was calculated for varying doses of drug A when combined with a constant dose of drug B. This allowed an assessment of whether drug B affected the potency of drug A or shifted its intrinsic activity. All plots were generated using Prism GraphPad software.

### Preparation of Protein Lysates and Western Blotting

Cell extracts were prepared by detergent lysis [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1% NP40, 0.5% Na-deoxycholate, 0.1% SDS, containing protease (P8340, Sigma, St. Louis, MO) and phosphatase (P5726, Sigma) inhibitor cocktails]. The soluble protein concentration was determined by micro-bovine serum albumin assay (Pierce, Rockford, IL). Protein immunodetection was done by electrophoretic transfer of SDS-PAGE separated proteins to nitrocellulose, incubation with antibody, and chemiluminescent second step detection (PicoWest, Pierce). The antibodies included EGFR, phospho-EGFR (Y1068), phospho-p42/p44, phospho-Akt (473), phospho-Akt (308), total Akt, phospho-S6 (235/236), and total S6. All antibodies were obtained from Cell Signaling Technologies (Danvers, MA).

For analysis of the effects of erlotinib and rapamycin on the phosphorylation of downstream signaling proteins, cell lines were grown to ~70% confluence, at which time erlotinib and/or rapamycin was added at the indicated concentration, and cells were incubated at 37°C for 2 hours. Where indicated, 10 ng/mL EGF ligand was added for 5 minutes. The medium was removed, cells were washed twice with PBS, and cells were lysed as previously described.

### Xenograft Studies in Nude Mice

Female CD-1 *nu/nu* mice (Charles River Laboratories, Wilmington, MA) were implanted with harvested Calu6 NSCLC tumor cells in a single s.c. site on the flank of the

mice in the axillary region. Tumors were allowed to grow to  $200 \pm 50 \text{ mm}^3$ , at which time the animals were sorted into treatment groups of eight animals per group based on weight ( $\pm 1 \text{ g}$  body weight) and tattooed on the tail for permanent identification. Tumor volumes and body weights were determined twice weekly. The tumor volume was determined by measuring in two directions with vernier calipers and calculated using the following formula: tumor volume =  $(\text{length} \times \text{width}^2) / 2$ . The data were plotted as the percent change in mean values of tumor volume and body weight for each group. The percent tumor growth inhibition was determined as  $(1 - W_t - W_c) \times 100$ , where  $W_t$  is the median tumor volume of the treated group at time  $x$  and  $W_c$  is the median tumor volume of the control group at time  $x$ . Tarceva was dosed in a 6% Captisol (CyDex, Inc., Lenexa, KS) in water for injection solution and all control animals were dosed with an equal volume of the vehicle. Tarceva animals were dosed by oral gavage once a day for 14 days and the percent tumor growth inhibition measured on day 15. Rapamycin (LC Laboratories, Woburn, MA) was dosed in 4% ethanol/5% PEG400/5% Tween 80 in water for injection solution. Rapamycin animals were dosed by i.p. injection on days 1 and 8 and all control animals were dosed with an equal volume of the vehicle.

## Results

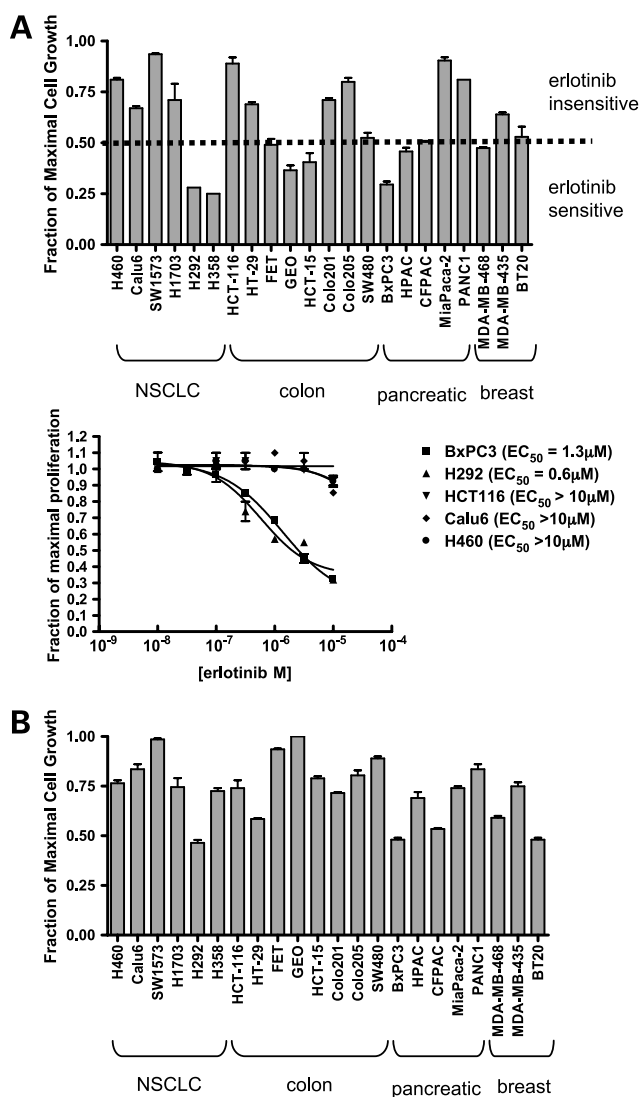
The sensitivities of 22 cell lines derived from four tumor types (NSCLC, colon, pancreatic, and breast) to erlotinib were determined. The mutation profiles of these cell lines for EGFR, K-Ras, PI3K, and phosphatase and tensin homologue (PTEN) are shown in Table 1. All NSCLC cell lines and the colorectal cancer tumor cell lines HCT-116, HT-29, HCT-15, Colo201, and Colo205 were determined to have wild-type EGFR (24). The EGFR mutation status of other cell lines is unknown; however, EGFR mutation status has been investigated for these other tumor types and none have been found. For example, in a collection of 40 pancreatic tumors and 20 colorectal tumors, all were found to have wild-type EGFR (24). Therefore, we suspect that the pancreatic and colorectal tumor cell lines in this panel are of wild-type EGFR. Mutations in K-Ras, PI3K, and PTEN are described for 10 of 22, 4 of 22, and 1 of 22 cell lines, respectively (25). Maximal growth inhibition by  $10 \mu\text{mol/L}$  erlotinib was chosen as the criteria to rank the sensitivities of cell lines. Previous reports have shown that growth inhibition by  $10 \mu\text{mol/L}$  erlotinib *in vitro* closely mirrors the percent tumor growth inhibition derived from *in vivo* xenograft experiments, showing that this dose is biologically relevant (26). Ten micromolar also represents a plasma concentration that is achievable in nonsmoking patients dosed with erlotinib, and therefore this is a clinically relevant concentration (27). The ability of  $10 \mu\text{mol/L}$  erlotinib to inhibit the growth of these cell lines is shown in Fig. 1A (*top*). The cell lines displayed a range of sensitivities to erlotinib, and the cutoff criterion for defining sensitivity

**Table 1. Mutation status EGFR, K-Ras, PI3K, and PTEN in 22 tumor cell lines**

Cell line	Tumor type	EGFR	K-Ras	PI3K	PTEN
H460	NSCLC	WT	Mutant	Mutant	WT
Calu6	NSCLC	WT	Mutant	WT	WT
SW1573	NSCLC	WT	Mutant	Mutant	WT
H1703	NSCLC	WT	WT	WT	WT
H292	NSCLC	WT	WT	WT	WT
H358	NSCLC	WT	Mutant	WT	WT
HCT-116	CRC	ND	Mutant	Mutant	WT
HT-29	CRC	ND	WT	WT	WT
FET	CRC	ND	ND	WT	ND
GEO	CRC	ND	ND	WT	ND
HCT-15	CRC	WT	Mutant	Mutant	WT
Colo201	CRC	ND	WT	WT	WT
Colo205	CRC	ND	WT	WT	WT
SW480	CRC	ND	Mutant	WT	WT
BxPC3	Pancreatic	ND	WT	WT	WT
HPAC	Pancreatic	ND	Mutant	ND	ND
CFPAC	Pancreatic	ND	Mutant	WT	WT
MiaPaca-2	Pancreatic	ND	Mutant	ND	ND
Panc1	Pancreatic	ND	Mutant	ND	ND
MDA-MB-468	Breast	ND	WT	WT	Null
MDA-MB-435	Breast	ND	WT	WT	WT
BT20	Breast	ND	WT	Mutant	WT

Abbreviations: WT, wild-type; CRC, colorectal cancer.

was a maximal growth inhibition of at least 50%. The effects of varying concentrations of erlotinib for two erlotinib-sensitive (BxPC3 and H292) and three erlotinib-insensitive (Calu6, H460, and HCT-116) cell lines are shown in Fig. 1A (*bottom*), showing that growth inhibition is dose dependent. The cell line panel displayed varying sensitivities to growth inhibition by rapamycin; however, no cell line tested showed maximal growth inhibition  $>50\%$  (Fig. 1B). This highlights the potential for the mTOR-ricor or S6K-IRS feedback loops to limit the antiproliferative and antiapoptotic effects for inhibiting mTOR-raptor alone. We sought to analyze the effect of erlotinib and rapamycin on the mitogen-activated protein kinase and mTOR pathways in a group of erlotinib-sensitive and erlotinib-insensitive cell lines. We selected the two erlotinib-sensitive cell lines (H292 and BxPC3) and three erlotinib-insensitive cell lines (H460, Calu6, and HCT-116) for further investigation. Xenograft experiments have previously confirmed our *in vitro* classification of sensitivity for this group (previous publication and data not shown; ref. 26). We chose phosphorylation of Erk-1/2 (p42/p44) and S6 as readouts for the activities of the mitogen-activated protein kinase and mTOR pathways, respectively. In both sensitive and insensitive cell lines, erlotinib could inhibit EGFR phosphorylation, indicating that erlotinib could still bind EGFR in relatively insensitive cell lines (data not shown). In the two sensitive cell lines, we find that erlotinib can efficiently inhibit the phosphorylation of Erk, Akt, and S6, whereas in the insensitive cell lines we



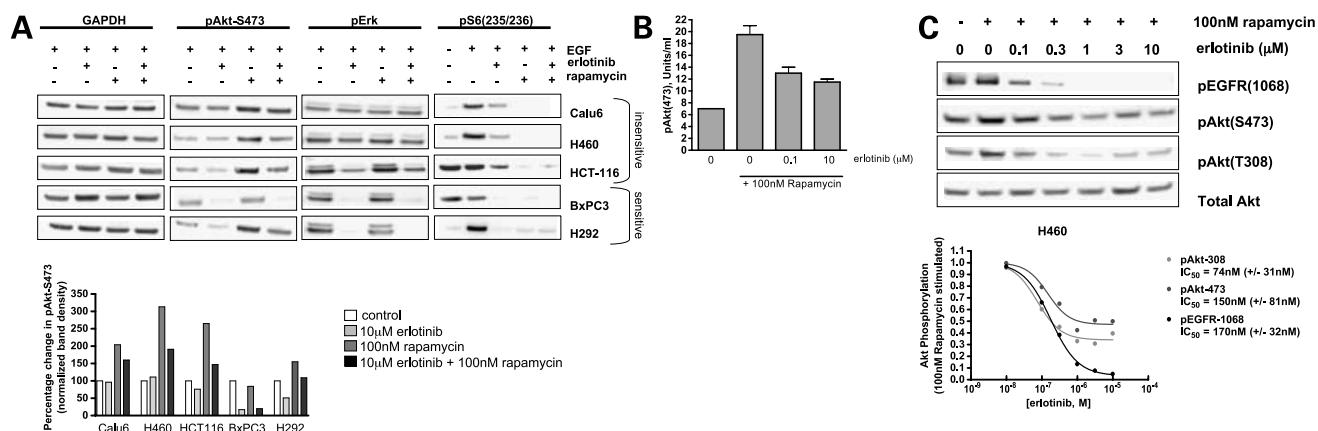
**Figure 1.** **A, top**, sensitivity of 22 cell lines derived from four tumor types to growth inhibition by erlotinib (10  $\mu\text{mol/L}$ ). Data are expressed as maximal cell growth at 72 h. A 50% inhibition in maximal cell growth was our cutoff criteria for distinguishing sensitive from insensitive cell lines. **Bottom**, effects of varying concentrations of erlotinib on the growth inhibition of BxPC3, H292, H460, Calu6, and HCT-116 cells. **B**, sensitivity of the 22 cell lines to growth inhibition by rapamycin (10 nmol/L). Data are expressed as maximal cell growth at 72 h. Results shown are typical of three or more independent experiments.

observe a less pronounced inhibition of these kinases (Fig. 2A, top). Phosphorylated levels of S6 could not be inhibited by erlotinib below basal levels. These results are consistent with previous reports describing signaling pathways in other cell lines that are sensitive or insensitive to EGFR inhibitors (8, 28).

Whereas erlotinib was very effective in inhibiting S6 phosphorylation only in the three sensitive cell lines, rapamycin could fully inhibit the phosphorylation of S6 in both sensitive and insensitive cell lines (Fig. 2A, top).

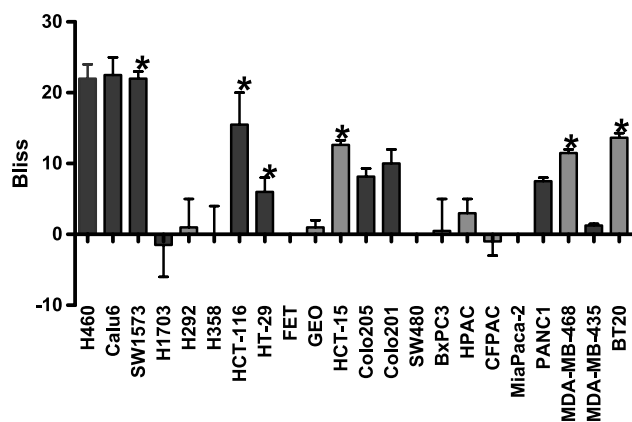
However, this was accompanied by an increase in Akt-S473 phosphorylation in four of five cell lines tested (Calu6, H460, HCT-116, and H292). Band density analysis of this experiment shows that rapamycin stimulated Akt phosphorylation >2-fold for Calu6, H460, and HCT-116 cells (Fig. 2A, bottom). In this specific experiment, rapamycin concentration was 100 nmol/L; however, we have observed similar results even with as low as 10 nmol/L rapamycin (data not shown). It is likely that this enhancement of Akt activity promotes cell survival and could limit the use of rapamycin for inhibiting cell growth as a single agent. However, combination with erlotinib could inhibit rapamycin-stimulated Akt-S473 phosphorylation (Fig. 2A). To ensure that changes in Akt-S473 phosphorylation by rapamycin and erlotinib were genuine, and not due to variability in lysate preparations for Western blotting, we measured the effect of rapamycin and erlotinib on Akt-S473 phosphorylation by ELISA capture assay for H460 cells (Fig. 2B). As previously shown by Western blotting, we observed a similar increase in Akt-S473 phosphorylation by rapamycin of  $\sim 3$ -fold (Fig. 2B). Moreover, erlotinib was effective at attenuating this phosphorylation. We measured the effects of varying concentrations of erlotinib on rapamycin-stimulated Akt phosphorylation at both the Akt-S473 and Akt-T308 sites. Rapamycin increased Akt phosphorylation at both of these sites, and erlotinib could dose-dependently inhibit this phosphorylation with  $\text{IC}_{50}$  values of  $\sim 74$  nmol/L ( $\pm 31$  nmol/L) and 170 nmol/L ( $\pm 81$  nmol/L) for the Akt-T308 and Akt-S473 sites, respectively (Fig. 2C). This is consistent with the potency of erlotinib for inhibiting EGFR phosphorylation,  $\text{IC}_{50}$  of  $\sim 170$  nmol/L ( $\pm 32$  nmol/L). Collectively, these results show that in select cell lines, it is only with the combination of rapamycin and erlotinib that inhibition of S6 phosphorylation can be achieved without enhanced phosphorylation of Akt.

We suspected that the modulation of the cell signaling pathways observed for the erlotinib and rapamycin combination would render synergistic antiproliferative and antiapoptotic responses. We determined the effect that combining rapamycin with erlotinib had on cell proliferation using two criteria: (a) potency and (b) maximal efficacy. Our assessment of additivity, synergy, or antagonism was based on the Bliss additivism model described in Materials and Methods. We chose this model over isobologram or combination index analyses as it would allow us to evaluate the nature of drug interactions even in cases where the maximal inhibition by rapamycin or erlotinib as single agents was low enough such that a reliable  $\text{IC}_{50}$  value could not be obtained. For cell lines that are less sensitive to erlotinib as a single agent, the  $\text{IC}_{50}$  value is often  $>10$   $\mu\text{mol/L}$ . In addition, as both isobologram and combination index analyses are based on a single criteria,  $\text{IC}_{50}$  value, and do not directly reflect variability within data, they do not necessarily always indicate whether the extent of synergy is significant. The Bliss additivism model would also allow us to evaluate changes



**Figure 2.** *A, top*, effect of erlotinib and rapamycin on pEGFR, pErk, and pS6 in a panel of two sensitive and three insensitive cell lines. Cells were treated with 10  $\mu$ M/L erlotinib, 100 nmol/L rapamycin, or DMSO vehicle alone for 2 h. Lysates were prepared and run on gels for Western blot with phosphorylation-specific antibodies. *Bottom*, analysis of band density for phosphorylated Akt-S473 for each cell line under control conditions or when cells were treated with 10  $\mu$ M/L erlotinib, 100 nmol/L rapamycin, or the combination. **B**, effect of rapamycin treatment (100 nmol/L) on pAkt-S473 levels in H460 cells, alone and in the presence of 0.1 or 10  $\mu$ M/L erlotinib, as determined with a pAkt-S473 ELISA capture assay. **C**, effects of rapamycin (100 nmol/L) plus varying concentrations of erlotinib on the phosphorylation of EGFR(1068), Akt(473), or Akt(308) as determined by Western blotting with phosphorylation-specific antibodies (*top*). Analysis of band density of phosphorylation specific bands (normalized to total Akt levels) for pEGFR, pAkt-S473, and pAkt-T308. The IC<sub>50</sub> values of erlotinib for inhibiting these phosphorylation states are indicated (*bottom*).

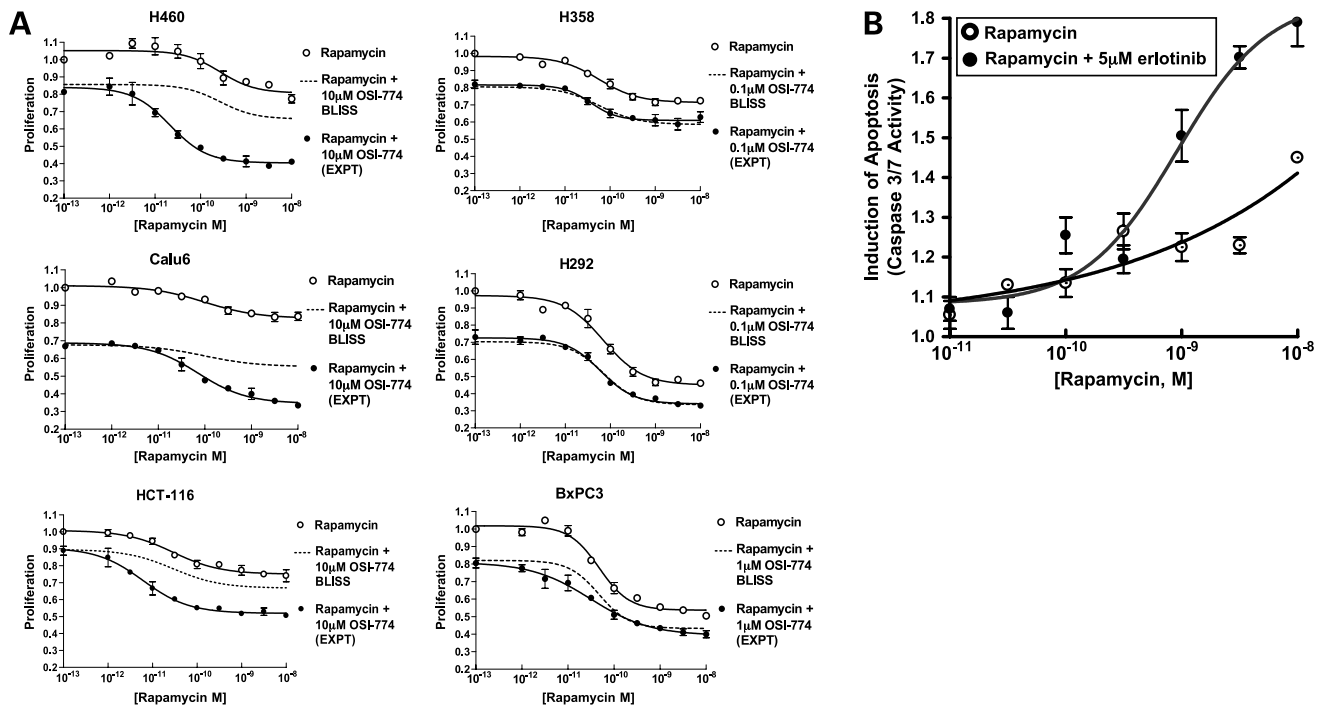
in maximal efficacy. Both isobologram and combination index analyses reflect only changes in potency, but shifts in intrinsic efficacy also have the potential to be clinically meaningful. This approach has previously been described for high-throughput studies involving drug combinations (29). The Bliss analysis of the combination data is summarized in Fig. 3. Here the data are expressed as percentage decrease in cell growth above what would be expected if the combination was strictly additive in nature. Bliss = 0 would indicate the combination was directly additive; Bliss > 0 would indicate the percentage increase in maximal inhibition above additivity (synergy); and Bliss < 0 would indicate the percentage decrease in maximal



**Figure 3.** Sensitivity of cell lines to the combination of erlotinib and rapamycin. Synergy, as noted by a positive Bliss value, was observed in 12 of 22 cell lines. The calculation of Bliss is described in Materials and Methods.

inhibition below additivity (antagonism). These two targeted agents act synergistically to inhibit cell growth in 12 of 22 cell lines, including all five cell lines (marked by an asterisk) that harbor mutations in either PI3K or PTEN. The remaining 10 cell lines showed pure additivity, and in no cell line tested was the combination antagonistic. HCT-116 and MDA-MB-468 were among those that showed a synergistic response to the combination of erlotinib with rapamycin. Both of these cell lines have been reported to contain genetic defects that result in activation of mTOR that is partially independent of growth factor signaling. HCT-116 has a mutation in PI3K, leading to constitutive activity that is partially growth factor independent. MDA-MB-468 has been reported to have a deletion of PTEN (10, 11). The loss of this PI3K pathway inhibitor leads to partial activation of the Akt-mTOR pathway that is independent of EGFR. By targeting the Akt-mTOR pathway downstream of these mutations, the combination with rapamycin effectively overcomes these genetic alterations. Indeed, rapamycin can fully shut down this pathway as shown by its ability to fully block S6 phosphorylation (Fig. 2A).

The effects of varying concentrations of rapamycin on growth inhibition in the presence and absence of a single concentration of erlotinib are shown in Fig. 4A. In this figure, the Bliss analysis shows the theoretical curve that would be expected if the combination of erlotinib and rapamycin was purely additive in nature. For the three sensitive cell lines (H292, H358, and BxPC3), Bliss analysis shows that erlotinib is exactly additive with rapamycin. Isobologram analysis of this data confirmed additivity of the combination (data not shown). For the three erlotinib insensitive cell lines, we find that the combination shows synergy, as reflected by both an increase in potency and a gain in maximal inhibition. For example, H460 cells show



**Figure 4.** **A**, effects of varying concentrations of rapamycin on the proliferation of three sensitive cell lines (H358, H292, and BxPC3) and three insensitive cell lines (H460, Calu6, and HCT-116) in the presence or absence of erlotinib (at indicated concentrations). *Dashed line*, Bliss additivity curve, representing the theoretical expectation if the combined effects of erlotinib with rapamycin were exactly additive. Representative of three or more independent experiments. **B**, effect of varying concentrations of rapamycin, alone or in the presence or absence of 5 μmol/L erlotinib, on apoptosis in Calu6 cells. Apoptosis was evaluated by measuring caspase-3/caspase-7 activity 48 h after addition of compounds. Results shown are typical of three independent experiments.

an increase in maximal efficacy of ~60% (34–56%) as well as a >10-fold shift in potency (267–21 pmol/L) when rapamycin is combined with erlotinib.

We hypothesize that the synergy for rapamycin with erlotinib observed in the cell growth assay might be attributed, at least in part, to the ability of erlotinib to promote apoptosis by decreasing the Akt activity that was stimulated by rapamycin. We therefore measured the effects of varying concentrations of rapamycin on the induction of apoptosis for Calu6 cells in the presence and absence of 5 μmol/L erlotinib (Fig. 4B). Compared with cells treated with rapamycin alone, we observed a significant increase in apoptosis in cells treated with the combination of erlotinib and rapamycin.

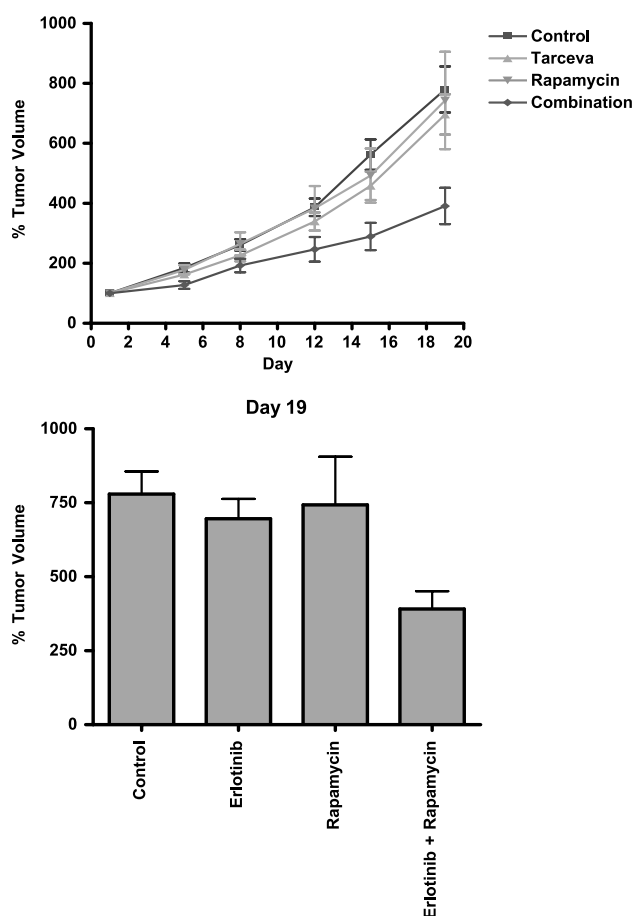
We sought to establish whether our *in vitro* observations of synergy would extend to a xenograft model *in vivo* and so we tested the combination of erlotinib and rapamycin in a Calu6 xenograft model. The Calu6 model was selected because of the previously confirmed lack of sensitivity to erlotinib as a single agent both *in vivo* and *in vitro* because this cell line exhibited robust synergy for the rapamycin and erlotinib combination *in vitro* and because of the propensity for this cell line to readily form tumors in xenograft experiments. As cell culture studies had suggested, neither erlotinib nor rapamycin had a substantial effect on inhibiting tumor growth when they were

administered as single agents. However, when these two targeted agents were combined, they show synergistic effects in inhibiting tumor growth (Fig. 5). On day 19, there is a 56% reduction in tumor growth with the erlotinib and rapamycin combination whereas there is no statistically significant reduction in tumor growth by either drug alone. By day 19, tumors in control animals as well as tumors in animals treated with either erlotinib or rapamycin alone had grown to the point that animals had to be sacrificed whereas animals receiving the combination continued to survive for more than 4 weeks (data not shown). These data show the usefulness for combining rapamycin with EGFR inhibitors across multiple tumor types.

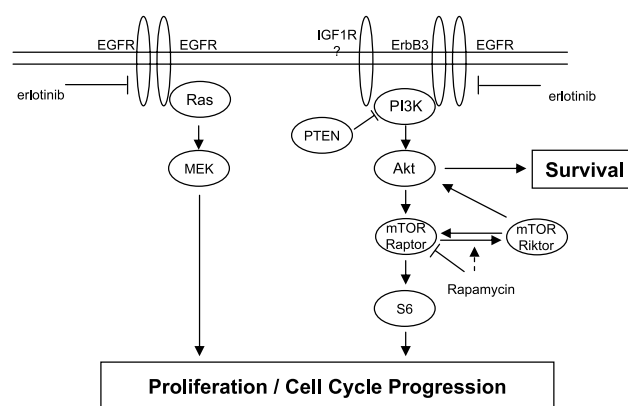
## Discussion

Molecular targeted agents acting on the EGFR can inhibit a number of different signaling cascades within the cell. For inhibitors of the EGFR, down-regulation of the PI3K-Akt pathway seems to track with sensitivity to growth inhibition (8, 9, 28). Mechanisms leading to EGFR-independent activity of the Akt pathway may include, among other things, other growth factor receptors such as IGF-1R and fibroblast growth factor receptor, specific mutations such as constitutively activating PI3K mutations, or loss of PTEN activity (7). Such mechanisms are illustrated in Fig. 6.

Therefore, in certain tumor cell lines, intervention at multiple points in a signaling cascade may be necessary to most effectively inhibit cell growth and survival. Herein, we find that in cell lines sensitive to growth inhibition by erlotinib, there is corresponding inhibition of Erk, Akt, and S6 activity. The activation state of Erk and Akt was unaffected by erlotinib in the three cell lines less sensitive to the antiproliferative effects of erlotinib, and the phosphorylation of S6 could not be inhibited further than basal (absence of exogenous EGF) levels. The three erlotinib-insensitive cell lines shown in this panel all harbor mutations that constitutively activate K-Ras. Therefore, Erk activity might be controlled, at least to some extent, by this growth factor-independent mechanism. However, for other cell lines that harbor K-Ras mutations, we were able to achieve Erk inhibition by erlotinib (9); therefore, the growth factor independence for Erk activity may not extend to all cell lines with mutations in K-Ras. The erlotinib-insensitive cell line HCT-116 harbors a mutation in PI3K (H1047R), indicating growth factor independence



**Figure 5.** Combination of erlotinib with rapamycin in a Calu6 xenograft model. Mice harboring tumors of the NSCLC cell line Calu6 were dosed with erlotinib (100 mg/kg daily), rapamycin (4 mg/kg, days 1, 8, and 15), or the combination of erlotinib and rapamycin. Percentage change in tumor volume.



**Figure 6.** Cartoon describing signaling pathways that modulate the mitogen-activated protein kinase and mTOR pathways leading to cell cycle progression and proliferation.

for Akt and the inability of erlotinib to inhibit Akt. Most of the cell lines tested were relatively insensitive to growth inhibition by rapamycin alone. This is not surprising given the relatively limited clinical efficacy observed for rapamycin analogues as single agents (30). Two regulatory loops have been described for rapamycin effects that provide mechanistic insight into this limited efficacy and provide a rationale for combinations. These regulatory loops include (a) a shift in equilibrium from the rapamycin-sensitive mTOR-Raptor complex to the rapamycin-insensitive mTOR-Rictor complex, and (b) effects on the S6K-IRS-IGF-IR autoinhibitory feedback loop (31–33). Therefore, whereas rapamycin as a single agent might be effective in inhibiting some of the activities in the Akt pathway, such as blocking S6 proliferative signaling, it stimulates others, by promoting cell survival signals at the level of Akt phosphorylation. Herein, we find that for both erlotinib-sensitive and erlotinib-insensitive cell lines, rapamycin was able to completely inhibit phosphorylation of S6; however, we observed an accompanying increase in Akt-S473 phosphorylation in five cell lines tested. In a recent report describing the effects of the rapamycin analogue RAD001 in human tumors, it was shown that Akt phosphorylation increases in response to treatment, indicating that these *in vitro* observations translate into the clinic (33). These observations provide us with some understanding of the relative insensitivity to growth inhibition by rapamycin alone in our cell line panel. Surprisingly, although erlotinib had no effect on basal Akt phosphorylation in insensitive cell lines, it was able to inhibit rapamycin-stimulated Akt phosphorylation. Therefore, when rapamycin was combined with erlotinib, we were able to achieve blockade of both S6 and Akt. There may be a threshold for Akt activity that is required for erlotinib inhibition. Alternatively, we have found that phosphorylation of select tyrosine sites on IRS1 is EGFR mediated, and erlotinib can inhibit this (data not shown). Therefore, similar to what has been observed with inhibitors of the IGF-IR, it is conceivable that erlotinib

also attenuates rapamycin-stimulated Akt phosphorylation by interfering with the S6K-IRS1-PI3K feedback loop. The exact mechanism by which erlotinib can inhibit rapamycin-stimulated Akt phosphorylation but not affect basal Akt phosphorylation requires further investigation.

The modulation of signaling cascades achieved by the erlotinib and rapamycin combination achieved a synergistic effect in inhibiting cell growth and promoting apoptosis. We observed synergy for the rapamycin and erlotinib combination in 12 of 22 cell lines tested. This is reflected in both a shift in potency and an increase in maximal inhibition. Five of the 22 cell lines in the panel harbor mutations in PI3K or PTEN, and all of these exhibited synergy for the rapamycin and erlotinib combination. Other cell lines that showed synergistic responses to this combination are described as reliant on receptor tyrosine kinase signaling independent of the EGFR. For example, the colorectal tumor cell line Colo205 has been reported to be partially dependent on IGF-IR signaling (34). Previously, it has been shown that inhibiting rapamycin-stimulated Akt phosphorylation by an alternative mechanism, specifically through inhibition of IGF-IR, in select cell lines also generates synergy for growth inhibition (33). Therefore, we believe that the ability of erlotinib to attenuate rapamycin-stimulated Akt phosphorylation underlies the mechanism of synergy observed for the erlotinib and rapamycin combination.

We further characterized the rapamycin and erlotinib combination in a Calu6 xenograft model. This model was chosen due to its low sensitivity to erlotinib as a single agent *in vitro* and *in vivo* and for its propensity to readily form tumors in xenograft models. Neither rapamycin nor erlotinib showed significant reduction in tumor growth as single agents in the Calu6 xenograft model but showed pronounced reduction in tumor growth when dosed in combination.

Unlike cytotoxic chemotherapies that often share similar toxicities, limiting their use in combination, many molecular targeted agents have nonoverlapping toxicity profiles. Thus, cocktails of molecular targeted agents that block multiple signaling pathways in cancer cells may offer more effective and well-tolerated regimens in the clinic. The ability of specific combinations of targeted agents to synergize may also allow for lower dosing of each single agent. The coadministration of rapamycin or rapamycin analogues, RAD001 (Novartis, Basel, Switzerland) and CCI-779 (Wyeth, Madison, NJ), with erlotinib is currently being conducted clinically in trials for glioblastoma, breast cancer, and NSCLC (35–37). Our results provide preclinical support for the broad usefulness of this combination across multiple types of solid tumors.

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