Dependence on Phosphoinositide 3-Kinase and RAS-RAF Pathways Drive the Activity of RAF265, a Novel RAF/VEGFR2 Inhibitor, and RAD001 (Everolimus) in Combination

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Abstract

Activation of phosphatidylinositol-3-kinase (PI3K)-AKT and Kirsten rat sarcoma viral oncogene homologue (KRAS) can induce cellular immortalization, proliferation, and resistance to anticancer therapeutics such as epidermal growth factor receptor inhibitors or chemotherapy. This study assessed the consequences of inhibiting these two pathways in tumor cells with activation of KRAS, PI3K-AKT, or both. We investigated whether the combination of a novel RAF/vascular endothelial growth factor receptor inhibitor, RAF265, with a mammalian target of rapamycin (mTOR) inhibitor, RAD001 (everolimus), could lead to enhanced antitumoral effects in vitro and in vivo. To address this question, we used cell lines with different status regarding KRAS, PIK3CA, and BRAF mutations, using immunoblotting to evaluate the inhibitors, and MTT and clonogenic assays for effects on cell viability and proliferation. Subcutaneous xenografts were used to assess the activity of the combination in vivo. RAD001 inhibited mTOR downstream signaling in all cell lines, whereas RAF265 inhibited RAF downstream signaling only in BRAF mutant cells. In vitro, addition of RAF265 to RAD001 led to decreased AKT, S6, and Eukaryotic translation initiation factor 4E binding protein 1 phosphorylation in HCT116 cells. In vitro and in vivo, RAD001 addition enhanced the antitumoral effect of RAF265 in HCT116 and H460 cells (both KRAS mut, PIK3CA mut); in contrast, the combination of RAF265 and RAD001 yielded no additional activity in A549 and MDAMB231 cells. The combination of RAF and mTOR inhibitors is effective for enhancing antitumoral effects in cells with deregulation of both RAS-RAF and PI3K, possibly through the cross-inhibition of 4E binding protein 1 and S6 protein. Mol Cancer Ther; 9(2); 358–68. ©2010 AACR.

Introduction

Growth factor receptors play a major role in cell survival and proliferation. Two important downstream pathways are the phosphatidylinositol 3-kinase (PI3K)-AKT-mammalian target of rapamycin (mTOR) pathway and the RAS-RAF-mitogen-activated protein (MAP)/extracellular signal-related kinase (ERK) kinase (MEK)-ERK pathway. These pathways regulate transcription factors and other proteins involved in cell proliferation, survival, motility, and differentiation. In many human cancers, genetic and epigenetic mechanisms have deregulated major components of the RAS and AKT signaling network (1).

RAS is one of the most important molecules in the growth factor downstream signaling pathway. It can activate the serine/threonine kinase RAF, ERK1 and ERK2, and several nuclear proteins to promote cell proliferation. RAS genes, especially KRAS (homologous to the oncogene of the Kirsten rat sarcoma virus), have been implicated in the pathogenesis and prognosis of several cancers. In many tumors, a point mutation leading to the loss of GTPase activity is associated with transforming activation of the protein, resulting in continued proliferation signal. Overall, KRAS mutations are found in ~30% of human cancers, mainly in pancreatic, colorectal, endometrial, biliary tract, lung, and cervical cancers (2).

RAS plays an essential role in tumor maintenance and is therefore an appropriate target for anticancer therapy. Different pharmacologic strategies have been developed to inhibit KRAS oncogenic activation, including inhibition of its association with the plasma membrane (prenylation and postprenylation inhibitors), downstream signaling (kinase inhibitor), upstream pathways (kinase inhibitor and monoclonal antibody), and protein expression of RAS or other components of the pathway (small interfering RNA and antisense oligonucleotides; ref. 3). However,
several of these therapeutic agents have yielded disappointing results (4). RAS upstream pathway inhibition is based mostly on epidermal growth factor receptor (EGFR) inhibition, which is ineffective when administered alone in patients with colorectal (5–8) or lung cancer (9) harboring KRAS mutations. EGFR-targeted therapies are now being tested in combination with other therapies to overcome this KRAS-related resistance (10). Finally, pharmacologic inhibition of RAS downstream pathways with kinase inhibitors remains promising.

mTOR is a highly conserved serine-threonine kinase that recognizes stress signals (e.g., nutrients and energy depletion, oxidative and hypoxic stress, proliferative and survival signals) through the PI3K-AKT pathway. mTOR signaling occurs through the phosphorylation of substrates p70 ribosomal S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E binding protein 1 (4EBP1). Phosphorylation of 4EBP1 releases eukaryotic initiation factor 4E, permitting the initiation of cap-dependent protein translation. mTOR activation also results in glucose transporter 1 (GLUT1) mRNA translation and protein expression. mTOR signaling pathways are dysregulated in a variety of human malignancies, making mTOR an attractive target for anticancer therapy (11). RAD001 (everolimus) is an orally bioavailable mTOR inhibitor that binds with high affinity to its intracellular receptor FKBP12, resulting in a complex that interacts with mTOR to inhibit downstream signaling events, including phosphorylation of S6 protein, phosphorylation of 4EBP1, and expression of GLUT1 protein (12, 13).

The RAS and AKT signaling network intersects at various points, conferring high redundancy on this network (1), with complex positive and negative feedback controls and bifurcations at several levels (14–17). For example, AKT-mediated phosphorylation of RAF shifts the cellular response in a human breast cancer cell line from cell cycle arrest to proliferation (15). Such observations suggest a molecular basis for cross-talk between the two signaling pathways at the level of RAF and AKT, and that combined RAF and mTOR inhibition may be promising for overcoming the intrinsic plasticity of the RAS-AKT network. We tested this hypothesis by investigating the effect of the combination of a novel RAF/vascular EGFR (VEGFR) inhibitor, RAF265, with the rapamycin derivative RAD001, in cell lines with different KRAS, PI3K, and RAF mutational status.

### Materials and Methods

#### Compounds

Novartis Pharma AG synthesized and provided RAF265 and RAD001. Stock solutions (20 mmol/L) of these compounds were made in DMSO (Sigma) and diluted in PBS (Invitrogen) shortly before use. Solutions were then diluted in the culture medium of each cell line to achieve the indicated final concentrations.

#### Cell Lines

We used human adenocarcinoma and large cell carcinoma cell lines with different mutations of the RAS-RAF and PI3K-AKT pathways (Table 1). Human A549 and H460 lung, HT29 and HCT 116 colon, and MDAMB231 breast cancer cell lines were purchased from the American Type Culture Collection. Cell lines were cultured in complete medium, consisting of 10% (v/v) fetal bovine serum, 2 mmol/L-glutamine, and 50 units/mL penicillin-streptomycin in the medium recommended by the American Type Culture Collection (all from Invitrogen). Cells were grown at 37°C and 5% CO2 in an incubator. All cell lines were tested as mycoplasm free using the 4′,6-diamidino-2-phenylindole assay.

#### Immunoblotting

Immunoblotting was done to identify inhibitor activity and the upstream and downstream consequences of inhibition. After plating of 1.5 × 10^5 cells in six-well plates for 24 h, cells were treated with RAD001 (1 nmol/L), RAF265 (concentrations of 1–10 μmol/L), or concurrently with both at a fixed dose of RAD001 and increasing doses of RAF265 to achieve final concentrations of 1 mmol/L and 10 μmol/L, respectively. After 24 h, cell extracts were prepared by detergent lysis. The soluble protein concentration was determined by the microbovine serum albumin assay. Protein immunodetection was achieved by electrophoretic transfer of SDS-PAGE-separated proteins to nitrocellulose, incubation with the appropriate antibody,

| Table 1. RAS, RAF, and PI3K mutational status of the selected cell lines |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| **RAS-RAF pathway** | **BRAF** | **PI3K-Akt pathway** | **PTEN** |
| KRAS | G12S mutant | Wt | Wt |
| H460 (lung LC) | G61H mutant | Wt | E545K mutant |
| HCT116 (colon adk) | G13D mutant | Wt | H1047R mutant |
| HT29 (colon adk) | Wt | V599E mutant | Wt |
| MDAMB231 (breast adk) | G13D mutant | G463V mutant | Wt |

Abbreviations: Adk, adenocarcinoma; LC, large cell carcinoma; wt, wild-type.
and chemoluminescent second-step detection. All antibodies were obtained from Cell Signaling Technology.

**Cell Viability Assay**

The MTT assay and Bliss additivism model were used to assess the effect of the combination on cell viability. In each well of a 96-well plate, 1 × 10⁴ cells were grown in 200 μL of medium. After 24 h, RAD001, RAF265, or the combination was added to achieve a final concentration of 0.1 to 10 nmol/L and 0.1 to 10 μmol/L, respectively. After 48 h of treatment, 20 μL of 5 mg mL⁻¹ MTT (Organics Research, Inc.) solution in PBS was added to each well. After 4 h, supernatant was removed and formazan crystals were discarded in 200 μL of DMSO. Absorbance was then measured at 595 nm using an absorbance plate reader (Bio-Rad Microplate Reader). Data are expressed as the percentage of viable cells in treated relative to nontreated conditions.

**Analysis of Additivity and Synergy**

The Bliss additivism model was used to classify the effect of combining RAD001 and RAF265 as synergistic, additive, or antagonistic. A theoretical curve was calculated for combined inhibition using the equation $E_{\text{Bliss}} = EA + EB - EA \times EB$, in which $EA$ and $EB$ are the fractional inhibitions obtained by drug A alone or 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 $E_{\text{observed}}$, the experimentally measured fractional inhibition, is less than $E_{\text{Bliss}}$, the combination is said to be antagonistic, and $\delta = E_{\text{observed}} - E_{\text{Bliss}}$ is a negative value. If the experimentally measured fractional inhibition is greater than $E_{\text{Bliss}}$, the combination is said to be synergistic, and $\delta$ is a positive value. The data are expressed as the percentage decrease in cell viability above what would be expected if the combination were strictly additive (18, 19).

**Clonogenic Assay**

Cell proliferation was assessed by clonogenic assay. Cells were seeded in six-well plates at a density of 200 to 800 (A549, H460, HT29, and HCT116) or 1,600 to 6,400 (MDAMB231) cells in 2 mL of medium per well. After 4 h, they were treated with vehicle, RAD001, RAF265, or the combination to achieve a final concentration of 1 and 5 μmol/L, respectively. After 24 h, the medium was changed to stop the treatment. After 10 to 14 d, cells were stained with crystal violet, and colonies up to 50 cells were counted. Plating efficiency, surviving fraction, and colony mean size were determined for each condition.

**In vivo Experiments**

We also tested the efficacy of the combination in vivo. A total of 3 × 10⁴ A549, H460, HCT116, or MDAMB231 cells were injected s.c. into the flank region of 6-wk-old female athymic mice (Janvier). When tumors reached 50 mm³, the mice were randomized into four groups ($n = 7$ / group) for the following treatment: vehicle, RAF265 (12 mg/kg daily), RAD001 (12 mg/kg daily), or both. All drug were administered over 14 d (6 d on, 2 d off, 6 d on), and the drug combination was administered concurrently. Control mice received the respective vehicles of both drugs. Animal weight and tumor volumes were taken twice weekly and expressed relative to initial tumor volume. Tumors were measured until achieving a relative volume of 10 times the initial volume, and the time to this end point was noted. Drug efficacy was assessed based on the tumor growth curve, growth delay, and tumor volume inhibition percentage. The tumor growth curve was designed to depict the evolution of the relative tumor size over time. For statistical comparison of tumor volumes in treated versus control mice, the Student’s $t$ test (two sided) and ANOVA were used. Growth delay was defined as the time to achieve a relative volume of 10 times the initial volume (T10) in experimental groups compared with control group. The tumor volume inhibition percentage (TVI%) was calculated as: TVI% = 100 − (mean TV treated / mean TV control) × 100 on day 15 after the last day of treatment. Toxic effects of the drug treatment were assessed as body weight loss percentage and lethal toxicity, assessed as deaths occurring in treated mice before the death of the first control mouse (20).

**Immunohistochemistry**

During the in vivo experiment, one animal of each group was dedicated to immunohistochemistry experiments and sacrificed after 5 d of treatment. Tumors were resected, fixed in a formalin-free alcoholic-based fixative (Finefix), and embedded in paraffin. CC1 antigen retrieval buffer (Ventana Medical Systems) was used for anti-cleaved caspase-3, phospho-EGFR, phospho-AKT, mouse CD34, and Glut1 staining, and CC2 antigen retrieval buffer (Ventana Medical Systems) was used for anti–phospho-VEGFR2 staining. All immunohistochemical stainings were done on an automated immunostainer (Benchmark; Ventana) using the Ventana I-view 3,3′-diaminobenzidine detection kit with the following antibodies: anti-mouse CD34 (rat monoclonal; MEC14.7; Hycult Biotechnology; dilution, 1:50), anti-phospho-AKT (rabbit monoclonal; 4060; Cell Signaling Technology; dilution, 1:50) anti-glut1 (rabbit polyclonal; A3536; DAKO Corp.; dilution, 1:400), anti-phospho-EGFR (rabbit polyclonal; 4404; Cell Signaling Technology; dilution, 1:25), anti-phospho-VEGFR2 (rabbit monoclonal; 2478; Cell Signaling Technology; dilution, 1:300), and anti–cleaved caspase-3 (rabbit polyclonal; 9661; Cell Signaling Technology; dilution, 1:400). Slides were counterstained with hematoxylin. Immunostaining was evaluated on whole standard tissue sections of mouse subcutaneous xenografts. Five medium-power fields per section were viewed, and tumors were given a semiquantitative score of 0, <50% (+), >50% (++), or 100% (+++) positive tumor cells. The staining positivity of cells was defined as a membranous staining (anti-CD34, anti–phospho-EGFR, anti–phospho-VEGFR2, anti-glut1), cytoplasmic staining (anti–cleaved caspase-3), or both nuclear and cytoplasmic stainings (anti–phospho-Akt). A qualified
pathologist with no knowledge of the clinicopathologic variables evaluated the specimens.

Results

Addition of RAD001 Enhanced the Cytotoxic Effect of RAF265 in the HCT116 Cell Line In vitro

We investigated the inhibition of targeted kinase by RAD001 and RAF265 in the context of selected KRAS, BRAF, and phosphoinositide-3-kinase, catalytic α poly-peptide (PIK3CA) mutations, assessing phosphorylation of downstream proteins by immunoblotting in selected cell lines (Fig. 1). Exposure to RAD001 at a concentration of 1 nmol/L led to a decrease in phosphorylation of S6 ribosomal protein at serines 235 and 236 in all tested cell lines. Exposure to RAD001 was not linked to any modification in MEK phosphorylation at serines 217 and 221. Exposure to RAF265 at concentrations ranging from 1 to 10 μmol/L decreased MEK phosphorylation in BRAF-mutated cell lines. Interestingly, exposure of non-BRAF–mutated cell lines to 1 μmol/L of RAF265 paradoxically increased the phosphorylation of MEK, but this change was reversible when RAF265 concentration reached 5 μmol/L. In the HCT116, HT29, and MDAMB231 cell lines, increasing concentrations of RAF265 were associated with decreased phosphorylation of S6 ribosomal protein, an mTOR downstream effector. This unexpected effect has not been explained yet, and several hypotheses should be tested, including a transient effect linked to the anti-proliferative activity of the combination, a downstream effect following VEGFR inhibition by RAF265, or a cross-inhibition of S6 protein by the RAF-MEK pathway.

We evaluated drug efficacy in vitro using the MTT viability assay and clonogenic proliferation assay. The MTT assay revealed that in HCT116, HT-29, and MDAMB231 cells, RAD001 alone (0.1–10 nmol/L) failed to decrease cell viability while achieving a decrease of 30% in cell viability in A549 cells. In HT29 and MDAMB231 cells, RAF265 alone showed significant activity with IC_{20} values of 1 to 3 μmol/L and IC_{50} values of 5 to 10 μmol/L. In A549 and HCT116 cells, IC_{20} values were 1 μmol/L for both, but RAF265 concentrations up to 10 μmol/L did not reach IC_{50} values. However, in the presence of 1 nmol/L RAD001, the IC_{50} for RAF265 was 5 μmol/L in A549 cells and 10 μmol/L in HCT116 cells (Fig. 2A).

We used the Bliss additivism model to classify the effect of combining RAD001 and RAF265 as synergistic, additive, or antagonistic. Δ was positive in HCT116 cells, signaling a synergy between RAD001 and RAF265. Δ was negative or close to zero in A549, HT29, and MDAMB231 cells, indicating no synergy between the drugs in these cell lines. Therefore, the effect of the combination was found to be synergistic in HCT116 (KRAS mut, PIK3CA mut) cells but not in the other selected cell lines (Supplementary Fig. S1A).

Notably, exposure to RAD001 led to a small decrease in clonogenic survival in only the HCT116, HT29, and MDAMB231 cell lines. In the same experiments, RAF265 led to a significant decrease in clonogenic survival in all tested cell lines. When compared with the MTT assay findings, these results indicate that RAF265 induces a dominant effect on clonogenic survival compared with the MTT assay. This finding is consistent with the dominant role of the RAF-ERK-MEK pathway in cell proliferation. Interestingly, addition of RAF265 to RAD001 induced a small but significant decrease in cell proliferation only in
the A549 and HT29 cell lines (Fig. 2B). No significant difference was found in the size of the colonies between cells exposed to each compound alone or to the combination (Supplementary Fig. S1A and B). The different mechanisms involved in each assay likely explain the discrepancy between the MTT and clonogenic assay results, i.e., short-term cytotoxicity in the MTT assay and long-term proliferation and clonogenic survival in the clonogenic assay.

Combination of RAD001 and RAF265 Is Well Tolerated In vivo and Has an Additive Effect in the HCT116 Cell Line

In single-compound efficacy studies, optimal dosing of RAD001 and RAF265 was 5 to 12 mg/kg daily (21) and 30 mg/kg every two days (22), respectively. However, combination tolerability studies in nontumor-bearing mice defined dose-limiting toxicity as a 10% weight loss.
with the combination of RAD001 at a dose of 12 mg/kg daily and RAF265 at a dose of 20 mg/kg every two days. Therefore, the combination of RAF265 at a dose of 12 mg/kg qd and RAD001 at a dose of 12 mg/kg qd seemed to be the maximal tolerated dose. In our studies, RAD001 and RAF265 were both given at a dose of 12 mg/kg qd, alone or concurrently, over 6 days. After a 2-day stop, the compounds were given for another 6 days, and the treatment was then stopped. Under these conditions, no death or major toxicity occurred, and no animal lost >10% of its initial body weight (data not shown).

To confirm the potential of the combination of RAF265 and RAD001, we chose to test in vivo the antitumor effect of the combination in HCT116 xenografts (KRAS mut, PIK3CA mut). We then compared HCT116 sensitivity with that of A549 (KRAS mut) and MDAMB231 (KRAS mut, BRAF mut). In HCT116 xenografts, RAD001 or RAF265 given alone showed 60% to 65% and 71% to 72% TVI%, respectively. Time to achieve a relative tumor volume of 10 times the initial tumor volume (T10) was 20 days in the control group, 25 days in the RAD001 group, 25 days in the RAF265 group, and 35 days in the combination group. Therefore, the tumor growth delay was 5 days with each compound alone, and 15 days with the combination, indicating an additive effect of the combination in vivo (Supplementary Fig. S1D and E; Fig. 3). Conversely, the combination of RAF265 and RAD001 showed no additional activity in A549 and MDAMB231 cells.

Addition of RAF265 to RAD001 in HCT116 Cells Leads to Moderately Decreased AKT, S6 Protein, and 4EBP1 Phosphorylation

We performed immunoblotting to investigate the cellular effects of the RAD001/RAF265 combination in vitro. The efficacy of this combination may be linked to the inhibition of upstream and/or downstream signaling, coding for survival, and/or proliferative information. Regarding upstream signaling, mTOR inhibition leads to enhanced AKT phosphorylation in mammalian cells (11), a negative feedback loop that may involve growth factor receptors and PI3K signalization. In addition, AKT phosphorylation may lead to cell survival and ionizing radiation resistance. Therefore, we investigated whether addition of RAF-MAPK inhibition to mTOR inhibition...
interferes with AKT phosphorylation. Exposure to RAD001 was associated with an increase in AKT phosphorylation in MDAMB231 (KRAS mut, BRAF mut) but not in the A549 or HCT116 cell lines. Exposure to RAF265 was associated with decreased AKT phosphorylation in A549 and HCT116 cells but not in MDAMB231 cells. Exposure to the combination of RAD001 and RAF265 in HCT116 cells led to a slight inhibition of AKT phosphorylation, compared with each drug given alone, but not in A549 or MDAMB231 cells. Dose-effect immunoblotting confirmed these findings with a RAD001 concentration of 1 nmol/L and RAF265 concentrations of 5 or 10 μmol/L (Fig. 4A).

We have already noted that RAF265 exposure led to the decreased phosphorylation of the mTOR downstream effector S6 in HCT116, HT29, and MDAMB231 cells (Fig. 1). To investigate the mechanism of the RAD001/RAF265 combination efficacy, we studied the phosphorylation of another downstream protein, 4EBP1. This binding protein plays a key role in translation initiation because its phosphorylation releases eukaryotic initiation factor 4E, permitting initiation of cap-dependent protein translation. In HCT116 cells, phosphorylation of 4EBP1 at four different phosphorylation sites (thr 37, thr 46, ser 65, and thr 70) seemed to be decreased more notably after exposure to the combination rather than after exposure to each compound alone. A similar outcome was not observed in either A549 (KRAS mut) or MDAMB231 (KRAS mut, BRAF mut) cell lines (Fig. 4B).

Combination of RAF265 and RAD001 in HCT116 Xenografts Led to Increased Caspase-3 Activation and Decreased GLUT1 Expression without Change in CD34 Staining

The combination of RAD001 and RAF265 significantly enhanced the activation of caspase-3 in HCT116 and MDAMB231 but not in A549 xenografts. Interestingly,
RAD001 alone did not lead to an increase in AKT phosphorylation in A549, HCT116, or MDAMB231 xenografts. The combination of RAD001 and RAF265 significantly increased AKT phosphorylation in MDAMB231 xenografts but not in A549 or HCT116 xenografts. In these cell lines, the level of AKT phosphorylation remained too low to be detected by immunohistochemistry (Fig. 4C).

Vasculogenesis-targeted immunoblotting was done to dissect the mechanism of the effects of combined RAD001/RAF265 in vivo. Phosphorylation of VEGFR was not detected in HCT116 xenografts in any group. CD34 staining remained stable after the administration of RAF265, RAD001, or the combination. Interestingly, GLUT1 expression, as assessed by immunoblotting, decreased in the RAF265 and combination groups (Fig. 4D). Decreased GLUT1 expression may be linked to hypoxia, nonspecific glucose starvation, an antitumoral effect, or intracellular inhibition of the mTOR pathway. The lack of decrease in CD34 or phospho-VEGFR2 expression suggests that the decreased GLUT1 expression is not attributable to oxygen starvation. The lack of decreased GLUT1 expression after the administration of RAD001 alone, together with no additional decrease in GLUT1 expression after addition of RAF265 to RAD001, argues against the role of nonspecific glucose starvation or antitumoral effects. Therefore, the decrease in GLUT1 expression after exposure to RAF265 may be linked to the cross-inhibition of mTOR downstream effectors, rather than to nonspecific or VEGFR-driven antiangiogenic effects.

Confirmation of the Efficacy of the Combination of RAF265 and RAD001 in a Cell Line with both KRAS and PIK3CA Mutational Background

To confirm the need for concomitant mutations of KRAS and PIK3CA for optimal efficacy of the combination, we selected H460, a lung cancer cell line with specific mutation of oncogenic KRAS and concomitant mutation of PIK3CA. In the MTT assay, addition of RAD001 to RAF265 led to decreased H460 cell viability compared with each drug given alone (Fig. 5A). Using the Bliss additivism model, we found that the combination was not synergistic (Supplementary Fig. S1A). In the clonogenic assay, fewer colonies were found with the combination than with the drug alone or the predictive sum of both drugs, suggesting synergistic inhibition of cell proliferation and clonogenic survival in this cell line (Supplementary Fig. S2A; Fig. 5B).

In vivo, the combination of RAD001 and RAF265 had an enhanced effect compared with each compound given alone to mice harboring H460 xenografts. The tumor growth curve showed a limited effect of RAD001 or RAF265 given alone, but the combination of RAD001 and RAF265 induced a significantly enhanced and sustained cytostatic effect in H460 xenografts, confirmed by tumor growth curves, increased TVI%, and delayed T10 (Supplementary Fig. S2B and C; Fig. 5C). Therefore, the combination of RAF265 and RAD001 is efficient in xenografts with mutations of both KRAS and PIK3CA. Together, these data may suggest that concomitant mutations of KRAS and PIK3CA underlie the effectiveness of the RAD001/RAF265 combination, both in vitro and in vivo.

Discussion

KRAS mutations remain a major therapeutic challenge in oncology, and specific treatments require further investigation. Tyrosine kinase inhibitor combinations may be an interesting option in the setting of KRAS-mutated tumors, with different combinations being available. Legrier et al. (1) have shown synergism of the combination of concurrent low-dose MEK and mTOR inhibitor therapy in vivo in A549 (KRAS G12S) but not in H157 (KRAS G12R and PTEN G251C) xenografts. In a genetically engineered mouse model of lung adenocarcinomas, Engelman et al. (23) found that cancers driven by mutant KRAS (G12D) did not substantially respond to dual pan-PI3K and mTOR inhibitor NVP-BEZ235 used as a single agent, but did respond when NVP-BEZ235 was combined with a MEK inhibitor, ARRY-142886. In a third study, She et al. (24) tested concomitant MEK and PI3K inhibitor in vivo in PI3K-deficient tumor cells. In tumor cells with PTEN deletion and significant activation of MAPK, inhibition of PI3K-AKT signaling alone was insufficient to induce marked apoptosis, but inhibition of both pathways had synergistic effects, probably through additive inhibition of BAD phosphorylation. In a fourth study, Yu et al. (25) tested the antiproliferative effects of the PI3K inhibitors WAY-266176 and WAY-266175 in different cancer cell lines and established that heightened PI3K-AKT-mTOR signaling was linked to a sensitive phenotype. In HCT116 cells, concomitant PIK3CA and K-RAS mutations correlated with a resistant phenotype. However, a profoundly augmented growth suppression and apoptosis were achieved in resistant cells by combination treatment with WAY-266176/WAY-266175 and a MEK1 kinase inhibitor, CI-1040 or U0126 (25). Furthermore, the combination of the dual RAF/VEGFR inhibitor sorafenib with the mTOR inhibitor rapamycin has been reported to synergistically inhibit melanoma cell growth and induce enhanced apoptosis in vitro (26).

We found that the addition of RAF265 to RAD001 did not decrease cell viability or cell proliferation in cell lines with mutant KRAS and wild-type PIK3CA. Therefore, our results do not confirm the previous findings of Legrier et al. (1) and Engelman et al. (23). This discrepancy may have several explanations, including different pathway intersections, intrinsic signal plasticity, and downstream signaling differences between RAF and MEK inhibitors on the one hand, and PIK3CA and mTOR inhibitors on the other hand. Conversely, we found that the combination of RAF265 and RAD001 was efficient in cells with concomitant PIK3CA and KRAS mutations, confirming the findings of She et al. (24) and Yu et al. (25). Compared with their results, we describe similar findings with the upstream inhibition of the RAF-MEK-ERK pathway and
Figure 5. Confirmatory studies in H460 cells: the combination of RAF265 and RAD001 is additive in H460 cells. A, effect of RAF265 and RAD001 on H460 cell viability. Addition of RAD001 to RAF265 led to an enhanced cytotoxic effect in H460 cells (t test, $P = 0.02$).

B, clonogenic assay in H460 cells. Compared with vehicle, RAD001 failed to decrease the clonogenic survival of H460 cells ($P = 0.4$), whereas RAF265 induced a significant decrease ($P = 0.01$). Addition of RAD001 to RAF265 increased its antiproliferative effect (combination versus RAD001 or RAF265 alone, $P = 0.003$ and $P = 0.016$, respectively).

C, relative tumor growth curve. In H460 xenografts, RAD001 and RAF265 given alone exhibited limited effect. However, the combination of RAD001 and RAF265 significantly delayed tumor growth (ANOVA, $P < 0.001$) compared with vehicle or to each compound given alone.
the downstream inhibition of the PI3K-AKT-mTOR pathway, and we offer three important observations relevant to our findings. First, RAF inhibitors have a chemical structure that allows the cross-inhibition of VEGFR2 and may contribute antiangiogenic properties to intracellular signal inhibition. Second, mTOR inhibition occurs downstream of PI3K and may interfere with other growth factor receptor proliferation signals. Third, RAD001 is already available in the clinic and combines good tolerance profile with high potency, and RAF265 is currently being tested in a phase I study in patients with locally advanced or metastatic melanoma (ClinicalTrials.gov Identifier NCT00304525).

Our findings suggest that the combination of RAD001 and RAF265 is effective in the setting of concomitant mutations of both the RAS-RAF-MAPK and PI3K-AKT-mTOR pathways. Three different mechanisms may mediate this efficacy, inferred from our current results. First, cross-inhibition of 4EBP1 phosphorylation induces a shift toward the hypophosphorylated form of 4EBP1. This shift correlates with a reduced level of cap-dependent translation that permits the 4EBP1 sequestration of eIF4E (27). Second, the inhibition of the negative feedback loop by RAF265 decreases RAD001-induced AKT hyperphosphorylation and may interfere with AKT-associated survival signaling (28). Third, the combination of RAF265 and RAD001 may lead to decreased GLUT1 expression. However, these data require further investigation, including experiments involving genetic inhibition of AKT using small interfering RNA, and ongoing investigations should clarify the respective roles of AKT, S6 protein, 4EBP1, GLUT1, and VEGFR2 in the observed effects.

Together, our results identify the deregulation of the RAS-RAF-MEK-ERK pathway as a dominant determinant in cancer cell resistance to mTOR inhibitors. Recent studies have outlined the existence of oncogenic PIK3CA mutations concomitantly with EGFR, KRAS, or BRAF mutations. In non–small-cell lung cancer, mutation and amplification of PI3K have recently been reported to be involved in as much as 20% of lung tumors (29). Interestingly, if EGFR, KRAS, RAF, and HER2 mutations are mutually exclusive, PIK3CA mutations can be associated with a mutation in one of these four proteins (29, 30). To date, the exact frequency of double mutations remains unknown either in primary lung tumors or in distant metastases, and significant discrepancies between both sites have already been suggested for EGFR (31) and KRAS mutations (32, 33). In colorectal cancer, a recent population-based study of 886 patients with colon adenocarcinomas found mutations in KRAS, BRAF, and/or PIK3CA in 316 (56%) of the 586 tumors studied. KRAS is the most commonly mutated gene in this pathway, with mutations in 35% to 45% of colorectal adenocarcinomas; mutations in PIK3CA (<20%) and BRAF (<15%) are less common. Again, even if KRAS and BRAF mutations seem to be mutually exclusive, mutations in PIK3CA and KRAS may coexist within the same tumor (34).

Conclusion

A combination of the RAF/VEGFR2 inhibitor RAF265 with the mTOR inhibitor RAD001 is an effective strategy to enhance cytotoxic and antiproliferative effects on cells with deregulation of both the RAS-RAF and PI3K-PTEN pathways. Our results indicate that RAD001 and RAF265 inhibit their targets as well as downstream effectors thought to be in other pathways, providing evidence for cross-talk between the different signaling pathways studied. Therefore, concomitant PI3K-AKT-mTOR and RAF-MAPK inhibition is a promising strategy to overcome concomitant KRAS and PIK3CA oncogene activation. Together, these findings argue for the combination of targeted therapies in selected tumors. Further studies are needed to confirm the potential of this particular association in terms of tolerance and efficacy and to establish a translational project to select patients with both PIK3CA- and KRAS-mutated tumors who may benefit from combination therapy with RAF265 and RAD001.

Disclosure of Potential Conflicts of Interest

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Dependence on Phosphoinositide 3-Kinase and RAS-RAF Pathways Drive the Activity of RAF265, a Novel RAF/VEGFR2 Inhibitor, and RAD001 (Everolimus) in Combination

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