Overcoming IGF1R/IR Resistance through Inhibition of MEK Signaling in Colorectal Cancer Models

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Abstract

Purpose: Results from clinical trials involving resistance to molecularly targeted therapies have revealed the importance of rational single-agent and combination treatment strategies. In this study, we tested the efficacy of a type 1 insulin-like growth factor receptor (IGF1R)/insulin receptor (IR) tyrosine kinase inhibitor, OSI-906, in combination with a mitogen–activated protein (MAP)–ERK kinase (MEK) 1/2 inhibitor based on evidence that the MAP kinase pathway was upregulated in colorectal cancer cell lines that were resistant to OSI-906.

Experimental Design: The antiproliferative effects of OSI-906 and the MEK 1/2 inhibitor U0126 were analyzed both as single agents and in combination in 13 colorectal cancer cell lines in vitro. Apoptosis, downstream effector proteins, and cell cycle were also assessed. In addition, the efficacy of OSI-906 combined with the MEK 1/2 inhibitor selumetinib (AZD6244, ARRY-142886) was evaluated in vivo using human colorectal cancer xenograft models.

Results: The combination of OSI-906 and U0126 resulted in synergistic effects in 11 of 13 colorectal cancer cell lines tested. This synergy was variably associated with apoptosis or cell-cycle arrest in addition to molecular effects on prosurvival pathways. The synergy was also reflected in the in vivo xenograft studies following treatment with the combination of OSI-906 and selumetinib.

Conclusions: Results from this study demonstrate synergistic antiproliferative effects in response to the combination of OSI-906 with an MEK 1/2 inhibitor in colorectal cancer cell line models both in vitro and in vivo, which supports the rational combination of OSI-906 with an MEK inhibitor in patients with colorectal cancer. Clin Cancer Res; 19(22); 6219–29. ©2013 AACR.

Introduction

The insulin-like growth factor (IGF) system involves complex regulatory networks, which can control numerous developmental and physiological functions including growth, mitosis, apoptosis, and differentiation (1–3). These diverse biological effects are primarily controlled through interactions between the ligands (IGF-1 and IGF-2) and the type 1 IGF receptor (IGF1R; ref. 4). The IGF ligands are modulated by the activity of IGF binding proteins (IGFBP) found in the circulation and extracellular fluids. IGFBPs bind and sequester IGF ligands controlling their availability for receptors. IGF-1 and IGF-2 are usually found in complex with IGFBP-3 and the acid-labile subunit. Both IGF ligands elicit signals through IGF1R, and IGF-2 can additionally signal through the insulin receptor (IR; refs. 2, 5). Following ligand binding, the intracellular tyrosine kinase domain of IGF1R is auto-phosphorylated before recruitment of 2 adaptor proteins, SRC homology and collagen domain and insulin receptor substrate 1. Subsequent phosphorylation of these adaptor proteins leads to recruitment of other factors, Grb2/SOS and PI3 kinase, thereby activating the mitogen-activated protein kinase (MAPK) and PI3K signaling cascades, respectively. These pathways ultimately regulate transcription factors to elicit changes in gene expression, which mediate the biological effects of the IGF system (5).

Many years of research have culminated in a strong association between the IGF1R pathway and cancer. The IGF system has been implicated in multiple aspects of the tumor environment including growth, evasion of apoptosis, and metastasis (6, 7). Epidemiological data suggest that...
increased levels of circulating IGF-1 result in higher risk for colon, breast, lung, and prostate cancers, which comprise the most prevalent and deadly types of cancer (5, 8). Furthermore, several studies have specifically linked the IGF system to colorectal cancer. For example, it has been reported that IGF-2 is overexpressed in colorectal cancer compared with normal colonic mucosa (3, 9, 10). In addition, high levels of IGF1R and circulating IGF-1 have been linked to metastasis of colorectal cancer (11, 12). Altogether these data suggest that IGF1R is an attractive therapeutic target for several tumor types including colorectal cancer.

Because of its implication in several tumor types, the IGF1R has been one of the most intensely studied therapeutic targets in cancer research. Nearly 30 compounds targeting IGF1R are being investigated both in vitro and in phase I, II, and III clinical trials. These compounds include both antibodies against IGF1R and inhibitors of the IGF1R intracellular tyrosine kinase domain (13). The tyrosine kinase inhibitor (TKI), OSI-906, is one of these agents. OSI-906 is a selective and orally bioavailable IGF1R/IR TKI, which exhibits potent ligand-dependent inhibition of phosphorylation of IGF1R and IR. Furthermore, OSI-906 has been shown to prevent ligand-induced activation of downstream pathways including pAkt, pERK1/2, and p-p70S6K. Phase I and II clinical trials involving OSI-906 are currently in progress (14).

Our prior in vitro data demonstrated the effect of OSI-906 on 27 colorectal cancer cell lines. Six cell lines were found sensitive and 21 cell lines resistant to OSI-906. The sensitivity profiles of these cell lines were further confirmed through in vivo xenograft studies (15). The major clinical challenge of drug resistance in developmental cancer therapeutics necessitates investigation into patient-selective single agent and rational combination therapeutic strategies. For that reason we previously performed pathway enrichment analysis of basal gene expression to identify expression differences between the colorectal cancer cell lines that were sensitive or resistant to OSI-906. This analysis revealed RAS/RAF/MAPK signaling pathway as one of the top enriched pathways in colorectal cancer cell lines that were resistant to OSI-906 (15). Therefore, in this study, we examined the efficacy of OSI-906 in combination with an MAP–ERK kinase (MEK) 1/2 inhibitor, either U0126 or selumetinib (AZD6244, ARRY-142886) against colorectal cancer cell lines. Based upon our prior analysis, we hypothesized that the interaction between OSI-906 and an MEK inhibitor would be synergistic in colorectal cancer cell lines that are resistant to OSI-906. Interestingly, we found that this combination was synergistic regardless of sensitivity to OSI-906. Our results suggest that the combination of OSI-906 with an MEK inhibitor represents a rational and potentially active therapeutic strategy in patients with colorectal cancer.

Materials and Methods

Drugs

Selumetinib was generously provided by AstraZeneca Pharmaceutical and the National Cancer Institute, NIH. OSI-906 was generously provided by OSI Pharmaceuticals, LLC/Astellas, and the National Cancer Institute, NIH. U0126 was obtained from Promega. Both OSI-906 and U0126 were dissolved in dimethyl sulfoxide at 10 mmol/L, and stored at −20°C. For in vivo studies, OSI-906 was dissolved in 25 µmol/L tartaric acid and selumetinib was dissolved in 80%, 0.5% methylcellulose/20% Tween 80 for in vivo use.

Cell lines and culture

Twelve of the human colorectal cancer cell lines were obtained from the American Type Culture Collection. GEO cells were provided by Dr. Fortunato Ciardiello (Cattedra di Oncologia Medica, Dipartimento Medico-Chirurgico di Internistica Clinica e Sperimentale “F Magrassi e A Lanzara,” Seconda Universita degli Studi di Napoli, Naples, Italy). GEO cells were cultured in DMEM/F12. All other cells were routinely cultured in RPMI 1640. All medium was supplemented with 10% FBS, 1% penicillin–streptomycin, and 1% MEM nonessential amino acids. All cells were kept at 37°C under an atmosphere containing 5% CO2. Cells were routinely tested for the presence of mycoplasma (MycoAlert; Cambrex BioScience).

Proliferation and combination effects

Cell proliferation was analyzed using the sulforhodamine B (SRB) method (16). Cells in a logarithmic growth phase were transferred to 96-well flat bottom plates with lids. One hundred microliter cell suspensions containing 5,000 viable cells were plated into each well, and cells were allowed to attach overnight before drug exposure. All cell lines were exposed to OSI-906 (0.3, 0.6, and 1.2 µmol/L) and U0126 (0.5, 1.0, and 5.0 µmol/L) alone or in all possible combinations for 72 hours. Doses were chosen based on previous published studies with these compounds (15, 17). After exposure to drugs, cells were fixed with cold 10% trichloroacetic acid for 30 minutes at 4°C. Cells were washed with water and stained with 0.4% SRB (MP Biomedicals) for 20
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minutes at room temperature, after that cells were washed again with 1% acetic acid and bound SRB was solubilized with 10 mmol/L Tris at room temperature. The optical density was read on a plate reader (Biotek Synergy 2) set at an absorbance wavelength 565 nm. Cell proliferation curves were derived from the raw optical density data and results of combinations were analyzed by the Chou and Talay method (18) using the CalcuSyn software program (Biosoft). For each combination, the combination index (CI) was calculated with synergy indicated by a CI < 1, additivity by a CI = 1, and antagonism by a CI > 1.

Caspase 3/7 activity

Cells were seeded in 96-well white-walled plates at 5,000 to 10,000 cells/well and allowed to attach for 24 hours before drug exposure. Cells were then exposed to OSI-906 (0.6 and 1.2 μmol/L) and U0126 (2.5 and 5.0 μmol/L) alone and in combination for 12, 24, 48, and 72 hours. Caspases 3 and 7 were measured using a luminometric Caspase-Glo 3/7 assay (Promega) according to manufacturer’s protocol using a plate reader (Biotek Synergy 2). For statistical analysis, each single agent was compared with the appropriate combination and a T test was performed.

Flow cytometric analysis of cell-cycle distribution

Cells were plated in 6-well plates and incubated overnight. The cells were then treated with both OSI-906 (1.2 μmol/L) and U0126 (5 μmol/L) alone or in combination. After 24 hours, cells were collected using trypsin and rinsed once in PBS. Cells were then re-suspended in Krishan’s stain and allowed to incubate for at least 12 hours at 4°C before analysis by the University of Colorado Cancer Center Flow Cytometry Core Facility.

Immunoprecipitation and immunoblotting

Five different colorectal cancer cell lines were seeded into 6-well plates, allowed to attach overnight, and exposed for 6 hours to OSI-906 (1.2 μmol/L) and U0126 (5 μmol/L) alone or in combination. After treatment, cells were rinsed with PBS and scraped into RIPA lysis buffer containing protease inhibitors, EDTA, NaF, and sodium orthovanadate. Total protein was quantified using the BioRad DC Protein Assay (BioRad). Total protein (40 μg) was loaded on a 4% to 12% gradient gel, electrophoresed, and then transferred to a nitrocellulose membrane using the I-Blot (Promega) according to a 4% to 12% gradient gel, electrophoresed, and then transferred to a nitrocellulose membrane using the I-Blot (Promega) according to manufacturer’s protocol. The following primary antibodies were used: pAkt, Akt, pERK, ERK, p66RPA, S6RPA, p4EBP1, 4EBP1, pMDM2, MDM2, PARP, LC3B, or Actin. (All from Cell Signaling except MDM2 from Santa Cruz Biotechnology.) Blots were then washed 3 × 10 minutes in 1× PBS containing 0.1% Tween-20 and incubated with the appropriate secondary goat anti-rabbit and goat anti-mouse immunoglobulin G (H + L) DyLight conjugated antibodies (Thermo Scientific) at a 1:15,000 dilution for 1 hour at room temperature. Following 3 × 20 minute washes, blots were developed using the Odyssey Infrared Imaging System (LI-COR Biosciences).

In vivo xenograft studies

Five to six-week-old female athymic nude mice (Harlan Sprague-Dawley) were used. Mice were caged in groups of 5, kept on a 12-hour light/dark cycle, and provided with sterilized food and water ad libitum. Animals were allowed to acclimate for at least 7 days before any handling. For the colorectal cancer cell line–derived xenografts, SW480 or HCT15 cells in logarithmic growth phase were harvested with 0.25% Trypsin/EDTA, pelleted by centrifugation, resuspended in a 1:1 mixture of serum-free RPMI 1640 and Matrigel (BD Biosciences), and injected (5 × 106 cells in 100 μL) subcutaneously into the flank of 20 mice (8–10 evaluable tumor per group). The patient–derived xenograft CUCRC006 was generated as previously described (17). Briefly, a peritoneal metastasis tumor specimen was collected at the time of surgery from a consenting patient at the University of Colorado Hospital. Tumor material not needed for histopathologic analysis was cut into pieces 2 to 3 mm3 in size, coated in Matrigel, and implanted subcutaneously. After tumors were expanded through the F3 generation, they were injected into the left and right flanks of approximately 60 mice (30 evaluable tumors per group). When the average tumor size of patient- or cell line–derived xenografts reached a volume of approximately 150 mm3, mice were randomized into the vehicle group, OSI-906 group, selumetinib group, or combination group. Mice were monitored daily for signs of toxicity and weighed twice weekly. Treatment was administered once daily (40 mg/kg OSI-906) or twice daily (25 mg/kg selumetinib) by oral gavage, and tumor size was evaluated twice per week with caliper measurements, using the Study Director software package (Studyslog Systems). Tumor volume was calculated with the equation: volume = (length × width2) / 2. Tumor growth inhibition (TGI) was calculated from the average tumor volume of the treated (Vt) and vehicle (Vv) groups, with the equation: TGI = 1 − (Vt/Vv).

Xenograft studies were conducted in accordance with the NIH guidelines for the care and use of laboratory animals in a facility accredited by the American Association for Accreditation of Laboratory Animal Care, and received approval from University of Colorado Institutional Animal Care and Use Committee before initiation. Collection of patient tumor tissue at the University of Colorado hospital was performed under a Colorado Multi-Institutional Review Board approved protocol.

Immunohistochemistry

Immunohistochemistry was performed in the University of Colorado Cancer Center Pathology Core Lab. Five-micrometer-thick paraffin sections were deparaffinized, antigens unmasked, and immunohistochemically stained for Ki-67 [Neomarkers/Thermo Scientific; rabbit monoclonal SP6; cat# RM-9106-SO; dilution 1:500 in TBST + 1% BSA (w/v)]. Antigens were revealed in pH 9.5 BORG.
solution (Biocare Medical) for 5 minutes at 125°C (22 psi; Decloaking chamber, Biocare) with a 10-minute ambient cool down. Immunodetection was performed on the NexES stainer (Ventana Medical Systems) at an operating temperature of 37°C with a primary antibody incubation time of 32 minutes. Antibodies were detected with a modified I-VIEW DAB (Ventana) detection kit. The I-VIEW secondary antibody and enzyme were replaced with a species-specific rabbit conjugated polymer (Rabbit ImmPress; cat# MP-7401; Vector Labs; full strength in place of the secondary antibody, 50% strength diluted in PBS pH 7.6 in place of the SA-HRP, 8 minutes each). All sections were counterstained in Acidified Harris hematoxylin for 1.5 minutes, blued in 1% ammonium hydroxide (v/v), dehydrated in graded alcohols, cleared in xylene, and coverglass mounted using synthetic resin.

Statistical methods
Statistical analysis was performed using GraphPad Prism Software. For comparisons of 2 groups, an unpaired t test was performed. All comparisons with P < 0.05 are considered statistical significant. A ANOVA was used to determine whether the means were significantly different between the groups. If the overall means were significantly different, we carried out a pair-wise comparison. The P values were adjusted using Tukey’s method for multiple comparisons. SE of the mean was indicated for each value by a bar.

Results
The effects of OSI-906 and U0126 as single agents on proliferation of colorectal cancer cell lines
Initially, a panel of 27 colorectal cancer cell lines was exposed to OSI-906 (0–5 μmol/L). Cell lines considered sensitive (S) to OSI-906 had IC50 < 1.5 μmol/L and resistant (R) cell lines had no IC50 found up to 5 μmol/L (15). As shown in Fig. 1A and Supplementary Fig. S1A, 13 of these cell lines were further investigated for this study. Similarly, a panel of the same 13 colorectal cancer cell lines was exposed to U0126 (0–20 μmol/L; Fig. 1B and Supplementary Fig. S1B). We did not consider any of the cell lines sensitive to U0126 as IC50 values were largely >1.5 μmol/L. Mutational status for KRAS, BRAF, and PIK3CA did not correlate to sensitivity to either agent.

The interaction between OSI-906 and U0126 is synergistic in colorectal cancer cell lines
Previously, Affymetrix U133 Plus 2.0 gene arrays were performed on 4 sensitive and 5 OSI-906–resistant colorectal cancer cell lines to assess basal gene expression. Pathway enrichment analysis of this data revealed that the RAS/RAF/MAPK signaling pathway was among the top enriched pathways in OSI-906–resistant colorectal cancer cell lines (15). The core genes upregulated were found at different levels throughout the classical MAPK pathway including ligands, receptors, and key downstream signaling proteins. For example, among the core genes found upregulated were: FGF, PDGF, FGFR, RAS, RAF1, and MEK2 (Fig. 2). We therefore hypothesized that the combination of U0126 with OSI-906 would induce synergistic antiproliferative effects in colorectal cancer cell lines that were resistant to OSI-906. Thirteen colorectal cancer cell lines were chosen for exposure to the combination with a range of responsiveness to OSI-906 or U0126. OSI-906 and U0126 displayed a synergistic interaction in 11 of the 13 colorectal cancer cell lines tested regardless of sensitivity to either agent (Fig. 3 and Supplementary Figs. S2–S4). In these 11 cell lines, nearly all CI values were less than 1 and several cell lines displayed strong synergy. For example, Colo205 colorectal cancer cells exhibited a range of CI values between 0.09 and 0.40 following exposure to all combinations of OSI-906 and U0126. Two other cell lines, RKO and GEO, showed variable combination effects (CI values ranging from 0.7 to 1.6).
Apoptosis is enhanced following exposure to the combination of OSI-906 and U0126 in colorectal cancer cell lines

Several studies have demonstrated induction of apoptosis in response to inhibition of the MEK pathway (19–21). Therefore, caspase 3/7 activity was measured in all 13 colorectal cancer cell lines to identify whether there was induction of apoptosis following exposure to U0126 and/or the combination. Interestingly, only the Colo205 cell line that is inherently sensitive to OSI-906 demonstrated a synergistic induction of apoptosis following combination treatment, which was associated with PARP cleavage (Fig. 4 and Supplementary Figs. S5 and S6).

G₀–G₁ cell-cycle arrest is induced in colorectal cancer cells exposed to OSI-906, U0126, and the combination

Inhibitors of IGF-1R and RAS/RAF/MAPK signaling pathways are known to impact cell cycle through G₀–G₁ arrest (19, 22–26). Thus, assessment of cell-cycle distribution was carried out using flow cytometric analysis in the colorectal cancer cell lines. Five colorectal cancer cell lines were chosen for this analysis, including Colo205 and LS513 cells, which were associated with apoptosis in response to combination treatment, and the SW480 and HCT15 cell lines that exhibited strong synergy, but no apoptosis. In general, we observed G₀–G₁ arrest with OSI-906 and U0126 that was maintained in the combination (Supplementary Fig. S7). No differential effects on the cell cycle were observed to differentiate the effects of synergy associated with proliferation.

The combination of OSI-906 and U0126 inhibits prosurvival pathways and affects MDM2 phosphorylation

OSI-906 and U0126 inhibit well-characterized downstream effects on their respective pathways. OSI-906 has been shown to decrease IGF1R, IR, AKT, ERK, and p70S6K phosphorylation (14), whereas U0126 inhibits phosphorylation of MEK and ERK (27, 28). To validate the effects of these 2 agents and look for evidence of synergy, modulation of downstream targets in the MAPK and PI3K pathways were analyzed (Fig. 5A and Supplementary Fig. S8). Phosphorylation of AKT or ERK was decreased with exposure to OSI-906 or U0126, respectively (Fig. 5); although this was variable among the cell lines and there were more consistent effects on p-ERK (Supplementary Fig. S8). Interestingly, these effects were not observed in the RKO cells that were resistant to OSI-906 and did not exhibit synergy in the combination. A striking effect observed in the sensitive cell lines undergoing apoptosis with the combination (Colo205, LS513) was the loss of pS6RP with a subtle decrease in 4eBP1. Because of the reported relationship
between the PI3K/AKT/mTOR pathway and MDM2 regulation (29), we also assessed MDM2 phosphorylation following a 24-hour exposure to the combination. We observed a modest decrease in pMDM2 in the combination in the 2 sensitive cell lines exhibiting apoptosis, but not in the other cell lines exhibiting additivity or synergy but not apoptosis.

**Confirmation of antitumor effects using OSI-906 and selumetinib in vivo**

To further investigate this combination, we next conducted in vivo experiments. Selumetinib was used for the in vivo studies as this agent has better antitumor effects in vivo and is available through the Cancer Therapy Evaluation Program (CTEP). The combination was initially tested in HCT15 and SW480 colorectal cancer cell line–derived xenografts. In the HCT15 xenograft model, neither OSI-906 nor selumetinib as a monotherapy had a statistically significant effect on tumor growth when compared with vehicle, after 20 days of treatment (TGI = 38% and 48% for OSI-906 and selumetinib, respectively). However, as depicted in Fig. 6A, treatment with the combination resulted in statistically significant tumor growth inhibition (TGI = 81%, P = 0.0007) as compared with vehicle. A similar effect was observed in the SW480 model but to a lesser extent (Supplementary Fig. S9).

We also tested the combination in a patient-derived tumor xenograft (PDTX) model. CUCRC006 (KRASMT/BRAFWT, and PIK3CAWT) responded very similarly to the HCT15 xenograft (Fig. 6B). After treatment for 62 days, neither single agent demonstrated a statistically significant effect on tumor growth (TGI = 4.8% and 28% for OSI-906 and AZD-6244, respectively) nor the combination treatment did when compared with the vehicle or either single-agent group (TGI = 64%, P < 0.0002). As a comparison, this PDTX model had a TGI of 34% for irinotecan (data not shown). Concordant with these results was IHC staining for % of Ki67 positive cells: 90% (vehicle), 75% (selumetinib), 80% (OSI-906), and 30% for the combination (data not shown).

**Discussion**

Several laboratory and epidemiological studies have revealed the importance of the IGF signaling pathway in colorectal cancer (30). Although initial clinical trials involving IGF1R inhibitors were encouraging, the latest combination studies have shown varied results (13, 31, 32). In recent clinical trials with other molecularly targeted agents, low response rates and acquired resistance have been observed. Evidence of resistance has already been discovered following treatment with inhibitors of BCR-ABL, and EGFR, among others (33). Therefore, multiple studies are investigating the mechanisms of resistance to specific
targeted agents, including our previous investigation into predictive biomarkers for sensitivity to OSI-906 (15, 34–36). These studies can also provide evidence for rational combinations, which may result in more effective clinical therapeutic regimens and successful patient outcomes.

The combination of an IGF1R inhibitor with an MEK inhibitor has recently generated wide interest, yet few studies of this type have been published to date. Buck and colleagues (37) briefly explored the combination of PQIP (IGF1R-TKI) and PD98059 (MEK1 inhibitor) in colorectal, pancreatic, and lung cancer cell lines using the Bliss additivity model. In addition, examination of the combination of an IGF1R antibody with U0126 has been assessed in hematopoietic cells (38). Based on previous study, we investigated the combination of an IGF1R/IR tyrosine kinase inhibitor, OSI-906, and an MEK 1/2 inhibitor (either U0126 or selumetinib) in OSI-906–resistant colorectal cancer cell lines (15). We hypothesized that cell lines resistant to OSI-906 would be more likely to have a synergistic response when used in combination. However, this study revealed sensitivity/resistance to OSI-906 did not predict synergistic response. We additionally evaluated trends in U0126 sensitivity, and mutational statuses of KRAS, BRAF, and PIK3CA (previously sequenced in our colorectal cancer cell lines; ref. 15). However, there were no apparent trends that predicted a synergistic response.

To further delineate the interaction between these drugs in combination, we first assessed induction of apoptosis. Bertrand and colleagues (38) reported that apoptosis was enhanced in response to the combination of an IGF1R antibody and U0126 in hematopoietic stem cells. Two colorectal cancer cell lines of our 13 tested similarly indicated an induction of apoptosis in response to the combination of OSI-906 and U0126. However, only one of them (Colo205), which is inherently sensitive to OSI-906 demonstrated synergistic induction of apoptosis.

Because of the lack of apoptosis and yet the presence of synergy, cell-cycle effects were also assessed by flow cytometry. Several studies have shown induction of G0–G1 arrest following exposure to IGF1R and MEK inhibitors as single agents (19, 22–26). However, evaluation of the cell cycle following treatment with both an IGF1R inhibitor and an MEK inhibitor in combination has not been published. Our cell-cycle analysis did not generally reveal mechanisms of synergy. Combination treatment did not demonstrate any increase in cell-cycle arrest as compared with single-agent effects with the exception of one cell line. In the OSI-906–resistant cell line, HCT15 cells did exhibit a small induction of G0–G1 arrest in combination treated cells. Similar results have been recently described with combinations of PIK3CA and MEK inhibitors where there was an enhanced cell-cycle arrest at G0–G1 following exposure to IGF1R and MEK inhibitors as single agents (19, 22–26). However, evaluation of the cell cycle following treatment with both an IGF1R inhibitor and an MEK inhibitor in combination has not been published. Our cell-cycle analysis did not generally reveal mechanisms of synergy. Combination treatment did not demonstrate any increase in cell-cycle arrest as compared with single-agent effects with the exception of one cell line. In the OSI-906–resistant cell line, HCT15 cells did exhibit a small induction of G0–G1 arrest in combination treated cells. Similar results have been recently described with combinations of PIK3CA and MEK inhibitors where there was an enhanced cell-cycle arrest at G0–G1 following combination treatments (39–42). One possible explanation for the lack of cell-cycle arrest observed in this study could be the fact that 3 of 5 cell lines evaluated exhibited strong single-agent effects with 90% arrest in G0–G1.

Given that both OSI-906 and U0126 are inhibitors of prosurvival signaling cascades, their actions as such have been characterized in previous reports (14, 27, 28). In our colorectal cancer cell lines it seems that IGF1R inhibition primarily acts through the PI3K/AKT/mTOR pathway and not the RAS/RAF/MAPK signaling pathway, as expected; whereas U0126 exhibited inhibition of MEK/ERK. Combination effects on phosphorylation of both AKT and ERK were comparable to those observed in single-agent exposures. Interestingly, dephosphorylation of S6RP was...
observed when the drugs were used in combination, which has been previously described (43). However, others have noted activation of AKT by MEK inhibitors and ERK activation by PI3K inhibitors (43–47). These data clearly support the fact that there is cross-talk between the RAS/RAF/MAPK and PI3K/AKT/mTOR signaling pathways; however, this relationship is not clearly defined and perhaps is cell-context dependent.

Several studies have reported MDM2-mediated apoptosis in response to inhibiting PI3K/AKT/mTOR signaling pathway (29, 48, 49). In this study, 2 of the cell lines (Colo205 and LS513) demonstrated a reduction of MDM2 in response to combination treatment and interestingly, both are wild type for PIK3CA. These results are intriguing and warrant further study into the PI3K dependence of the p53/MDM2 axis.

Finally, we tested the efficacy of OSI-906 with a clinical MEK 1/2 inhibitor, selumetinib, in athymic nude mice. We conducted the in vivo studies using both HCT15 and SW480 cell lines, which both displayed synergy without apoptosis in response to the combination of OSI-906 and U0126 in vitro. Despite this lack of apoptosis in vitro, the combination of OSI-906 and selumetinib demonstrated striking tumor growth inhibition following 30 days of treatment in mice. This was confirmed with a reduction in Ki67 staining. Next both agents were tested in our PDTX model. These PDTX models have certain advantages over cancer cell line–derived xenograft models (50). They closely recapitulate the heterogeneity of human tumors and are excellent models to study stromal–tumor interactions and drug efficacy testing (50). Our PDTX model confirmed our previous findings that the combination had significant antitumor activity when compared with the vehicle or either single agent, and the Ki-67 staining was, again, markedly reduced in the combination. These in vivo studies would rationally support clinical investigation into the combination of an IGF1R inhibitor and an MEK inhibitor in patients with colorectal cancer.

In summary, initial results from clinical trials involving IGF1R inhibitors are mixed (13, 31, 32), and likely reflect either the lack of patient selection strategies and/or recognition of compensatory resistance pathways. Thus, studies such as this are needed to develop rational, mechanism-based combinations that may be translated to the clinic and yield improved benefit for patients. One ongoing challenge...
is how to select patients for combination therapy because the magnitude of synergistic effects observed in preclinical models can vary widely and insights into mechanisms of apoptosis may not be apparent. However, as next-generation sequencing analysis becomes more integrated into the characterization of preclinical models and patient response profiles, the ability to select particular molecular subtypes for rational combinations such as IGF1R/IR and MEK blockade in colorectal cancer may become possible.

Disclosure of Potential Conflicts of Interest
S.G. Eckhardt has commercial research grant in AstraZeneca. No potential conflicts of interest were disclosed by the other authors.

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Conception and design: S.A. Flanigan, T.M. Pitts, A.C. Tan, S.G. Eckhardt, S. Leong
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