

## The Insulin-like Growth Factor I Receptor/Insulin Receptor Tyrosine Kinase Inhibitor PQIP Exhibits Enhanced Antitumor Effects in Combination with Chemotherapy Against Colorectal Cancer Models

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### Abstract

**Purpose:** There is growing evidence implicating the importance of the insulin-like growth factor (IGF) pathway in colorectal cancer based upon the results of population studies and preclinical experiments. However, the combination of an IGF-I receptor (IGF-IR) inhibitor with standard colorectal cancer chemotherapies has not yet been evaluated. In this study, we investigated the interaction between PQIP, the dual IGF-IR/insulin receptor tyrosine kinase inhibitor, and standard chemotherapies in colorectal cancer cell line models.

**Experimental Design:** The antiproliferative effects of PQIP, as a single agent and in combination with 5-fluorouracil, oxaliplatin, or SN38, were analyzed against four colorectal cancer cell lines. Downstream effector proteins, apoptosis, and cell cycle were also assessed in the combination of PQIP and SN-38. Lastly, the efficacy of OSI-906 (a derivative of PQIP) combined with irinotecan was further tested using a human colorectal cancer xenograft model.

**Results:** Treatment with the combination of PQIP and each of three chemotherapies resulted in an enhanced decrease in proliferation of all four colorectal cancer cell lines compared with single-agent treatment. This inhibition was not associated with a significant induction of apoptosis, but was accompanied by cell cycle arrest and changes in phosphorylation of Akt. Interestingly, antitumor activity between PQIP and SN-38 *in vitro* was also reflected in the human colorectal cancer xenograft model.

**Conclusions:** Combination treatment with PQIP, the dual IGF-IR/insulin receptor tyrosine kinase inhibitor, and standard colorectal cancer chemotherapy resulted in enhanced antiproliferative effects against colorectal cancer cell line models, providing a scientific rationale for the testing of OSI-906 and standard colorectal cancer treatment regimens. *Clin Cancer Res*; 16(22); 5436–46. ©2010 AACR.

The insulin-like growth factor I (IGF-I) signaling pathway is a key regulator of normal cell proliferation, differentiation, and apoptosis (1, 2). The IGF systems consists of multiple circulating ligands (IGF-I, IGF-II, and insulin) interacting with the IGF-I receptor (IGF-IR), which then leads to signaling through various downstream pathways including the phosphoinositide 3-kinase (PI3K)/Akt pathway inducing stimulation of mTOR and S6 kinase, and activation of the mitogen-activated protein kinase (MAPK) pathway through Ras (2).

In the past two decades, there has been growing evidence implicating the importance of the IGF pathway in the devel-

opment and progression of cancers (reviewed in refs. 3–5). Clinical studies have shown that there is an overexpression of IGF-IR in cancer cells as compared with normal tissue, and that IGF-IR is ubiquitously expressed in cancerous tissues (6–9). Although there are no specific mutations in IGF receptors or ligands that have been identified in cancer, there is clear evidence of epigenetic alterations, whereas elevated IGF-I and IGF-IR signaling are associated with an increased risk of a wide range of cancers, including breast, prostate, and colon (10–13). Conversely, the reduction of IGF-IR signaling has been associated with tumor growth inhibition, reduced metastasis, and enhancement of the effects of other cancer therapy (6, 14, 15). The IGF/IGF-IR pathway has also been shown to have extensive cross-talk with the estrogen receptor, epidermal growth factor receptor (EGFR), and human epidermal growth factor receptor 2 (HER2) signaling, and plays an important role in the resistance mechanism of cytotoxic drugs, and hormonal and EGFR/HER2-targeted agents (16–18). Taken together, these data confirm the role of IGF-IR signaling in neoplasia and therapeutic resistance.

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### Translational Relevance

Insulin-like growth factor I receptor (IGF-IR) inhibitors have now entered clinical trials. The majority of these inhibitors, like other newly developed targeted agents, will eventually be incorporated into combination regimens with traditional chemotherapy. This article is the first to report on the combination of an IGF-IR/insulin receptor tyrosine kinase inhibitor (TKI) with standard chemotherapy in colorectal cancer cell line models *in vitro* and *in vivo*. These results provide the scientific rationale for an IGF-IR/insulin receptor TKI and chemotherapy combination and have led to an investigator-initiated phase I/Ib clinical trial assessing the combination of irinotecan and the dual IGF-IR/insulin receptor TKI OSI-906 in patients with advanced colorectal cancer.

In colorectal cancer, numerous epidemiologic studies have supported the relationship between the IGF system and the risk of colon cancers (11, 19–21). The IGF-IR is overexpressed  $\geq 5$ -fold higher in colorectal cancers than in adjacent normal tissue (9). In a retrospective study, higher expression of IGF-IR was associated with higher grade and stage of colorectal tumors, whereas, the gene for the IGF-II ligand is the single most overexpressed gene in colorectal cancer (7, 22). Therefore, inhibition of the IGF-IR pathway is an attractive therapeutic strategy for colorectal cancer.

The majority of inhibitors of IGF-IR in clinical studies are antibodies or tyrosine kinase inhibitors (TKI). Significant homology exists between the intracellular domains of IGF-IR and the insulin receptor (23), and because of this homology there were concerns that TKIs would increase the incidence of hyperglycemia as compared with antibodies. Early clinical results have not supported this hypothesis, and this cross-activity is now thought to theoretically expand the antitumor activity of TKIs (24–26). The insulin receptor is frequently overexpressed in cancer, and stimulation of the insulin receptor by insulin and IGF-II enhances mitogenesis in cancer cells (27–29). Therefore, TKIs of IGF-IR, like PQIP, may be more beneficial due to their ability to inhibit hybrid IGF-IR/insulin receptor and insulin receptor signaling.

In this study, we examined a novel, potent, and dual IGF-IR/insulin receptor TKI, *cis*-3-[3-(4-methyl-piperazin-1-yl)-cyclobutyl]-1-(2-phenyl-quinolin-7-yl)-imidazo[1,5-a]pyrazin-8-ylamine (PQIP). PQIP displayed a cellular  $IC_{50}$  of 19 nmol/L for inhibition of autophosphorylation of human IGF-IR and a cellular  $IC_{50}$  of 261 nmol/L for inhibition of insulin receptor (23). In preclinical studies, PQIP abolished the activity of ligand-induced activation of downstream phosphorylated Akt and phosphorylated extracellular signal-regulated kinase (ERK) 1/2 in both IGF-IR–transfected fibrosarcoma cells and GEO human

colorectal cancer cell lines (23). *In vivo*, PQIP exhibited robust antitumor activity in GEO xenografts (23).

Because the majority of newly developed targeted agents in colorectal cancer have eventually been incorporated into traditional chemotherapy regimens and there is evidence that the IGF pathway is important in resistance to chemotherapy, we examined PQIP in combination with standard colon chemotherapy agents (5-fluorouracil, SN38, and oxaliplatin) in colorectal cancer cell line models.

### Materials and Methods

#### Drugs

PQIP is a 1,3-disubstituted-8-amino-imidazopyrazine derivative. PQIP was obtained from OSI Pharmaceuticals. The agent was dissolved in DMSO at 10 mmol/L and stored at  $-20^{\circ}\text{C}$  for use in *in vitro* assays. OSI-906 is an updated derivative of PQIP, with the structure slightly changed to optimize adsorption distribution metabolism and excretion properties while maintaining target potency (30). For *in vivo* studies, OSI-906 was obtained from OSI Pharmaceuticals and was dissolved in 25 mmol/L tartaric acid. Oxaliplatin, 5-fluorouracil (FU), and irinotecan were obtained from the University of Colorado Pharmacy. SN38 (the active metabolite of irinotecan) was kindly provided by Dr. Daniel Gustafson (Department of Clinical Sciences, Clinical Pharmacology, Colorado State University, Fort Collins, CO) for *in vitro* studies.

#### Cell lines and culture

Twenty-eight human colorectal cancer cell lines were obtained from the American Type Culture Collection. The 29th cell line used, GEO, was 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% fetal bovine serum, 1% penicillin-streptomycin, and 1% MEM nonessential amino acids. All cells were kept at  $37^{\circ}\text{C}$  under an atmosphere containing 5%  $\text{CO}_2$ . Cells were routinely tested for the presence of mycoplasma (MycoAlert, Cambrex Bio Science)."

#### Proliferation and combination effects

Cell proliferation was analyzed using the sulforhodamine B (SRB) assay (31). Cells in a logarithmic growth phase were transferred to 96-well flat bottom plates with lids. One hundred microliter cell suspensions containing 2,000 viable cells were plated into each well, and cells were allowed to attach overnight before drug exposure. Colorectal cancer cell lines were exposed to 0 to 5  $\mu\text{mol/L}$  PQIP for 72 hours. In addition, all cell lines were exposed to 0 to 10 nmol/L SN38, 0 to 10  $\mu\text{mol/L}$  oxaliplatin, or 0 to 50  $\mu\text{mol/L}$  FU for 72 hours. Four colorectal cancer cell lines (HT29, LS513, HCT116, and RKO) were chosen for exposure to PQIP in combination with SN38, oxaliplatin,

or FU. In all combinations, PQIP and chemotherapy were added together for 72 hours. After exposure, 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 minutes at room temperature, after which cells were washed again with 1% acetic acid and the bound SRB was solubilized with 10 mmol/L Tris at room temperature. The optical density was measured on a plate reader (Biotek Synergy 2) set at an absorbance wavelength of 565 nm. Cell proliferation curves were derived from the raw optical density data and results of combinations were analyzed by the Chou and Talalay method using the Calcsyn software program (Biosoft; ref. 32). In each combination, a parameter called the combination index (CI) was calculated from each level of cytotoxicity. According to this method, synergy is indicated by a CI < 1, additivity by a CI = 1, and antagonism by a CI > 1.

#### Immunoprecipitation and immunoblotting

The HT29, HCT116, RKO, and LS513 colorectal cancer cell lines were seeded into 6-well plates, allowed to attach overnight, and exposed to appropriate compounds. After treatment cells were rinsed with PBS and scraped into radioimmunoprecipitation assay lysis buffer containing protease inhibitors, EDTA, NaF, and sodium orthovanadate. Total protein was quantified using the BioRad Dc Protein Assay (BioRad). Total protein (30 µg) was electrophoresed on a 4% to 20% gradient SDS-polyacrylamide gel then electrophoretically transferred to Immobilon-P (Millipore). Membranes were blocked for 1 hour in 5% nonfat dry milk (BioRad) in TBS-Tween (0.1%) prior to overnight incubation at 4°C with one of the following primary antibodies: pAkt, Akt, pS6RP, S6RP, pERK, ERK, pY, pCDK2, CDK2, poly(ADP-ribose) polymerase (PARP), or actin (Cell Signaling Technology). Blots were then washed 3 × 20 minutes in TBS-Tween (0.1%) and were incubated with the appropriate secondary anti-rabbit or anti-mouse IgG1 horseradish peroxidase (HRP)-linked antibody at 1:20,000 (Jackson ImmunoResearch) for 1 hour at room temperature. After three additional washes, the blots were developed by Immobilon Western Chemiluminescent HRP substrate (Millipore). For immunoprecipitation, IGFIRβ (Cell Signaling Technology) was used prior to immunoblotting.

#### Receptor tyrosine kinase proteome array

Cells were plated in 6-well plates and incubated overnight. Following a 24-hour exposure to PQIP (1.0 µmol/L), cells were rinsed once with PBS and solubilized with NP-40 lysis buffer [1%NP-40, 20 mmol/L Tris-HCl (pH 8.0), 137 mmol/L NaCl, 10% glycerol, 2 mmol/L EDTA, 1 mmol/L sodium orthovanadate, 10 µg/mL aprotinin, 10 µg/mL leupeptin]. Lysates were gently rocked at 4°C for 30 minutes, then microcentrifuged for 5 minutes at 14,000 × g. Supernatants were transferred to clean microcentrifuge tubes and total protein was quantified (as previously described). Two hundred micrograms of

lysates were diluted and incubated with the Human Phospho-RTK Proteome profiler array (R&D systems) according to the manufacturer's protocol.

#### Flow cytometric analysis of cell cycle distribution

Cells were plated in 6-well plates and incubated overnight. The cells were then exposed to both PQIP (1.0 µmol/L) and SN38 (4.0 nmol/L) alone or in combination. After 24 hours cells were collected using trypsin, rinsed with PBS, resuspended in Krishan's stain, and allowed to incubate for at least 24 hours at 4°C before analysis by the University of Colorado Cancer Center Flow Cytometry Core Facility.

#### Caspase 3/7 activity

Cells were seeded in 96-well white-walled plates at 2,000 cells/well and allowed to attach for 24 hours prior to drug exposure. Cells were then exposed to PQIP (0.1 µmol/L and 0.4 µmol/L) and SN38 (4 nmol/L and 8 nmol/L) alone and in combination for 6, 12, 24, 48, and 72 hours. Caspases 3 and 7 were measured using a luminometric Caspase-Glo 3/7 assay (Promega) according to the manufacturer's protocol using a plate reader (Biotek Synergy 2).

#### 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. Colorectal cancer cells (RKO) in a logarithmic growth phase were harvested and resuspended in a 1:1 mixture of serum-free RPMI 1640 and Matrigel (BD Biosciences). Five million cells per mouse were injected s.c. into the flank using a 23-gauge needle. Mice were monitored daily for signs of toxicity and were weighed twice weekly. Tumor size was evaluated twice per week by caliper measurements using the Study Director Program (South San Francisco). Tumor volume was calculated using the following formula: volume = (length × width<sup>2</sup>)/0.52. When tumors reached 150 to 300 mm<sup>3</sup> mice were randomized into 4 groups with at least 10 tumors per group. The mice were then treated for 14 days with OSI-906 (40 mg/kg) oral daily, irinotecan (50 mg/kg) i.p. once weekly, or the combination of OSI-906 and irinotecan. These doses were selected in previously published experiments (33–35).

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 prior to initiation.

#### Statistical methods

To determine the statistical significance between the groups in the xenograft studies, a two-sided unpaired *t*-test was done using GraphPad Prism Software. Differences were considered significant at *P* < 0.05.

## Results

### Effect of PQIP on 29 colorectal cancer cell lines

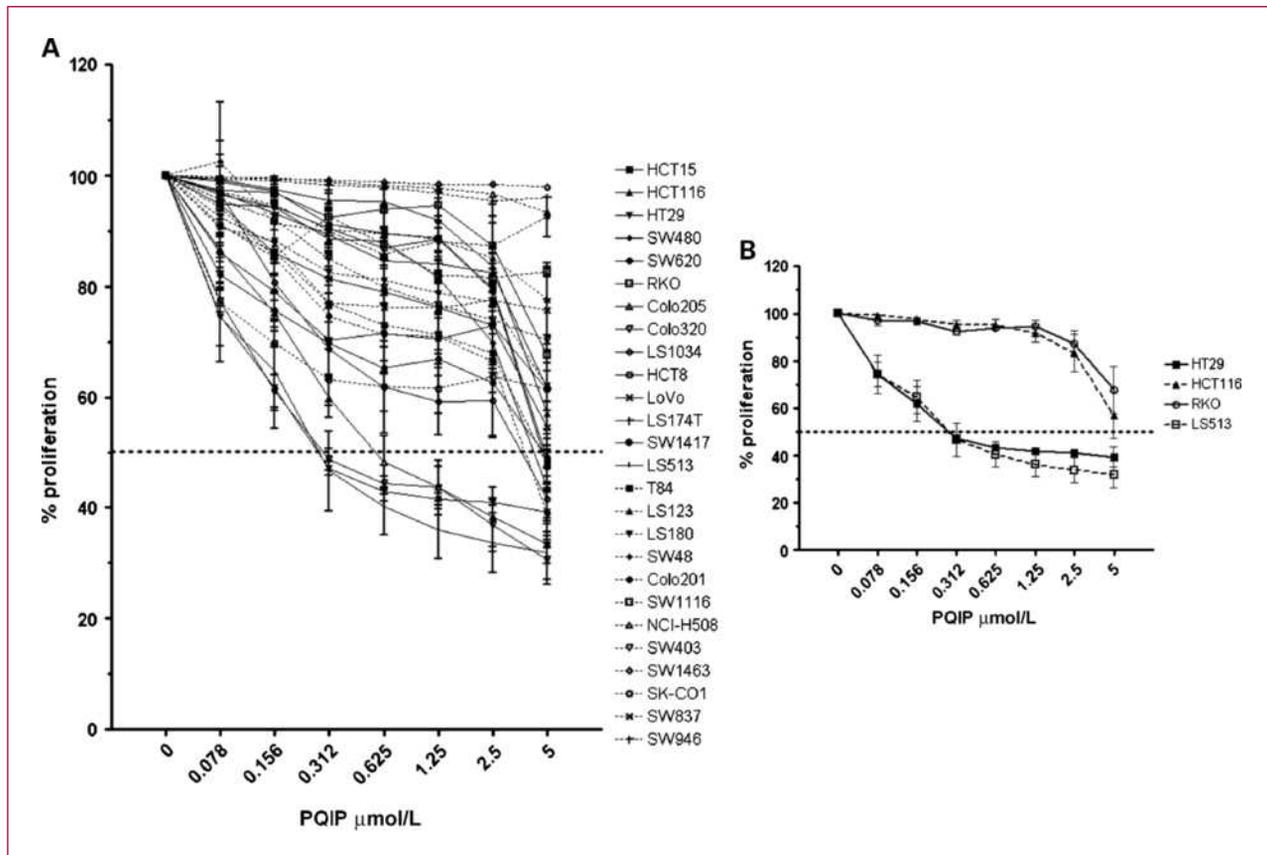
The antiproliferative effect of single-agent PQIP at a range of concentrations was evaluated against a panel of 29 colorectal cancer cell lines *in vitro* using the SRB assay. Following a 72-hour exposure to PQIP, the colorectal cancer cell lines displayed differential sensitivity with  $IC_{50}$  values ranging from 0.3  $\mu\text{mol/L}$  to  $>5 \mu\text{mol/L}$  (Fig. 1A). Doses above 5  $\mu\text{mol/L}$  were not tested because they are not clinically relevant, based upon available pharmacokinetics data in the phase I studies (23). For segregation, we deemed cell lines with an  $IC_{50} \leq 0.5 \mu\text{mol/L}$  as sensitive and cell lines with an  $IC_{50} \geq 5.0 \mu\text{mol/L}$  as resistant. Eight cell lines had an  $IC_{50} < 5.0 \mu\text{mol/L}$  but only five were considered sensitive by our criteria. Four cell lines, two sensitive (HT29, LS513;  $IC_{50} \approx 0.3 \mu\text{mol/L}$ ), and two resistant (HCT116, RKO;  $IC_{50} > 5 \mu\text{mol/L}$ ) were chosen for further experimentation (Fig. 1B).

### PQIP inhibits IGF-IR, insulin receptor, and downstream pathways

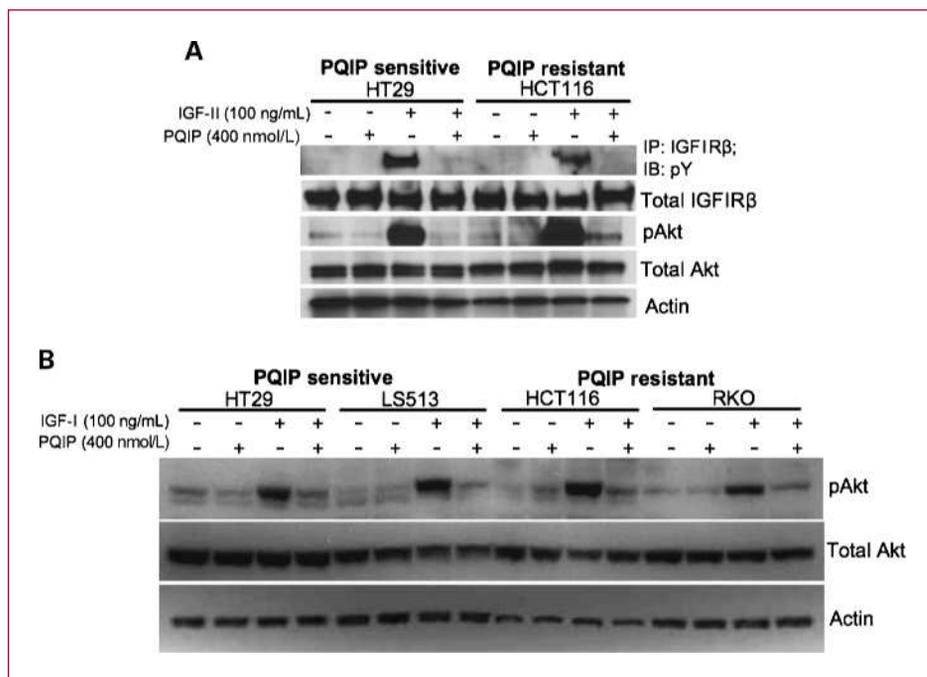
To validate the activity of PQIP, we analyzed phosphorylation of IGF-IR and the insulin receptor in the sensitive

and resistant colorectal cancer cell lines. In both the sensitive (HT29) and resistant (HCT116) serum-starved cells, a 3-hour exposure to PQIP almost fully inhibited IGF-II-induced tyrosine autophosphorylation of IGF-IR as determined by immunoprecipitation and immunoblotting analysis (Fig. 2A). We similarly verified the effects of PQIP on activity of the downstream effector Akt in two sensitive and two resistant cell lines. As depicted in Fig. 2B, a 3-hour exposure to PQIP inhibited IGF-I- and IGF-II-induced phosphorylation of Akt. Interestingly, phosphorylation of S6 ribosomal protein was inhibited by PQIP in a ligand-independent manner, whereas there was no effect on ERK phosphorylation despite ligand stimulation (Supplementary Fig. S1).

To assess the activity of PQIP on other receptors, we evaluated its effects on an array of 42 different receptor tyrosine kinases (RTK). The proteome arrays showed that phosphorylated IGF-IR was decreased upon 24-hour exposure to PQIP in all colorectal cancer cell lines tested. As expected, phosphorylation of the insulin receptor was also decreased following exposure to PQIP, albeit to a lesser extent than IGF-IR (Supplementary Fig. S2). No other RTK on the array was significantly affected by exposure to PQIP (Supplementary Fig. S2). It was noted that assessment of



**Fig. 1.** A, effect of PQIP on proliferation after 72 hours in a panel of 29 colorectal cancer cell lines. B, effect of PQIP on the four cell lines chosen for further experiments.



**Fig. 2.** Effect of PQIP on IGF-IR and downstream intracellular pathways. Cells were stimulated with IGF-II (A) or IGF-I (B) for 10 minutes at the end of a 3-hour exposure to PQIP. IP, immunoprecipitation; IB, immunoblot.

target or downstream effector pathways was not sufficient to segregate the colorectal cancer cell lines according to responsiveness to PQIP.

#### Enhanced antitumor effects are exhibited by PQIP in combination with standard chemotherapies for colorectal cancer

Studies have shown that chemoresistant colorectal cancer cells may be susceptible to inhibition by IGF-IR (36). There are currently three standard chemotherapy agents used for colorectal cancer treatment: irinotecan, oxaliplatin, and FU. Because it is likely that new targeted therapies will be incorporated into conventional chemotherapy regimens, we evaluated the interaction of PQIP with SN38 (the active metabolite of irinotecan), oxaliplatin, or FU in two sensitive (HT29, LS513) and three resistant (HCT116, RKO) colorectal cancer cell lines using the SRB assay. Cells were exposed for 72 hours to varying doses of each compound alone and in all possible combinations. We chose three concentrations of each compound based on the sensitivity profiles of the cell lines (Supplementary Fig. S3). Cells were exposed to PQIP (0.1, 0.4, 1.6  $\mu\text{mol/L}$ ), SN38 (2.0, 4.0, 8.0  $\text{nmol/L}$ ), oxaliplatin (0.5, 1.0, 5.0  $\mu\text{mol/L}$ ), and FU (3.125, 6.25, 12.5  $\mu\text{mol/L}$ ). In the highly sensitive cell lines, HT29 and LS513, strong synergy was shown, with nearly all CI between 0.2 and 1.0, in all combinations of PQIP with chemotherapy (SN38, oxaliplatin, and FU; Fig. 3 and Supplementary Fig. S4). Among the highly resistant HCT116 and RKO cells, the combinations were primarily additive with some CI values showing synergy (Fig. 3 and Supplementary Fig. S4). The combination of PQIP and SN38 was chosen to further evaluate the mechanism of antitumor effects.

#### The interaction between PQIP and SN38 does not significantly induce apoptosis

First, apoptosis was evaluated to determine whether the synergistic interactions between PQIP and irinotecan led to induction of programmed cell death. Apoptosis, in response to PQIP and SN38 alone or in combination, was measured by both caspase 3/7 activity and PARP cleavage. Although there was a slight increase in caspase 3/7 activity in the sensitive LS513 cell line after 6-, 12-, and 24-hour exposures to PQIP (0.1 and 0.4  $\mu\text{mol/L}$ ), SN38 (4.0 and 8.0  $\text{nmol/L}$ ), or any of the possible combinations (Supplementary Fig. S5A-C), this increase was not statistically significant when compared with the appropriate single-agent controls.

This lack of apoptosis was validated through evaluation of PARP cleavage by Western blotting. No increase in cleaved PARP was seen after a 24-hour exposure to PQIP (0.4  $\mu\text{mol/L}$ ), SN38 (8.0  $\text{nmol/L}$ ), or the combination (data not shown). These data suggest that the enhanced antitumor effects of the chemotherapy combinations with PQIP are mediated primarily through antiproliferative, rather than proapoptotic effects.

#### Effects of PQIP and SN38 on the cell cycle

Some IGF-IR TKIs have been shown to induce a  $G_0$ - $G_1$  cell cycle arrest, whereas SN38 is known to induce either S phase or  $G_2$ -M phase arrest (37-40). Therefore, assessment of the cell cycle was carried out by flow cytometric and Western blotting analysis. We first examined cell cycle distribution of the four colorectal cancer cell lines (sensitive lines HT29 and LS513; resistant lines HCT116 and RKO) following exposure to SN38 (4  $\text{nmol/L}$ ) and PQIP (1.0  $\mu\text{mol/L}$ ), alone and in combination. Cells were

exposed to compounds for 24 hours and analyzed by flow cytometry. As depicted in Fig. 4, the sensitive HT29 and LS513 cells displayed a robust induction of G<sub>0</sub>-G<sub>1</sub> arrest in response to PQIP ( $P < 0.05$ ), which was not observed in the resistant HCT116 and RKO cells. By contrast, all cells underwent a G<sub>2</sub>-M induction following exposure to SN38 ( $P < 0.05$  for all cell lines), with perhaps the greatest induction observed in the resistant HCT116 and RKO cells ( $P < 0.001$  for resistant cell lines). Interestingly, the only consistent difference between the sensitive and the resistant cell lines in the combination studies was that the sensitive cells exhibited a distribution of the cell cycle that was closer to untreated cells, whereas the resistant cells maintained the effects of SN38 in the combination.

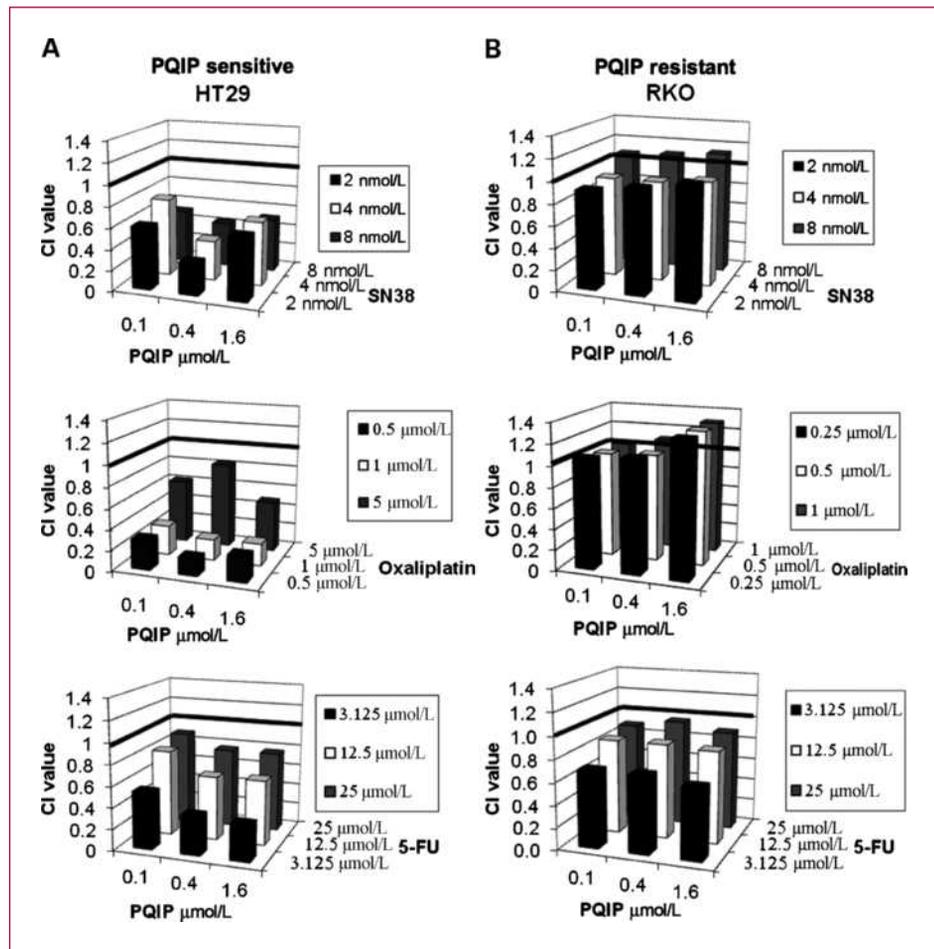
Following flow cytometric cell cycle distribution, specific proteins were evaluated through immunoblotting. We evaluated phosphorylated and total CDK2 levels to validate the G<sub>0</sub>-G<sub>1</sub> arrest seen in response to PQIP. All four colorectal cancer cell lines were exposed to PQIP (1.0 μmol/L) and SN38 (4.0 nmol/L), alone and in combination, and were assessed after 24 hours. As expected, phosphorylated CDK2 was decreased after 24-hour expo-

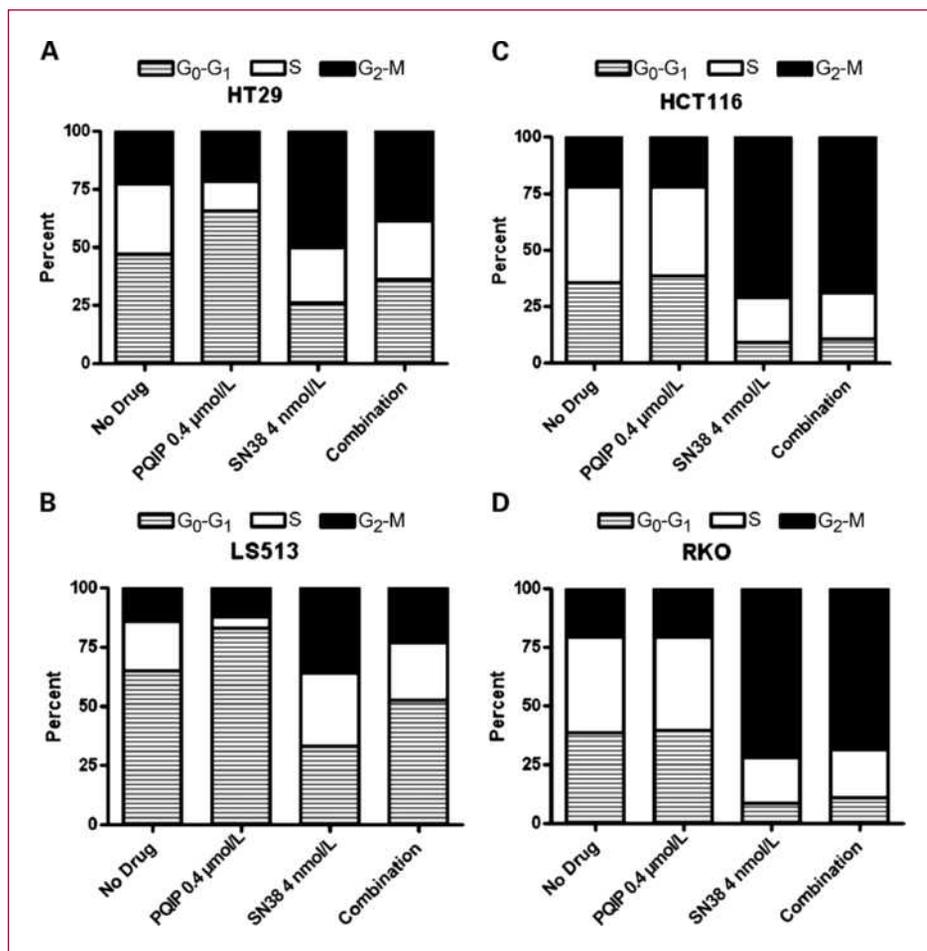
sure to PQIP in the sensitive HT29 and LS513 cells (these cells showed a decrease in both phosphorylated and total CDK2, which was not observed in the resistant HCT116 or RKO cells, or after SN38; Fig. 5A). We similarly evaluated the effects of PQIP, SN38, and the combination on cyclins D1 and E, and p21 (Fig. 5B). These results are more difficult to interpret, but indicate that SN38 induced cyclin D1, E, and p21, and was maintained in the combination in both resistant cell lines and to a lesser degree in one sensitive cell line.

**Phosphorylation of Akt is upregulated in response to chemotherapy**

Previous studies conducted by us and others have shown that exposure to DNA-interactive chemotherapeutic agents can activate prosurvival pathways, such as PI3K and MAPK, in colorectal cancer cells (41, 42). We next did immunoblotting analysis to assess the ability of SN38 to induce prosurvival pathways associated with IGF-IR. Cells were exposed to PQIP (1.0 μmol/L) and SN38 (4.0 nmol/L), both alone and in combination for 6 hours. As anticipated, SN38 induced p-Akt in three of the four cell lines (the resistant RKO cell line exhibited

**Fig. 3.** Effect of PQIP combined with one of three different chemotherapies in one sensitive colorectal cancer cell line, HT29 (A), and one resistant colorectal cancer cell line, RKO (B). Cells were exposed to PQIP and SN38, oxaliplatin, or FU for 72 hours. CI values were generated by Calcsyn, the Chou and Talalay mathematical method for assessing drug interactions.





**Fig. 4.** Effect of PQIP and SN38 on four colon cancer cell lines. Cells were exposed to agents for 24 hours, and analyzed by flow cytometry after staining with Krishans.

maximal p-Akt at baseline that did not change with treatment; Supplementary Fig. S6). Somewhat erratically, there was a reduction in the stimulatory effect of SN38 on p-Akt with the combination in the sensitive LS513 and resistant HCT116 cells, but not in the sensitive HT29 and resistant RKO cells. These data indicate that, at least in this short-term assay, modulation of p-Akt was not a consistent effect of the combination leading to synergy.

#### Confirmation of the antitumor effects of irinotecan and OSI-906 *in vivo*

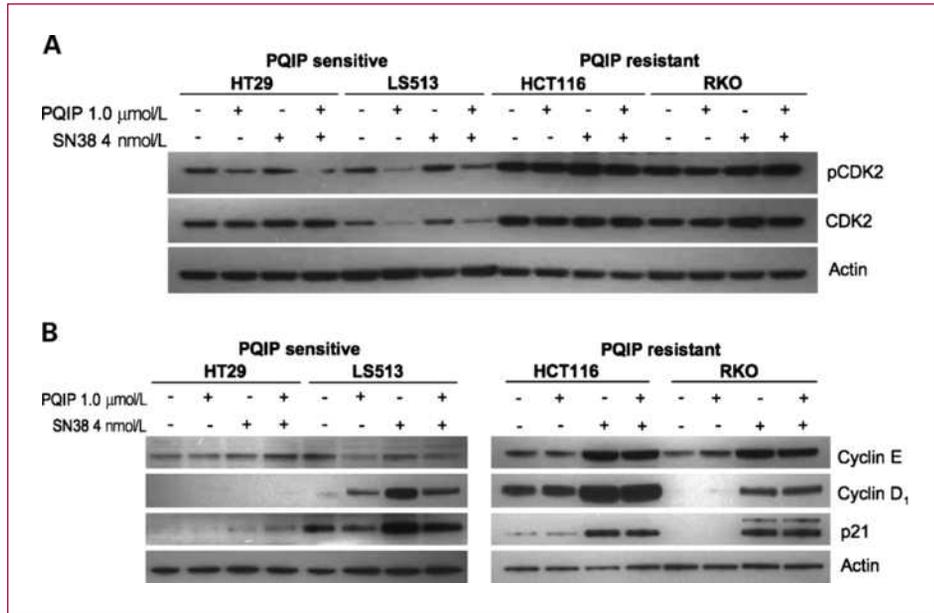
Lastly, we tested the efficacy of this combination *in vivo*, to further validate our *in vitro* results and to support the use of this combination in patients. The effects of OSI-906 (the clinical derivative of PQIP) and irinotecan (a prodrug, which is biologically converted to SN38) were evaluated using a human colorectal cancer xenograft model. For our analysis, we used the most stringent model, RKO cells that were resistant to PQIP and showed the least synergy *in vitro*. As shown in Fig. 6, OSI-906 (40 mg/kg) did not significantly inhibit tumor growth at 14 days of treatment [25% tumor growth inhibition

(TGI)], corresponding to the results we observed *in vitro* with PQIP. Although single-agent irinotecan (50 mg/kg) significantly inhibited tumor growth compared with the vehicle (59% TGI;  $P < 0.001$ ), the combination of OSI-906 and irinotecan led to even greater tumor growth inhibition (85% TGI;  $P < 0.01$ ). For the combination, the average tumor volume (in  $\text{mm}^3$ ) at day 14 was only slightly higher than the average tumor volume at day 0, suggesting almost complete growth inhibition in the combination group. In addition, there was no significant loss in body weight observed, compared with controls, indicating little or no toxicity with this combination (data not shown). Despite the caveats of preclinical models, these results indicate that this combination warrants clinical testing and may be active even in tumors that are inherently resistant to OSI-906.

#### Discussion

The importance of the IGF-IR pathway has already been implicated in colon cancer based on results of several population studies and *in vitro* and *in vivo* experiments (reviewed in ref. 43). Several IGF-IR inhibitors are now

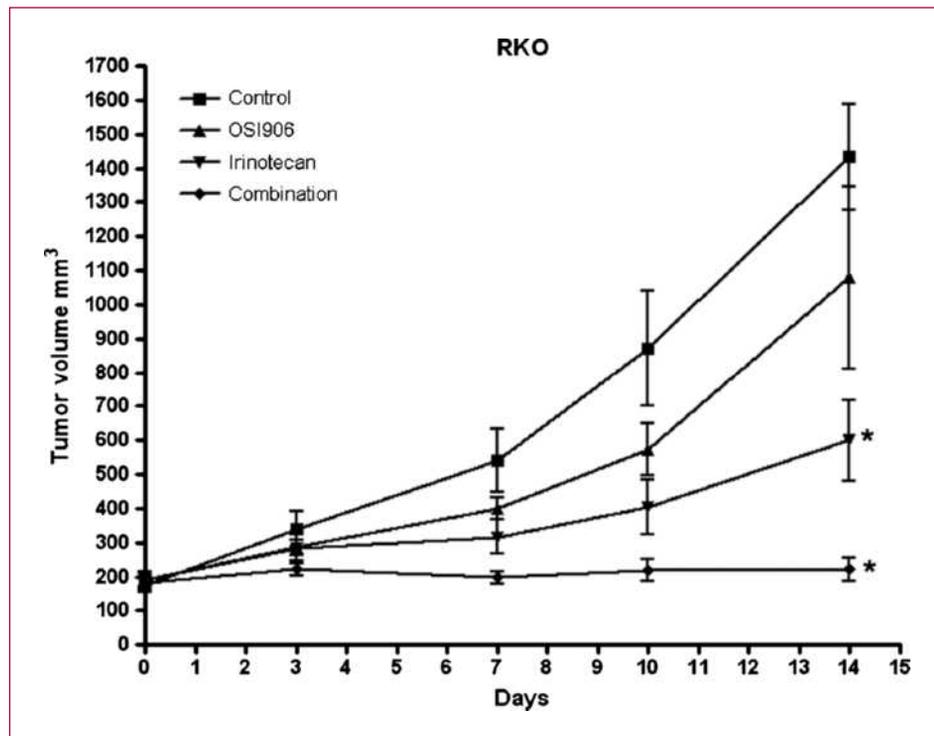
**Fig. 5.** Effect of PQIP and SN38 alone and in combination on two sensitive and two resistant colorectal cancer cell lines. Cells were exposed to PQIP and SN38 for 24 hours, and protein was isolated from cell lines for immunoblotting.



into clinical trials, but their role in the treatment paradigm of colon cancer has not been evaluated. The aim of this study was therefore to examine the effects of PQIP, the dual IGF-IR/insulin receptor TKI, as a single agent and in combination with standard colon cancer chemotherapy agents (FU, irinotecan, and oxaliplatin) in colorectal cancer cell lines.

In this study PQIP was tested in our bank of 29 colorectal cancer cell lines, identifying 5 sensitive cell lines with  $IC_{50}$  values  $<0.5 \mu\text{mol/L}$ . Mutational analysis of several genes, including *EGFR*, *KRAS*, *TP53*, *BRAF*, *PTEN*, and *PI3CA* (mutational statuses obtained from <http://www.sanger.ac.uk/genetics/CGP/CellLines/>), did not predict sensitivity to PQIP in these colon cancer cell lines,

**Fig. 6.** Effect of OSI-906 and irinotecan alone and in combination in athymic nude mice. Mice were treated with drugs for 14 days. Tumors treated with the combination of OSI-906 and irinotecan were significantly inhibited in comparison to the control, OSI-906, and irinotecan groups ( $P < 0.01$ ). \*, significant difference between combination treatment and single-agent or untreated controls.



although there was a trend towards the presence of a mutant *KRAS* being associated with resistance to OSI-906 (35). In the same study, it was shown through fluorescence *in situ* hybridization that gene amplification of IGF-IR was not correlated with sensitivity to OSI-906, whereas an unbalanced IGF-IR copy number gain (based on ploidy) was, thereby identifying one of several potential predictive biomarkers for this agent in colorectal cancer (35). At concentrations reflective of an  $IC_{50}$ , PQIP exerts its actions by inhibiting the IGF-I- and IGF-II-induced phosphorylation of Akt and S6 ribosomal protein but not the phosphorylation of ERK. Similarly, Ji et al. found that much higher concentrations of PQIP are needed to inhibit p-ERK than are necessary to inhibit p-Akt and p-S6 in GEO colorectal cancer cells (23). These data suggest that IGF-IR is primarily signaling through the PI3K pathway in colon cancer, and that high levels of activated ERK may be related to PQIP resistance. This observation has been reported in other tumor types and may lend mechanistic support to published reports of the beneficial anticancer effects of dual inhibition of EGFR and IGF-IR (44, 45).

The combination of a dual IGF-IR/insulin receptor TKI inhibitor and chemotherapy is not a novel concept. For example, in breast cancer it has been shown that increased IGF-IR signaling in cell lines treated with chemotherapy leads to a decrease in apoptosis (46, 47). Additionally, it has been shown that both oxaliplatin- and FU-resistant colon cancer cell lines express high levels of IGF-IR (36). Despite the growing preclinical support for IGF-IR and chemotherapy combinations, data regarding these combinations for colorectal cancer are lacking. In this study, our data showed that regardless of inherent sensitivity to PQIP, the interaction between chemotherapy and PQIP was enhanced. Identifying the mechanism of this enhanced interaction has been challenging and remains elusive. In other studies of osteosarcoma and breast cancer, it has been shown that apoptosis induced by chemotherapy is enhanced following the addition of an IGF-IR inhibitor (47, 48). Our data, however, did not show any significant increase in apoptosis following PQIP and SN38 treatment, indicating that induction of programmed cell death, at least in the timeframe assessed, was not involved.

To further delineate the interaction of these agents alone and in combination, we assessed both the phenotypic and molecular components of the cell cycle. Although no definitive conclusions can be drawn, there were some interesting findings that warrant further study. By flow cytometry, we found that the sensitive cells underwent a  $G_0$ - $G_1$  arrest that was not observed in the resistant cells. Although all cells underwent some degree of  $G_2$ -M arrest with SN38, this effect was pronounced in the resistant cells and was maintained in the combination. Effects exerted by both SN38 and IGF-IR inhibitors have been reported previously in colorectal cancer cell lines (37, 39, 40), yet cell cycle analysis of this combination has not been published to date. It is interesting, however, that the PQIP-resistant cells exhib-

ited a higher phosphorylated and total CDK2 baseline protein expression than the sensitive cells and more robust induction of cyclins D1 and E, and p21 by SN38 that was unaffected by the combination. From these data, it may be worthwhile to further investigate the role of CDK2 as a predictive or pharmacodynamic biomarker for OSI-906 or the combination.

We also examined phosphorylation of Akt because cancer cells are known to activate prosurvival pathways in response to treatment with DNA-damaging agents, such as oxaliplatin (41, 42). Consistent with this, our results showed that SN38 produced an increase in prosurvival signaling as shown by the activation of Akt. Although reduction of p-Akt with the combination was observed in the LS513 (sensitive) and HCT116 (resistant) cells, this was not consistent among all cell lines, despite the observation of synergy, and thus is difficult to interpret mechanistically. However, these results do also contribute to the observation that IGF-IR inhibition may reverse the chemoresistant phenotype and warrant further study (36).

To provide the highest stringency, we conducted the *in vivo* study using the RKO cells that are highly resistant ( $IC_{50}$  not reached at 5  $\mu$ mol/L) to OSI-906 and primarily exhibited additivity when exposed to the combination of SN38 and PQIP *in vitro*. Despite this, against the RKO xenograft, the combination of OSI-906 and irinotecan suppressed tumor growth greater than either agent alone with almost complete tumor growth inhibition at the end of the study (14 days). Although pure speculation, it is intriguing to consider whether the enhanced activity *in vivo* could be due to effects on the tumor microenvironment and/or a cancer stem cell phenotype (36). Nonetheless, this *in vivo* experiment contributes to the growing evidence that IGF-IR inhibitors may be effective at potentiating the effects of standard chemotherapy, particularly in patients with colorectal cancer (36, 46–48).

In summary, the IGF-IR pathway seems to be a scientifically valid target for anticancer therapy, although single-agent trials have yielded limited activity and recent results of combination studies are mixed (49). Clearly, the path forward will require tools for patient selection and better insight into the mechanistic interactions between these agents and standard or targeted therapy. The data presented here have provided the rationale for a clinical trial of the combination of OSI-906 and irinotecan, which is ongoing and includes patient selection tools as well as pharmacodynamic biomarkers.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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