Molecular Cancer Therapeutics

Epithelial–Mesenchymal Transition Predicts Sensitivity to the Dual IGF-1R/IR Inhibitor OSI-906 in Hepatocellular Carcinoma Cell Lines

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Abstract

A growing body of data indicates that inhibiting the type 1 insulin-like growth factor receptor (IGF-1R) might be an effective treatment strategy for hepatocellular carcinoma (HCC). OSI-906 is a dual IGF-1R/IR kinase inhibitor currently in phase II clinical development for HCC. However, biomarkers are lacking to help identify patients with HCC who are more likely to benefit from OSI-906 treatment. We sought to determine the effect of OSI-906 on proliferation against a panel of 21 HCC cell lines and to investigate molecular determinants of responsiveness to OSI-906. We identified a subset of HCC cell lines that was sensitive to OSI-906, and sensitivity is associated with elevated phosphorylation levels of IGF-1R and IR and greater inhibition of AKT signaling. Dual targeting of both receptors seems to be important for maximal inhibition as treatment with a selective IGF-1R–neutralizing antibody was associated with increased IR signaling, whereas OSI-906 fully inhibited both phosphorylated IR and IGF-1R and resulted in greater inhibition of the IRS/AKT pathway. Epithelial–mesenchymal transition (EMT) seems to predict HCC cell sensitivity to OSI-906, as the epithelial phenotype is strongly associated with expression of *IGF-2* and *IR*, activation of IGF-1R and IR, and sensitivity to OSI-906, alone or in combination with erlotinib. Induction of EMT upon treatment with TGFβ reduced sensitivity to OSI-906. Collectively, these data support the concept for dual IGF-1R/IR targeting in HCC, where EMT status and expressions of *IGF-2* and *IR* may be used to identify those patients who are most likely to benefit from treatment with an IGF-1R/IR dual inhibitor. *Mol Cancer Ther; 11(2); 503–13. ©2011 AACR.*

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer, affecting more than 600,000 patients worldwide and is the third leading cause of cancer-related death (1). The prognosis for patients with HCC remains poor, and the current overall 5-year survival rate (9%) is only modestly better than that recorded more than 3 decades ago (4%). The most common predisposing condition for HCC is cirrhosis, primarily due to hepatitis B (HBV) or hepatitis C infection or heavy alcohol consumption (1, 2). Hepatitis C infections have increased to epidemic numbers in recent years, and it is expected that this will translate to an increased number of HCC cases. Current treatment options for patients with HCC include cytotoxic chemotherapeutics such as doxorubicin and the recently approved multikinase inhibitor sorafenib (3). Although sorafenib is regarded as the current standard-of-care for patients with advanced HCC, the majority of cancers progress on therapy. There is a clear unmet need for patients who either cannot tolerate the side effects of sorafenib or whose cancers progress on treatment, and understanding other signaling pathways that may be effectively targeted for the treatment of HCC is an important goal.

There is a growing body of data to support the hypothesis that targeting the type 1 insulin-like growth factor receptor (IGF-1R) might be an especially effective strategy for the treatment of a number of tumor types including HCC (4–7). IGF-1R is a receptor tyrosine kinase (RTK) and a critical mediator of tumor cell proliferation and survival. This receptor, activated upon binding of the cognate ligands IGF-1 or IGF-2, transduces signals to the PI3K/AKT signaling pathway through the adaptor proteins insulin substrates 1/2 (IRS-1/2). IGF-1R activity is required for oncogenic transformation by a number of oncogenes including RAS and can promote tumor formation *in vivo* (8, 9). For HCC, elevated phosphorylation of IGF-1R is observed in a subgroup of tumor cells but not in adjacent cirrhotic tissue (10). Preclinical studies have shown that for a number of tumor types, blockade of IGF-1R signaling results in reduced proliferation of tumor cells *in vitro* and growth *in vivo* (10–15). For HCC, neutralizing antibodies directed against either IGF-1R or IGF-2 have been shown to inhibit tumor cell proliferation,

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anchorage-independent growth, and the growth of xenograft tumors (10, 16).

There are also data to support a role for the insulin receptor in tumor cell proliferation and survival (17–19). Insulin can promote the proliferation of tumor cells *in vitro*, and the growth of xenograft tumors in mice is attenuated when circulating insulin levels are reduced through ablation of pancreatic islet cells (20, 21). The IR-A fetal splice variant is tumorigenic in a mouse mammary tumor model (22). Human tumor cells, including those representing HCC, frequently coexpress both IGF-1R and IR, presenting the potential for cross-talk between these receptors. We have recently reported that compensatory cross-talk between IGF-1R and IR can occur in tumor cells; wherein inhibition of either IGF-1R or IR results in increased phosphorylation of the alternate receptor (22). In a subset of tumor models, inhibition of both receptors within this axis seems to be required for maximal inhibition of IRS-1 phosphorylation and antitumor activity. Dual dependence on IGF-1R and IR may be centered on aberrant regulation of IGF-2 because this ligand can activate both IGF-1R and IR-A variant. A subset of HCC tumor cells has been shown to express both IGF-2 and IR. *IGF-2* is one of a group of imprinted genes that are expressed from only one parental allele, however, loss of imprinting at this locus occurs in a subset of tumors, leading to elevated IGF-2 levels through bi-allelic gene expression (23–26). In *IGF-2* transgenic mice, HCC is among the most frequent cancers (27). Elevated *IGF-2* expression in a subset of human HCC tumors is accompanied by reactivation of fetal promoters leading to bi-allelic ligand expression for this normally imprinted gene (26, 28–30). Elevated plasma levels of IGF-2 are also reported for a subset of patients with HCC (31). Collectively, these data indicate that small-molecule inhibitors such as OSI-906 that target both IGF-1R and IR may have the potential for greater activity against HCC tumors than IGF-1R–specific antibodies. Although these studies have highlighted the potential importance for both IGF-1R and IR signaling in HCC tumors, the activity of small-molecule dual tyrosine kinase inhibitors (TKI) of IGF-1R/IR has yet to be evaluated in HCC preclinical models. Furthermore, the identification of biomarkers that could be used to identify specific patient populations likely to receive the most benefit from treatment with IGF-1R/IR inhibitors has yet to be realized.

We assessed the activity of the dual IGF-1R/IR TKI OSI-906 across a broad panel of HCC tumor cell lines. A subset of HCC tumor cell lines was sensitive to OSI-906 in proliferation assays, and where inhibition of the AKT pathway, but not the extracellular signal-regulated kinase (ERK) pathway, was associated with sensitivity. Moreover, OSI-906 exhibited enhanced activity against the IRS-1/AKT survival pathway compared with an anti-IGF-1R–specific antibody, where treatment resulted in a compensatory increase in IR phosphorylation. These data show that dual inhibition of both IGF-1R and IR may be important for maximal inhibition of tumor cell proliferation and survival signaling pathways. We also determined that there is a significant correlation between expression of molecular markers associated with epithelial–mesenchymal transition (EMT) status and OSI-906 sensitivity in HCC tumor cells, where epithelial tumor cells express significantly higher levels of *IGF-2* and *IR* and were more sensitive to OSI-906 than mesenchymal tumor cells. Induction of EMT in epithelial HCC tumor cells was also associated with loss of sensitivity to OSI-906. Collectively, these data provide rationale for targeting the IGF-1R/IR pathway in HCC, where EMT biomarkers may be useful to identify those patients who are most likely to benefit from treatment.

Materials and Methods

IGF-1R/IR inhibitors

OSI-906 was synthesized as previously described (32). Both OSI-906 and erlotinib were dissolved in dimethyl sulfoxide for use in *in vitro* cellular assays. Structures of OSI-906 and erlotinib are shown in Fig. 2A. IGF-1R–neutralizing antibody MAB391 was purchased from R&D Systems.

Cell cultures

Twenty-one HCC cell lines from purchased from either American Type Culture Collection (HepG2, Hep3B, PLC/PRF/5, Sk-Hep1, SNU-182, SNU-387, SNU-398, SNU-423, SNU-449, and SNU-475) or Health Science Research Resources Bank (Japan; Huh-1, Huh-6, Huh-7, HLE, HLF, Jhh-1, Jhh-2, Jhh-4, Jhh-5, Jhh-6, and Jhh-7). Cell lines were banked upon receipt and passaged for fewer than 6 months before use without further authentication. All the cell lines are maintained in media as described by the vendors. For growth inhibition assays, cells were plated and allowed to proliferate for 24 hours. After 24 hours, cells had reached approximately 15% confluency, at which time serial dilutions of OSI-906 were added and the cells grown for a further 72 hours. Cell viability was assayed using the CellTiter-Glo reagent (Promega Corp.).

Preparation of protein lysates and Western blotting

Cells were rinsed with PBS and lysed in RIPA buffer (Sigma; #R0278) containing protease and phosphatase inhibitor cocktails (Sigma; #P2850, P8340, P5726). Cell lysates were cleared by centrifugation and subjected to Western blotting. Antibodies included IGF-1R (Santa Cruz), IR (Santa Cruz), phospho-p42/p44 (Cell Signaling Technologies), phospho-Akt (S473; Cell Signaling Technologies), phospho-Akt (T308; Cell Signaling Technologies), phospho-PRAS40 (Cell Signaling Technologies), E-cadherin (Santa Cruz #sc21791), ErbB3 (Santa Cruz #sc285), vimentin (BD Pharmingen #550513), and Zeb1 (Santa Cruz #sc25388).

Analysis of phosphoproteins

Proteome Profiler arrays containing capture antibodies for 42 RTKs were from R&D systems. Phospho-IR and phospho-IRS-1 were measured with Insulin Signaling Panel kit (#K15151C) from Meso Scale Discovery. The cells were lysed and assays were carried out according to manufacturer's protocols.

TaqMan assays

Total RNA was isolated with RNeasy kit (Qiagen) and treated with RNase-free DNase. Reverse transcription was carried out with SuperScript III First-Strand Synthesis system (Invitrogen). The gene expression assays for *IGF-2*, *IGF-1*, *IGF-1R*, and *IR* were obtained from Applied Biosystems, and primers for IR-A isoform were designed as previously described (22). Quantitation of relative gene expression was conducted as described by the manufacturer using 50 ng of template. For EMT gene expression, cDNA was loaded on Custom TaqMan Array 384-Well Micro Fluidic Cards (Applied Biosystems), which were preloaded with primers for 19 EMT genes, and quantitative PCR (qPCR) was run on 7900 HT Fast Real-Time PCR system (Applied Biosystems).

EMT studies

Cells were grown in medium supplemented with 10 ng/mL TGFβ (EMD Biosciences #616450) for 10 days with replating every 3 to 4 days. The cells were then lysed for qPCR, Western blotting, or CellTiter-Glo proliferation assays (Promega). The 12 mesenchymal genes used for heatmaps are *ACTN1*, *SPARC*, *ITGB3*, *PLAUR*, *CDH2*, *SNAI1*, *SNAI2*, *TWIST1*, *VCAN*, *VIM*, *ZEB1*, and *ZEB2* and the 7 epithelial genes are *CDH1*, *CLDN3*, *ERBB3*, *MTA3*, *MAP7*, *TJP3*, and *OCLN*.

Relative gene expression and statistical analysis

To determine relative expression across cell lines, amplification of IGF axis gene was compared with that of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene to have ΔC_t values. The relative expression is calculated as the $2^{(-\Delta C_t)}$ values from each cell line divided by the lowest $2^{(-\Delta C_t)}$ value among 21 HCC cell lines. Error bars were SDs generated from at least triplicates in each experiment. The ΔC_t values were used to calculate correlation with EC_{50} values. To calculate the correlation between EMT and sensitivity, or *IR* and *IGF-2* expressions, epithelial and mesenchymal phenotypes are represented by 1 and 0, respectively. Pearson correlation coefficient (r) was used to measure the strength of linear dependence. For area under curve (AUC) analyses, the significant differences between AUC values of HUH-1, HepG2, and JHH-5 cells with TGFβ treatment and those with mock treatment were analyzed with 2-sided Mann–Whitney test.

Analysis of additivity and synergy

The Bliss additivism model was used to classify the effect of combining erlotinib and OSI-906 as additive or synergistic, and the Bliss curve was calculated as described previously (33).

Results

A subset of HCC human tumor cell lines is sensitive to OSI-906

OSI-906 is a selective, orally bioavailable, dual inhibitor of IGF-1R and IR and is currently in advanced clinical development (32, 34). We assessed the sensitivity to OSI-906 in a panel of 21 HCC tumor cell lines (Table 1). The proliferation of 7 HCC cell lines (HepG2, Hep3B, Huh-1, Huh-6, Huh-7, Jhh-5, and Jhh-7) was inhibited by at least 40% at 5 μmol/L OSI-906 with $EC_{50} < 1$ μmol/L, and these cell lines were classified as sensitive to OSI-906. Previous studies have shown that HBV gene products may promote increased signaling through the IGF signaling axis through upregulation of bioavailable ligand (35). However, we found that only 3 of 7 OSI-906 sensitive tumor cell lines and 50% (7 of 14) insensitive HCC cell lines exhibited positive HBV status. These data indicate that HBV infection might be one, but not the only, path by which tumor cells acquire dependence on IGF-1R/IR signaling.

Table 1. Sensitivity of 21 HCC cell lines to OSI-906

HCC cell lines	OSI-906 sensitivity (EC_{50}), μmol/L	HBV	EMT status
HepG2	0.22	−	E
Hep3B	0.35	+	E
Huh-1	0.18	+	E
Huh-6	0.25	−	E
Huh-7	0.37	−	E
Jhh-5	0.19	−	E
Jhh-7	0.94	+	E
PLC/PRF/5	>10	+	E
Jhh-1	>10	−	E
Jhh-4	>10	−	M
SNU-182	>10	+	M
SNU-449	>10	+	M
Jhh-6	>10	−	M
SNU-398	>10	+	M
HLE	>10	−	M
Jhh-2	>10	−	M
Sk-Hep1	>10	−	M
HLF	>10	−	M
SNU-387	>10	+	M
SNU-423	>10	+	M
SNU-475	>10	+	M

NOTE: Twenty-one HCC cell lines were treated with serial dilution of OSI-906 for 72 hours, and proliferation assays were carried out using CellTiter-Glo Kit (Promega). EC_{50} values were derived from proliferation assays. Information about HBV status was obtained through vendors.

Inhibition of AKT activation is associated with sensitivity to OSI-906

We determined the signaling mechanisms associated with sensitivity to OSI-906 in HCC cell lines. Because IGF-1R and IR are involved in regulation of the phosphoinositide 3-kinase (PI3K) and ERK pathways, the effect of OSI-906 on the phosphorylation of AKT and ERK1/2 was assessed by immunoblotting in a panel of 3 sensitive and 4 insensitive HCC cell lines (Fig. 1A). Although OSI-906 did not affect ERK1/2 phosphorylation in any of these HCC cell lines, it reduced AKT phosphorylation in all 3 sensitive cell lines but had little to no effect on AKT phosphorylation in each of the insensitive cell lines, indicating that this pathway is alternately regulated. Therefore, sensitivity to OSI-906 in human HCC cell lines is likely mediated through inhibition of the AKT survival pathway.

Dual inhibition of IGF-1R and IR in HCC cells

For a subset of tumor types, data indicate that signaling through both IGF-1R and IR may promote tumor growth. We investigated the phosphorylation status of IGF-1R and IR in HCC cell lines (Fig. 1B). Compared with other tumor cell lines we tested (22), all 6 HCC cell lines showed higher IR phosphorylation than IGF-1R, suggesting the significance of IR activity in HCC. Furthermore, all 3 HCC cell lines (HepG2, Hep3B, and HuH-7) that are sensitive to OSI-906 had much higher phosphorylation levels of both IGF-1R and IR than insensitive cell lines (Fig. 1B). This suggests that sensitivity to OSI-906 associates with activation of both IGF-1R and IR in HCC cell lines. Recently, we reported that in some tumor cell lines, inhibition of IGF-1R signaling by neutralizing antibodies was associated with a compensatory increase in IR signaling (22), and dual inhibition of both IGF-1R and IR was required for maximal inhibition of tumor growth for tumors where both IGF-1R and IR were phosphorylated. Compensatory signaling between IGF-1R and IR was bidirectional as knockdown of IR was associated with increased phosphorylation of IGF-1R. To test whether the IR pathway can also compensate for the loss of IGF-1R activities in HCC

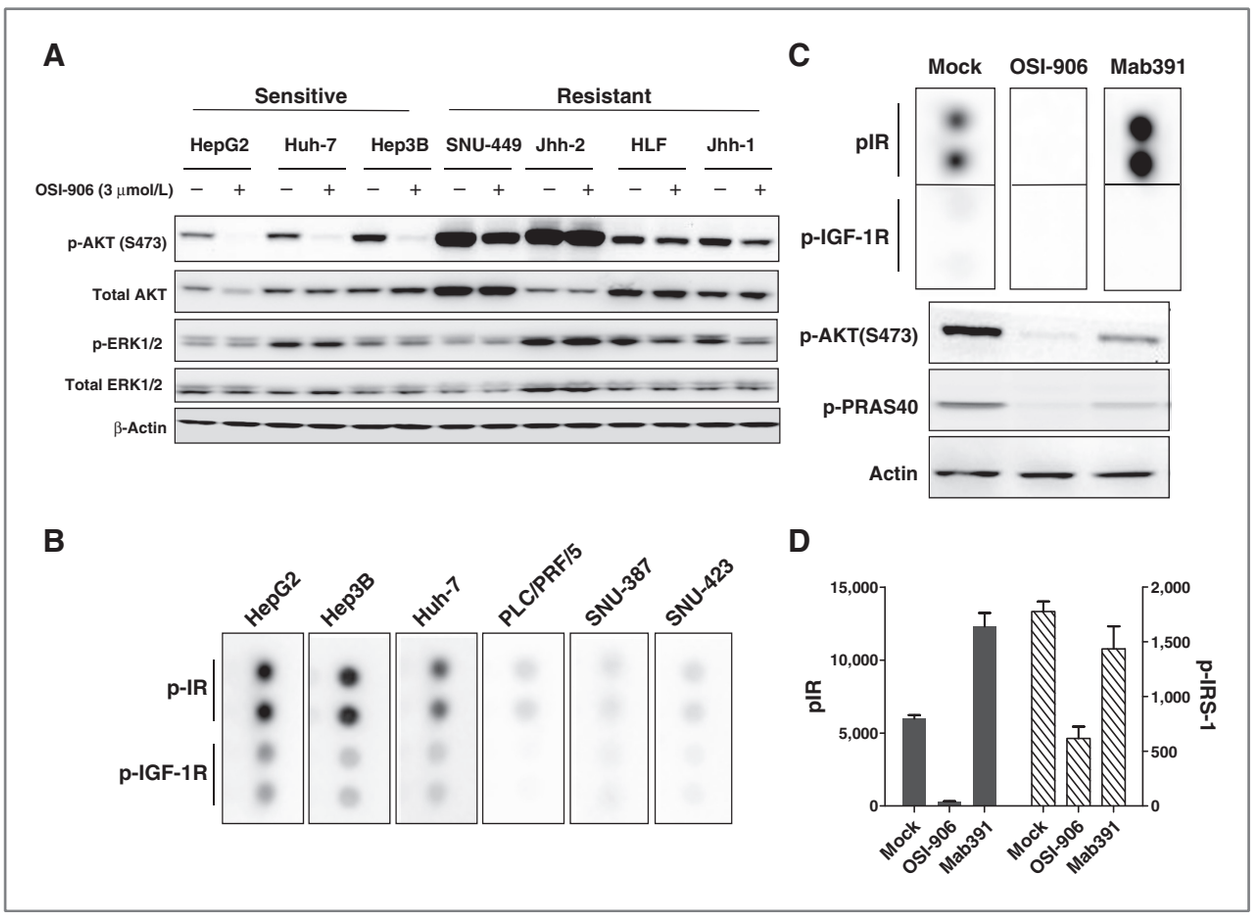

Figure 1. Inhibition of IR and AKT activation and sensitivity to OSI-906. A, HCC cell lines were treated with OSI-906 (3 μmol/L) for 20 hours before lysis for Western blotting. B, phosphorylation levels of IR and IGF-1R in select HCC cell lines were assessed by RTK array. Equal amounts of cell lysates were used for this study. C, IR can compensate for the loss of IGF-1R activity in HCC cell lines. Huh-7 cells were treated with mock (dimethyl sulfoxide), OSI-906 (3 μmol/L), or Mab391 (10 μg/mL) for 20 hours before lysis for RTK array and immunoblotting with p-AKT and p-PRAS40 antibodies or MSD Insulin signaling panel kit (D) for simultaneous measurement of phospho-IR (left) and phospho-IRS-1 (right). SD is shown in D.

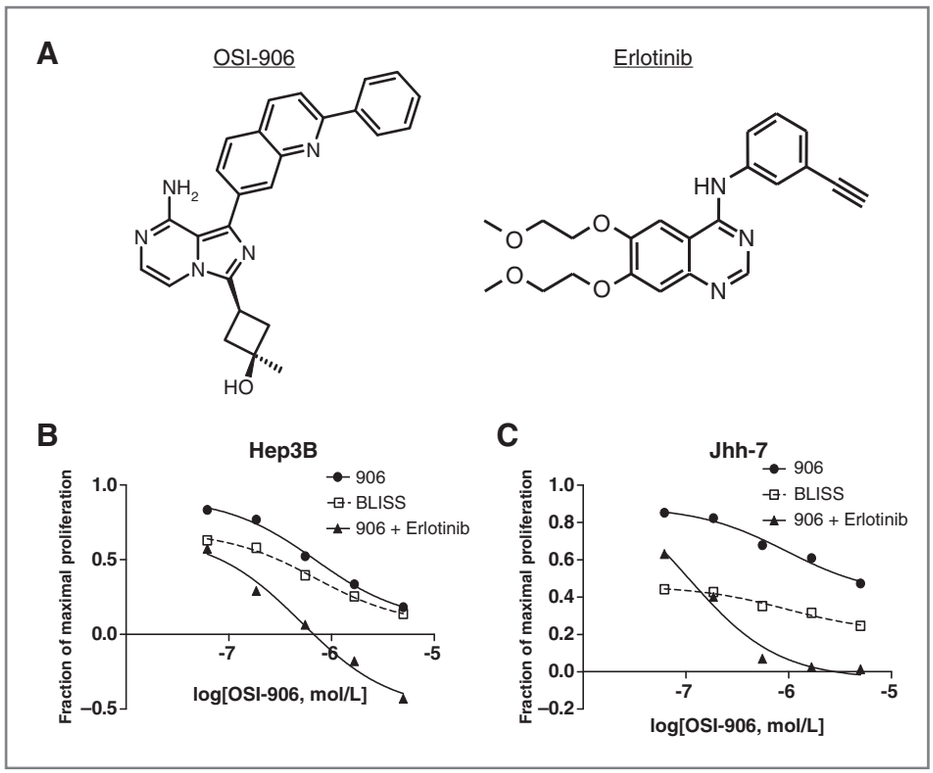

Figure 2. Combination of OSI-906 and erlotinib confers synergy in epithelial HCC cells. A, structures of OSI-906 and erlotinib. Two sensitive epithelial cell lines Hep3B (B) and Jhh-7 (C) were treated serial dilution of OSI-906 in the presence of erlotinib for 72 hours, and sensitivity to treatment was measured by proliferation assays. The concentrations of erlotinib used were determined beforehand to inhibit about 50% proliferation (Hep3B, 3.3 μmol/L) or maximal concentration (10 μmol/L) was used (Jhh-7).

cell lines, we treated OSI-906 sensitive HuH-7 cells with either the anti–IGF-1R antibody Mab391 or OSI-906 and monitored the phosphorylation status of IR and IGF-1R (Fig. 1C). OSI-906 fully inhibited phosphorylation of both IGF-1R and IR. In contrast, specific inhibition of IGF-1R phosphorylation upon treatment with Mab391 was accompanied by an increase in IR phosphorylation, indicating the potential for compensatory signaling. We investigated differential inhibition of IGF-1R/IR on downstream signaling, including IRS-1, AKT, and PRAS40 phosphorylation, for OSI-906 compared with Mab391. Immunoblotting showed that phosphorylation of AKT and the AKT substrate PRAS40 was inhibited to a greater extent by OSI-906 than by Mab391 (Fig. 1C). Furthermore, while OSI-906 fully inhibited IR and IRS-1 phosphorylation, the induced IR phosphorylation upon Mab391 treatment was associated with an inability to inhibit IRS-1 tyrosine phosphorylation to the same extent as OSI-906 (Fig. 1D). These data indicate that signaling through the IRS-1/AKT pathway may also be mediated by IR in HCC tumor cells, and inhibition of IR is needed to achieve maximal inhibition of this cellular survival pathway in HCC.

Blocking compensatory pathways increases sensitivity to OSI-906

It was shown previously that a subset of HCC tumor cells was sensitive to epidermal growth factor receptor (EGFR) antagonists including erlotinib (36), which can also inhibit AKT phosphorylation. In addition, several studies have shown that cross-talk between the IGF and EGF signaling pathways can occur, resulting in limited sensitivity when either pathway is targeted individually. Therefore, we hypothesized that the combination of erlotinib and OSI-906 (Fig. 2A) might be especially efficacious at inhibiting select HCC cell proliferation. A panel of HCC tumor cell lines exhibiting varying sensitivity to OSI-906 was treated with the combination of erlotinib and OSI-906, and effects of compound treatment were monitored by cell proliferation assays (Fig. 2). Addition of erlotinib further increased sensitivity of Hep3B, Jhh-7 cells, 2 OSI-906–sensitive HCC cell lines (Fig. 2B and C). We assessed combination synergy using the BLISS method, which would allow us to capture cooperative effects both on potency and maximal activity (33). Using this approach, we find the combination of erlotinib and OSI-906 to be synergistic in these tumor cell lines. Moreover, the combination of erlotinib and OSI-906 also showed good synergy for inhibiting cell proliferation for Jhh-1 and PLC/PRF/5 cells, 2 HCC cell lines that are not sensitive to OSI-906 as a single agent (data not shown). Taken together, these data indicate that the activity for OSI-906 may be augmented in HCC tumor cells through combination with an EGFR antagonist.

IGF axis gene expression in HCC cell lines

We sought to determine whether expression of genes within the IGF axis might be predictive of sensitivity to OSI-906 within the panel of HCC tumor cell lines. Expression levels of *IGF-1*, *IGF-2*, *IGF-1R*, and *IR* were

examined by quantitative real-time PCR for all HCC tumor cell lines within the panel (Fig. 3). Although we found no significant correlation between OSI-906 sensitivity and expression of either IGF-1 or IGF-1R (Fig. 3B and C), the expression levels of both *IGF-2* and *IR* were significantly positively correlated with OSI-906 sensitivity (Fig. 3A and D). Five of 6 cell lines within the top quartile for *IGF-2* expression within the panel are sensitive to OSI-906 (Fig. 3A). In contrast, none of the tumor cell lines with *IGF-2* expression in the lowest quartile were sensitive to OSI-906. Consistent with these findings for a role of IR in mediating IRS-1/AKT signaling in HCC tumor cell lines, IR expression levels were also higher in cell lines sensitive to OSI-906 (Fig. 3D). Three of 5 cell lines within the top quartile for IR expression were sensitive to OSI-906, whereas all within the lowest quartile were insensitive to OSI-906 (Fig. 3D). Expression of *IR-A* isoform showed similar correlation with sensitivity to OSI-906 (correlation coefficient, $r = 0.43$, $P = 0.05$, data not shown). Collectively, these data support the potential use of IGF-2/IR ligand/receptor pair expression levels as a potential biomarker for OSI-906 activity in HCC.

EMT status is associated with sensitivity of HCC cell lines to OSI-906

A challenge in optimizing the use of molecular-targeted therapies including IGF-1R/IR and EGFR inhibitors has been identification of specific patient populations that will receive the most benefit upon treatment. Recent reports have described molecular markers associated with EMT as predictive of sensitivity to inhibitors of RTKs including EGFR and IGF-1R (36–40). EMT is an evolutionarily conserved developmental process and has been increasingly recognized to occur during the progression of various carcinomas and it seems to play an important role in drug resistance (41–43). EMT is facilitated by transcriptional reprogramming and characterized by the combined loss of epithelial cell junction proteins such as E-cadherin and the gain of mesenchymal markers such as vimentin. Epithelial and mesenchymal cells can be distinguished by expression of a number of classical markers. Within a

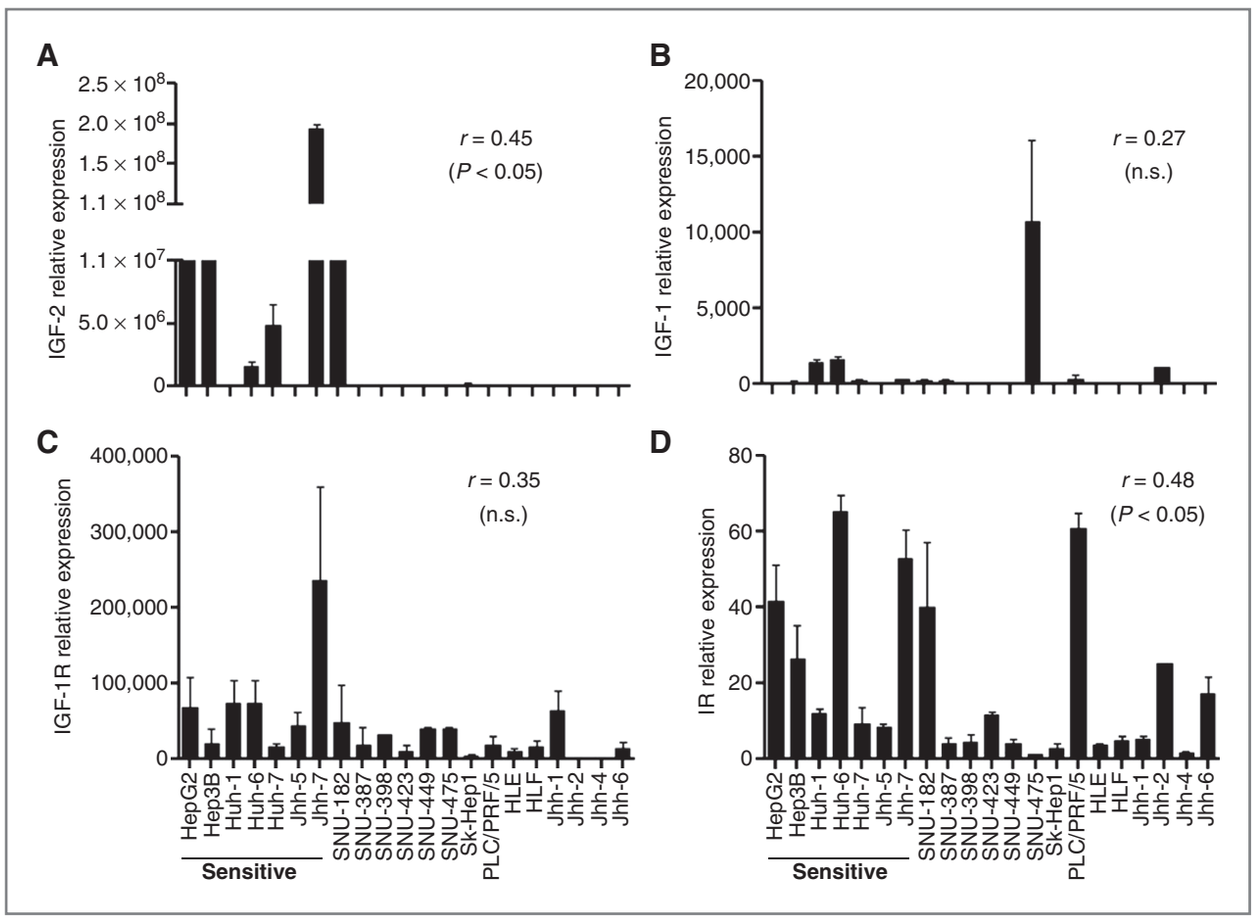

Figure 3. IGF axis expression in HCC cell lines predicts sensitivity to OSI-906. Total RNA was isolated from 21 HCC cell lines and expressions of *IGF-2* (A), *IGF-1* (B), *IGF-1R* (C), and *IR* (D) were measured by quantitative real-time PCR. The ΔC_t values of each gene were used to calculate the Pearson correlation coefficient (*r*) values. *P* values more than 0.05 are indicated as not significant (n.s.).

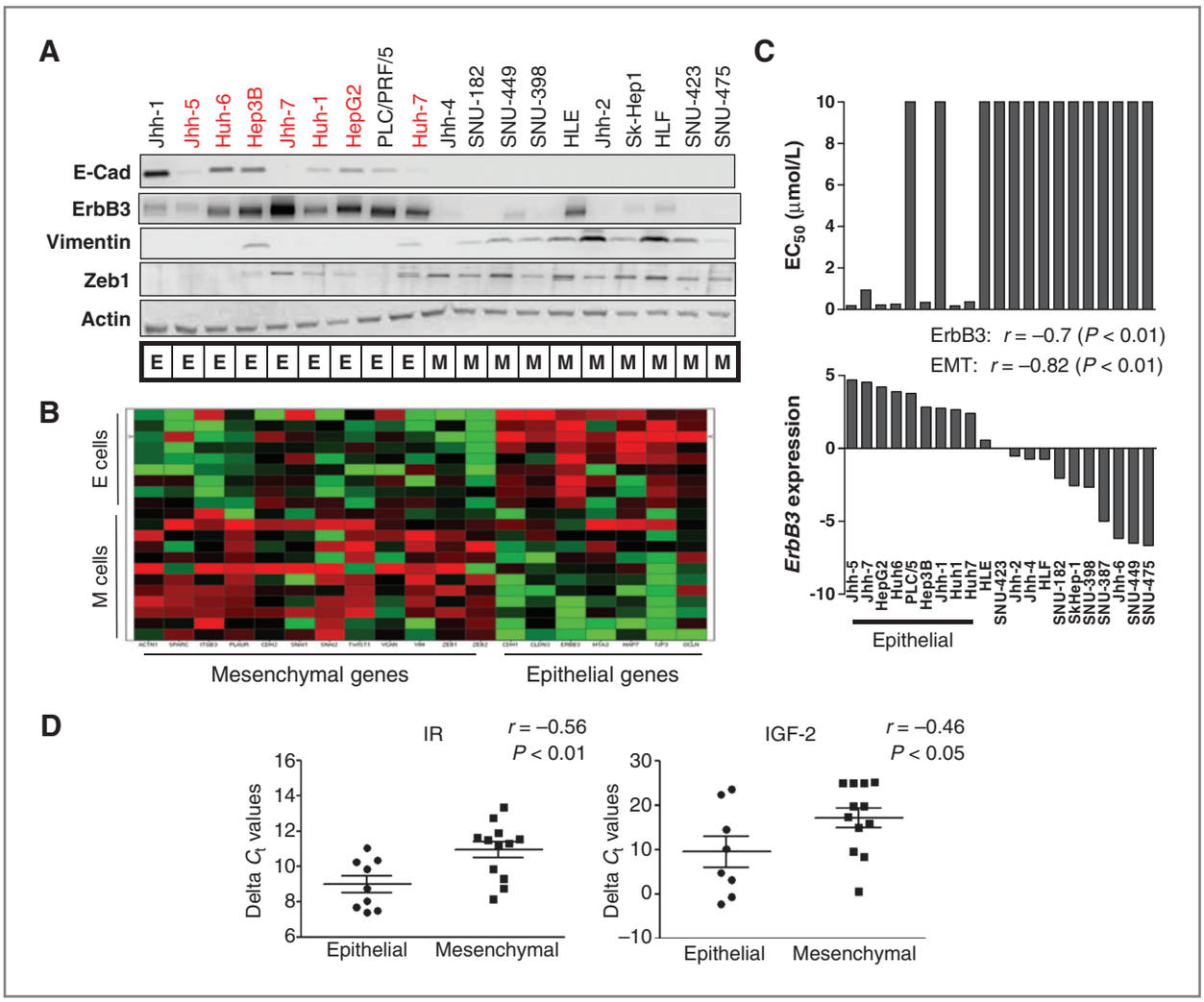

Figure 4. EMT predicts sensitivity to OSI-906 in HCC cells. A, analysis of EMT markers by Western blotting. Cell lysates from HCC cell lines were used for direct Western blotting of 2 epithelia markers (E-cadherin and ErbB3), 2 mesenchymal markers (vimentin and Zeb), phospho-AKT, and actin as loading control. Sensitive cell lines are indicated in red. B, heatmap of 19 EMT genes of HCC cell lines. Total RNA was isolated and used for real-time PCR. ΔC_t value of each gene was used to generate the heatmap. Within heatmap, red and green colors indicate higher and lower than the mean expression value across 21 HCC cells, respectively. C, *ErbB3* expression and EMT correlated with sensitivity to OSI-906. Top, EC_{50} from proliferation assays; bottom, *ErbB3* relative expression. The ΔC_t values of each gene were used to calculate the Pearson correlation coefficient values. D, EMT correlates with *IR* and *IGF-2* expression. The ΔC_t values of *IR* (left) or *IGF-2* (right) of HCC cell lines were grouped according to their epithelial or mesenchymal status and were used to calculate correlation coefficient (*r*) or *P* values between EMT and *IR* or *IGF-2* expression.

number of tumor types including non–small cell lung cancer (NSCLC), pancreatic cancer (PaCa), and HCC, EGFR TKIs are more active against tumor cells that maintain expression of epithelial markers than those that have undergone EMT and gained properties characteristic of cells of mesenchymal origin (36–38, 44). We have also recently reported that for several tumor types, including NSCLC, increased phosphorylation of IGF-1R/IR and sensitivity to OSI-906 is associated with an epithelial phenotype (39).

We sought to determine whether sensitivity to OSI-906 is associated with the EMT status for HCC tumor cell lines (Fig. 4). We assessed protein expression levels of 2 epithelial markers, E-cadherin and ErbB3, and 2 mesenchymal markers, vimentin and Zeb, in HCC cell lines by immunoblotting (Fig. 4A). Nine cell lines expressed detectable levels of both epithelial markers or expressed detectable levels of one epithelial marker and lacked expression of either mesenchymal marker and these were classified as epithelial tumor cells. Twelve cell lines showed low expression of E-cadherin and ErbB3 and high expression of vimentin and Zeb and these were classified as mesenchymal cells. To further assess the EMT characteristics for cell lines within this panel, we evaluated the expression of a group of 19 genes (12 mesenchymal genes and 7 epithelial genes) that are commonly recognized as E or M genes (45). Expression levels of these 19 genes in the 21 HCC cell line panel were measured as an additional

assessment of EMT status for each HCC cell line (Fig. 4B). Consistent with results from immunoblotting studies, the 9 epithelial cell lines generally expressed higher levels of epithelial genes and lower levels of mesenchymal genes, whereas the 12 mesenchymal cell lines showed the opposite pattern. Importantly, we found that 7 of 9 epithelial cell lines and none of the 12 mesenchymal cells were sensitive to OSI-906 (Fig. 4A, Table 1), indicating the potential for EMT biomarkers to be predictive of OSI-906 sensitivity. Indeed, EMT status strongly correlated with sensitivity to OSI-906 in HCC tumor cells (correlation coefficient, $r = -0.82$, $P < 0.01$). Specific EMT biomarkers were also significantly correlated with OSI-906 sensitivity. For example, expression levels of the genes *ErbB3* and *E-cadherin* highly positively correlated with cell sensitivity to OSI-906 with correlation coefficients of -0.7 and -0.57, respectively (Fig. 4C, data not shown).

Because *IR* or *IGF-2* expression and EMT correlate with sensitivity to OSI-906, we investigated the relationship between EMT status and *IR* or *IGF-2* expression. Epithelial cells expressed significantly more *IR* and *IGF-2* mRNA than mesenchymal cells, and EMT status significantly correlated with *IR* (correlation coefficient, $r = 0.56$, $P < 0.01$) and *IGF-2* mRNA expression (correlation coefficient, $r = 0.46$, $P < 0.05$; Fig. 4D). Taken together, these data indicate that protein and gene markers associated with EMT status can predict for sensitivity to OSI-906 in HCC cells.

EMT can modulate sensitivity to OSI-906 in HCC cell lines

In preclinical models, treatment of tumor cells with ligands such as TGFβ can induce EMT, resulting in reduced expression of epithelial genes and increased expression of mesenchymal genes (45, 46). To further validate the hypothesis that EMT status can be used as a molecular determinant of sensitivity to OSI-906 for HCC tumor cells, we assessed the impact of TGFβ-induced EMT on OSI-906 sensitivity for 3 epithelial cell lines (HUH-1, JHH-5, and HepG2 cells; Fig. 5). TGFβ treatment resulted in decreased expression of either E-cadherin or ErbB3 and/or increased expression of vimentin, indicative of EMT (Fig. 5A). Analyses of expression of 19 EMT genes also showed that TGFβ treatment generally increased expression levels of mesenchymal genes and decreased expression of epithelial genes (Fig. 5B). TGFβ treatment drastically decreased sensitivity to OSI-906 in all 3 cell lines tested without obvious effect on *IR* or *IGF-2* expression (Fig. 5C, data not shown). These indicate that EMT can modulate HCC cell sensitivity to OSI-906, providing further validation for EMT status as a determinant of sensitivity to OSI-906 in HCC.

Discussion

We investigated the determinants of sensitivity to the selective dual IGF-1R/IR inhibitor OSI-906 in HCC tumor cell lines. A subset of HCC cell lines exhibited activation of both IGF-1R and IR and showed high sensitivity to OSI-906 in proliferation assays, where sensitivity to OSI-906 was associated with inhibition of the AKT pathway. Signaling through alternate RTKs has emerged as a major molecular mechanism of resistance toward inhibitors targeting a single RTK. Cross-talk can occur even within an RTK family, and we have previously shown that dual inhibition of IGF-1R and IR was important for OSI-906 activity across a number of different tumor types including NSCLC, colorectal carcinoma, and sarcomas, where blockade of either receptor individually resulted in increased activity through the alternate receptor. Our studies indicate that signaling through IR may play an important role in HCC as IR is coactivated with IGF-1R. Importantly, we show that the IR-A fetal variant is expressed by HCC tumor cell lines sensitive to OSI-906. For these cells, treatment with an IGF-1R–specific antibody resulted in increased phosphorylation of IR and lack of complete inhibition of the IRS/AKT pathway. In contrast, dual inhibition of IGF-1R and IR upon treatment with OSI-906 resulted in complete suppression of the phosphorylation states for both of these receptors, and this was associated with complete inhibition of the IRS/AKT pathway.

Recently, we reported that coexpression of ligand–receptor pairs within the IGF axis is associated with sensitivity to OSI-906 in lung, colon, and breast cancer cell lines (22). In HCC cell lines, while expression of neither *IGF-1R* nor *IGF-1* showed significant correlation with OSI-906 sensitivity, expression of both *IR*, notably *IR-A*, and *IGF-2* was significantly positively correlated with greater sensitivity to OSI-906. Among different tissues, liver has one of the highest expression levels of IR (47). IR may be activated by either insulin or IGF-2, and IGF-2 may be especially potent for activating the IR-A variant. IGF-2 expression also seems to be a major determinant of sensitivity to OSI-906 for HCC tumor cell lines. Eighty-three percent of cells (5 of 6) with high (top quartile) *IGF-2* expression are sensitive to OSI-906, and 93% of insensitive cells (13 of 14) showed *IGF-2* expression levels within the bottom quartile for the group. Collectively, these data indicate that IGF-2 activation of IR may be an important pathway for HCC tumor cells and explain why blockade of IGF-1R alone is only moderately effective, whereas OSI-906 exhibits robust activity against select tumor cell lines in this setting. IGF ligands may be secreted into blood by tumors, and serum IGF-2 levels correlate with HCC stages (31). This presents the possibility for evaluating serum IGF-2 levels in the clinic for predicting response to OSI-906 in patients with HCC.

Tumor cells with an EMT status exhibited higher expression levels of both *IR* and *IGF-2*. More than 50% of epithelial cells (5 of 9) had high *IGF-2* expression, whereas only one of 12 mesenchymal cell lines expressed high levels of *IGF-2*. Analysis showed that the epithelial phenotype and *IGF-2* or *IR* expression had significant correlation ($r = 0.46$, $P < 0.05$; $r = 0.56$, $P < 0.01$,

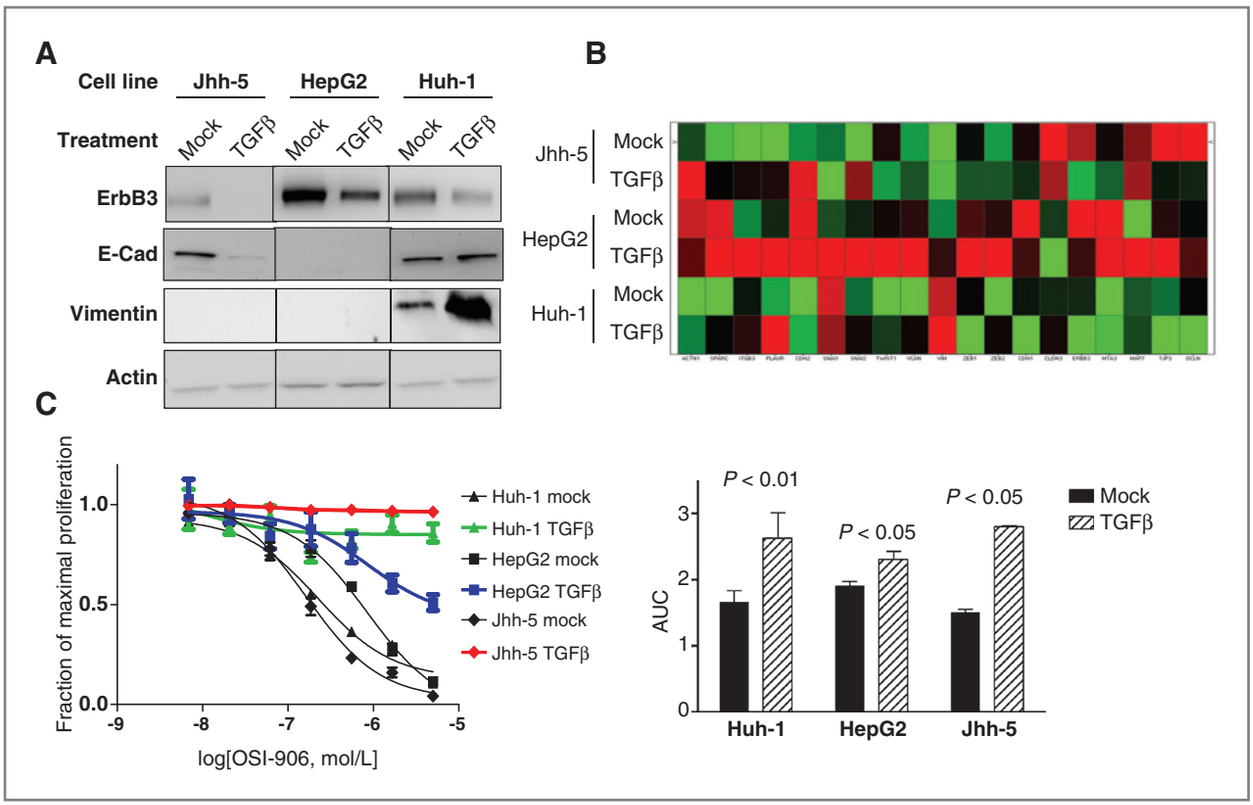

Figure 5. EMT in HCC cells decreases sensitivity to OSI-906. Huh-1, Jhh-5, and HepG2 cells were treated with TGFβ (10 ng/mL) for 10 days and subject to 3 analyses. A, TGFβ induced change in EMT markers. Western blotting was carried out to analyze expressions of E-cadherin, ErbB3, and vimentin. B, TGFβ treatment changed expressions of 19 EMT genes. Total RNA was isolated from treated or mock-treated cells, and used for real-time PCR. The ΔC_t value of each of the 19 EMT genes was used to generate heatmap. Red and green colors indicate higher and lower than the mean expression value, respectively. C, the effect of E → M transition on HCC proliferation. After 10-day treatment, different amount of OSI-906 was added to cell medium for 72 hours and then cell proliferation was measured by CellTiter-Glo Kit (left). The significant overall effect of TGFβ treatment on sensitivity to OSI-906 (overall difference between 2 curves) was analyzed with AUC analysis of all 3 cell lines (right).

respectively). EMT markers predicted for sensitivity to OSI-906 treatment in HCC tumor cells. In this study, EMT status was assigned using either the expression of a group of 19 genes with known involvement in EMT or protein expression of 4 EMT protein markers. Seventy-eight percent (7 of 9) of epithelial HCC tumor cells and none of mesenchymal cells were sensitive to OSI-906 treatment. The majority of OSI-906 sensitive cells expressed high levels of epithelial genes and low levels of mesenchymal genes. Consistent with EMT as a molecular determinant of sensitivity, EMT induced by TGFβ treatment resulted in significantly decreased sensitivity to OSI-906. One epithelial marker that we find predictive of OSI-906 sensitivity is ErbB3. ErbB3 has been shown to be repressed by factors including the transcription factor Snail when tumor cells undergo EMT (48). Data indicate that IGF-1R/IR and EGFR/ErbB3 are coexpressed and activated in tumor cells that exhibit an epithelial phenotype. There is also cross-talk between IGF-1R/IR and EGFR/ErbB3, and the combination of OSI-906 and the EGFR inhibitor erlotinib was synergistic in epithelial HCC cells.

Clinically, HCC tumors have been classified into several subgroups depending on their gene expression profiles (10, 49, 50). One subgroup, the proliferation subgroup is enriched in tumors with high *IGF-2* expression and high levels of IGF-1R/IR phosphorylation (10). As suggested by studies with HCC tumor cell lines, this proliferation subgroup could be particularly responsive to OSI-906 therapy. Previous reports showed mutual exclusivity between HCC tumors with high *IGF-2* expression and those with induction of IFN-regulated genes (51), suggesting that the inflammation subgroup may be less likely to respond to OSI-906. Another subgroup showed strong overexpression of TGFβ-regulated genes and activation of Wnt pathways, suggesting that this subgroup may be enriched in mesenchymal tumors. These together with our results showing decreased sensitivity upon TGFβ treatment suggest that the Wnt subgroup is likely to be less sensitive to OSI-906 treatment.

Evidence indicates that there is compensatory cross-talk among RTKs, such as EGFR, IGF-1R, IR, and others (22, 39, 52–54). Coinhibition of 2 or more RTKs may be required to fully inhibit AKT and achieve maximal efficacy. We provided evidence for compensatory IR signaling in HCC tumor cells upon treatment with an

IGF-1R–specific antibody, which was associated with reduced blockade of IRS/AKT signaling compared with OSI-906, which dually targeted both IGF-1R and IR. HCC tumor cells exhibit a marked elevated level of p-IR, which could be driven by IGF-2 signaling. We also found evidence for cross-talk between RTK families in HCC tumor cells. In this study, we provide evidence of synergy for the combination of OSI-906 and the EGFR inhibitor erlotinib (36). The combination of erlotinib and OSI-906 showed synergistic inhibition of cell proliferation for select epithelial HCC tumor cells but not for mesenchymal tumor cells.

The prognosis for patients with HCC is poor, partly, due to the limited options available, and additional therapies are clearly needed. Our data provide proof of concept for an IGF-1R/IR dual inhibitor, such as OSI-906, for the treatment of patients with HCC. Furthermore, biomarkers including EMT status and *IGF-2* and *IR* expression could be useful in ensuring that patients receive the appropriate therapy in a timely manner. Exploratory analysis of biomarkers associated with IGF signaling and EMT may be helpful in understanding OSI-906 in this setting.

Disclosure of Potential Conflicts of Interest

All authors are employees of OSI Pharmaceuticals Inc., a wholly owned subsidiary of Astellas U.S. holdings. H. Zhao and E. Buck have ownership interest (including patents).

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