Molecular Cancer Therapeutics

ERK Phosphorylation Is Predictive of Resistance to IGF-1R Inhibition in Small Cell Lung Cancer

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Abstract

New therapies are critically needed to improve the outcome for patients with small cell lung cancer (SCLC). Insulin-like growth factor 1 receptor (IGF-1R) inhibition is a potential treatment strategy for SCLC: the IGF-1R pathway is commonly upregulated in SCLC and has been associated with inhibition of apoptosis and stimulation of proliferation through downstream signaling pathways, including phosphatidylinositol-3-kinase-Akt and mitogen-activated protein kinase. To evaluate potential determinants of response to IGF-1R inhibition, we assessed the relative sensitivity of 19 SCLC cell lines to OSI-906, a small molecule inhibitor of IGF-1R, and the closely related insulin receptor. Approximately one third of these cell lines were sensitive to OSI-906, with an $IC_{50} < 1$ μmol/L. Cell line expression of *IGF-1R*, *IR*, *IGF-1*, *IGF-2*, *IGFBP3*, and *IGFBP6* did not correlate with sensitivity to OSI-906. Interestingly, OSI-906 sensitive lines expressed significantly lower levels of baseline phospho-ERK relative to resistant lines ($P = 0.006$). OSI-906 treatment resulted in dose-dependent inhibition of phospho-IGF-1R and phospho-Akt in both sensitive and resistant cell lines, but induced apoptosis and cell-cycle arrest only in sensitive lines. We tested the *in vivo* efficacy of OSI-906 using an NCI-H187 xenograft model and two SCLC patient xenografts in mice. OSI-906 treatment resulted in 50% tumor growth inhibition in NCI-H187 and 30% inhibition in the primary patient xenograft models compared with mock-treated animals. Taken together our data support IGF-1R inhibition as a viable treatment strategy for a defined subset of SCLC and suggest that low pretreatment levels of phospho-ERK may be indicative of sensitivity to this therapeutic approach. *Mol Cancer Ther; 12(6); 1131–9. ©2013 AACR.*

Introduction

Lung cancer is the leading cause of cancer death in the United States in both men and women (1). Approximately 15% of all lung cancers are of small cell histology, characterized by exceptionally rapid proliferation and early dissemination to metastatic sites. Although standard of care platinum-based chemotherapy induces responses in up to 80% of newly diagnosed small cell lung cancer (SCLC) cases, these responses are of short duration and the median survival in patients with advanced SCLC is less than 1 year from the time of diagnosis (2). New therapeutic approaches are needed to overcome clinical resistance and improve long-term survival.

Signaling through growth factor receptor tyrosine kinase pathways is important for tumor growth and progression. Receptor engagement can lead to activation of downstream signal transduction cascades involved in cell proliferation and survival, typically including the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K)-Akt pathways. Aberrant activation of receptor tyrosine kinases, including the insulin-like growth factor 1 receptor (IGF-1R), has been implicated in development and growth of many types of cancer. Thus, these receptors have become a particularly attractive set of drug targets (3).

Several lines of evidence suggest that IGF-1R signaling in particular contributes to malignant transformation and cancer growth and survival. Overexpression of IGF-1R in murine and human fibroblasts promotes oncogenic transformation (4). IGF-1R is required for SV40 large tumor antigen transformation of mouse embryonic fibroblasts (5). Elevated IGF ligand levels have been detected in patient samples from many tumor types (6). High IGF ligand levels have been implicated in the promotion of tumor growth in several mouse models of cancer and lead to increased risk for prostate, breast, and lung cancers (7–9). It has also been shown that IGF-1R upregulation can lead to chemotherapeutic resistance (10).

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These findings have prompted extensive development of IGF-1R targeted inhibitors, including monoclonal antibodies specific to IGF-1R and small molecule receptor tyrosine kinase (RTK) inhibitors of the insulin receptor (IR) family, which are being explored in preclinical models and early phase clinical trials. OSI-906, or *cis*-3-[8-amino-1-(2-phenyl-quinolin-7-yl)-imidazo[1,5-a]pyrazin-3-yl]-1-methyl-cyclobutanol (Fig. 1), is a potent and specific small molecule tyrosine kinase inhibitor of the IR family. Testing against a panel of more than 40 kinases revealed OSI-906 to be a selective inhibitor of IGF-1R (35 nmol/L) and IR (75 nmol/L) with all other kinases having an IC_{50} greater than 10 μmol/L. OSI-906 has significant antiproliferative effects and results in successful blocking of IGF-1R signaling and IGF-1R-dependent downstream activation of PI3K-Akt and MAPK pathways in several cancer cell types. It has also been shown to inhibit growth of several tumor types *in vivo* (11). Currently OSI-906 is being evaluated clinically in patients with many cancer types, including colorectal, ovarian, and lung cancer (12).

With regard to SCLC, IGF-1R has been reported to be highly expressed in both cell lines and patient tumors (13–17). Signaling through the IGF-1R pathway has been implicated in the development and growth of SCLC (14, 18, 19). Several reports have suggested that IGF-1R inhibitors, including both monoclonal antibodies and RTK inhibitors, can limit growth and induce apoptosis in SCLC cell lines (20–22). However, only a small number of cell lines have been assessed and determinants of sensitivity and resistance have not been explored. In addition, there have been no reports on the *in vivo* efficacy of IGF-1R inhibitors in SCLC. We examined a large panel of SCLC cell lines, including 3 cell lines derived from primary patient tumors, to define determinants of OSI-906 activity and identify possible predictive biomarkers of OSI-906 sensitivity. This analysis was complemented with *in vivo* efficacy studies in SCLC patient-derived xenograft mouse models. Our data show that IGF-1R inhibition may be a viable treatment strategy for a molecularly defined subset of SCLC.

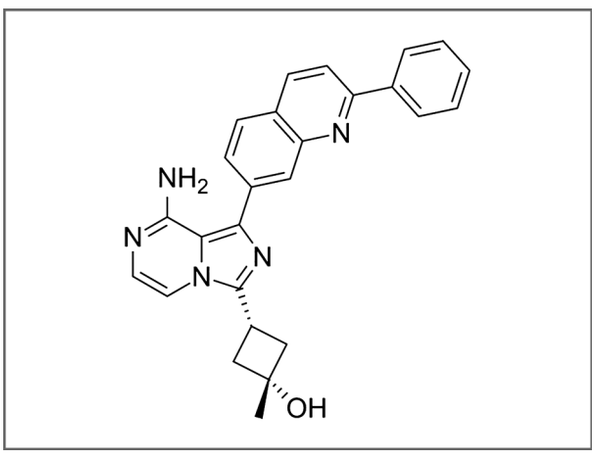

Figure 1. Chemical structure of OSI-906.

Materials and Methods

Cell lines

The following cell lines were authenticated by short tandem repeat analysis using the StemElite ID System (Promega): H69, H82, H146, H187, H209, H345, H446, H510A, H526, H720, H774, H1092, and H1930. All cell lines were grown and maintained in conditions recommended by American Type Culture Collection.

MTS cell proliferation assays

Cells were plated in quadruplicate and treated with OSI-906 for 72 hours at doses ranging from 10 nmol/L to 10 μmol/L. MTS cell proliferation assays were conducted following manufacturer instructions using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega). IC_{50} values were extrapolated from dose–response curves using the GraphPad Prism program. OSI-906 sensitivity was defined as an IC_{50} less than 1 μmol/L. This threshold was functionally defined based on an estimate of maximal predicted tumor drug levels achievable *in vivo*: OSI-906 plasma C_{max} after a 50 mg/kg oral dose in mice (as used in our xenograft experiments) is 2.68 μmol/L (11).

Western blots

Protein was isolated from cell pellets and tumor tissue using RIPA buffer with protease and phosphatase inhibitor cocktails (Sigma) and run on 10% or 12% Bis-Tris gels with MOPS running buffer (Invitrogen). Membranes were probed with antibodies for phospho-IGF-1Rβ (Y1135/6)/IRβ (Y1150/1), phospho-Akt (S473), phospho-ERK (T202/Y204), phospho-P70S6K, IR, AKT, ERK (Cell Signaling), IGF-1Rβ, GAPDH, and β-Actin (Santa Cruz) following manufacturer instructions. Relative protein levels were measured using the BioRad Quantity One software and calculated by normalizing to control (either GAPDH or β-actin). Differences between the sensitive and resistant groups were analyzed using the Wilcoxon rank sum test.

Reverse transcription PCR

RNA was isolated from cells using the Qiagen RNeasy Mini kit and reverse transcribed using Qiagen Quantitect Reverse Transcription Kit. PCR was done using conditions and primers specific for *IGF-1*, *IGF-2* (23), *IGFBP3* (24), *IGFBP6* (25), and *GAPDH* (26) as previously reported. Products were analyzed on 1.5% agarose gels.

Cell viability assays and MEK inhibitors

Cells were plated in quadruplicate and treated with OSI-906 and/or AZD6244 for 72 hours at doses indicated. Cell viability was measured using a CellTiter-Glo Luminescent Cell Viability Assay (Promega) on a compatible luminometer plate reader. The MEK inhibitors U0126 (#S1102), GSK1120212 (#S2673), and AZD6244 (#S1008) were purchased from Selleck Chemicals.

Overexpression of a constitutively active MEK mutant

Gateway cloning was used to introduce a constitutively active MEK mutant (MEKDD) into the destination plasmid pLenti6/V5-DEST (Life Technologies). The plasmid pDONR223-MEKDD was obtained from Dr. David Root through Addgene (Addgene plasmid #31202) and was generated as previously described (27). Lentiviral particles were generated using a 3-plasmid system and infected as per the RNAi Consortium Library Production and Performance Protocols, Broad Institute (28).

Twenty-four hours after infection, NCI-H187 cells were maintained in media containing blasticidin. Protein was isolated and screened for phospho-ERK expression as described earlier. Cells were treated with several doses of OSI-906 and assayed by the MTS cell proliferation assay to determine IC_{50} values as described earlier.

Flow cytometry

Cells were treated with OSI-906 or vehicle control (dimethyl sulfoxide) for 72 hours before flow cytometry analysis. For active caspase-3 assessment, cells were fixed and permeabilized using a Cytofix/Cytoperm kit (BD Biosciences), then incubated with FITC labeled antiactive caspase-3 antibody (BD Biosciences) following manufacturer instructions and analyzed by flow cytometry. For cell cycle analysis, cells were fixed and permeabilized with ethanol. A phosphate/citric acid buffer was used to elute fragmented DNA. Cells were stained with propidium iodide and analyzed for DNA content by flow cytometry. Mitotic trapping with nocodazole (100 ng/mL) was conducted 24 hours before cell collection.

In vivo studies

NCI-H187 cells (8×10^6), or SCLC patient derived tumor cells, LX33 and LX36 (3×10^6) were suspended in PBS and matrigel (BD Biosciences). Cell suspensions were injected subcutaneously on right-hind flanks of female Nu/Nu 6 to 8-week-old mice (Charles River). Drug treatments began when tumor sizes reached 200 mm^3. Mice were treated daily with 50 mg/kg OSI-906 (orally) or mock treated with an equal volume of 25 mmol/L tartaric acid vehicle for 17 to 21 days. Tumors were measured with a manual caliper every 3 days and volumes were calculated using the formula: tumor weight (mg) = [length (mm) $\times$ width^2 (mm^2)]/2. To test whether treatment inhibited tumor growth, we used a mixed effects linear model comparing tumor growth between treatment and control groups, modeling the log of tumor size over time and including an interaction term. One mouse was excluded from the control group in H187 because of a tumor that failed to establish. Tumors were harvested for Western blot analysis 24 hours after the final dose. For the pharmacodynamics study in LX33, cells were injected into Nu/Nu mice as described earlier and treated for 3 days after tumors reached 200 to 500 mm^3. Tumors were harvested 2 hours after the last treatment on day 3. Protein was isolated from tumors and analyzed by Western blot as described earlier.

Table 1. OSI-906 IC_{50} values determined from MTS cell proliferation assays

Cell line	IC_{50} value (μmol/L)
H69	3.7
H82	2.3
H128	7.2
H146	2.8
H187	0.250
H209	5.7
H345	3.9
H446	4.5
H526	0.9
H720	0.43
H774	3.4
H847	0.28
H1092	4.2
H1930	7
DMS114	>1
H510A	>1
LX22	2.1
LX33	0.2
LX36	0.1

Results

We analyzed 19 SCLC cell lines, including 3 cell lines derived from primary patient tumors, for sensitivity to OSI-906. Cell lines were plated in quadruplicate and treated with doses of OSI-906 ranging from 10 nmol/L to 10 μmol/L for 72 hours. MTS assays were conducted and IC_{50} values were extrapolated from dose–response curves (Table 1). Six of 19 cell lines (30%) were sensitive to OSI-906, using the criterion of $IC_{50} < 1$ μmol/L to define sensitivity. This extended panel of sensitive lines and the 70% of lines with relative OSI-906 resistance provided an opportunity to explore possible biomarker determinants of OSI-906 sensitivity. Taking a candidate approach, we hypothesized that expression of the primary receptors affected by OSI-906 (IGF-1R and IR), or activity of key factors in the known downstream signaling pathways influenced by these receptors (notably MAPK and PI3K-Akt pathway components) might be associated with sensitivity to OSI-906.

All cell lines were screened by Western blot for expression levels of IGF-1R and IR, and for downstream targets, phosphorylated-Akt and phosphorylated-ERK. There was no correlation between phospho-IGF-1R ($P = 0.3$), phospho-Akt ($P = 0.2$), IGF-1R ($P = 0.2$), IR ($P = 0.1$), total Akt ($P = 0.5$), and total ERK ($P = 0.2$), and sensitivity to OSI-906 (Fig. 2A–C, Supplementary Fig. S1A–S1D). However, OSI-906 sensitive cell lines expressed significantly lower levels of phospho-ERK at baseline than resistant cell

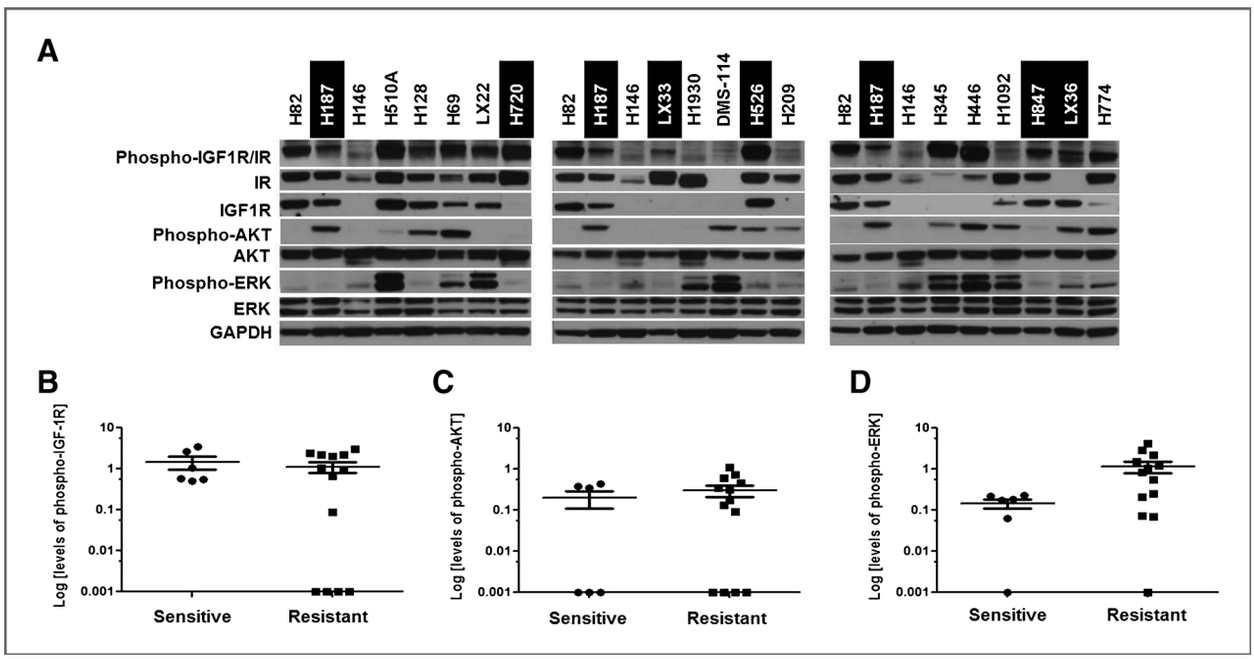

Figure 2. Expression levels of phosphorylated ERK correlates with sensitivity to OSI-906. A, protein was harvested from SCLC cell lines and cell lines derived from primary patient tumors (LX) and analyzed by Western blot. Cell lines with IC_{50} values less than 1 μmol/L are designated as sensitive and are highlighted in black. Data shown are representative of several independent experiments. B–D, from the Western blot shown in A, band intensities for proteins were measured on a densitometer using Bio-Rad Quantity One Software. Relative levels of each protein were calculated by normalizing to GAPDH. There was no statistically significant difference between sensitive and resistant lines for phospho-IGF-1R (B; $P = 0.3$) and phospho-Akt levels (C; $P = 0.2$). Resistant cell lines expressed significantly higher levels of phospho-ERK than sensitive cell lines (D; $P = 0.006$). There were also no significant differences for total protein levels of IGF-1R, IR, AKT, and ERK (Supplementary Fig. S2).

lines (Fig. 2A and D; $P = 0.006$). Cell lines were screened for expression levels of genes encoding IGF ligands, IGF-1 and IGF-2, and IGF binding proteins, IGFBP3 and IGFBP6, by reverse transcription PCR (RT-PCR). There was no evident correlation with any of these factors and sensitivity to OSI-906 (Fig. 3). Together these studies suggest that high baseline MAPK pathway activity, as determined by ERK phosphorylation, but not the absolute expression levels of other factors assessed at either RNA or protein levels, may be a determinant of OSI-906 resistance.

We next sought to further define the biological effects of OSI-906–targeted inhibition on a subset of sensitive and resistant lines, in the presence and absence of the primary ligand for the receptor, and in the presence and absence of other exogenous growth factors present in typical cell culture media. Interestingly, stimulation of both OSI-906 sensitive and resistant cell lines with IGF-1 ligand resulted in similarly strong activation of the IGF-1R pathway and downstream signaling components in both serum starved (Fig. 4A) and nonserum starved conditions (Fig. 4B, Supplementary Fig. S2). In addition, treatment of both sensitive (H187, H526, LX33, LX36, shown highlighted in black) and resistant lines (H1092, H1930, H128, H774) with OSI-906 resulted in dose-dependent inhibition of IGF-1R phosphorylation and of downstream signaling pathway components including phospho-Akt and phospho-P70S6K (Fig. 4A and B, Supplementary Fig. S2). Surprisingly,

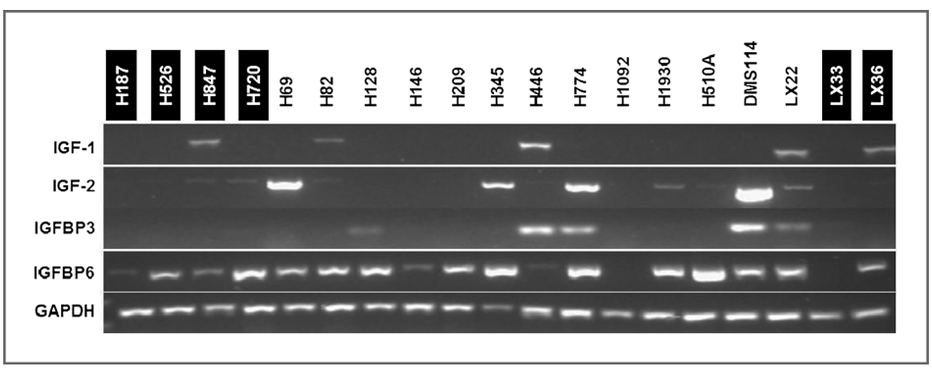

Figure 3. Expression of IGF-1R ligands and binding proteins does not correlate with sensitivity to OSI-906 in SCLC cell lines. RNA was isolated and reverse transcribed from SCLC cell lines and cell lines derived from primary patient tumors (LX) for use in RT-PCR expression analyses. Data shown are representative of several independent experiments.

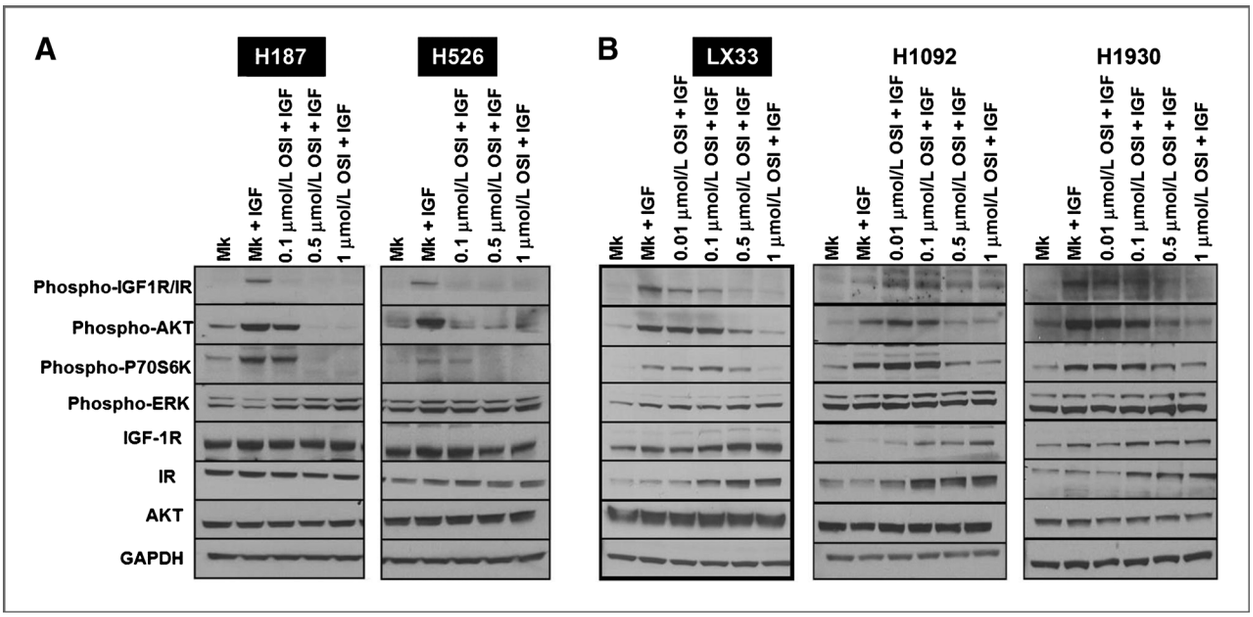

Figure 4. Treatment with OSI-906 results in inhibition of phosphorylated IGF-1R and phosphorylated AKT, but not phosphorylated ERK, in both sensitive and resistant cell lines. A, serum starved cells (H187 and H526) were treated with increasing doses of OSI-906 for 2 hours and then stimulated with IGF-1 (50 ng/mL) for 15 minutes before harvesting. B, cells grown in complete media (LX33, H1092, and H1930) were treated with increasing doses of OSI-906 for 24 hours then stimulated with IGF-1 (50 ng/mL) for 15 minutes before harvesting. Protein was analyzed by Western blot. Similar results were obtained using LX36, H128, and H774 cell lines (Supplementary Fig. S3). OSI-906–sensitive cell lines are highlighted in black. Data shown are representative of several independent experiments.

no inhibition of phospho-ERK was observed, and in some cases (H187, a sensitive line), a counterintuitive upregulation of ERK phosphorylation was observed with increasing concentrations of OSI-906 (Fig. 4A). Taking these data together, although baseline activation state of the MAPK pathway may correlate with OSI-906 sensitivity in SCLC, we observed similar suppression of Akt signaling and no evident suppression of MAPK signaling in response to OSI-906 in sensitive and resistant SCLC lines.

Because several MEK inhibitors are in clinical development and given our findings above, we decided to test whether the inhibition of ERK phosphorylation in OSI-906–resistant lines could alter OSI-906 response. In 3 resistant lines with varying levels of baseline phospho-ERK, multiple MEK inhibitors were able to effectively suppress phospho-ERK (Supplementary Fig. S3A). However, MEK inhibition did not increase sensitivity to OSI-906 in any lines tested, sensitive or resistant, and in fact may be slightly antagonistic (Supplementary Fig. S3B and S3C). Furthermore, the expression of a constitutively active mutant form of MEK in one of the most OSI-906–sensitive cell lines, H187, did not confer therapeutic resistance to OSI-906 (Supplementary Fig. S3D). Therefore, although it seems that high baseline levels of phospho-ERK may correlate with relative intrinsic resistance to OSI-906, ERK phosphorylation alone does not affect resistance, arguing against combination treatment approaches with MEK inhibitors as a therapeutic approach to overcome OSI-906 resistance.

The MAPK and PI3K-Akt pathways are known regulators of both cell proliferation and apoptotic induction. To further explore the differential responses of sensitive and resistant cell lines, we conducted flow cytometry to evaluate the effects of OSI-906 on induction of apoptosis and cell-cycle arrest in small cell lung cancer. OSI-906–sensitive cell lines, H187 and LX36, treated with increasing levels of drug showed a dose-dependent increase in active-caspase 3, although no increase in caspase-3 activation was seen in the resistant H1930 and LX22 cell lines (Fig. 5A). Consistent with this, cell-cycle analysis showed a marked increase in the sub-G_1 population of apoptotic cells in H187 and LX36 upon treatment with OSI-906, but not in LX22 and H1930 (Fig. 5B). In addition, a growth arrest phenotype was seen in H187 cells, but not in LX22 as shown by an increased G_1 peak in H187 cells treated with OSI-906 and nocodozole compared with controls (Supplementary Fig. S4).

Finally, we pursued analysis of OSI-906 efficacy in small cell lung cancer *in vivo*. To evaluate whether OSI-906 could have single-agent anticancer activity in tumors predicted by *in vitro* analysis to be responsive, we administered daily oral OSI-906 versus vehicle control to a series of human SCLC xenograft models, including a xenograft established from a sensitive cell line (NCI-H187), as well as 2 primary SCLC xenografts, LX33 and LX36. Treatment of tumor-bearing mice with OSI-906 resulted in significant tumor growth inhibition in all 3 models (Fig. 6A–C; $P \leq 0.001$ for all 3).

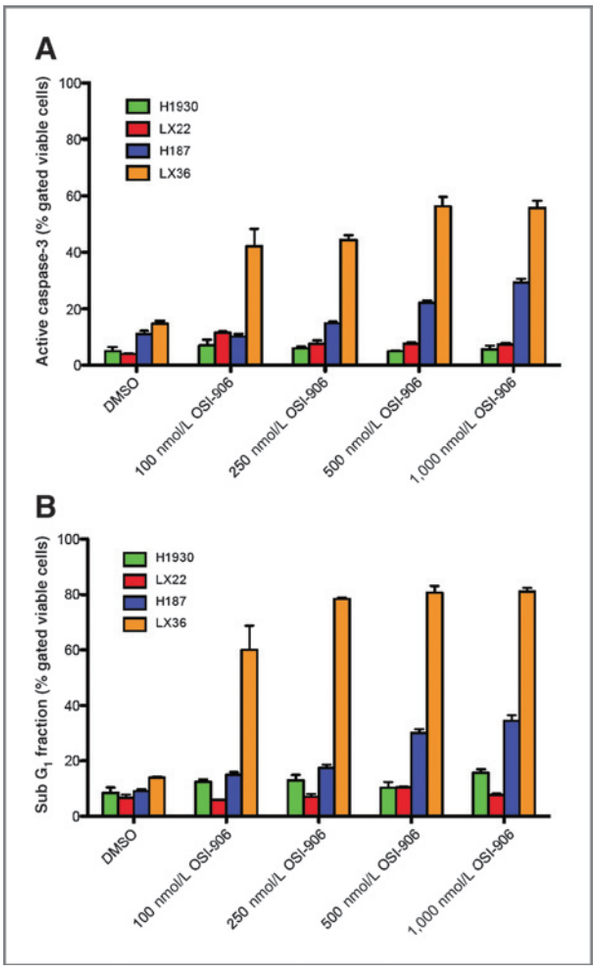

Figure 5. OSI-906 induces apoptosis in sensitive cell lines, but not in resistant cell lines. A, OSI-906 treatment increases active caspase-3 in sensitive cell lines H187 and LX36, but not in resistant cell lines H1930 and LX22. Cells were treated with OSI-906 for 72 hours and then fixed, permeabilized, and stained for active caspase-3. Samples were analyzed by flow cytometry. B, OSI-906 treatment induces an increase in the sub-G_1 population of cells in sensitive cell lines. Cells were treated with OSI-906 for 72 hours and then ethanol fixed and stained with propidium iodide for flow cytometry analysis. Percent sub-G_1 cells were measured and shown in the graph. Data shown are from 3 independent replicates.

Despite inhibition of tumor growth, tumors in treated animals in all 3 models began to progress over the course of treatment. To evaluate intertumor heterogeneity and to assess whether disease progression was associated with loss of target suppression or alteration in Akt or MAPK signaling pathways, individual tumors were harvested from both control and OSI-906–treated animals, and used for Western blotting (Fig. 6D–F). In at least 2 of the models (H187 and LX36), OSI-906–mediated inhibition of IGF-1R/IR phosphorylation seems to be maintained ($P = 0.02$ and 0.03, respectively); in contrast, in LX33 tumors, phospho-IGF-1R/IR levels in OSI-906–treated tumors were not significantly different from controls. Phospho-Akt levels were similarly variable in progressive tumors from OSI-906–treated animals: significantly lower in tumors from OSI-906–treated animals in H187 and LX33 ($P < 0.05$ for each) but not in LX36. In agreement with our *in vitro* data, phospho-ERK levels in OSI-906–treated animals were generally similar to levels seen in tumors from control-treated animals, showing statistically significant inhibition only in LX33.

Discussion

Several major pharmaceutical companies have been concurrently developing inhibitors of IGF-1R, and many of the lead compounds are in active clinical trials. An area of ongoing controversy concerns the relative merits of IGF-1R monoclonal antibodies versus small molecule inhibitors of the IGF-1R kinase domain. One purported advantage of the antibody approach is specificity: these agents can potently inhibit IGF-1R without "off target" inhibition of the IR, theoretically leading to less disruption of normal insulin function and glucose regulation: small molecule RTK inhibitors of IGF-1R (including OSI-906) generally also inhibit IR.

However, data from clinical trials involving small molecule RTKs show that the hyperglycemic side effects of these inhibitors are manageable (29). In addition, perhaps because of feedback signaling or because of IGF-1R:IR receptor dimerization, hyperinsulinemia and hyperglycemia is also seen with IGF-1R monoclonal antibodies (29–31). Most importantly, the ability of small molecule RTKs to inhibit both IGF-1R and IR may have a therapeutic advantage. IR, like IGF-1R, has been shown to promote tumor cell survival and proliferation (32–34). Upon IGF-1R knockdown, an upregulation in insulin signaling was observed in several cell types, suggesting compensatory mechanisms between these pathways (35, 36). Buck and colleagues showed that knockdown of IR using shRNA or inhibition of IGF-1R using a monoclonal antibody resulted in upregulation of phosphorylation of the reciprocal receptor in cancer cell lines coexpressing both receptors. Treatment of these cell lines with OSI-906 resulted in superior pathway inhibition and enhanced efficacy in tumor xenograft models relative to monoclonal antibody inhibition of IGF-1R (37).

Prior reports of IGF-1R signaling in SCLC have generally involved a small number of cell lines, have not defined potential correlates of therapeutic sensitivity, and have not included *in vivo* testing. Here, we sought to take a broader look at the efficacy of OSI-906 across a larger array of SCLC models, including cell lines and primary xenografts. In a panel of 19 SCLC cell lines, we have showed that OSI-906 treatment resulted in significant inhibition of cell proliferation in about 30% of SCLC tumor lines. Profiling of our panel of SCLC lines for expression of IGF-1R axis components identified no significant correlation between receptor or ligand expression and sensitivity to OSI-906. Previous studies in other tumor types have yielded conflicting results, with some suggesting that IGF-1R and IGF-1 expression levels correlate with sensitivity to IGF-1R inhibitors, and others suggesting the

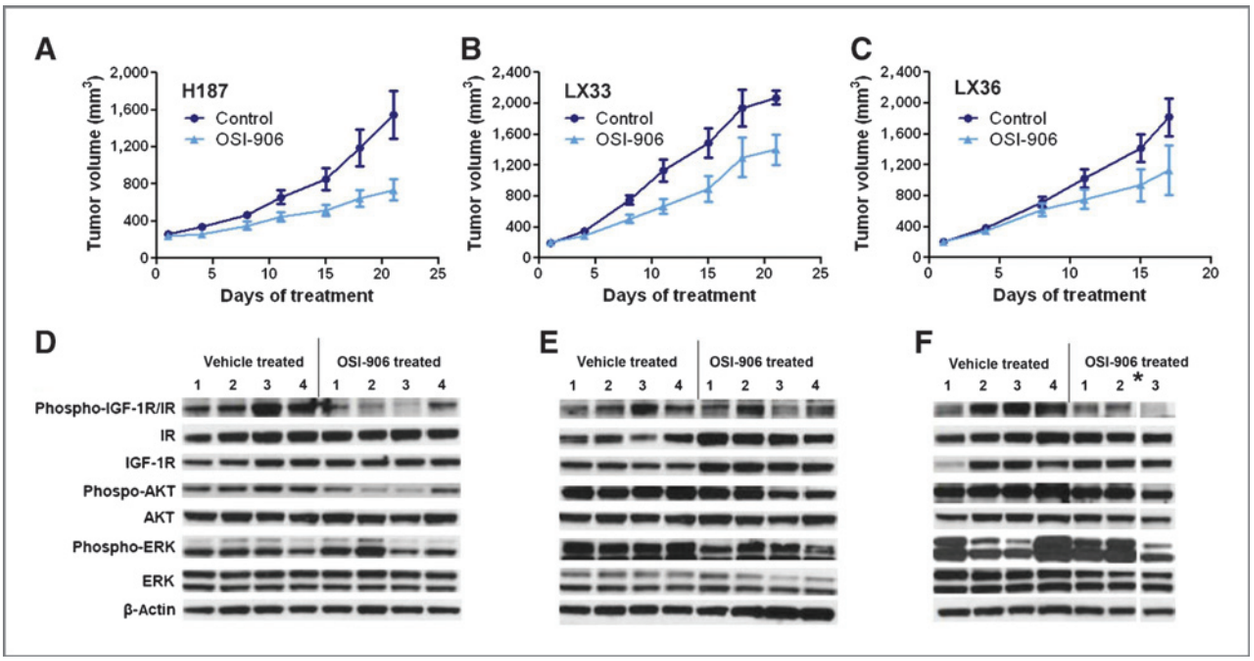

Figure 6. OSI-906 treatment leads to tumor growth inhibition in NCI-H187 and primary patient xenograft mouse models. A–C, Nu/Nu mice xenografted with H187 (A), LX33 (B), or LX36 (C) were treated daily with OSI-906 (50 mg/kg, orally) or vehicle (equivalent volume of 25 mmol/L tartaric acid) for 17 to 21 days (cohorts of 6–7 per treatment arm). Tumor volumes were measured every 3 days. Group mean and SEM for each measurement are shown. OSI-906 resulted in significant tumor growth inhibition compared with controls in all 3 models ($P \leq 0.001$ in all 3 models). D–F, Western blots of tumors harvested at end of study from mice implanted with H187 (D), LX33 (E), and LX36 (F). (Note: * indicates one failed sample: tumor harvested from an OSI-906 treated mouse harboring an LX36 tumor showed no detectable protein in the β-actin blot, and is excluded.)

converse (38–41). Whether IGF-1R expression correlates with sensitivity to IGF-1R inhibition may vary by cancer type, and by type of IGF-1R inhibitor. Going further, we also measured the expression levels of IGF binding proteins, IGFBP3 and IGFBP6 because prior data suggested an association between higher expression levels of these and resistance to IGF-1R inhibition (40). Again, in our panel of SCLC cell lines, we observed no evident correlation between expression of genes encoding either of these binding proteins and sensitivity to OSI-906.

We also tested whether activation of downstream signaling through phospho-Akt and phospho-ERK had any correlation with OSI-906 sensitivity. Although no relationship was seen with phospho-Akt, we were able to define a statistically significant difference between sensitivity and resistance to OSI-906 with regard to phospho-ERK levels. OSI-906–resistant cell lines expressed significantly higher levels of phospho-ERK than sensitive cell lines. This novel observation is supported by data from a microarray study of OSI-906 sensitivity in colorectal cancer cell lines revealing frequent upregulation of MAPK pathway activity in resistant lines (42).

We assessed the changes in key signaling pathways in response to OSI-906 in both sensitive and resistant cell lines. In both sensitive and resistant cell lines, we were able to similarly stimulate the IGF-1R pathway with IGF-1 ligand and inhibit IGF-1R signaling (IGF-1R downregulation and phospho-Akt downregulation) with OSI-906 in a dose-dependent manner. Previous data with an IGF-1R monoclonal antibody had suggested that sensitivity of cell lines to IGF-1R inhibition correlated with downregulation of phospho-Akt (21), but this correlation was not observed in our data set of SCLC lines treated with OSI-906. Interestingly, despite the correlation between OSI-906 resistance and baseline phospho-ERK levels, we observed no downregulation of phospho-ERK levels in response to OSI-906 in either sensitive or resistant cell lines. Taken together, these data suggest a model in which OSI-906 sensitive tumors are primarily reliant on the PI3K-Akt pathway for survival, while OSI-resistant tumors are preferentially reliant on the MAPK pathway. This model, however, is not supported by our observation of the lack of combinatorial efficacy of OSI-906 with targeted MEK inhibition. OSI-906–resistant cell lines show strong basal activity of the MAPK pathway for survival, but this seems to be a correlate, rather than a determinant of resistance. Thus, although baseline ERK activation seems to be a statistically significant predictor of OSI-906 resistance, the therapeutic implications of this association are not clear.

To complement the MTS-based sensitivity data and to show that differential sensitivity to OSI-906 reflected cytotoxicity rather than just inhibition of metabolic activity, we also evaluated apoptotic induction in response to OSI-906 in both sensitive and resistant cell lines. Both measurement of cleaved caspase-3 positive cells and the sub-G_1 population in OSI-906–treated cell lines revealed

robust induction of apoptosis in sensitive cell lines, but not in resistant cell lines. In addition, a cell-cycle arrest phenotype was seen in sensitive cell lines. Although the apoptotic induction observed *in vitro* translated into only partial antitumor suppression *in vivo*, the lowering of the apoptotic threshold suggests that OSI-906 might substantially increase the efficacy of standard cytotoxics used in combination against a responsive subset of SCLC.

Our *in vivo* analysis of OSI-906 included, for the first time, assessment of activity against primary xenograft tumors. We believe there are advantages to testing therapies for SCLC using the preclinical model of primary patient-derived xenografts as a predictor of ultimate clinical utility (43, 44). Notably, efficacy in these models in response to another novel therapeutic, a Bcl-2 inhibitor, was more consistent with the observed clinical activity in SCLC than responses obtained using traditional cell line xenografts (45–47). The xenograft data presented here support that OSI-906 has significant anticancer activity against SCLC tumors *in vivo*. The detailed *in vitro* analyses define a subset of SCLC that may be particularly responsive to this agent, and a larger cohort in which this strategy would be predicted to have minimal activity.

These data justify the exploration of IGF-1R inhibition in SCLC in the clinical setting, and the potential biomarkers defined here will need to be validated in that context. Analysis of relative phospho-ERK levels in pretreatment tumor biopsies of patients enrolling on these studies would be of interest, and may help focus phase II studies of OSI-906 and other IGF-1R inhibitors. Data presented herein would suggest that tumors showing increased levels of phospho-ERK would be relatively resistant to this type of therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: R.L. Zinn, C.M. Rudin
Development of methodology: R.L. Zinn, C.M. Rudin
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E.E. Gardner, I. Dobromilskaya, C.M. Rudin
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References

1. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. CA Cancer J Clin 2012;62:10–29.
2. Jackman DM, Johnson BE. Small-cell lung cancer. Lancet 2005;366:1385–96.
3. LeRoith D, Roberts CT Jr. The insulin-like growth factor system and cancer. Cancer Lett 2003;195:127–37.
4. Kaleko M, Rutter WJ, Miller AD. Overexpression of the human insulin-like growth factor I receptor promotes ligand-dependent neoplastic transformation. Mol Cell Biol 1990;10:464–73.
5. Sell C, Rubini M, Rubin R, Liu JP, Efstratiadis A, Baserga R. Simian virus 40 large tumor antigen is unable to transform mouse embryonic fibroblasts lacking type 1 insulin-like growth factor receptor. Proc Natl Acad Sci U S A 1993;90:11217–21.
6. Samani AA, Yakar S, LeRoith D, Brodt P. The role of the IGF system in cancer growth and metastasis: overview and recent insights. Endocr Rev 2007;28:20–47.
7. Renehan AG, Zwahlen M, Minder C, O'Dwyer ST, Shalet SM, Egger M. Insulin-like growth factor (IGF)-I, IGF binding protein-3, and cancer risk: systematic review and meta-regression analysis. Lancet 2004;363:1346–53.
8. Yakar S, Leroith D, Brodt P. The role of the growth hormone/insulin-like growth factor axis in tumor growth and progression: lessons from animal models. Cytokine Growth Factor Rev 2005;16:407–20.
9. Yu H, Spitz MR, Mistry J, Gu J, Hong WK, Wu X. Plasma levels of insulin-like growth factor-I and lung cancer risk: a case-control analysis. J Natl Cancer Inst 1999;91:151–6.
10. Casa AJ, Dearth RK, Litzenburger BC, Lee AV, Cui X. The type I insulin-like growth factor receptor pathway: a key player in cancer therapeutic resistance. Front Biosci 2008;13:3273–87.
11. Mulvihill MJ, Cooke A, Rosenfeld-Franklin M, Buck E, Foreman K, Landfair D, et al. Discovery of OSI-906: a selective and orally efficacious dual inhibitor of the IGF-1 receptor and insulin receptor. Future Med Chem 2009;1:1153–71.
12. URL http://clinicaltrial.gov/ct2/results?term=OSI-906&Search=Search; accessed 04/01/2013.
13. Macaulay VM, Everard MJ, Teale JD, Trott PA, Van Wyk JJ, Smith IE, et al. Autocrine function for insulin-like growth factor I in human small cell lung cancer cell lines and fresh tumor cells. Cancer Res 1990;50:2511–7.
14. Nakanishi Y, Mulshine JL, Kasprzyk PG, Natale RB, Maneckjee R, Avis I, et al. Insulin-like growth factor-I can mediate autocrine proliferation of human small cell lung cancer cell lines *in vitro*. J Clin Invest 1988;82:354–9.
15. Niklinski J, Furman M. Clinical tumour markers in lung cancer. Eur J Cancer Prev 1995;4:129–38.
16. Quinn KA, Treston AM, Unsworth EJ, Miller MJ, Vos M, Grimley C, et al. Insulin-like growth factor expression in human cancer cell lines. J Biol Chem 1996;271:11477–83.
17. Badzio A, Wynes MW, Dziadziuszko R, Merrick DT, Pardo M, Rzyman W, et al. Increased insulin-like growth factor 1 receptor protein expression and gene copy number in small cell lung cancer. J Thorac Oncol 2010;5:1905–11.
18. Reeve JG, Morgan J, Schwander J, Bleehen NM. Role for membrane and secreted insulin-like growth factor-binding protein-2 in the

regulation of insulin-like growth factor action in lung tumors. Cancer Res 1993;53:4680–5.
19. Rotsch M, Maasberg M, Erbil C, Jaques G, Worsch U, Havemann K. Characterization of insulin-like growth factor I receptors and growth effects in human lung cancer cell lines. J Cancer Res Clin Oncol 1992;118:502–8.
20. Warshamana-Greene GS, Litz J, Buchdunger E, Garcia-Echeverria C, Hofmann F, Krystal GW. The insulin-like growth factor-I receptor kinase inhibitor, NVP-ADW742, sensitizes small cell lung cancer cell lines to the effects of chemotherapy. Clin Cancer Res 2005;11: 1563–71.
21. Yeh J, Litz J, Hauck P, Ludwig DL, Krystal GW. Selective inhibition of SCLC growth by the A12 anti-IGF-1R monoclonal antibody correlates with inhibition of Akt. Lung Cancer 2008;60:166–74.
22. Warshamana-Greene GS, Litz J, Buchdunger E, Hofmann F, Garcia-Echeverria C, Krystal GW. The insulin-like growth factor-I (IGF-I) receptor kinase inhibitor NVP-ADW742, in combination with STI571, delineates a spectrum of dependence of small cell lung cancer on IGF-I and stem cell factor signaling. Mol Cancer Ther 2004;3:527–35.
23. Ji QS, Mulvihill MJ, Rosenfeld-Franklin M, Cooke A, Feng L, Mak G, et al. A novel, potent, and selective insulin-like growth factor-I receptor kinase inhibitor blocks insulin-like growth factor-I receptor signaling *in vitro* and inhibits insulin-like growth factor-I receptor dependent tumor growth *in vivo*. Mol Cancer Ther 2007;6:2158–67.
24. Chun SY, Chen F, Washburn JG, MacDonald JW, Innes KL, Zhao R, et al. CDX2 promotes anchorage-independent growth by transcriptional repression of IGFBP-3. Oncogene 2007;26:4725–9.
25. Wirtz MK, Samples JR, Xu H, Severson T, Acott TS. Expression profile and genome location of cDNA clones from an infant human trabecular meshwork cell library. Invest Ophthalmol Vis Sci 2002;43: 3698–704.
26. Zinn RL, Pruitt K, Eguchi S, Baylin SB, Herman JG. hTERT is expressed in cancer cell lines despite promoter DNA methylation by preservation of unmethylated DNA and active chromatin around the transcription start site. Cancer Res 2007;67:194–201.
27. Yang X, Boehm JS, Salehi-Ashtiani K, Hao T, Shen Y, Lubonja R, et al. A public genome-scale lentiviral expression library of human ORFs. Nat Methods 2011;8:659–61.
28. Moffat J, Grueneberg DA, Yang X, Kim SY, Kloepfer AM, Hinkle G, et al. A lentiviral RNAi library for human and mouse genes applied to an arrayed viral high-content screen. Cell 2006;124:1283–98.
29. Haluska P, Worden F, Olmos D, Yin D, Schteingart D, Batzel GN, et al. Safety, tolerability, and pharmacokinetics of the anti-IGF-1R monoclonal antibody figitumumab in patients with refractory adrenocortical carcinoma. Cancer Chemother Pharmacol 2010;65: 765–73.
30. Kim JG, Kang MJ, Yoon YK, Kim HP, Park J, Song SH, et al. Heterodimerization of glycosylated insulin-like growth factor-1 receptors and insulin receptors in cancer cells sensitive to anti-IGF1R antibody. PLoS One 2012;7:e33322.
31. Yin D, Sleight B, Alvey C, Hansson AG, Bello A. Pharmacokinetics and pharmacodynamics of figitumumab, a monoclonal antibody targeting the insulin-like growth factor 1 receptor, in healthy participants. J Clin Pharmacol 2012 Jan 18. [Epub ahead of print.]
32. Belfiore A. The role of insulin receptor isoforms and hybrid insulin/IGF-I receptors in human cancer. Curr Pharm Des 2007;13:671–86.
33. Giorgino F, Belfiore A, Milazzo G, Costantino A, Maddux B, Whittaker J, et al. Overexpression of insulin receptors in fibroblast and ovary cells induces a ligand-mediated transformed phenotype. Mol Endocrinol 1991;5:452–9.
34. Frittitta L, Vigneri R, Stampfer MR, Goldfine ID. Insulin receptor overexpression in 184B5 human mammary epithelial cells induces a ligand-dependent transformed phenotype. J Cell Biochem 1995;57:666–9.
35. Zhang H, Pelzer AM, Kiang DT, Yee D. Down-regulation of type I insulin-like growth factor receptor increases sensitivity of breast cancer cells to insulin. Cancer Res 2007;67:391–7.
36. Fulzele K, DiGirolamo DJ, Liu Z, Xu J, Messina JL, Clemens TL. Disruption of the insulin-like growth factor type 1 receptor in osteoblasts enhances insulin signaling and action. J Biol Chem 2007;282: 25649–58.
37. Buck E, Gokhale PC, Koujak S, Brown E, Eyzaguirre A, Tao N, et al. Compensatory insulin receptor (IR) activation on inhibition of insulin-like growth factor-1 receptor (IGF-1R): rationale for cotargeting IGF-1R and IR in cancer. Mol Cancer Ther 2010;9:2652–64.
38. Gong Y, Yao E, Shen R, Goel A, Arcila M, Teruya-Feldstein J, et al. High expression levels of total IGF-1R and sensitivity of NSCLC cells *in vitro* to an anti-IGF-1R antibody (R1507). PLoS One 2009;4:e7273.
39. Cao L, Yu Y, Darko I, Currier D, Mayeenuddin LH, Wan X, et al. Addiction to elevated insulin-like growth factor I receptor and initial modulation of the AKT pathway define the responsiveness of rhabdomyosarcoma to the targeting antibody. Cancer Res 2008;68: 8039–48.
40. Huang F, Greer A, Hurlburt W, Han X, Hafezi R, Wittenberg GM, et al. The mechanisms of differential sensitivity to an insulin-like growth factor-1 receptor inhibitor (BMS-536924) and rationale for combining with EGFR/HER2 inhibitors. Cancer Res 2009;69:161–70.
41. Mitsiades CS, Mitsiades NS, McMullan CJ, Poulaki V, Shringarpure R, Akiyama M, et al. Inhibition of the insulin-like growth factor receptor-1 tyrosine kinase activity as a therapeutic strategy for multiple myeloma, other hematologic malignancies, and solid tumors. Cancer Cell 2004;5:221–30.
42. Pitts TM, Tan AC, Kulikowski GN, Tentler JJ, Brown AM, Flanigan SA, et al. Development of an integrated genomic classifier for a novel agent in colorectal cancer: approach to individualized therapy in early development. Clin Cancer Res 2010;16:3193–204.
43. Tentler JJ, Tan AC, Weekes CD, Jimeno A, Leong S, Pitts TM, et al. Patient-derived tumour xenografts as models for oncology drug development. Nat Rev Clin Oncol 2012;9:338–50.
44. Daniel VC, Marchionni L, Hierman JS, Rhodes JT, Devereux WL, Rudin CM, et al. A primary xenograft model of small-cell lung cancer reveals irreversible changes in gene expression imposed by culture *in vitro*. Cancer Res 2009;69:3364–73.
45. Oltersdorf T, Elmore SW, Shoemaker AR, Armstrong RC, Augeri DJ, Belli BA, et al. An inhibitor of Bcl-2 family proteins induces regression of solid tumours. Nature 2005;435:677–81.
46. Hann CL, Daniel VC, Sugar EA, Dobromilskaya I, Murphy SC, Cope L, et al. Therapeutic efficacy of ABT-737, a selective inhibitor of BCL-2, in small cell lung cancer. Cancer Res 2008;68:2321–8.
47. Rudin CM, Hann CL, Garon EB, Ribeiro de Oliveira M, Bonomi PD, Camidge DR, et al. Phase II study of single-agent navitoclax (ABT-263) and biomarker correlates in patients with relapsed small cell lung cancer. Clin Cancer Res 2012;18:3163–9.