ERK phosphorylation is predictive of resistance to IGF-1R inhibition in Small Cell Lung Cancer

Rebekah L. Zinn, Eric E. Gardner, Luigi Marchionni, Sara C. Murphy, Irina Dobromilskaya, Christine L. Hann, Charles M. Rudin

Department of Oncology, The Sidney Kimmel Comprehensive Cancer Center at the Johns Hopkins University School of Medicine, Baltimore, MD

Running Title: Targeting IGF-1R in small cell lung cancer

Key Words: IGF-1R, ERK, AKT, primary xenograft, small cell lung cancer

Abbreviations list: SCLC: small cell lung cancer; IGF-1R: insulin-like growth factor 1 receptor; IR: insulin receptor; MAPK: mitogen activated protein kinase; PI3K: phosphatidylinositol-3 kinase; MTS: (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium); RTK: receptor tyrosine kinase; DMSO: dimethyl sulfoxide

Grant support: Flight Attendant Medical Research Institute Center of Excellence Award (C.M. Rudin), Burroughs Wellcome Fund Clinical Scientist Award in Translational Research (C.M. Rudin), and the Anti-Cancer Drug Development Training Grant NIH T32 CA009243 (R.L. Zinn)

Conflict of interest: The authors have no conflicts of interest with regard to the contents of this paper.

Correspondence: Charles M. Rudin, MD PhD
Cancer Research Building 2, Room 544
1550 Orleans Street, Baltimore, MD 21287
Phone: 410-502-0678
Fax: 410-502-0677
E-mail: rudin@jhmi.edu

Word Count: 3951
Tables: 1
Figures: 6
Abstract

New therapies are critically needed to improve the outcome for patients with small cell lung cancer (SCLC). 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 PI3K-Akt and MAPK. 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 (IR). Approximately one third of these cell lines were sensitive to OSI-906, with an IC50 < 1 μM. 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 to 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.
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 SCLC cases, these responses are of short duration and the median survival in patients with advanced SCLC is less than one 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).

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 family, which are being explored in preclinical models and early phase clinical trials. OSI-906, or \textit{cis}-3-[8-amino-1-(2-phenyl-quinolin-7-yl)-imidazo[1,5-a]pyrazin-3-yl]-1-methyl-cyclobutanol (Figure 1), is a potent and specific small molecule tyrosine kinase inhibitor of the insulin receptor (IR) family. Testing against a panel of more than 40 kinases revealed OSI-906 to be a selective inhibitor of IGF-1R (35 nM) and IR (75 nM) with all other kinases having an IC\textsubscript{50} greater than 10 µM. OSI-906 has significant anti-proliferative 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 \textit{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 \textit{in vivo} efficacy of IGF-1R inhibitors in SCLC. We examined a large panel of SCLC cell lines, including three 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 \textit{in vivo} efficacy studies in SCLC patient derived xenograft mouse models. Our data demonstrate that IGF-1R inhibition may be a viable treatment strategy for a molecularly-defined subset of SCLC.
Materials and Methods

Cell Lines. The following cell lines were authenticated by short tandem repeat (STR) 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 nM to 10 µM. MTS cell proliferation assays were performed 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 µM. 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, see below) is 2.68 µM (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.
**RT-PCR.** RNA was isolated from cells using the Qiagen RNeasy Mini kit and reverse transcribed using Qiagen Quantitect Reverse Transcription kit. PCR was performed 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 three-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 above. Cells were treated with several doses of OSI-906 and assayed by the MTS Cell Proliferation assay in order to determine IC$_{50}$ values as described above.

**Flow cytometry.** Cells were treated with OSI-906 or vehicle control (DMSO) 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 anti-active 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 (100ng/mL) was performed 24 hours before cell collection.

**In vivo studies.** NCI-H187 cells (8 × 10⁶), or SCLC patient derived tumor cells, LX33 and LX36 (3 × 10⁶) were suspended in PBS and matrigel (BD Biosciences). Cell suspensions were injected subcutaneously on right-hind flanks of female Nu/Nu 6-8 week old mice (Charles River). Drug treatments began when tumor sizes reached 200 mm³. Mice were treated daily with 50 mg/kg OSI-906 (PO) or mock treated with an equal volume of 25 mM tartaric acid vehicle for 17-21 days. Tumors were measured with a manual caliper every 3 days and volumes were calculated using the formula: tumor weight (mg) = [tumor volume = length (mm) × width² (mm²)]/2. In order 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 due to a tumor that failed to establish. Tumors were harvested for western blot analysis 24 hours after the final dose. For the pharmacodynamics (PD) study in LX33, cells were injected into Nu/Nu mice as described above and treated for 3 days after tumors reached 200-500 mm³. Tumors were harvested 2 hours after the last treatment on day 3. Protein was isolated from tumors and analyzed by Western blot as described above.
Results

We analyzed 19 SCLC cell lines, including three 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 nM to 10 µM for 72 hrs. MTS assays were performed and IC₅₀ values were extrapolated from dose response curves (Table 1). Six of 19 cell lines (30%) were sensitive to OSI-906, using the criterion of IC₅₀< 1µM 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 (Figure 2A-C, Supplemental Figure 1A-D). However, OSI-906 sensitive cell lines expressed significantly lower levels of phospho-ERK at baseline than resistant cell lines (Figure 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 RT-PCR. There was no evident correlation with any of these factors and sensitivity to OSI-906 (Figure 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 (Figure 4A) and non-serum starved conditions (Figure 4B, Supplemental Figure 2). 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 (Figures 4A & B, Supplemental Figure 2). Surprisingly, 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 (Figure 4A). Taking these data together, while 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 three resistant lines with varying levels of baseline phospho-ERK, multiple MEK inhibitors were able to effectively suppress phospho-ERK (Supplemental Figure 3A). However, MEK inhibition did not increase sensitivity to OSI-906 in
any lines tested, sensitive or resistant, and in fact may be slightly antagonistic (Supplemental Figure 3B & C). Further, 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 (Supplemental Figure 3D). Therefore, while it appears 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 performed 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, while no increase in caspase-3 activation was seen in the resistant H1930 and LX22 cell lines (Figure 5A). Consistent with this, cell cycle analysis showed a marked increase in the sub-G1 population of apoptotic cells in H187 and LX36 upon treatment with OSI-906, but not in LX22 and H1930 (Figure 5B). In addition, a growth arrest phenotype was seen in H187 cells, but not in LX22 as demonstrated by an increased G1 peak in H187 cells treated with OSI-906 and nocodazole compared to controls (Supplemental Figure 4).

Finally, we pursued analysis of OSI-906 efficacy in small cell lung cancer in vivo. To evaluate whether OSI-906 could have single agent anti-cancer activity in tumors predicted by in vitro analysis to be responsive, we administered daily oral OSI-906 vs. vehicle control to a series of human SCLC xenograft models, including a xenograft established from a sensitive cell line (NCI-H187), as well as two primary SCLC xenografts, LX33 and LX36. Treatment of tumor-
bearing mice with OSI-906 resulted in significant tumor growth inhibition in all three models (Figure 6 A-C; p ≤ 0.002 for all three).

Despite inhibition of tumor growth, tumors in treated animals in all three models began to progress over the course of treatment. To evaluate inter-tumor 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 (Figures 6 D-F). In at least two of the models (H187 and LX36), OSI-906-mediated inhibition of IGF-1R/IR phosphorylation appeared to be maintained (p= 0.02 and p=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, demonstrating 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 vs. 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 demonstrate that the hyperglycemic side effects of these inhibitors are manageable (29). In addition, perhaps due to feedback signaling or due to 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 et al. 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 co-expressing 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 demonstrated 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 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 since 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. While 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 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 demonstrate strong basal activity of the MAPK pathway for survival, but this appears to be a correlate, rather than a determinant of resistance. Thus while baseline ERK activation appears 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 demonstrate 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-G1 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. While the apoptotic induction observed in vitro translated into only partial anti-tumor 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 \textit{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 anti-cancer activity against SCLC tumors \textit{in vivo}. The detailed \textit{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 demonstrating increased levels of phospho-ERK would be relatively resistant to this type of therapy.

\textbf{Acknowledgments}

The authors would like to thank Elizabeth Buck (Astellas Pharma), Blake Aftab, Timothy Burns, John Poirier, and Frank Vendetti (Rudin Laboratory) for helpful discussions. We would also like to thank Nick Connis for technical assistance.
Grant Support

Flight Attendant Medical Research Institute Center of Excellence Award (C.M. Rudin), Burroughs Wellcome Fund Clinical Scientist Award in Translational Research (C.M. Rudin), and the Anti-Cancer Drug Development Training Grant NIH T32 CA009243 (R.L. Zinn)
References


12. NIH. Clinicaltrials.gov.


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


Table 1. OSI-906 IC$_{50}$ values determined from MTS Cell Proliferation Assays.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>IC$_{50}$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>H69</td>
<td>3.7 µM</td>
</tr>
<tr>
<td>H82</td>
<td>2.3 µM</td>
</tr>
<tr>
<td>H128</td>
<td>7.2 µM</td>
</tr>
<tr>
<td>H146</td>
<td>2.8 µM</td>
</tr>
<tr>
<td>H187</td>
<td>0.250 µM</td>
</tr>
<tr>
<td>H209</td>
<td>5.7 µM</td>
</tr>
<tr>
<td>H345</td>
<td>3.9 µM</td>
</tr>
<tr>
<td>H446</td>
<td>4.5 µM</td>
</tr>
<tr>
<td>H526</td>
<td>0.9 µM</td>
</tr>
<tr>
<td>H720</td>
<td>0.43 µM</td>
</tr>
<tr>
<td>H774</td>
<td>3.4 µM</td>
</tr>
<tr>
<td>H847</td>
<td>0.28 µM</td>
</tr>
<tr>
<td>H1092</td>
<td>4.2 µM</td>
</tr>
<tr>
<td>H1930</td>
<td>7 µM</td>
</tr>
<tr>
<td>DMS114</td>
<td>&gt;1 µM</td>
</tr>
<tr>
<td>H510A</td>
<td>&gt;1 µM</td>
</tr>
<tr>
<td>LX22</td>
<td>2.1 µM</td>
</tr>
<tr>
<td>LX33</td>
<td>0.2 µM</td>
</tr>
<tr>
<td>LX36</td>
<td>0.1 µM</td>
</tr>
</tbody>
</table>
Figure Legends

Figure 1. Chemical structure of OSI-906.

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 IC50 values less than 1 µM 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 statically 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 (See Supplemental Figure 2).

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 hrs and then stimulated with IGF-1 (50 ng/mL) for 15 min. before harvesting. B. Cells grown in complete media (LX33, H1092 and H1930) were treated with increasing doses of OSI-906 for 24 hrs then stimulated with IGF-1 (50 ng/mL) for 15 min. before harvesting. Protein was
analyzed by Western blot. Similar results were obtained using LX36, H128, and H774 cell lines (Supplemental Figure 3). OSI-906-sensitive cell lines are highlighted in black. Data shown are representative of several independent experiments.

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 hrs 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-G1 population of cells in sensitive cell lines. Cells were treated with OSI-906 for 72 hrs and then ethanol fixed and stained with propidium iodide for flow cytometry analysis. Percent sub-G1 cells were measured and shown in the graph. Data shown are from three independent replicates.

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, PO) or vehicle (equivalent volume of 25mM tartaric acid) for 17-21 days (cohorts of 6 – 7 per treatment arm). Tumor volumes were measured every three days. Group mean and standard error of mean (SEM) for each measurement are shown. OSI-906 resulted in significant tumor growth inhibition compared to controls in all three models (p ≤ 0.001 in all three 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.)
Figure 2

A.

B.

C.

D.
Figure 5

A. 

B.
Figure 6
Molecular Cancer Therapeutics

ERK phosphorylation is predictive of resistance to IGF-1R inhibition in small cell lung cancer


Mol Cancer Ther Published OnlineFirst March 20, 2013.

Updated version
Access the most recent version of this article at: doi:10.1158/1535-7163.MCT-12-0618

Supplementary Material
Access the most recent supplemental material at: http://mct.aacrjournals.org/content/suppl/2013/03/20/1535-7163.MCT-12-0618.DC1

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.