OSI-930: A Novel Selective Inhibitor of Kit and Kinase Insert Domain Receptor Tyrosine Kinases with Antitumor Activity in Mouse Xenograft Models


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Abstract

OSI-930 is a novel inhibitor of the receptor tyrosine kinases Kit and kinase insert domain receptor (KDR), which is currently being evaluated in clinical studies. OSI-930 selectively inhibits Kit and KDR with similar potency in intact cells and also inhibits these targets in vivo following oral dosing. We have investigated the relationships between the potency observed in cell-based assays in vitro, the plasma exposure levels achieved following oral dosing, the time course of target inhibition in vivo, and antitumor activity of OSI-930 in xenograft models. In the mutant Kit–expressing HMC-1 xenograft model, prolonged inhibition of Kit was achieved at oral doses between 10 and 50 mg/kg and this dose range was associated with antitumor activity. Similarly, prolonged inhibition of wild-type Kit in the NCI-H326 xenograft model was observed at oral doses of 100 to 200 mg/kg, which was the dose level associated with significant antitumor activity in this model as well as in the majority of other xenograft models tested. The data suggest that antitumor activity of OSI-930 in mouse xenograft models is observed at dose levels that maintain a significant level of inhibition of the molecular targets of OSI-930 for a prolonged period. Furthermore, pharmacokinetic evaluation of the plasma exposure levels of OSI-930 at these effective dose levels provides an estimate of the target plasma concentrations that may be required to achieve prolonged inhibition of Kit and KDR in humans and which would therefore be expected to yield a therapeutic benefit in future clinical evaluations of OSI-930.

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Introduction

Inhibition of protein kinases has recently emerged as a promising therapeutic approach in many types of human cancer and a variety of agents targeting several different protein kinase family members have now been shown to provide significant clinical benefit in certain indications (1–4). Examples of such molecular targeted therapies include small-molecule inhibitors such as the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor erlotinib (Tarceva, OSI-774), which was shown to increase patient survival in both non–small-cell lung cancer (5, 6) and pancreatic cancer, and imatinib (Gleevec), which has significant clinical benefit in chronic myelogenous leukemia and gastrointestinal stromal tumors (1, 7).

The receptor tyrosine kinases Kit and kinase insert domain receptor (KDR) are closely related members of the split kinase domain subfamily of tyrosine kinases, which also includes platelet-derived growth factor receptor (PDGFR)α/PDGFRβ and colony-stimulating factor-1 receptor (CSF-1R). Inhibition of Kit and KDR in vivo might be expected to result in antitumor effects through two distinct mechanisms in appropriate tumor types; i.e., direct effects on the tumor cell phenotype through inhibition of Kit and indirect effects via disruption of endothelial cell function by inhibition of KDR. Combination of these activities within the same molecule is predicted to result in more potent activity against a broader range of tumor types than a molecule with inhibitory activity against a single target. There is considerable evidence that expression of mutant alleles encoding constitutively active Kit receptor molecules is a major factor driving tumor growth in mast cell leukemias/mastocytosis and gastrointestinal stromal tumors (8–19). In addition, several reports have highlighted the potential for wild-type Kit to be involved in progression of other tumor types, including small-cell lung cancer (20–25). In such tumors, the proposed mechanism for Kit involvement is the generation of an autocrine/paracrine loop due to coexpression of ligand and receptor within the same tumor cell type. Alternatively, for tumors that express Kit but not stem cell factor, circulating stem cell factor levels may be sufficient to activate the Kit receptor when aberrantly expressed on tumor cells.

The tyrosine kinase activity of the receptor tyrosine kinase KDR is thought to be essential for promoting the formation of new blood vessels, both during development and in support of growing solid tumors (26), and is therefore considered a viable target for anticancer therapy. KDR is responsible for the formation, proliferation, and survival of endothelial cells in response to vascular endothelial growth factor (VEGF) ligand family members, and
VEGFR/KDR signaling is frequently up-regulated in the tumor vasculature resulting in increased formation of new blood vessels (27–30).

Several proof-of-concept studies have now been reported that support the concept of KDR inhibition as an anticancer approach, including a number of studies using small-molecule inhibitors of KDR to inhibit solid tumor growth in mouse xenograft models (31–40). Several ongoing or completed clinical trials have also explored the role of KDR in a variety of human cancers through either small-molecule KDR inhibitors (41–43) or antibody approaches (2, 44–47).

We have recently identified a series of novel 2,3-substituted thiophenes with potent inhibitory activity against the tyrosine kinases Kit and KDR, and OSI-930 (Fig. 1) has emerged from this series as a clinical candidate. In this report, the in vitro and in vivo pharmacologic properties of OSI-930 are described. OSI-930 potently inhibits Kit and KDR in intact cells and the promising pharmacokinetic and pharmacodynamic properties of the compound, together with the broad antitumor activity observed in preclinical models, suggest that OSI-930 may provide clinical benefit in a broad range of tumor types.

Materials and Methods

Synthesis of OSI-930. OSI-930 [N-(4-trifluoromethoxyphenyl) 3-[quinolin-4-ylmethyl]amino]thiophene-2-carboxamide] is a heterocyclic anthranilamide analogue synthesized by the methods described in patent application number WO 2004/063330. Compound identity and purity (>98%) was verified by 1H and 13C nuclear magnetic resonance, mass spectrometry (MS), and high-performance liquid chromatography using Bruker Avance 400, Waters Micromass ZQ, and Waters LC Module I Plus instruments, respectively. OSI-930 was dissolved in DMSO at 10 mmol/L for use in biochemical or cellular assays done in vitro. For in vivo studies, OSI-930 was dissolved in the indicated vehicle (either Labrafil M 1944 CS [Gattefosse] or polyethylene glycol (PEG)-400/water [50:50]) at an appropriate concentration to deliver the desired dose at 20 mL/kg by oral gavage.

Cell lines. HMC-1 mast cell leukemia cells (48) were obtained from Dr. Joseph Butterfield (Mayo Clinic, Rochester, MN) and were maintained in Iscove’s modified Dulbecco’s medium supplemented with 10% FCS, 1% l-glutamine, and 1.2 mmol/L L-α-monomethylcysteine. WBA small-cell lung cancer cells were a kind gift from Dr. Geoff Krystal (Virginia Commonwealth University, Richmond, VA) and were maintained in RPMI 1640 supplemented with 10% FCS. Human umbilical vascular endothelial cells (HUVEC) were obtained from Cambrex (East Rutherford, NJ) and maintained in EGM2 medium. The BxPc3-A1 cell line used is a variant of the BxPc3 cell line with growth characteristics, which was derived by subculturing tumor-derived tissue fragments by serial passage in nude mice.

Antibodies. The following antibodies were used for immunoprecipitation or as the capture antibody in ELISA assays: Kit (AB-6/MS-289, Lab Vision Corp., Fremont, CA), KDR (AF357, R&D Systems, Minneapolis, MN), and PDGFRα (AF1042, R&D Systems). The following antibodies were used for immunohistology: Kit (AB-6/MB-1518, Lab Vision Corp.), pKit (3391, Cell Signaling Technology, Beverly, MA), antiphosphotyrosine-horseradish peroxidase (HRP) conjugate (pT205/pT206,S355, Clontech, San Diego, CA), KDR (c-1158/SE-504, Santa Cruz Biotechnology, Santa Cruz, CA), PDGFRα (pK272/pT273, R&D Systems), Akt (9272, Cell Signaling Technology), Erk1/2 (9102, Cell Signaling Technology), pErk1/2(S202/S204), pS6235/236(2211, Cell Signaling Technology), p70S6K (9202, Cell Signaling Technology), p-p70S6K(2492, Cell Signaling Technology), p-Akt(473), p-Akt473(9272, Cell Signaling Technology), p-GSK3β(9232, Cell Signaling Technology), p-MAPK (9102, Cell Signaling Technology), p-MEK-1/ERK-5 (9106, Cell Signaling Technology), p-AKT(473), p-AKT473(9272, Cell Signaling Technology), cell signaling Technology, and pS6(2212, Cell Signaling Technology). CD31 antibody used for immunohistochemical analysis was clone MEC13.3 from BD Pharmingen (San Diego, CA).

Animals. Female CD-1 and athymic nude nu/nu CD-1 mice (6-8 weeks, 25-29 g) were obtained from Charles River Laboratories (Wilmington, MA). Animals were allowed to acclimate for a minimum of 1 week before initiation of a study. Throughout the studies, animals were allowed sterile rodent chow and water ad libitum and immunocompromised animals were maintained under specific pathogen-free conditions. All animal studies were conducted at OSI facilities with the approval of the Institutional Animal Care and Use Committee in an American Association for Accreditation of Laboratory Animal Care–accredited vivarium and in accordance with the Institute of Laboratory Animal Research guidelines (Guide for the Care and Use of Laboratory Animals, NIH, Bethesda, MD).

Protein kinase assays. Protein kinase assays were either done in-house by ELISA-based assay methods (Kit, KDR, PDGFRα, and PDGFRβ) or at Upstate by a radiometric method (KinaseProfiler service). In-house ELISA assays used poly(Glu,Tyr) (Sigma, St. Louis, MO) as the substrate bound to the surface of 96-well assay plates; phosphorylation was then detected using an antiphosphotyrosine antibody conjugated to HRP. The bound antibody was then quantitated using ABTS as the peroxidase substrate by measuring the absorbance at 405/490 nm. All assays used purified recombinant kinase catalytic domains that were either expressed in insect cells or in bacteria. The Kit and EGFR protein used for in-house assays were prepared internally; other enzymes were obtained from Upstate (PDGFRα and PDGFRβ) or ProQinase (Freiburg, Germany) (KDR). Recombinant Kit protein was expressed as an NH2-terminal glutathione S-transferase fusion protein in insect cells and was initially purified as a nonphosphorylated (nonactivated) enzyme with a relatively high Km for ATP (400 μmol/L). In some assays, an activated (tyrosine phosphorylated) form of the enzyme was prepared by incubation with 1 mmol/L ATP for 1 hour at 30 °C. The phosphorylated protein was then passed through a desalting column to remove the majority of the ATP and stored at −80 °C in buffer containing 50% glycerol. The resultant preparation had a considerably higher specific activity and a lower Km for ATP (25 μmol/L) than the initial non-phosphorylated preparation. The inhibition of Kit autophosphorylation by OSI-930 was assayed by incubation of the nonphosphorylated enzyme at 30 °C in the presence of 200 μmol/L ATP and various concentrations of OSI-930. The reaction was stopped by removal of aliquots into SDS-PAGE sample buffer followed by heating to 100 °C for 5 minutes. The degree of phosphorylation of Kit was then determined by immunoblotting for both total Kit and phosphorylated Kit.

Mechanistic assays of protein kinase inhibition in intact cells. Cells were seeded the day before use into 96-well plates for quantitative 96-well ELISA-based assays of the cellular effects of OSI-930 or into 10-cm dishes for analysis by immunoblotting. The cells were treated with various concentrations of compound for 3 hours before lysis (the final concentration of DMSO in the assay was 0.1%), and as required, the appropriate ligand was added for the final 15 minutes of the compound treatment period (100 ng/mL stem cell factor (R&D Systems), 50 ng/mL VEGF165 (R&D Systems), and 25 ng/mL PDGF-BB (R&D Systems))). Lysates were then prepared in buffer containing 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 10% glycerol, 1% Triton X-100, 0.5 mmol/L EDTA, 1 μg/mL leupeptin, 1 μg/mL aprotinin, and 1 mmol/L sodium orthovanadate. ELISA-based assays of target protein phosphorylation were done by transferring lysates into a second 96-well plate that was precoated with the appropriate capture antibody. The captured target proteins were then probed with an antiphosphotyrosine antibody-HRP conjugate using a chemiluminescent HRP substrate (Pierce) for detection by...

Figure 1. Structure of OSI-930.
luminometry. In experiments done to evaluate the effect of plasma protein binding of OSI-930 on its ability to affect cellular processes, purified human plasma proteins albumin (Sigma) and α1-acid glycoprotein (Sigma) were incorporated into the quantitative 96-well assays at concentrations approximating those found in vivo (3% and 0.05%, respectively). In these experiments, plasma proteins were added to the cell culture medium before compound addition and the DMSO stock solution of OSI-930 was also initially diluted into cell culture medium containing plasma proteins to ensure preequilibration of compound binding to plasma protein.

For immunoblotting analysis, lysates were cleared of insoluble material by centrifugation at 15,000 × g for 5 minutes at 4°C and the resultant supernatant was subjected to immunoprecipitation with the appropriate antibody coupled to Protein G-Sepharose beads (Amersham Biosciences, Piscataway, NJ), followed by SDS-PAGE and immunoblotting with the same antiphosphotyrosine antibody-HRP conjugate and chemiluminescent detection. Alternatively, for highly abundant protein targets (S6, Erk, and p70S6K), lysates were analyzed directly by SDS-PAGE and immunoblotting.

Phenotypic assays in intact cells. For assays of cell proliferation and apoptosis, cells were seeded into 96-well plates and incubated for 2 to 3 days in the presence of OSI-930 at various concentrations. Inhibition of cell growth was determined by luminescent quantitation of the intracellular ATP content using CellTiterGlo (Promega, Madison, WI). Induction of caspase-dependent apoptosis by OSI-930 was quantitated by an enzymatic caspase 3/7 assay (CaspaseGlo, Promega). Inhibition of angiogenesis by OSI-930 was monitored using the rat aortic ring endothelial sprout outgrowth assay. Sections of aorta were prepared from CO2-anesthetized male rats and cultured in vitro in a collagen matrix in the presence or absence of OSI-930. The collagen matrix was prepared from type I rat tail collagen (Sigma) solubilized in 0.1% acetic acid at 3 mg/mL, which was combined with 0.125 volume collagen buffer (0.05 N NaOH, 200 mmol/L HEPES, 260 mmol/L NaHCO3), 0.125 volume of 10× medium 199 (PF; Sigma), 0.0125 volume of 1 mol/L NaOH, and 1% GlutaMax (Life Technologies). Aortic rings were embedded in 0.4 mL of this matrix in six-well plates, to which 0.5 mL endothelial basal medium (Cambrex) and the appropriate amount of OSI-930 was added; the rings were then incubated for 10 days (medium was replaced every 2-3 days) and the resultant angiogenic sprout outgrowth was digitally quantitated from images at 40× magnification by measurement of the sprout-containing area within a series of concentric rings around the aortic tissue area.

Pharmacokinetic analysis of OSI-930. Terminal blood samples in EDTA were taken by cardiac puncture and plasma samples were extracted by protein precipitation with methanol (50 μL plasma + 200 μL methanol) followed by centrifugation (10,000 × g for 10 minutes at 4°C). Extracted plasma samples were analyzed by high-performance liquid chromatography-Ms/MS using calibration and quality control samples prepared in blank mouse plasma. All pharmacokinetic variables were obtained by noncompartmental modeling of the concentration-time data.

Pharmacodynamic analysis of Kit and KDR inhibition in vivo. Female nu/nu CD-1 mice were implanted s.c. with cells from HMC-1 or NCI-H526 cell lines harvested from cell culture flasks and tumors were established to 250 ± 50 mm3 in volume before dosing. The mice were then treated daily orally with OSI-930 or vehicle and both tumors and plasma were collected at appropriate time points for analysis of Kit phosphorylation (tumor samples) and OSI-930 concentrations (tumor and plasma samples). The phosphorylation status of Kit was determined by immunoprecipitation of total Kit followed by immunoblotting for both phospho-Kit (Y719) and total Kit. Comparison of immunoblotting band intensities yielded a ratio of phosphorylated Kit and total Kit protein for each sample (n = 4 tumors per time point). The effect of OSI-930 was established by comparison of this ratio with that obtained from the vehicle control dosed animals.

The effect of KDR inhibition by OSI-930 in vivo was evaluated by monitoring estrogen-induced mouse uterine edema following OSI-930 dosing. Female BALB/c mice were hormonally synchronized by s.c. injection with pregnant mare serum gonadotropin (15 IU), followed 48 hours later by s.c. injection of human chorionic gonadotropin (HCG, 20 IU). At 24 hours after HCG injection, animals were administered either vehicle or OSI-930 by oral gavage, and 2 hours later were injected with estradiol (25 μg, i.p.) to induce uterine swelling. At 2.5 hours after estradiol injection, animals were euthanized and the wet weight of the uterus was determined. Following incubation in an oven at 30°C overnight, the dry uterine weights were measured to establish the percentage of uterine weight present as water.

For immunohistochemical analysis of tumor blood vessel content, tumors were removed from CD-1 nu/nu mice following daily oral dosing for 3 consecutive days with either vehicle or OSI-930. Tumors were removed and frozen and 5-μm cryostat sections of tumor tissue were prepared and stained for CD31 (platelet/endothelial cell adhesion molecule 1) content.

Tumor xenograft growth inhibition studies. Cells were harvested from cell culture flasks during exponential cell growth, washed twice with sterile PBS, counted, and resuspended in PBS to a suitable concentration before s.c. implantation in the right flank of nu/nu CD-1 mice. Tumors were established to 200 ± 50 mm3 in size before randomization into treatment groups of eight mice each for efficacy studies; OSI-930 or vehicle was then administered orally as indicated. Body weights were determined twice weekly along with tumor volume [V = length × (width)2 / 2] measurements using Vernier calipers for the duration of the study. Tumor growth inhibition (TGI) was determined by the following formula: % TGI = 100(1 − Wt / Wc), where Wt is the median tumor volume of the treated group and Wc is the median tumor volume of the control group. Tumor growth inhibition of ≥42% is considered significant. Growth delay is calculated as T − C, where T and C are the times in days for median tumor size in the treated (T) and control (C) groups to reach 500% of the initial tumor volume. Cures are excluded from this calculation.

Results and Discussion

Kinase inhibition profile of OSI-930 in vitro. OSI-930 potently inhibited the activity of recombinant kinase domains derived from the closely related receptor tyrosine kinases Kit and KDR in vitro when assayed at ATP concentrations approximating the Ka values (Table 1). Both phosphorylated (activated) and nonphosphorylated (nonactivated) forms of Kit were inhibited by OSI-930 when assayed using poly(Glu:Tyr) as the substrate, suggesting that multiple activation/phosphorylation states of Kit can be inhibited by OSI-930; the IC50 values for Kit kinase inhibition by OSI-930 were 80 nmol/L (activated Kit) and 629 nmol/L (nonactivated Kit) when assayed at ATP concentrations approximating the respective Ka value for each form of the enzyme (Table 1). In addition, OSI-930 inhibited with very high potency (IC50 < 15 nmol/L) autophosphorylation of the nonactivated form of the enzyme in the presence of 200 μmol/L ATP (Supplementary Fig. S1; Table 1). In view of the ability of OSI-930 to inhibit the activity of Kit in cellular systems with IC50 values of ~10 nmol/L (Fig. 2; Table 2), it seems that monitoring autophosphorylation of the enzyme provides a more accurate estimate of the potency of Kit inhibition by OSI-930 than assays done in an ELISA format with the artificial substrate poly(Glu:Tyr). The molecular basis for inhibition of Kit by OSI-930 has been examined by determining a co-crystal structure of OSI-930 bound to the kinase domain of the nonactivated form of Kit. The structure obtained showed that the compound was bound to the enzyme in an inactive conformation through noncovalent interactions to the ATP-binding site within the kinase domain (data not shown). Consistent with the observation that OSI-930 was observed interacting with the ATP-binding pocket of Kit, the IC50 for inhibition of Kit by OSI-930 was higher when kinase assays were done at higher ATP concentrations due to competition for binding to the same site. No significant inhibition was observed with the majority of additional protein kinases tested in vitro although OSI-930 inhibited the activity of PDGFRα, β, Flt1, and CSF-1R (which are closely related to Kit and KDR) and two more distantly-related enzymes, Lck and c-Raf (Table 1).
Kinase inhibition by OSI-930 in intact cells. Inhibition of Kit by OSI-930 was evaluated in cells expressing exclusively wild-type Kit (NCI-H526 small-cell lung cancer) as well as in the HMC-1 (mast cell leukemia) cell line that expresses a mutant constitutively active form of Kit resembling the type of mutation most commonly observed in gastrointestinal stromal tumors (V560G juxtamembrane mutant within exon 11). OSI-930 inhibited both wild-type and V560G mutant forms of Kit, as well as KDR and PDGFRα, in intact cell-based assays with IC_{50} values of <10 nmol/L (Fig. 2; Table 2). The potency of inhibition of PDGFRβ by OSI-930 in intact cells was somewhat surprising in view of the relatively weak inhibition of this target in biochemical assays. However, there are significant differences between the two assay formats that may account for this apparent discrepancy [e.g., use of a truncated protein construct in the biochemical assay versus the full-length protein in the cell-based assay and use of receptor autophosphorylation as the readout in the cell-based assay versus poly(Glu:Tyr) phosphorylation in the biochemical assay]. In contrast, in spite of the ability of OSI-930 to inhibit purified c-Raf in biochemical assays (Table 1), OSI-930 failed to inhibit significantly the phosphorylation of mitogen-activated protein kinase/extracellular signal-regulated kinase (Erk) kinase 1 (MEK1) in intact cells at concentrations up to 10 nmol/L (Table 2). Therefore, although the kinase profiling data suggested that OSI-930 may be a more potent inhibitor of c-Raf than PDGFRβ, it seems that the converse is true within intact cells in vitro and it is likely that OSI-930 is a considerably more potent inhibitor of PDGFRβ than c-Raf under physiologic conditions in vivo. These observations highlight the need to establish the selectivity profiles of kinase inhibitors within the physiologic context of intact cells to gain a more accurate understanding of the potential influence of potential target kinases on the in vivo effects of pharmacologic agents. Thus, it is clear from our studies (Supplementary Fig. S1; Table 1) that the kinase inhibition profiling data obtained in assays done with purified proteins in vitro is influenced by a number of assay variables, and it is not always clear which condition is likely to accurately reflect conditions within the cell. These conditions include the concentration of ATP, the precise kinase domain construct used in the assay, the choice of substrate (e.g., autophosphorylation versus exogenous substrate phosphorylation), and the activation state of the kinase (particularly as it relates to the phosphorylation state and conformation of the activation-loop within the kinase domain). Similar conclusions can be drawn from studies on inhibition of the Abl tyrosine kinase domain by imatinib, which was found to inhibit potently only the nonphosphorylated form of Abl, whereas the activated fully phosphorylated form of the enzyme was relatively insensitive to imatinib (49). Because imatinib potently inhibits Abl-dependent phosphorylation events within intact cells (50, 51), it is clear that only in vitro assays done with the nonphosphorylated form of the enzyme accurately reflected the ability of imatinib to influence signaling events downstream of Abl tyrosine kinase under physiologic conditions. In general, obtaining biochemical selectivity data for novel kinase inhibitors is a useful starting point for indicating potentially relevant physiologic targets, but it is clear that such data provide only limited information on the ability of novel compounds to interact with a selection of potential targets in vivo. Therefore, selectivity data obtained with purified enzymes should ideally be evaluated in light of supporting quantitative assays in cellular systems when attempting to assess the potential involvement of inhibition of different target enzymes in the pharmacologic activities of a novel pharmacologic agent.

To determine the potential effect of plasma protein binding on the interactions between OSI-930 and its target proteins within intact cells, the ability of OSI-930 to inhibit its targets was assessed in the presence of physiologic concentrations of the human plasma proteins albumin and chiral acid glycoprotein. This analysis provides an estimate of therapeutically relevant target plasma concentrations of OSI-930 for in vivo studies. Plasma protein inclusion into the assays increased the IC_{50} values by 6.1- to 11.9-fold (Fig. 2; Table 2), suggesting that significant inhibition of the target enzymes in vivo may result from exposure of the tumor tissue to 0.1 to 1 nmol/L OSI-930.

Inhibition of signaling events downstream of Kit by OSI-930 in intact cells. The downstream consequences of Kit inhibition were examined by immunoblot analysis of signaling proteins in cells expressing mutant Kit (HMC-1) or wild-type Kit (H526). Inhibition of Kit by OSI-930 in intact cells was associated with potent reduction in the level of phospho-Erk, phospho-Akt, phospho-p70S6K, and phospho-S6 (Fig. 2). These effects were generally observed with a similar concentration dependence for OSI-930, which also corresponded to the concentrations required to inhibit Kit phosphorylation. The data suggest that these signaling events are closely linked to the level of activation of Kit in both mutant Kit– and wild-type Kit–expressing cells. An exception was S6 phosphorylation in H526 cells where significantly higher concentrations of OSI-930 were required to achieve a significant reduction in spite of the potent reduction in phosphorylation of the upstream kinase p70S6K (Fig. 2). The

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### Table 1. Protein kinase inhibition profile of OSI-930

<table>
<thead>
<tr>
<th>Protein kinase</th>
<th>[ATP] (µmol/L) IC_{50} (nmol/L)</th>
<th>OSI-930 IC_{50} (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kit (activated)</td>
<td>25</td>
<td>80</td>
</tr>
<tr>
<td>Kit (nonactivated)</td>
<td>400</td>
<td>629</td>
</tr>
<tr>
<td>Kit (autophosphorylation)</td>
<td>200 (0.5 × K_{m})</td>
<td>&lt;15</td>
</tr>
<tr>
<td>KDR</td>
<td>20</td>
<td>9</td>
</tr>
<tr>
<td>PDGFRβ</td>
<td>200</td>
<td>6,900</td>
</tr>
<tr>
<td>PDGFRα</td>
<td>120</td>
<td>3,408</td>
</tr>
<tr>
<td>Flt1</td>
<td>200</td>
<td>8</td>
</tr>
<tr>
<td>CSF-1R</td>
<td>200</td>
<td>15</td>
</tr>
<tr>
<td>c-Raf</td>
<td>45</td>
<td>41</td>
</tr>
<tr>
<td>Flt3</td>
<td>200</td>
<td>1,303</td>
</tr>
<tr>
<td>Lck</td>
<td>90</td>
<td>22</td>
</tr>
<tr>
<td>Abl</td>
<td>10</td>
<td>4,738</td>
</tr>
<tr>
<td>EGFR</td>
<td>10</td>
<td>&gt;10 µmol/L</td>
</tr>
<tr>
<td>Aurora-A</td>
<td>15</td>
<td>&gt;10 µmol/L</td>
</tr>
<tr>
<td>Axl</td>
<td>90</td>
<td>&gt;10 µmol/L</td>
</tr>
<tr>
<td>Cyclin-dependent kinase 2/cyclin A</td>
<td>45</td>
<td>&gt;10 µmol/L</td>
</tr>
<tr>
<td>I-κB kinase</td>
<td>10</td>
<td>&gt;10 µmol/L</td>
</tr>
<tr>
<td>MEK1</td>
<td>10</td>
<td>&gt;10 µmol/L</td>
</tr>
<tr>
<td>Phosphoinositide-dependent kinase 1</td>
<td>10</td>
<td>&gt;10 µmol/L</td>
</tr>
<tr>
<td>Protein kinase Bγ</td>
<td>200</td>
<td>&gt;10 µmol/L</td>
</tr>
<tr>
<td>Tie-2</td>
<td>200</td>
<td>&gt;10 µmol/L</td>
</tr>
</tbody>
</table>

NOTE: Inhibition of protein kinases by OSI-930 was assessed using recombinant purified enzymes in the presence of ATP at the concentrations indicated; unless otherwise stated, these concentrations approximate the K_{m} value for each enzyme. IC_{50} values were determined from the sigmoidal dose-response plot of percent inhibition versus log_{10} compound concentration (Xlfit 3.0, IDBS).
explanation for this difference between H526 and HMC-1 cells is unclear but the requirement for higher concentrations of OSI-930 to reduce S6 phosphorylation was also observed in a second wild-type Kit–expressing cell line (WBA; data not shown). One possible explanation for these observed differences in kinetics of dephosphorylation could be that the turnover rate of S6 phosphorylation is relatively slow in the small-cell lung cancer cell lines compared with HMC-1 cells, perhaps reflecting lower levels of S6 protein phosphatases under the culture conditions used in these experiments. Alternatively, the degree of S6 phosphorylation may be regulated by different S6 protein kinases in HMC-1 and small-cell lung cancer lines because various members of both p90rsk and p70S6K enzyme families have been implicated in S6 phosphorylation in different cultured cell systems (52–54).

Phenotypic effects of OSI-930 in intact cells. OSI-930 inhibited proliferation and induced apoptosis in the HMC-1 cell line when

### Table 2. Inhibition of protein kinases in intact cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Ligand</th>
<th>Target</th>
<th>IC_{50} (nmol/L), no plasma proteins</th>
<th>IC_{50} (nmol/L), with plasma proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>H526</td>
<td>Stem cell factor</td>
<td>Kit (wild-type)</td>
<td>9.6</td>
<td>78.9</td>
</tr>
<tr>
<td>HMC-1</td>
<td>None</td>
<td>Kit (V560G)</td>
<td>9.5</td>
<td>58.1</td>
</tr>
<tr>
<td>HUVEC</td>
<td>VEGF</td>
<td>KDR</td>
<td>10.1</td>
<td>64.4</td>
</tr>
<tr>
<td>NIH-3T3</td>
<td>PDGF-BB</td>
<td>PDGFRβ</td>
<td>51.5</td>
<td>614.0</td>
</tr>
<tr>
<td>SK-MEL-2</td>
<td>None</td>
<td>Raf</td>
<td>&gt;10,000</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

NOTE: Inhibition of potential targets of OSI-930 was assessed in intact cells either in the absence or presence of physiologic concentrations of human plasma proteins albumin (3%) and α1-acid glycoprotein (0.05%). Cells were either serum starved for 3 hours in the presence of compound before ligand stimulation (H526, HUVEC, and NIH-3T3) or were treated with compound for 3 hours under standard growth conditions [i.e., in the presence of 10% FCS (HMC-1 and SK-MEL-2)]. The extent of inhibition of the target enzymes was then determined by monitoring effects of OSI-930 on ligand-stimulated receptor autophosphorylation (wild-type Kit, KDR, and PDGFRβ), constitutive mutant receptor autophosphorylation (V560G mutant Kit), or downstream substrate (MEK1) phosphorylation (Raf). IC_{50} values were determined from the sigmoidal dose-response plot of percent inhibition versus log\_10 compound concentration (Xlfit 3.0, IDBS). n.d., not determined.
cultured in vitro in the presence of 10% FCS (Supplementary Table S1). The concentration of OSI-930 that induced these phenotypic effects was comparable to that required to inhibit Kit phosphorylation in the HMC-1 cell line under the same culture conditions (Fig. 2; Table 2); therefore, HMC-1 cells seem to be highly dependent on Kit signaling for continued growth and survival in culture. In contrast, under normal culture conditions, growth of the COLO-205 cell line that does not express a constitutively active mutant receptor tyrosine kinase was insensitive to OSI-930 in culture at concentrations up to 20 μmol/L (Supplementary Table S1).

To assess the potential for KDR inhibition by OSI-930 to provide an antiangiogenic component in the antitumor activity of OSI-930, the effect of OSI-930 on endothelial cell function in an in vitro culture system was investigated. OSI-930 inhibited sprout formation from rat aortic rings cultured for 10 days in a collagen matrix, with a >50% reduction in sprout formation being observed at 100 nmol/L (Fig. 3; Supplementary Table S1). The data indicate that endothelial cell function is inhibited in vitro by 100 nmol/L OSI-930 and this activity of OSI-930 may contribute to the antitumor activity of OSI-930 in tumor xenograft efficacy studies.

**Pharmacokinetic/pharmacodynamic analysis of OSI-930 in the mutant Kit–expressing xenograft model HMC-1.** Pharmacokinetic analysis of OSI-930 in mice revealed that plasma exposure levels of OSI-930 (Cmax and area under the curve) increased approximately linearly with dose, up to a dose level of 300 mg/kg (Supplementary Fig. S2; Supplementary Table S2). Furthermore, bioavailability calculations using the median area under the curve following i.v. administration at 1 mg/kg indicate that the oral bioavailability of OSI-930 is ~100% in the mouse within the 5 to 300 mg/kg dose range. These in vivo properties have enabled extensive characterization of the in vivo efficacy of OSI-930 in mice using oral dosing within the 5 to 300 mg/kg dose range.

The ability of OSI-930 to inhibit its targets in vivo following oral dosing was initially evaluated by monitoring the level of tyrosine phosphorylation of Kit in lysates derived from HMC-1 tumor xenografts. Expression of the constitutively activated V560G mutant form of Kit in this cell line ensures that there is a constitutively high level of Kit receptor autophosphorylation within the tumor tissue. Inhibition of Kit activity in vivo can therefore be monitored readily by Kit immunoprecipitation followed by antiphosphotyrosine immunoblotting analysis of tumor lysates. Tumors and plasma were collected at various time points during a 24-hour period following oral dosing of HMC-1 tumor–bearing animals with OSI-930, and both the extent of phosphorylation of Kit and the associated plasma drug concentrations were determined. Analysis of these data revealed that the degree of inhibition of Kit phosphorylation correlated well with the plasma levels of the compound; i.e., phosphorylation was inhibited potently when plasma levels of OSI-930 were above the in vitro IC50 value for inhibition of Kit phosphorylation in the HMC-1 cell line when measured in the presence of plasma proteins (58 nmol/L; Fig. 4). Furthermore, OSI-930 suppressed Kit phosphorylation by >90% over a full 24-hour period following a single oral dose of 50 mg/kg. This pharmacodynamic effect translated into potent antitumor efficacy when OSI-930 was dosed for 17 days at 50 mg/kg in the HMC-1 model whereas lower doses of OSI-930 that resulted in incomplete inhibition of Kit during the 24-hour dosing period were less effective in inhibiting tumor growth (Fig. 4). The degree of inhibition of tumor growth therefore correlated well with the level of inhibition of Kit phosphorylation observed in the pharmacodynamic studies, suggesting that in the HMC-1 xenograft model tumor growth is highly dependent on Kit signaling. These data are also consistent with in vitro data obtained using the HMC-1 cell line in which OSI-930 potently inhibited cell proliferation and induced apoptosis at concentrations similar to those required to inhibit Kit phosphorylation under the same conditions (Table 2 and Supplementary Table S1).

**Pharmacodynamic analysis of OSI-930 in Kit-expressing small-cell lung cancer xenograft models.** The ability of OSI-930 to inhibit the wild-type Kit enzyme in vivo was investigated by oral dosing of animals bearing tumor xenografts from the Kit-expressing small-cell lung carcinoma line NCI-H526. The data showed that >80% inhibition of Kit phosphorylation can be maintained for up to 24 hours following a single dose of OSI-930 (Fig. 5A); however, in NCI-H526 tumors this degree of inhibition required administration of higher doses of OSI-930 (200 mg/kg) than in HMC-1 tumors (50 mg/kg). As described above for the HMC-1 model, there was again a good correlation between the dose levels required to achieve maximal inhibition of Kit phosphorylation at the 24-hour time point and the doses that resulted in maximal tumor growth inhibition in the NCI-H526 model (i.e., 200 mg/kg when administered on a daily oral dosing schedule; Fig. 5B). Taken together, these data suggest that the maximal antitumor effects of OSI-930 are associated with doses that result in extensive inhibition of the molecular targets of OSI-930 throughout the majority of the dosing period.

![Figure 3](image-url)
A second small-cell lung cancer model (WBA) was found to be highly sensitive to OSI-930 treatment in vivo in that 200 mg/kg OSI-930 was sufficient to induce tumor stasis that extended beyond the dosing period (Fig. 5C). In this model, immunohistochemical analysis of the tumor vasculature following dosing with OSI-930 indicated that these tumors contained a considerably reduced number of blood vessels compared with control animals, consistent with KDR inhibition contributing to the antitumor effects of OSI-930 (Fig. 5D). In contrast, the less sensitive NCI-H526 model failed to show such dramatic changes in the tumor vasculature, which may indicate that KDR-dependent angiogenesis plays a less significant role in tumor growth in this model.

To establish more directly the potential role of KDR inhibition by OSI-930 in the antitumor effects observed in vivo, the ability of OSI-930 to inhibit a physiologic KDR-dependent process was evaluated by monitoring the rapid swelling of the mouse uterus due to water uptake that occurs in response to estradiol (55, 56). The results indicate that oral dosing of OSI-930 inhibits uterine edema at efficacious dose levels, supporting the potential involvement of KDR inhibition in the antitumor effects of OSI-930 (Fig. 5E).

Antitumor activity of OSI-930 in a broad range of preclinical xenograft models. OSI-930 has been tested for antitumor activity in multiple tumor xenograft models and significant activity was observed in the majority of cases (Table 3). In most models, OSI-930 was administered daily at the maximally efficacious dose of 200 mg/kg by oral gavage for dosing periods ranging from 10 to 38 days. In the majority of tumor models tested, there was no significant body weight loss at 200 mg/kg, suggesting that OSI-930 is well tolerated with this dose and schedule, although body weight reductions (12-20%) were observed in some studies in the melanoma models SK-MEL-1 and SK-MEL-5. In these models, there were also body weight effects in vehicle control–treated animals (weight reduction or slower weight gain), suggesting that these effects are partially xenograft model dependent.

Tumor regressions were observed in seven of the xenograft models tested, which were derived from six different tumor types [i.e., mast cell leukemia (HMC-1), small-cell lung carcinoma (WBA), head and neck carcinoma (KB), gastric carcinoma (NCI-SNU-5), colon carcinoma (SW48 and COLO-205; Supplementary Fig. S3), and glioblastoma (U251); Supplementary Fig. S3]. The ability of OSI-930 to induce tumor regressions in preclinical models from several different tumor types indicates that OSI-930 may have broad clinical utility in the treatment of a range of human cancers. Furthermore, in four of these models (HMC-1, WBA, NCI-SNU-5, and COLO-205), durable cures were observed in some animals, highlighting the potential for OSI-930 to elicit potent antitumor effects in preclinical models.

Figure 4. Pharmacokinetic/pharmacodynamic relationship of OSI-930 in the HMC-1 tumor xenograft model and correlation with antitumor activity. A, pharmacokinetic analysis of OSI-930 for 24 hours following a single oral dose of 5, 10, 25, or 50 mg/kg OSI-930. Compound was dosed using PEG-400/water (1:1, v/v) as the vehicle. Plasma exposures are expressed relative to the IC50 for inhibition of Kit phosphorylation in the presence of plasma proteins. B, effect of oral dosing of OSI-930 on Kit phosphorylating content of HMC-1 tumor xenografts. C, quantitation of the extent of inhibition of Kit phosphorylation in HMC-1 tumor xenografts following oral dosing of OSI-930. D, inhibition of HMC-1 tumor xenograft growth with oral doses of 10, 25, and 50 mg/kg OSI-930; compound was administered daily on days 1 to 17.
In addition to the models described above where OSI-930 induced tumor regressions or durable cures, significant cytostatic antitumor effects were evident in several models. In these models, there were meaningful delays in the tumor growth period (i.e., delays at least equivalent to the number of days in the dosing period) and tumor growth inhibition was >42%. In total, six models responded to OSI-930 in a predominantly cytostatic manner, including the colon carcinoma models HT29, HCT-116, LS180, and DLD-1, the renal cell carcinoma model SN12C, and the small-cell lung carcinoma model NCI-H209 (Table 3). Certain additional xenograft models seemed to be insensitive to OSI-930 at the 200 mg/kg dose level (e.g., Caki-1 renal cell carcinoma; Table 3). The reasons for these differential antitumor effects of OSI-930 are not fully understood but are likely to be related to differences in the level of contribution of the molecular targets of OSI-930 to the growth of each cell line as a tumor xenograft in vivo. In the majority of cell lines tested in dose-response tumor growth inhibition studies, the effective dose level was 100 to 200 mg/kg/d; the plasma exposure levels of OSI-930 observed in efficacy studies at these dose levels (Supplementary Fig. S2; Supplementary Table S2) therefore provide an estimate of the target exposures for clinical evaluation of OSI-930 as a novel anticancer therapeutic.

The results outlined above suggest that OSI-930 may have significant antitumor activity in several tumor types and clinical evaluation of OSI-930 is now under way. Several additional novel therapeutic agents with target kinase inhibition profiles that overlap to some extent with that of OSI-930 are currently being evaluated clinically, the most advanced of which are imatinib, PTK-787, SU-11248, and BAY 43-9006. It is likely that differences in the selectivity profiles and pharmacokinetic/pharmacodynamic properties will result in each compound displaying a different spectrum of antitumor activity when tested against a range of tumor types in the clinic. For example, the ability of OSI-930 to inhibit both wild-type and mutant Kit with similar potency in intact cell systems provides the potential for OSI-930 to inhibit wild-type Kit–dependent tumor growth to a greater extent than imatinib, which was reported to inhibit mutant Kit with significantly greater potency than wild-type Kit (57, 58). Indeed, this difference in potency of imatinib between wild-type and mutant Kit enzymes correlates with the clinical observation that gastrointestinal stromal tumor patients expressing wild-type Kit are less responsive to imatinib treatment than gastrointestinal stromal tumor patients expressing mutant Kit (1). A recent study on the selectivity of kinase domain binding of a number of clinically tested kinase inhibitors suggested that there are many selectivity differences among PTK-787, SU-11248, BAY 43-9006, and imatinib (59). Imatinib and PTK-787 were found to be relatively selective for binding to only a few kinases whereas BAY 43-9006 and SU-11248 bound to many different kinases from several kinase subfamilies. Although the relevance of the various potential kinase targets identified within these in vitro selectivity profiles has not been established, either within a cellular context or in vivo, it is clear that these agents, and presumably also OSI-930, are likely to have selectivity profiles that can be

![Figure 5. Pharmacodynamic effects of OSI-930 and correlation with antitumor activity. A, the extent of inhibition of Kit phosphorylation in NCI-H526 tumor xenografts was determined at 24 hours following oral dosing of OSI-930 by immunoprecipitation and immunoblotting of the Kit protein from xenograft lysate samples. B, dose-dependent inhibition of NCI-H526 tumor xenograft growth by oral dosing of OSI-930; compound was administered daily on days 1 to 15. C, inhibition of WBA tumor xenograft growth by oral dosing of OSI-930 at 200 mg/kg/d for 38 days. D, CD31 staining of excised NCI-H526 and WBA tumors following dosing with vehicle (left) or 200 mg/kg OSI-930 (right) for 3 consecutive days. E, estradiol-induced uterine swelling was monitored following oral dosing of OSI-930 or vehicle by measuring wet and dry uterus weights; percentage of uterus weight as water.](https://www.aacrjournals.org)
Inhibition studies. OSI-930 elicited potent antitumor effects in 13
achieved and with the efficacy of OSI-930 in tumor growth
models
ability of OSI-930 to inhibit its target proteins in preclinical
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1.
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safety of trastuzumab as a single agent in first-line
treatment of HER2-overexpressing metastatic breast


Table 3. Antitumor activity of OSI-930 in a broad range of tumor types

<table>
<thead>
<tr>
<th>Tumor histotype</th>
<th>Cell line</th>
<th>Dosing duration (d)</th>
<th>Growth delay (d)*</th>
<th>%TGI†</th>
<th>%Regression ‡</th>
<th>Cures §</th>
<th>Kit expression¶</th>
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<tr>
<td>MCL</td>
<td>HMC-1†</td>
<td>10</td>
<td>NC</td>
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<td>&gt;51</td>
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<td>Head and neck</td>
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<td>Gastric</td>
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<td>35</td>
<td>95.2</td>
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<td>85.9</td>
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<td>Colon</td>
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<tr>
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<td>SN12C††</td>
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<td>15</td>
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<td>61.0</td>
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</table>

NOTE: OSI-930 was administered to tumor-bearing animals at 200 mg/kg/d in Labrafail, except for the HMC-1 study in which OSI-930 was dosed at 50 mg/kg/d in PEG-400/water (1:1, v/v). Antitumor efficacy was determined from comparison of median tumor sizes in growth-staged vehicle-treated and OSI-930-treated groups (n = 6–8 animals per group).

*Tumor growth delay refers to the additional time taken by the OSI-930-treated group to reach 500% of initial tumor size compared with the vehicle-treated control group (NC, not calculated).

†%TGI (% Tumor Growth Inhibition) = 100(1–Wt/W0), where Wt is the median tumor volume of the treated group at time t and W0 is the median tumor volume of the control group at time 0. TGI of ≥42% is significant.

‡%Regression refers to the maximal mean decrease in tumor size relative to the mean initial size, excluding any tumors designated as cures.

§Cures refers to the number of animals with nonmeasurable tumors determined at least 60 days following the final dose.

¶Relative levels of Kit expression determined by immunoblotting of tumor lysates (ND, not determined).

−Mutant Kit–expressing tumor—highly sensitive to OSI-930 at low dose levels.
−Very good response—high TGI and growth delays with significant regression and/or durable cures.
−Good response—TGI > 42% and growth delay equal to the number of days animals were dosed.
−Moderate response—TGI > 42% but growth delay less than the number of days animals were dosed.
−Not responsive—TGI < 42% and growth delay less than the number of days animals were dosed.

In summary, OSI-930 is a potent inhibitor of the Kit, KDR, and PDGFβ receptor tyrosine kinases in intact cells in vitro. The ability of OSI-930 to inhibit its target proteins in preclinical models in vivo can be correlated with the plasma drug levels achieved and with the efficacy of OSI-930 in tumor growth inhibition studies. OSI-930 elicited potent antitumor effects in 13 of 23 tumor xenograft models tested, which were derived from 7 different tumor histotypes. These observations suggest that OSI-930 may have clinical antitumor activity in a broad range of human tumor types.

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distinguished from each other. Furthermore, these differences in selectivity are likely to play a role in the toxicity profile as well as the antitumor activity profile displayed by these agents in the clinic.

In summary, OSI-930 is a potent inhibitor of the Kit, KDR, and PDGFβ receptor tyrosine kinases in intact cells in vitro. The ability of OSI-930 to inhibit its target proteins in preclinical models in vivo can be correlated with the plasma drug levels achieved and with the efficacy of OSI-930 in tumor growth inhibition studies. OSI-930 elicited potent antitumor effects in 13 of 23 tumor xenograft models tested, which were derived from 7 different tumor histotypes. These observations suggest that OSI-930 may have clinical antitumor activity in a broad range of human tumor types.

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OSI-930: A Novel Selective Inhibitor of Kit and Kinase Insert Domain Receptor Tyrosine Kinases with Antitumor Activity in Mouse Xenograft Models

Andrew J. Garton, Andrew P.A. Crew, Maryland Franklin, et al.


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