Indolinone Tyrosine Kinase Inhibitors Block Kit Activation and Growth of Small Cell Lung Cancer Cells

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ABSTRACT

Six indolinone tyrosine kinase inhibitors were characterized for their ability to inhibit Kit kinase and for their effects on the growth of small cell lung cancer (SCLC) cell lines. All of the six compounds were potent inhibitors of Kit kinase in a biochemical assay. A homology model of compound binding to the ATP binding site could account for the increased potency observed with the addition of a propionate moiety to the indolinone core but not the increase observed with addition of a chloride moiety. Although all of the compounds tested were potent in the biochemical assay, several exhibited significantly less potency in cellular kinase assays. Their effects on stem cell factor (SCF)-dependent Kit autophosphorylation and SCLC cell growth were also examined. Inhibition of SCF-stimulated Kit activation and cell growth in the HS26 cell line was dose-dependent. At concentrations that inhibited SCF-stimulated HS26 cell growth, there was little effect on insulin-like growth factor-1-stimulated growth, suggesting that these compounds exhibit reasonable selectivity for inhibition of Kit-mediated proliferation. Higher doses of the compounds were needed to inhibit serum-stimulated growth. Of the six compounds examined, SU5416 and SU6597 demonstrated the best cellular potency and, therefore, their effect on the growth of multiple SCLC cell lines in serum-containing media was examined. In addition to inhibiting proliferation, these compounds also induced significant cell death of several SCLC cell lines, but not of normal human diploid fibroblasts, in complete media. These observations suggest that Kit kinase inhibitors such as these may offer a new approach for inhibiting Kit-mediated proliferation of tumors such as SCLC, gastrointestinal stromal tumors, seminomas, and leukemias.

INTRODUCTION

The Kit receptor is a transmembrane protein of Mr 145,000 possessing an extracellular ligand-binding domain with five immunoglobulin-like motifs, a single transmembrane region, and a cytoplasmic domain that exhibits protein tyrosine kinase activity (1). Binding of SCF to the Kit receptor results in autophosphorylation on tyrosines located in the COOH-terminal tail region that results in activation of its kinase activity (2). These initial events are followed by SH2-mediated binding and tyrosine phosphorylation of various protein substrates, resulting in the activation of several signaling cascades that control cellular activities such as proliferation, apoptosis, and motility.

Kit is normally expressed in hemopoietic cells, melanocytes, other neural crest derivatives, and germ cells, as well as a variety of solid tumors (1, 3). Coexpression of both the Kit receptor and its ligand SCF has been reported in myeloid leukemia (4), neuroblastoma (5), breast tumors (6), colon tumors (7), gynecological tumors (8), testicular germ cell tumors (9), and at least 70% of SCLC cell lines and tumor specimens (10–13). These observations suggest the possibility of an autocrine growth loop involving the SCF/Kit system in these malignancies. In addition, mutations that cause constitutive activation of Kit have been reported in gastrointestinal stromal tumors, seminomas, and systemic mastocytosis associated with other hematological disorders such as acute myelogenous leukemia (14–16). The fact that Kit activation is likely to contribute to the development of the aforementioned tumors has led to efforts to block Kit-mediated mitogenic signaling.

One possible approach to interfering with Kit-mediated signaling would be to block the binding of SCF to Kit. Cohen et al. (5) showed that the functional blockade of Kit with a MoAb induced a reduction in growth of neuroblastoma cell lines. Similar results were obtained using breast tumor cell lines coexpressing SCF and Kit (6). Ricotti et al. (17) also reported that treatment of sarcoma cell lines with neutralizing Kit MoAb resulted in a significant increase in apoptosis and also demonstrated that treatment with c-kit antisense oligonucleotides resulted in growth inhibition. Introduction of mutations within the kinase domain that eliminate enzymatic activity, resulting in a dominant-negative receptor, is another way of abolishing signal transduction by the Kit receptor. Interference with a preexisting autocrine loop by transfection of the H209 SCLC cell line, which coexpresses high levels of Kit and SCF, with a kinase-defective c-kit gene construct resulted in a loss of growth factor independence (18).

Another attractive approach is to develop small molecule inhibitors that block the tyrosine kinase activity of the receptor. Screening studies have identified several classes of compounds as tyrosine kinase inhibitors (19–21). In addition to being potential antitumor agents, these compounds have proven to be useful research tools for understanding the physiological functions of specific tyrosine kinases. Certain tyrosine kinase inhibitors that block SCF-dependent proliferation have been reported. AG1295 and AG1296, quinoxalines that compete for ATP binding, have been shown to inhibit SCF-mediated Kit activation as well as SCF-mediated growth of SCLC cells (22). ST1571, a 2-phenylaminopyrimidine derivative originally designed as an ATP competitive inhibitor of Bcr-Abl (23), is also an effective Kit inhibitor and blocked SCF-mediated growth of SCLC (24). Substituted indolinones have been identified as a versatile scaffold for the development of protein kinase inhibitors (25). By altering the substituents on the pyrrole and oxindole rings, it is possible to tune the potency and selectivity of the compounds for inhibition of various kinases. These compounds have proven to be especially useful for inhibition of kinases of the PDGFR superfamily (25, 26) that includes the VEGF receptors and Kit. Activity against both the wild type and mutationaly activated Kit has been demonstrated for some members of this biochemical family (27). In this manuscript, we have examined the ability of six related indolinone-based compounds to inhibit Kit kinase and to block Kit-mediated responses and growth of SCLC cells. We have also attempted to correlate activity in both biochemical and cellular assays with unique structural features of these related compounds.

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The abbreviations used are: SCF, stem cell factor; SCLC, small cell lung cancer; MoAb, monoclonal antibody; IGFl, insulin-like growth factor-1; PDGFr, platelet-derived growth factor receptor; EGFr, epidermal growth factor receptor; FGFr, fibroblast growth factor receptor; IP, immunoprecipitation; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; Gyrb, B subunit of bacterial DNA gyrase.
MATERIALS AND METHODS

Compounds and Reagents. Kinase inhibitors SU6597, SU6668, SU6661, SU5416, SU6663, and SU5614 were synthesized at SUGEN, Inc. (South San Francisco, CA), as described previously (25, 26). Investigators who performed the studies in the SCLC system (G. W. K., S. H.) were blinded to the identity of the compounds until all of the studies were complete. All of the compound stock solutions were solubilized in DMSO, and the final concentration of test compounds in kinase buffer (25 mM HEPES, 100 mM NaCl, 5 mM MgCl₂, 2 mM l-glutamine, and 50 units/ml penicillin-50 units/ml streptomycin) was 0.1% (volume for volume). A plasmid containing the r24,000 subdomain of the GyrB was the generous gift of Drs. Michael A. Farrar and Roger M. Perlmutter (Howard Hughes Medical Institute, University of Washington, Seattle, WA). GyrB was fused with the cytoplasmic domain of human Kit and inserted into the mammalian expression vector pcDNA3-Neo (Promega, Inc., Madison, WI). Coumeymycin and novobiocin were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Culture and MTT Cellular Growth Assays. The SCLC cell lines used that express c-kit were H526, H510, H432, H209, and WBA, whereas H416 cells do not express the receptor (12). SCLC cells were grown in RPMI 1640 (Bio-Whitaker, Walkersville, MD) medium that was supplemented with 10% (volume for volume) heat-inactivated fetal bovine serum (Life Technologies, Inc.). All of the cells were maintained routinely in a humidified chamber at 37°C and 5% carbon dioxide in air. When grown in the absence of serum, 0.1% BSA (Sigma Chemical Co.) was added to the medium. Where indicated, serum-free medium was supplemented with saturating concentrations of recombinant SCF (100 ng/ml) or IGF-1 (20 ng/ml). Cells were treated with SCF and IGF-1 after preincubation in serum-free medium overnight. Cellular growth was assessed using the MTT (Sigma Chemical Co.) colorimetric dye reduction method. This assay is dependent on the ability of mitochondrial dehydrogenases within viable cells to reduce the MTT dye to a blue formazan product. The amount of dye reduced is directly correlated to the number of viable SCLC cells, as determined by other assays (28). Duplicate plates containing eight replicate wells/each assay condition were seeded at a density of 1 × 10⁴ cells in 0.1 ml of medium, and data were expressed as the change in absorbance at 540 nM over 72 h, relative to initial values obtained 3 h after plating.

GyrB/Kit Kinase Assays. Biochemical Kinetic autophosphorylation kinase assays were performed with immunoprecipitated GyrB/Kit. A confluent dish of CHO/GyrB-Kit cells were lysed in 2.5 ml of ice-cold radioimmunoprecipitation assay buffer containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1.4 µM E64, 10 µM bestatin, 1 µM leupeptin, 0.3 µM aprotonin, and 1 µM pepstatin A; Sigma Chemical Co.) and 20 mM EDTA and then diluted with an equal volume of 20 mM HEPS, 150 mM NaCl, 0.5% Triton X-100, and 10% glycerol. After centrifugation, the cleared lysate was incubated with protein A beads (175 µl; Pierce Chemical, Rockford, IL) and anti-Kit rabbit polyclonal antibodies (150 µl; Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at 4°C. Washed beads were incubated with the indicated concentration of test compounds in kinase buffer (25 mM HEPS, 100 mM NaCl, 5 mM MgCl₂, 100 µM EGTA, 5 µM leupeptin, 0.3 µM antipain, and 1 µM pepstatin A). Supernatants were removed and quantitated for activity using a p-Kit specific autophosphorylation assay (175). Briefly, lysates were diluted in kinase buffer (25 mM HEPS, 100 mM NaCl, 5 mM MgCl₂, 100 µM EGTA, 5 µM leupeptin, 0.3 µM antipain, and 1 µM pepstatin A) and incubated with the indicated concentration of test compounds. Blanks were incubated with a combination of 1% BSA and 0.1% DMSO. Where indicated, the kinase buffer was supplemented with increasing concentrations of SU5416, SU6663, SU6561, SU5614, SU5416, SU6668, SU6597, and SU5614. The autophosphorylation assay was performed using 125 mM [γ-32P]ATP (5000 Ci/mmol, 3000 Ci/mmol; DuPont NEN Life Sciences, Boston, MA) and 100 µM ATP. Lysates were incubated at room temperature for 30 min, and reactions were terminated by the addition of 3% trichloroacetic acid. Kinase assay products were separated by electrophoresis and visualized by autoradiography. Data were expressed relative to the autophosphorylation activity of the control (100% activity). Table 1 shows a list of the kinetics of compounds and their IC₅₀ values.

Table 1 Compound structure and biochemical kinase inhibitory activities

<table>
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<tr>
<th>Compound</th>
<th>Structure</th>
<th>Kit</th>
<th>VEGFr2</th>
<th>FGFr1</th>
<th>PDGFβ</th>
<th>EGFr</th>
<th>IGF1r</th>
<th>Zap70</th>
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<td>SU5416</td>
<td>![Image](86x342 to 172x397)</td>
<td>(32.3 ± 5.0) × 10⁻⁶</td>
<td>1.3 ± 0.8</td>
<td>4.2 ± 0.8</td>
<td>37.9 ± 8.9</td>
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<td>&gt;100</td>
<td>&gt;100</td>
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<tr>
<td>SU5614</td>
<td>![Image](86x402 to 169x463)</td>
<td>(28.3 ± 3.8) × 10⁻⁶</td>
<td>0.5 ± 0.2</td>
<td>3.1 ± 2.6</td>
<td>0.13 ± 0.13</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>SU6668</td>
<td>![Image](86x533 to 172x593)</td>
<td>(10.5 ± 1.3) × 10⁻⁶</td>
<td>3.9 ± 2.2</td>
<td>3.8 ± 1.2</td>
<td>0.10 ± 0.07</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<tr>
<td>SU6597</td>
<td>![Image](86x594 to 168x649)</td>
<td>(8.7 ± 0.9) × 10⁻⁶</td>
<td>3.3 ± 2.2</td>
<td>5.1 ± 3.3</td>
<td>(2.0 ± 0.6) × 10⁻³</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<tr>
<td>SU6663</td>
<td>![Image](86x654 to 148x701)</td>
<td>(14 ± 1.5) × 10⁻⁶</td>
<td>2.1 ± 0.4</td>
<td>3.7 ± 1.0</td>
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<td>&gt;100</td>
<td>&gt;100</td>
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<tr>
<td>SU5614</td>
<td>![Image](86x723 to 172x778)</td>
<td>(3.8 ± 0.2) × 10⁻⁶</td>
<td>2.9 ± 0.8</td>
<td>4.9 ± 0.2</td>
<td>1.0 ± 0.4 × 10⁻²</td>
<td>&gt;100</td>
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</table>

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>IC₅₀ (µM)</th>
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<tbody>
<tr>
<td>SU5416</td>
<td>![Image](86x342 to 172x397)</td>
<td>(n = 3)</td>
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<tr>
<td>SU5614</td>
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<tr>
<td>SU5614</td>
<td>![Image](86x723 to 172x778)</td>
<td>(n = 3)</td>
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and 5 mM MgCl₂ containing 5 μM EDTA and 1 mM orthovanadate. After centrifugation, Kit was immunoprecipitated from the supernatant, the amount of autophosphorylation was detected with antiphosphotyrosine antibodies (biotin-PY99; Santa Cruz Biotechnology) and enhanced chemiluminescence (Amersham, Arlington Heights, IL) reagents. The amount or autophosphorylation was measured with antibodies to phosphotyrosine. Lysates of compound and appropriate kinase buffer were added to the wells, and the kinase reaction was performed at twice the IC₅₀. The lysate, containing 1.0–1.5 mg of protein, was transferred to a clean microfuge tube, followed by IP with 10 μg of anti-Kit MoAb (K45; NeoMarkers, Fremont, CA) for 2 h at 4°C. For control experiments, 10 μg of nonimmune mouse IgG was used for IP. Immune complexes were harvested by the addition of protein A + G agarose beads for an additional 1-h incubation followed by centrifugation. Beads were then washed four times with lysis buffer and then once in cold PBS. The pellet was aspirated to dryness and boiled for 5 min in an equal volume of 2 × SDS sample loading buffer and centrifuged to obtain the supernatant, which was loaded onto a 10% SDS polyacrylamide gel. Western blotting was carried out using standard procedures. Staining was accomplished using an anti-phosphotyrosine MoAb (PY-20; Transduction Laboratories, Lexington, KY) and the anti-Kit 3D6 antibody (Boehringer-Mannheim, Indianapolis, IN). Visualization was accomplished using the enhanced chemiluminescence system.

Molecular Modeling. A homology model for the catalytic domain of Kit was generated using the Modeller program (Molecular Simulations, Inc., San Diego, CA). The “open form” of FGFR1 cocrystallized with SU6688 was used as reference, and the sequence alignment was based on that of Hanks and Quinn (31) with slight modifications. Because the sequence homology between FGFR1 and Kit is high (44%), the Kit model and FGFR1 crystal structures have very similar overall folding. The final Kit model was obtained after simple energy minimization. SU6597 was docked into the Kit model based on the crystal structure of SU6688 in FGFR1 (29).

RESULTS

Inhibition of Kinases in the PDGFr Superfamily. SU5416 (30) and SU6668 (29) are angiogenesis inhibitors that are currently under clinical evaluation for the treatment of human cancers. The other indolinone-based compounds evaluated in this study are structurally related to these two angiogenesis inhibitors (Table 1). The relative potencies of these compounds for inhibition of receptor tyrosine kinases in the PDGFr superfamily were compared in screening biochemical assays and are shown in Table 1. Although they demonstrate varying potencies toward the different family members as well as unrelated kinases, they tend to be selective inhibitors of Kit kinase (Table 1). None of the compounds inhibited EGFr kinase, and most demonstrated poor potency for inhibition of IGF-1r and Zap70 kinases. Of these compounds, only SU5416 and SU6668 were tested for inhibition of Abl kinase. SU5416 did not inhibit Abl kinase up to a concentration of 20 μM (the highest concentration tested), whereas SU6668 demonstrated an IC₅₀ value of 12.2 ± 1.3 μM. These observations confirm and extend previously reported activities for 3-substituted indolinones as kinase inhibitors (25, 26).
Substituted Indolinones Inhibit Kit Kinase with Varying Potencies. Farrar et al. (32) demonstrated that a fusion of the Raf kinase with a portion of GyrB produced a kinase, the activity of which was inducible by addition of the dimeric antibiotic, coumermycin. In contrast, the monomeric form of the antibiotic novobiocin did not activate the kinase. For this study, we have generated a DNA construct of a GyrB chimera containing the cytoplasmic domain of human Kit. Stable transfection of this DNA into CHO-K1 cells resulted in expression. Exposure of these CHO/GyrB-Kit cells to coumermycin induced kinase activity leading to maximal autophosphorylation after about 30 min (Fig. 1A). As expected, novobiocin was unable to activate the Kit kinase fusion protein.

Exposure of CHO/GyrB-Kit cells to various concentrations of kinase inhibitors during stimulation was tested to measure the ability of these inhibitors to affect Kit activity in cells. In parallel, the GyrB-Kit protein was immunoprecipitated and used in biochemical kinase assays to assess the biochemical potencies of these compounds. Fig. 1B illustrates a comparison of the biochemical and cellular potencies of these compounds for inhibition of Kit kinase autophosphorylation. Fig. 1C demonstrates that comparable amounts of protein were present in each lane and provides an internal control. SU5416 demonstrated nM biochemical potency for inhibition of Kit kinase. It was also effective at inhibiting Kit kinase in cells, where its apparent IC$_{50}$ value was less than 10-fold greater than that observed in the biochemical assay. The 5-Cl analogue of SU5416, SU5614, demonstrated slightly greater potency in both the biochemical and cellular assays than those observed for SU5416. SU6668 exhibited better potency in the biochemical Kit kinase assay than SU5416, with its 5-Cl analogue, SU6597, being even more potent. However, SU6668 and SU6597 displayed a significant discrepancy between their biochemical and cellular potencies, with their biochemical potencies more than 100-fold better than their cellular potencies. SU6663 demonstrated slightly better potency for inhibition of Kit kinase in the biochemical assay than was observed for SU5416, but it did not appear to be as potent as SU6668. Although its biochemical potency was slightly better than that for SU5416, its cellular potency was much weaker. There was also a large discrepancy between its biochemical and cellular potencies, as observed with SU6668 and SU6597. The discrepancy between biochemical and cellular potencies was even greater for SU6561 than for SU6663, because its IC$_{50}$ value in the biochemical assay was lower. The relative activity of SU6597 and SU6561, the two most active compounds in the biochemical kinase assay, can be more accurately estimated from the data shown in Fig. 1D, in which lower compound concentrations were used.
although addition of the 5-Cl enhanced biochemical potency toward Kit, it also enhanced activity against the more distantly related IGF-1r and Zap70 kinases.

Although addition of the carboxylate increased biochemical potency, it decreased cellular potency. The discrepancy between biochemical and cellular potency becomes even more apparent with SU6561 and SU6597, which contain both the carboxylate and the 5-Cl substituents and, therefore, are very potent biochemical Kit kinase inhibitors but work less well in cellular assays. Such weaker apparent potencies in cellular assays may result from such factors as membrane permeability, protein binding, and higher physiological ATP concentrations than those used in biochemical kinase assays.

Inhibition of SCF-induced Kit Autophosphorylation. To determine whether these tyrosine kinase inhibitors block SCF-induced Kit activation in tumor cells, we used H526 SCLC cells, which express high levels of Kit and low levels of endogenous SCF and can be stimulated by the addition of SCF in serum-free medium. These cells were incubated in serum-free medium overnight, followed by 30 min of incubation with vehicle or various concentrations of the compounds. They were then stimulated with 100 ng/ml SCF for 5 min and lysed for Kit IP. Phosphorylated Kit was detected on Western blots with an antiphosphotyrosine antibody. Representative Western blots for three compounds are illustrated in Fig. 3. Stimulation of H526 cells with SCF resulted in phosphorylation of a Mr 145,000–160,000 protein consistent with autophosphorylation of Kit. To confirm the identity of this band as Kit and to assure that equal amounts of Kit were immunoprecipitated, the Western blot was stripped and reblotted with an anti-Kit MoAb. The percentage change in Kit autophosphorylation was calculated as the ratio of the density of the tyrosine-phosphorylated Kit band divided by the density of the Kit band detected by Western blotting between samples analyzed on the same gel. Table 2 shows the concentration of the compounds necessary to produce a 50% inhibition of SCF-induced Kit autophosphorylation in H526 cells.

Indolinones Inhibit Cell Growth Stimulation by SCF. To evaluate the effects of these Kit inhibitors on SCF-stimulated growth of SCLC cells, H526 cells were exposed to increasing concentrations of drug in serum-free medium containing a saturating concentration of SCF (100 ng/ml). The relative change in viable cell number over 72 h in the absence of added growth factors (control) and in the presence of SCF or IGF-1 was measured by MTT assay. A saturating concentration of IGF-1 (20 ng/ml) was used as a control to define the specificity of these compounds.

Interestingly, all of the compounds blocked the SCF-stimulated cell growth in a dose-dependent fashion (Fig. 4). At a concentration of 5 μM, growth of H526 stimulated by the addition of SCF was completely inhibited by SU5416 and almost completely blocked by SU6597, whereas this effect was observed at 10 μM for SU6663 and SU6668. Increasing the concentration of SU5416 to 10 μM not only eliminated basal autocrine growth, presumably mediated by endogenously produced SCF, but also led to cell death, whereas up to 20

Table 2

<table>
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<tr>
<th>Compound</th>
<th>IC50 (μM)</th>
<th>SCF-stimulated growth</th>
<th>FBS-stimulated growth</th>
<th>SCF-induced Kit activation</th>
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<td>1.9</td>
<td>1.9</td>
<td>13.1</td>
<td>0.03</td>
</tr>
<tr>
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<td>ND*</td>
<td>ND*</td>
<td>ND*</td>
<td>ND*</td>
</tr>
<tr>
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<td>22.1</td>
<td>22.1</td>
<td>0.73</td>
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<tr>
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<td>15.0</td>
<td>24.0</td>
<td>24.0</td>
<td>0.25</td>
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</tbody>
</table>

* FBS, fetal bovine serum; ND, not determined.
μM SU6597, SU6663, and SU6668 was needed to have a similar effect. Although SU5614 significantly inhibited growth stimulated by SCF at 1 μM, this compound precipitated out of solution in tissue culture media at concentrations of 2 μM or greater, which accounts for the flat dose-response at higher concentrations. At 10 μM SU5416 and 20 μM for SU6597 and SU6663, growth inhibition was observed in the presence of IGF-1. This inhibition could be attributable to complete inhibition of background endogenous SCF-Kit autocrine growth and/or partial inhibition of IGF-1r signaling, because these concentrations approach or exceed the biochemical IC50 for the IGF-1r (Table 1). However, SU5416 concentrations up to 40 μM had no effect on IGF-1r-mediated Akt activation (data not shown). It is also interesting to note that the potency for inhibition of IGF-1-mediated growth does not correlate especially well with the ability to inhibit IGF-1r in the biochemical assay (Table 1). Although these observations make it less likely that the drugs are directly affecting IGF-1r signaling, an effect on other endogenous growth factor receptors and regulatory kinases cannot be excluded. Table 2 lists the potencies of the five soluble compounds as inhibitors of SCF-stimulated growth in H526 cells. The order of potency from most potent to least potent is SU5416, SU6597, SU6663, SU6668, and SU6561.

**Indolinones Inhibit Serum-stimulated Cell Growth.** To determine the ability of these compounds to inhibit growth in serum-containing medium, H526 cells were placed in medium containing 10% fetal bovine serum and increasing concentrations of test compound. Cell growth was measured over 72 h by MTT assay. Because little growth inhibition was observed in culture medium containing 5 μM or less (data not shown), the concentrations tested were from 10 to 40 μM. Fig. 5 illustrates that the treatment of serum-stimulated H526 cells with the compounds resulted in a dose-dependent growth inhibition. SU5416 and SU6597 inhibited H526 growth by more than 50% at concentrations between 10–20 μM, whereas SU6668, SU6661, and SU6663 required higher doses (concentrations between 20–30 μM) to achieve a similar inhibitory pattern. SU5614 was again found to be insoluble at concentrations greater than 1 μM and, therefore, results at higher concentrations cannot be interpreted. Calculated IC50 values for the soluble compounds are shown in Table 2, demonstrating that the order of potency of these compounds for inhibition of serum-stimulated growth is similar to that for SCF-stimulated growth. SU5416 appears to be the most potent inhibitor followed by SU6597.

**Indolinones Inhibit Growth of Multiple SCLC Cell Lines in Complete Medium.** The data represented in Figs. 4 and 5 demonstrate that SU5416 and SU6597 appeared to be consistently more potent inhibitors of cell growth than the other compounds. Therefore, we chose SU5416 and SU6597 for further evaluation of their growth inhibitory potential in serum-containing medium. Six SCLC cell lines, derived from both untreated and chemotherapy-treated patients and having both classic and variant features (33), were incubated in serum-containing medium for 72 h in the presence of increasing concentrations of drug, and cell growth was measured by MTT assay. All of the cell lines exhibited a dose-dependent growth inhibition, as illustrated in Fig. 6. At a concentration of 20 μM, SU5416 induced an
approximately 80% inhibition of H526, H146, and WBA growth and resulted in significant cell death of H209, H510, and H187 cells (Fig. 6A). A higher dose of SU6597 was required to induce growth inhibition of all of the six cell lines (Fig. 6B). At a concentration 30 μM, SU6597 inhibited more than 80% of H209 and H510 growth, whereas it had a little effect on H146 growth and no effect on WBA growth. The growth of all of the cell lines was completely inhibited at 40 μM SU6597. Interestingly, H146, which is the only one of these cell lines that does not express Kit, also displayed a moderate degree of growth inhibition in the presence of SU5416 and significant cell death in 40 μM SU6597. This effect could be explained by nonspecific inhibition of cell growth at high drug concentrations or inhibition of additional tyrosine kinases other than Kit that are required for SCLC cell growth.

To assess the possibility of nonspecific toxicity, the non-neoplastic MRC-5 pulmonary fibroblast cell line was exposed to increasing concentrations of both drugs while growing in complete medium. As illustrated in Fig. 7, MTT growth assays revealed that growth was moderately slowed by SU5416 and SU6597, especially at 30–40 μM concentrations. Cytotoxicity was absent, however, as evidenced by the cells remaining tightly adherent to the culture dish without morphological evidence of apoptosis or necrosis (data not shown). These data illustrate a clear differential cytotoxic effect of these compounds for SCLC cells relative to pulmonary fibroblasts.

**DISCUSSION**

Protein phosphorylation plays a critical role in the regulation of cell signaling and growth. In particular, protein tyrosine kinases have been shown to be key enzymes controlling the proliferation of cells. Growth factors acting in an autocrine, paracrine, or endocrine manner lead to persistently increased tyrosine kinase activity, which in turn can result in various diseases such as cancer and other proliferative disorders (34). The significance of tyrosine kinases in cell growth regulation makes them important targets for the development of specific inhibitors to serve both as potential chemotherapeutic agents and as pharmacological agents for defining the physiological roles of these enzymes.

We have investigated the effect of six related tyrosine kinase inhibitors on SCF-stimulated Kit tyrosine phosphorylation and SCF-induced growth stimulation in SCLC cells. The data demonstrate that they were able to block SCF-stimulated Kit autophosphorylation in a dose-dependent manner and selectively inhibit SCF-stimulated growth. IC50 values for inhibition of SCF-induced Kit phosphorylation in H526 SCLC cells were in reasonable agreement with the potency observed in the CHO cell assay. The IC50 values for inhibition of SCF-stimulated growth of H526 SCLC cells were 10–200-fold higher than those observed for inhibition of Kit phosphorylation.
Despite this differential, inhibition of SCF-mediated growth in serum-free medium appeared to be relatively specific, because little or no inhibition of IGF-1-mediated growth occurred at the IC\textsubscript{50} values for SCF-mediated growth. The differences between the IC\textsubscript{50} values for Kit phosphorylation and SCF-stimulated growth could be explained by several possible factors. They could be an indication that 50% inhibition of receptor autophosphorylation may not be sufficient to suppress growth, because it is not known how many functional receptors or phosphorylated tyrosines are needed to elicit a proliferative response. It may be a very small percentage of the total receptor capacity and, therefore, enough compound to inhibit autophosphorylation by 90% or more might be required to produce effects on cell growth. In addition, our results could be attributable to modifying factors in long-term culture, including nonspecific binding to media components, tissue culture plasticware, or cellular metabolism that lessens the effect of these compounds. Another possible explanation is that inhibition of another kinase in addition to Kit is required to inhibit cell growth, and this unidentified kinase requires a higher dose of these compounds for effective inhibition.

To determine whether these compounds could inhibit SCLC proliferation in an environment containing a complex mix of growth factors representative of \textit{in vivo} conditions, we tested their effects on H526 growth in medium containing 10% fetal bovine serum. The IC\textsubscript{50} values for serum-stimulated growth were somewhat higher than those observed for SCF-stimulated growth in serum-free medium. The higher IC\textsubscript{50} values could be attributable to the larger amount of proteins and lipids in 10% fetal bovine serum that could potentially bind the compounds. In addition to the low concentration of SCF present in serum, several other unidentified growth factors in serum could stimulate SCLC growth through other tyrosine kinase receptors and/or other signaling pathways involving tyrosine kinases. Therefore, an additional explanation for the less potent inhibitory effect of these compounds on serum-stimulated growth may be that higher concentrations may be necessary to inhibit these alternative tyrosine kinase-containing pathways. On the basis of their biochemical inhibitory profile (Table 1), it is possible that some inhibition of IGF-1r or FGFr signaling, for instance, may be necessary for optimal inhibition of serum-stimulated growth.

The two most potent inhibitors, based on inhibition of Kit phosphorylation and SCF-mediated growth, SU5416 and SU6597, were selected for study using six representative cell lines grown in serum-containing medium. We found that the compounds exhibited dose-dependent growth inhibition in all of the cell lines. Clearly, the response of SCLC cells in serum may not be entirely attributed to inhibition of Kit, because SU5416 and SU6597 also inhibited growth of the H146 cell line, which does not express Kit. However, the growth inhibition appeared to be relatively selective for SCLC, because only moderate growth suppression of MRC-5 cells by high concentrations of SU5416 and SU6597 was observed. Cytotoxicity was not observed for either compound in the MRC-5 assay. This is in marked contrast to the SCLC assays where 30–40 \textmu M concentrations of either drug induced extensive cytotoxicity. This cytotoxic effect is also in striking contrast to the results seen with the quinoxaline tyrosine kinase inhibitor AG1296 (22) and the 2-phenylaminopyrimidine compound STI571 (24), which inhibited growth without significant cytotoxicity in serum-containing medium. The broader spectrum of activity of SU5416 and SU6597 (Table 1) could account for the increase in cytotoxicity relative to these other inhibitors. In addition to the induction of selective cytotoxicity, these compounds have additional properties that theoretically could make them excellent agents for use in the treatment of SCLC. Both compounds exhibit activity against receptors that are involved in the induction of tumor angiogenesis (Table 1). It has already been demonstrated that \textit{in vivo} tumor growth can be significantly inhibited by SU5416 based solely on its ability to inhibit angiogenesis, because the drug had no significant effect on the \textit{in vitro} growth of the tumor cell lines tested (30). In SCLC, therefore, the \textit{in vivo} potency could be even greater, based on the ability of the drugs to inhibit kinases required for both growth and tumor angiogenesis. In addition, it would be expected that the development of drug resistance would be hampered by the fact that these agents block multiple kinases involved in regulating tumor growth that lie on two distinct biological pathways intrinsic to both the tumor and nonmalignant endothelial cells.

In summary, we have shown that indolomine tyrosine kinase inhibitors can be potent Kit kinase inhibitors and can block SCF-mediated Kit activation and SCF- and serum-stimulated growth of SCLC cells. To develop these compounds as therapeutic drugs for SCLC, their efficacy in animal models would have to be determined. However,
based on our results, it is reasonable to conclude these Kit inhibitors or their derivatives may have a role not only in the treatment of SCLC but also in the treatment of other cancers and human diseases where Kit activation occurs.

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Indolinone Tyrosine Kinase Inhibitors Block Kit Activation and Growth of Small Cell Lung Cancer Cells

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