[CANCER RESEARCH 61, 3660–3668, May 1, 2001]

Indolinone Tyrosine Kinase Inhibitors Block Kit Activation and Growth of Small Cell Lung Cancer Cells[1]

Geoffrey W. Krystal,[2] Sittisak Honsawek, David Kiewlich, Congxin Liang, Stefan Vasile, Li Sun, Gerald McMahon, and Kenneth E. Lipson

Departments of Internal Medicine [G. W. K.] and Microbiology/Immunology [G. W. K., S. H.], Virginia Commonwealth University, McGuire Veterans Affairs Medical Center, Richmond, Virginia 23249, and SUGEN, Incorporated, South San Francisco, California 94080 [D. K., C. L., S. V., L. S., G. M., K. E. L.]

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 H526 cell line was dose-dependent. At concentrations that inhibited SCF-stimulated H526 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 M_r 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[3] 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). STI571, a 2-phenylaminopryimidine 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 mutationally 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.

Received 6/15/00; accepted 3/13/01.

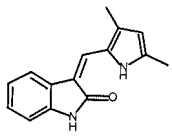
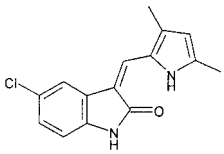
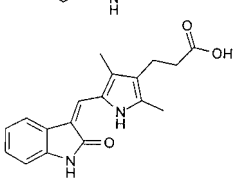
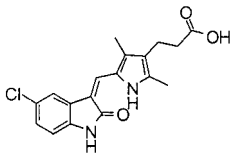
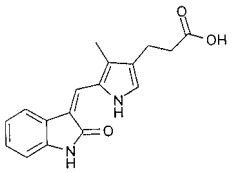
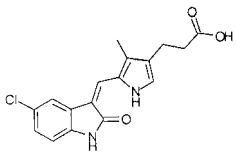
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported in part by a Merit Review Award from the Department of Veterans Affairs.

[2] To whom requests for reprints should be addressed, at Richmond Veterans Affairs Medical Center (111K), 1201 Broad Rock Boulevard, Richmond, VA 23249. Phone: (804) 675-5446; Fax: (804) 675-5447; E-mail: gkrystal@hsc.vcu.edu.

[3] The abbreviations used are: SCF, stem cell factor; SCLC, small cell lung cancer; MoAb, monoclonal antibody; IGF-1, 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-2yl)-2,5-diphenyl-tetrazolium bromide; GyrB, B subunit of bacterial DNA gyrase.

Table 1 *Compound structure and biochemical kinase inhibitory activities*

Compound	Structure	IC_{50} (μM) Kit	VEGFr2	FGFr1	PDGFrβ	EGFr	IGF1r	Zap70
SU5416		$(32.3 \pm 5.0) \times 10^{-3}$ ($n = 3$)	1.3 ± 0.8 ($n = 14$)	4.2 ± 0.8 ($n = 7$)	37.9 ± 8.9 ($n = 9$)	>100	19.5 ± 9.3 ($n = 3$)	20.7 ± 1.0 ($n = 3$)
SU5614		$(28.3 \pm 3.8) \times 10^{-3}$ ($n = 3$)	0.5 ± 0.2 ($n = 3$)	3.1 ± 2.6 ($n = 3$)	0.13 ± 0.13 ($n = 6$)	>100	6.7 ± 0.7 ($n = 3$)	2.7 ± 0.1 ($n = 3$)
SU6668		$(10.5 \pm 1.3) \times 10^{-3}$ ($n = 3$)	3.9 ± 2.2 ($n = 31$)	3.8 ± 1.2 ($n = 11$)	0.10 ± 0.07 ($n = 25$)	>100	28.6 ± 0.8 ($n = 3$)	28.4 ± 0.5 ($n = 3$)
SU6597		$(8.7 \pm 0.9) \times 10^{-8}$ (extrapolated) ($n = 3$)	3.3 ± 2.2 ($n = 7$)	5.1 ± 3.3 ($n = 4$)	$(2.0 \pm 0.6) \times 10^{-3}$ ($n = 6$)	>100	2.7 ± 0.1 ($n = 3$)	2.7 ± 0.1 ($n = 3$)
SU6663		$(14 \pm 1.5) \times 10^{-3}$ ($n = 3$)	2.1 ± 0.4 ($n = 3$)	3.7 ± 1.0 ($n = 3$)	0.14 ± 0.02 ($n = 9$)	>100	25.3 ± 1.3 ($n = 3$)	25.0 ± 0.2 ($n = 3$)
SU6561		$(3.8 \pm 0.2) \times 10^{-5}$ ($n = 3$)	2.9 ± 0.8 ($n = 8$)	4.9 ± 0.2 ($n = 3$)	$1.0 \pm 0.4 \times 10^{-2}$ ($n = 3$)	>100	9.3 ± 0.1 ($n = 2$)	7.6 ± 0.3 ($n = 3$)

MATERIALS AND METHODS

Compounds and Reagents. Kinase inhibitors SU6597, SU6668, SU6561, 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 DMSO in each assay was 0.1% (volume for volume). A plasmid containing the M_r 24,000 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 pCI-Neo (Promega, Inc., Madison, WI). Coumermycin 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 H146 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.), 2 mM L-glutamine, and 50 units/ml penicillin-50 units/ml streptomycin. Non-neoplastic MRC-5 pulmonary fibroblasts (ATCC CCL-171; Manassas, VA) were maintained in Eagle's minimum essential medium (Bio-Whitaker) supplemented with 10% heat-inactivated fetal bovine serum. CHO/GyrB-Kit cells (a stable clone of CHO-K1 cells expressing a GyrB-Kit chimeric protein) were grown in Ham's F12 medium containing 10% fetal bovine serum and 1 mg/ml G418 (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 1×10^4 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 Kit 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 aprotinin, and 1 μM pepstatin A; Sigma Chemical Co.) and 20 mM EDTA and then diluted with an equal volume of 20 mM HEPES, 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 HEPES, 100 mM NaCl, 5 mM $MgCl_2$,

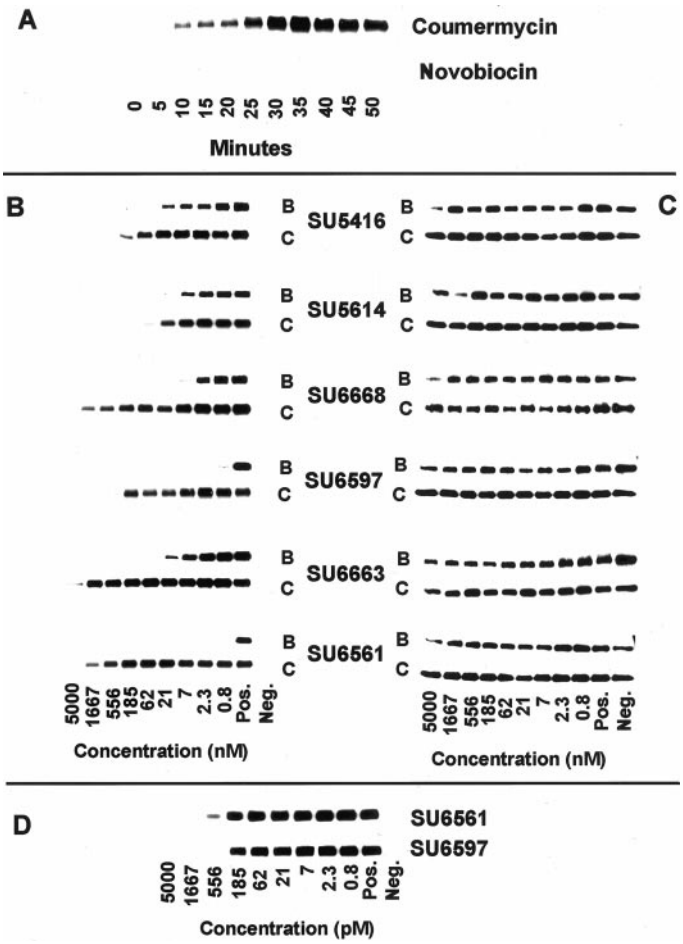


Fig. 1. Comparison of biochemical and cellular potency for inhibition of Kit kinase. *A*, serum-deprived CHO/GyrB-Kit cells (CHO-K1 cells expressing the GyrB-Kit chimera) were stimulated with 1 μM coumermycin or novobiocin for the indicated time. Kit was immunoprecipitated from cell lysates and resolved by SDS-PAGE, transferred to nitrocellulose, and phosphotyrosine was visualized with an antiphosphotyrosine antibody. *B*, for cellular assays (*C*), serum-deprived CHO/GyrB-Kit cells were incubated with the indicated concentration of kinase inhibitor for 2 h and then stimulated with 1 μM coumermycin for 30 min before cell lysis. In biochemical assays (*B*), the drug was added directly to the Kit IP before the *in vitro* kinase reaction. Kit IPs were then resolved by SDS-PAGE and analyzed as above. *C*, the blots from *B* were stripped, and Kit protein was visualized with anti-Kit antibodies. *D*, GyrB-Kit immunoprecipitated from serum deprived cells was incubated with the indicated concentrations of kinase inhibitor and an *in vitro* kinase assay was performed. Kit IPs were then resolved by SDS-PAGE and stained with an anti-pTyr antibody.

and 5 mM $MnCl_2$) containing 5 μM ATP for 30 min at room temperature. The kinase reaction was stopped by the addition of 2× reducing SDS-PAGE sample buffer and boiling. After fractionation by SDS-PAGE and transfer to nitrocellulose, the amount of autophosphorylation was detected with antiphosphotyrosine antibodies (biotin-PY99; Santa Cruz Biotechnology) and enhanced chemiluminescence (Amersham, Arlington Heights, IL) reagents. The amount of Kit protein loaded in each lane was visualized in stripped blots with anti-Kit goat polyclonal antibodies (Santa Cruz Biotechnology).

Cellular Kit kinase assays were performed with CHO/GyrB-Kit cells in 6-well plates. Cells were serum-starved in DMEM containing 0.1% BSA overnight and then incubated with the test compound for 2 h. Coumermycin (1 μM; final concentration) was added to the cells to initiate kinase dimerization. After 30 min of incubation, the cells were lysed in radioimmunoprecipitation assay buffer containing protease inhibitors, 20 mM EDTA, and 1 mM orthovanadate and then diluted in an equal volume of 20 mM HEPES, 150 mM NaCl, 0.5% Triton X-100, and 10% glycerol, containing protease inhibitors and orthovanadate. After centrifugation, Kit was immunoprecipitated from the cleared lysate, subjected to SDS-PAGE, transferred to nitrocellulose, and probed for phosphotyrosine and Kit protein, as described above.

Biochemical Kinase Assays. Biochemical kinase assays to evaluate the potency of compounds for inhibition of PDGFr, EGFr, and FGFr were performed as described previously (29, 30). In brief, all of the kinase assays were performed at twice the K_m for ATP to make the IC_{50} values comparable. The cytoplasmic domains of EGFr, FGFr1, or IGF-1r or the kinase domain of Zap70 were expressed in SF9 cells as a glutathione *S*-transferase fusion protein and purified on glutathione agarose columns. The purified protein was added to microtiter plate wells coated with poly-GluTyr (4:1), together with the test compound and the appropriate kinase buffer. The amount of substrate phosphorylation was then measured with antibodies to phosphotyrosine. Lysates of cells overexpressing PDGFrβ or GyrB/Kit were transferred to wells coated with antibodies to the COOH termini to immunopurify the kinase. The test compound and appropriate kinase buffer were added to the wells, and the amount or autophosphorylation was measured with antibodies to phosphotyrosine.

IP of Kit from SCLC. H526 cells were grown until confluence and then serum-starved overnight in serum-free medium. The cells were both pretreated with DMSO vehicle or various concentrations of inhibitors for 30 min and then either left unstimulated or stimulated with 100 ng/ml SCF for 5 min. They were then washed twice with cold PBS and lysed in a Dounce homogenizer with a tight-fitting pestle, using ice-cold lysis buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 1% NP40, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 0.2 mM Na_3VO_4, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 10 μg/ml leupeptin. Protein concentrations were determined by the BCA assay (Pierce). The lysate, containing 1.0–1.5 mg of protein, was transferred to a microfuge tube and centrifuged for 10 min at 10,000 × *g*. The supernatant 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 Modeler program (Molecular Simulations, Inc., San Diego, CA). The "open form" of FGFr1 cocrystallized with SU6668 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 SU6668 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 IC_{50} value of 12.2 ± 1.3 μM. These observations confirm and extend previously reported activities for 3-substituted indolinones as kinase inhibitors (25, 26).

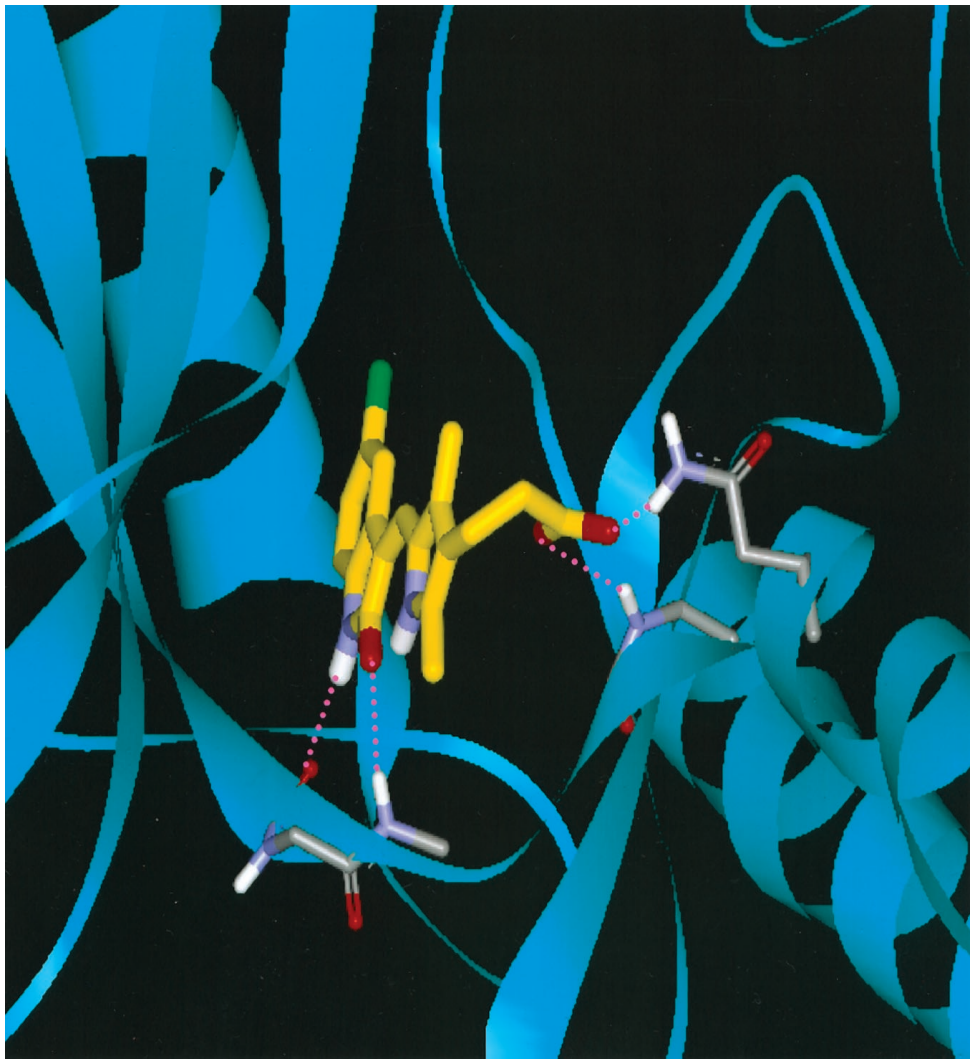


Fig. 2. Homology model of Kit kinase with SU6597 docked into the ATP-binding site. A homology model was made for the Kit kinase based on the crystal structure of FGFr1. SU6597 was docked into the ATP-binding site, based on the cocrystal of FGFr1 with SU6668 (29). Side chains for amino acids that are predicted to interact with the compound are shown on the ribbon structure.

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. 1*A*). 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. 1*B* illustrates a comparison of the biochemical and cellular potencies of these compounds for inhibition of Kit kinase autophosphorylation. Fig. 1*C* 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. 1*D*, in which lower compound concentrations were used.

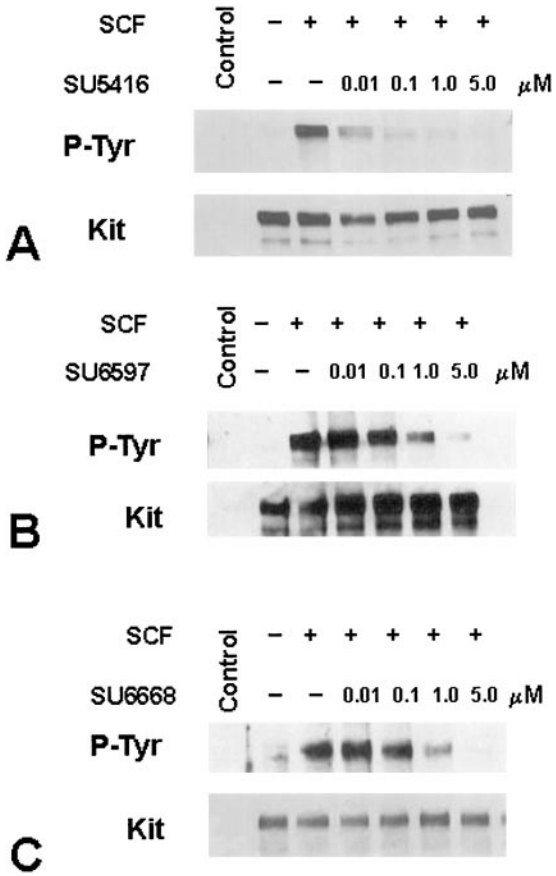


Fig. 3. Inhibition of SCF-stimulated Kit tyrosine phosphorylation. H526 cells were incubated in the indicated combinations of 100 ng/ml SCF and increasing concentrations of the kinase inhibitor and lysed, and a Kit IP was performed. The immunoprecipitate was Western blotted and stained with an antiphosphotyrosine antibody, resulting in the appearance of a M_r 145,000–160,000 band. The same blot was stripped and restained with an anti-Kit antibody. The *Control Lane* represents a mock IP using nonimmune mouse IgG. Results are illustrated for only three of the more potent compounds in growth assays and are representative of at least two independent experiments.

Kit Kinase Homology Model. To try to understand the differences in biochemical potency observed for the various compounds, a homology model for Kit kinase complexed with SU6597 was developed (Fig. 2). This model was based on the crystal structure of FGFr1 with SU6668 (29). As observed with other indolinone-based kinase inhibitors, the nitrogen and oxygen of the oxindole core form hydrogen bonds with the peptide backbone of Glu671 and Cys673. The carboxylate substituent can interact with the peptide amide of Asp677 and the side chain of Asn680. The ability of the carboxylate moiety to form additional contacts suggests a rationale for the observation that the acidic compounds tend to be more potent than SU5416. However, the homology model cannot explain why addition of the 5-Cl to the oxindole of SU6668 or SU6663 increases the potency of the compound for inhibition of Kit kinase.

Structure-Activity Relationship Summary. These results demonstrate that six indolinone compounds that had been previously identified as inhibitors of tyrosine kinases in the PDGFr superfamily are also potent inhibitors of Kit kinase. The biochemical potency of the compounds increased with the addition of a propionic acid moiety to the 4 position of the pyrrole or with the addition of Cl to the 5 position of the oxindole. Addition of both substituents dramatically increased biochemical potency. The homology model suggests a reason for the increases observed upon addition of the carboxylate but not for addition of the Cl. It also cannot explain the synergy observed upon addition of both substituents. It is also interesting to note that 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 M_r 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 restained 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 lead to cell death, whereas up to 20

Table 2 *Estimated IC_{50} values in H526 SCLC cells*

Compound	IC_{50} (μM)		
	SCF-stimulated growth	FBS-stimulated growth[a]	SCF-induced Kit activation
SU5416	1.9	13.1	0.01
SU5614	ND[a]	ND[a]	0.05
SU6668	8.5	22.1	0.73
SU6597	4.5	19.4	0.08
SU6663	7.4	23.8	0.48
SU6561	15.0	24.0	0.25

[a] FBS, fetal bovine serum; ND, not determined.

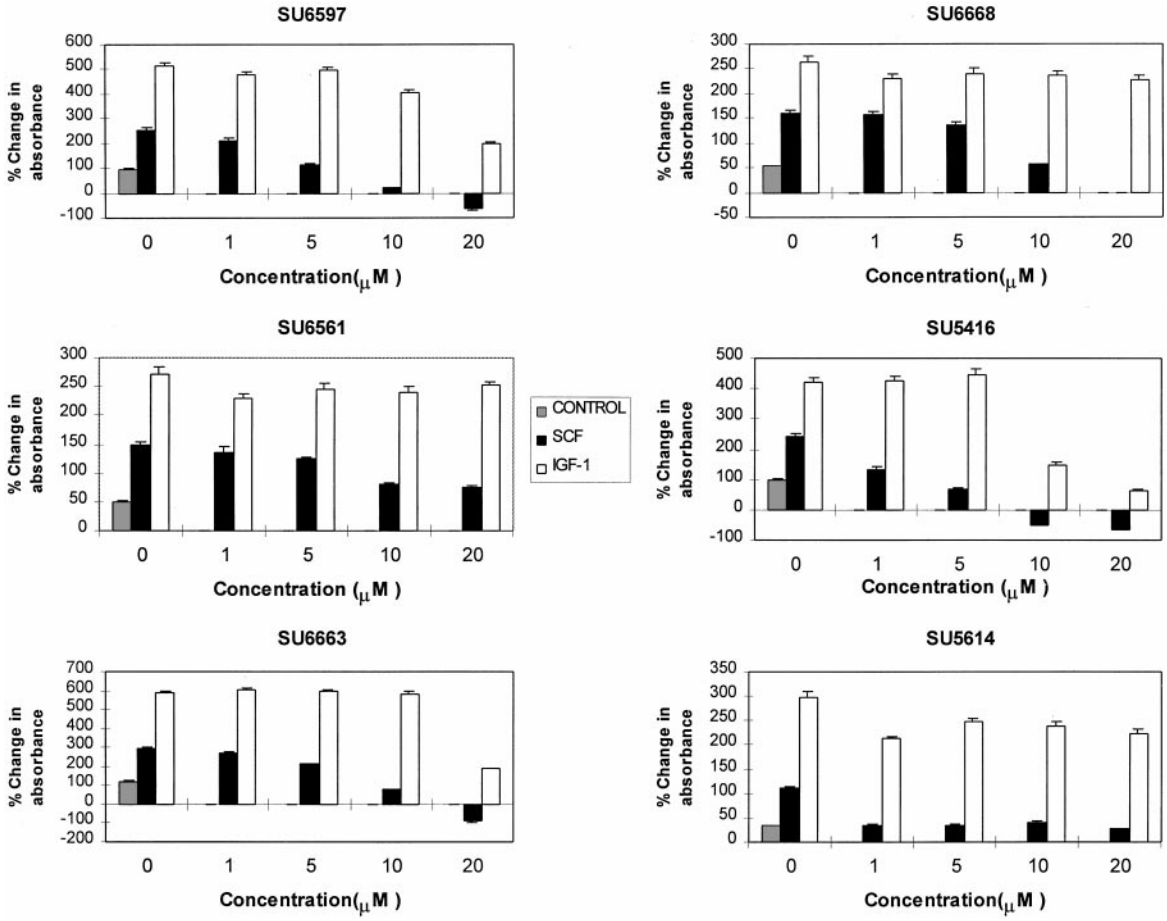


Fig. 4. Selective inhibition of SCF-mediated growth. H526 cells were incubated in serum-free medium overnight and then left unstimulated (*Control*) or stimulated with saturating concentration of SCF (100 ng/ml) or IGF-1 (20 ng/ml). The indicated concentration of drug was added 30 min before the growth factors. Cell growth was assessed using the MTT colorimetric dye method, measuring the change in absorbance over 72 h. *Error bars* indicate ±SD from the mean of eight replicate wells. Results are representative of at least two independent experiments. SU5614 precipitated out of solution at concentrations greater than 1 μM and, therefore, efficacy was not enhanced by higher drug concentrations.

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 IC_{50} for the IGF-1r (Table 1). However, SU5416 concentrations up to 40 μM had no effect on IGF-1-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, SU6561, 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 IC_{50} 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

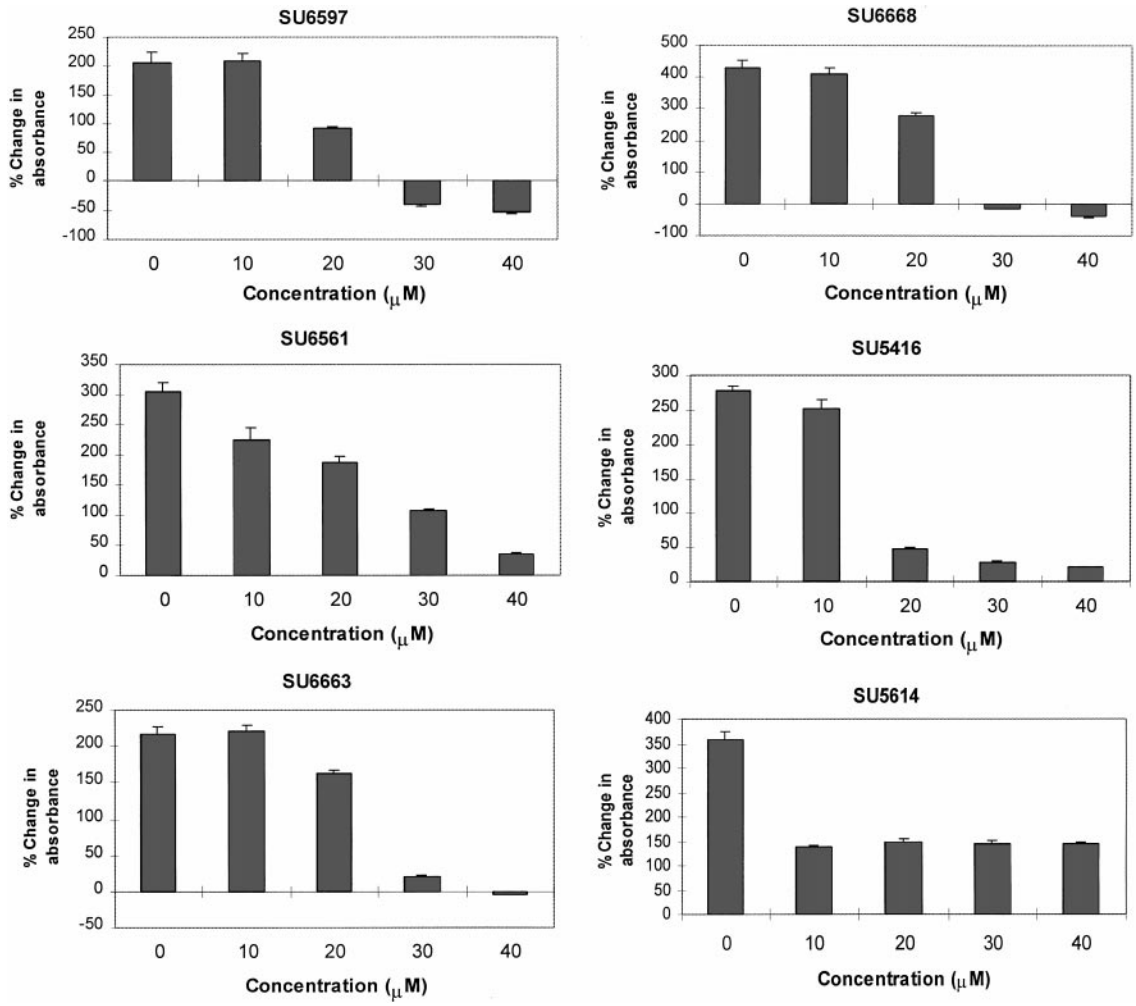


Fig. 5. Inhibition of H526 cell growth in serum-containing medium by indolinone tyrosine kinase inhibitors. H526 cells were incubated in medium containing 10% fetal bovine serum for 72 h in the presence of increasing concentrations of the indicated compounds and growth was measured by MTT assay. *Error bars* indicate ±SD from the mean of eight replicate wells. Results are representative of at least two independent experiments. SU5614 precipitated out of solution at concentrations greater than 1 μM, accounting for the flat-dose response observed.

approximately 80% inhibition of H526, H146, and WBA growth and resulted in significant cell death of H209, H510, and H187 cells (Fig. 6*A*). A higher dose of SU6597 was required to induce growth inhibition of all of the six cell lines (Fig. 6*B*). 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. IC_{50} 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 IC_{50} 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_{50} values for SCF-mediated growth. The differences between the IC_{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 *in vivo* conditions, we tested their effects on H526 growth in medium containing 10% fetal bovine serum. The IC_{50} values for serum-stimulated growth were somewhat higher than those observed for SCF-stimulated growth in serum-free medium. The higher IC_{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.

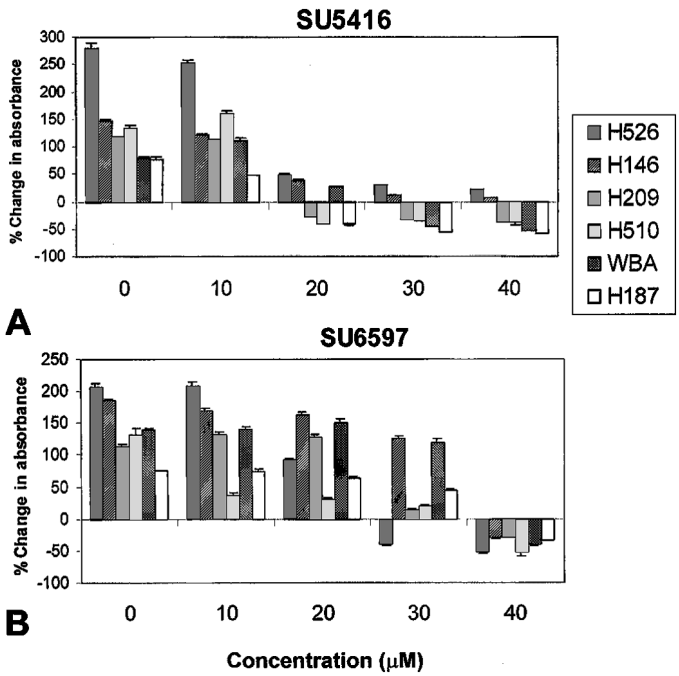


Fig. 6. Indolinone Kit inhibitors block SCLC growth in complete medium. Six representative cell lines were incubated in serum-containing medium for 72 h in the presence of increasing concentrations of SU5416 (*A*) and SU6597 (*B*). SCLC growth was determined by MTT assay. *Error bars* indicate ±SD from the mean of eight replicate wells. Results are representative of at least two independent experiments.

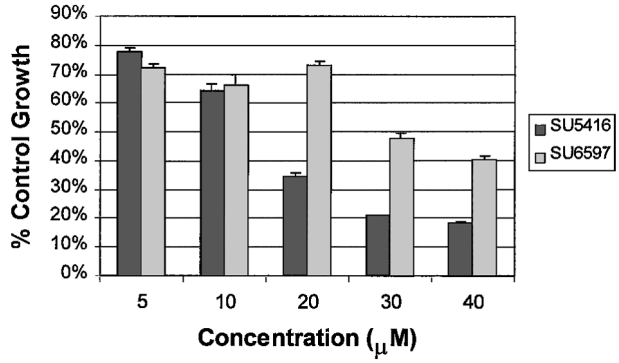


Fig. 7. The effect of SU5416 and SU6597 on the growth of MRC-5 pulmonary fibroblasts. MRC-5 cells were incubated in serum-containing medium for 72 h in the presence of increasing concentrations of SU5416 and SU6597. SCLC growth was determined by MTT assay and expressed as a percentage of the growth of control cells incubated in DMSO vehicle alone. *Error bars* indicate ±SD from the mean of eight replicate wells. Results are representative of three independent experiments.

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 μM 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 *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 *in vitro* growth of the tumor cell lines tested (30). In SCLC, therefore, the *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 indolinone 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.

REFERENCES

1. Broudy, V. C. Stem-cell factor and hematopoiesis. Blood, *90:* 1345–1364, 1997.
2. Lev, S., Blechman, J. M., Givol, D., and Yarden, Y. Steel factor and c-kit protooncogene: genetic lesson in signal transduction. Crit. Rev. Oncog., *5:* 141–168, 1994.
3. Ashman, L. K. The biology of stem-cell factor and its receptor C-kit. Int. J. Biochem. Cell Biol., *31:* 1037–1051, 1999.
4. Pietsch, T., Kyas, U., Steffens, U., Yakisan, E., Hadam, M. R., Ludwig, W. D., Zsebo, K. M., and Welte, K. Effects of human stem-cell factor (c-kit ligand) on proliferation of myeloid leukemia cells: heterogeneity in response and synergy with other hematopoietic growth factors. Blood, *80:* 1199–1206, 1992.
5. Cohen, P. S., Chan, J. P., Lipkunskaya, M., Biedler, J. L., and Seeger, R. C. Expression of stem-cell factor and c-kit in human neuroblastoma. The Children's Cancer Group. Blood, *84:* 3465–3472, 1994.
6. Hines, S. J., Organ, C., Kornstein, M. J., and Krystal, G. W. Coexpression of the c-kit and stem-cell factor genes in breast carcinoma. Cell Growth Differ., *6:* 769–779, 1995.
7. Toyota, M., Hinoda, Y., Takaoka, A., Makiguchi, Y., Takahashi, T., Itoh, F., Imai, K., and Yachi, A. Expression of c-*kit* and Kit ligand in human colon carcinoma cells. Tumor Biol., *14:* 295–302, 1993.
8. Inoue, M., Kyo, S., Fujita, M., Enomoto, T., and Kondoh, G. Coexpression of the c-kit receptor and the stem cell factor in gynecological tumors. Cancer Res., *54:* 3049–3053, 1994.
9. Strohmeyer, T., Peter, S., Hartmann, M., Munemitsu, S., Ackermann, R., Ullrich, A., and Slamon, D. J. Expression of the hst-1 and c-*kit* protooncogenes in human testicular germ cell tumors. Cancer Res., *51:* 1811–1816, 1991.
10. Sekido, Y., Obata, Y., Ueda, R., Hida, T., Suyama, M., Shimokata, K., Ariyoshi, Y., and Takahashi, T. Preferential expression of c-kit protooncogene transcripts in small cell lung cancer. Cancer Res., *51:* 2416–2419, 1991.
11. Hibi, K., Takahashi, T., Sekido, Y., Ueda, R., Hida, T., Ariyoshi, Y., Takagi, H., and Takahashi, T. Coexpression of the stem cell factor and the c-kit genes in small cell lung cancer. Oncogene, *6:* 2291–2296, 1991.
12. Plummer, H., III, Catlett, J., Leftwich, J., Armstrong, B., Carlson, P., Huff, T., and Krystal, G. W. c-*myc* expression correlates with suppression of c-*kit* proto-oncogenes expression in small cell lung cancer cell lines. Cancer Res., *53:* 4337–4342, 1993.
13. Rygarrd, K., Nakamura, T., and Spang-Thomsen, M. Expression of the proto-oncogenes c-*met* and c-*kit* and their ligand, hepatocyte growth factor/scatter factor and stem-cell factor in SCLC cell lines and xenografts. Br. J. Cancer, *67:* 37–46, 1993.
14. Nagata, H., Worobee, A. S., Oh, C. K., Chowdhury, B. A., Tannenbaum, S., Suzuki, Y., and Metcalfe, D. D. Identification of a point mutation in the catalytic domain of the proto-oncogene c-kit in peripheral blood mononuclear cells of patients with systemic mastocytosis with an associated hematologic disorder. Proc. Natl. Acad. Sci. USA, *92:* 10560–10564, 1995.
15. Hirota, S., Isozaki, K., Moriyama, Y., Hashimoto, K., Nishida, T., Ishiguro, S., Kawano, K., Hanada, M., Kurata, A., Takeda, M., Tunio, G. M., Matsuzawa, Y., Kanakura, Y., Shinomura, Y., and Kitamura, Y. Gain-of-function mutations of c-*kit* in human gastrointestinal stromal tumors. Science (Wash. DC), *279:* 577–580, 1998.
16. Tian, Q., Frierson, H. F., Krystal, G. W., and Moskaluk, C. A. Activating c-*kit* mutations in human germ cell tumors. Am. J. Pathol., *154:* 1643–1647, 1999.
17. Ricotti, E., Fagioli, F., Garelli, E., Linari, C., Crescenzio, N., Horenstein, A. L., Pistamiglio, P., Vai, S., Berger, M., di Montezemolo, L. C., Madon, E., and Basso, G. c-*kit* is expressed in soft tissue sarcoma of neuroectodermic origin and its ligand prevents apoptosis of neoplastic cells. Blood, *91:* 2397–2405, 1998.
18. Krystal, G. W., Hines, S., and Organ, C. Autocrine growth of small cell lung cancer mediated by coexpression of c-*kit* and stem-cell factor. Cancer Res., *56:* 370–376, 1996.
19. Levitzki, A., and Gazit, A. Tyrosine kinase inhibition: an approach to drug development. Science (Wash. DC), *267:* 1782–1788, 1995.
20. McMahon, G., Sun, L., Liang, C., and Tang, C. Protein kinase inhibitors: structural determinants for target specificity. Curr. Op. Drug Disc. Dev., *1:* 131–146, 1998.
21. Garcia-Echeverria, C., Traxler, P., and Evans, D. B. ATP site-directed competitive and irreversible inhibitors of protein kinases. Med. Res. Rev., *20:* 28–57, 2000.
22. Krystal, G. W., Carlson, P., and Litz, J. Induction of apoptosis and inhibition of small cell lung cancer growth by the quinoxaline tyrphostins. Cancer Res., *57:* 2203–2208, 1997.
23. Buchdunger, E., Zimmermann, J., Mett, H., Meyer, T., Muller, M., Druker, B. J., and Lydon, N. B. Inhibition of the Abl protein-tyrosine kinase *in vitro* and *in vivo* by a 2-phenylaminopyrimidine derivative. Cancer Res., *56:* 100–104, 1996.
24. Krystal, G. W., Honsawek, S., Litz, J., and Buchdunger, E. The selective tyrosine kinase inhibitor STI571 inhibits small cell lung cancer growth. Clin. Cancer Res., *6:* 3319–3326, 2000.
25. Sun, L., Tran, N., Tang, F., App, H., Hirth, P., McMahon, G., and Tang, C. Synthesis and biological evaluations of 3-substituted indolin-2-ones: a novel class of tyrosine kinase inhibitors that exhibit selectivity towards particular receptor tyrosine kinases. J. Med. Chem., *41:* 2588–2603, 1998.
26. Sun, L., Tran, N., Liang, C., Tang, F., Rice, A., Schreck, R., Waltz, K., Shawver, L. K., McMahon, G., and Tang, C. Design, synthesis, and evaluations of substituted 3-[(3- or 4-carboxyethylpyrrol-2-yl)methylidenyl]indolin-2-ones as inhibitors of VEGF, FGF, and PDGF receptor tyrosine kinases. J. Med. Chem., *42:* 5120–5130, 1999.
27. Ma, Y., Carter, E., Wang, X., Shu, C., McMahon, G., and Longley, B. J. Indolinone derivatives inhibit constitutively activated KIT mutants and kill neoplastic mast cells. J. Investig. Dermatol., *114:* 392–394, 2000.
28. Carmichael, J., Degraf, W. G., Gazdar, A. D., Minna, J. D., and Mitchell, J. B. Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. Cancer Res., *47:* 936–942, 1987.
29. Laird, A. D., Vajkoczy, P., Shawver, L. K., Thurnher, A., Liang, C., Mohammadi, M., Schlessinger, J., Ullrich, A., Hubbard, S. R., Blake, R. A., Fong, T. A. T., Strawn, L. M., Sun, L., Tang, C., Hawtin, R., Tang, F., Shenoy, N., Hirth, K. P., McMahon, G., and Cherrington, J. M. SU6668 is a potent anti-angiogenic and antitumor agent which induces regression of established tumors. Cancer Res., *60:* 4152–4160, 2000.
30. Fong, T. A. T., Shawver, L. K., Sun, L., Tang, C., App, H., Powell, T. J., Kim, Y. H., Schreck, R., Wang, X., Risau, W., Ullrich, Z., Hirth, K. P., and McMahon, G. SU5416 is a potent and selective inhibitor of the vascular endothelial growth factor receptor (Flk-1/KDR) that inhibits tyrosine kinase catalysis, tumor vascularization, and growth of multiple tumor types. Cancer Res., *59:* 99–106, 1999.
31. Hanks, S., and Quinn, A. M. Protein kinase catalytic domain sequence database: identification of conserved features of primary structure and classification of family members. Methods Enzymol., *200:* 38–62, 1991.
32. Farrar, M. A., Alberola-Ila, J., and Perlmutter, R. M. Activation of the Raf-1 kinase cascade by coumermycin-induced dimerization. Nature (Lond.), *383:* 178–181, 1996.
33. Carney, D., Gazdar, A., Bepler, G., Guccion, J., Marangos, P., Moody, T., Zweig, M., and Minna, J. Establishment and identification of small cell lung cancer cell lines having classic and variant features. Cancer Res., *45:* 2913–2923, 1985.
34. Bishop, J. M. The molecular genetics of cancer. Science (Wash. DC), *235:* 305–311, 1987.

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

Geoffrey W. Krystal, Sittisak Honsawek, David Kiewlich, et al.

Cancer Res 2001;61:3660-3668.