Effects of the bcr/abl Kinase Inhibitors AG957 and NSC 680410 on Chronic Myelogenous Leukemia Cells *In Vitro*†

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**ABSTRACT**

The tyrphostin AG957 (NSC 654705) inhibits p210bcr/abl, the transforming kinase responsible for most cases of chronic myelogenous leukemia (CML). The present studies were performed to determine the fate of AG957-treated cells and assess the selectivity of AG957 for CML progenitors. When K562 cells (derived from a patient with blast crisis CML) were treated with AG957, dose- and time-dependent p210bcr/abl down-regulation was followed by mitochondrial release of cytochrome c, activation of caspase-9 and caspase-3, and apoptotic morphological changes. These apoptotic changes were inhibited by transfection with cDNA encoding dominant negative caspase-9 but not dominant-negative FADD or blocking anti-Fas antibodies. In additional experiments, a 24-h AG957 exposure caused dose-dependent inhibition of K562 colony formation in soft agar. To extend these studies to clinical samples of CML, peripheral blood mononuclear cells from 10 chronic phase CML patients and normal controls were assayed for the growth of hematopoietic colonies *in vitro* in the presence of increasing concentrations of AG957. These assays demonstrated selectivity of AG957 for CML progenitors, with median IC50s (CML versus normal) of 7.3 versus >20 μM AG957 in granulocyte colony-forming cells (*P* < 0.001), 5.3 versus >20 μM in granulocyte/macrophage colony-forming cells (*P* < 0.05), and 15.5 versus > 20 μM in erythroid colony-forming cells (*P* > 0.05). The adamantyl ester of AG957 (NSC 680410) down-regulated p210bcr/abl in K562 cells and inhibited granulocyte colony formation in CML specimens at lower concentrations without enhanced toxicity in normal progenitors. These observations not only demonstrate that AG957-induced p210bcr/abl down-regulation is followed by activation of the cytochrome c/Apaf-1/caspase-9 pathway but also indicate that this class of kinase inhibitor exhibits selectivity worthy of further evaluation.

**INTRODUCTION**

The initial transforming event in the vast majority of cases of CML is a 9:22 chromosomal translocation that juxtaposes the c-abl gene on chromosome 9 with the bcr gene on chromosome 22 (reviewed in Refs. 1–3). The resulting p210bcr/abl fusion protein (p210bcr/abl) causes a disease resembling CML when expressed in transgenic animals (4–7). p210bcr/abl is a cytoplasmic protein (8, 9) that exhibits constitutive tyrosine kinase activity (10) and initiates signaling through multiple pathways, including the ras/raf/mitogen-activated protein kinase pathway and the CBL/PI3k/Akt pathway (reviewed in Refs. 3, 11–13). This p210bcr/abl-initiated signaling decreases the ability of a variety of stimuli to induce apoptosis *in vitro* (14–19), most likely by inhibiting release of cytochrome c to the cytosol and subsequent caspase activation (20–22).

In principle, p210bcr/abl-initiated signaling can be interrupted at a variety of points. For example, transfection with cDNA encoding a dominant-negative inhibitor of ras or its upstream activator Grb2 abrogates bcr/abl-initiated antiapoptotic signaling (19, 23, 24). Likewise, inhibition of PI3k by wortmannin reportedly inhibits proliferation of bcr/abl-transformed cell lines and CML cells *in vitro* (25).

Despite the ability to disrupt bcr/abl-initiated signaling at downstream points, the bcr/abl kinase itself remains an appealing target for selective chemotherapy because of its expression only in transformed cells (reviewed in Ref. 26). The nonspecific tyrosine kinase inhibitors erbastatin (27) or genistein (28) inhibit colony formation by CML progenitors *in vitro*, but these agents lack selectivity (28). The more selective inhibitor CGP57148B, which competes with ATP for binding to the active site of bcr/abl kinase (29, 30), also inhibits bcr/abl-positive cell lines and CML progenitors *in vitro* (29, 31). This agent has produced hematological remissions in some patients with chronic phase CML (32) and is about to enter Phase II testing in this disease.

An alternative approach to inhibiting protein kinases in-
volves the use of small molecules that alter the binding of peptide substrates rather than ATP. With this in mind, a chemically diverse group of agents generically termed tyrphostins have been synthesized and evaluated as potential inhibitors of various tyrosine kinases (33). Theoretical advantages of these compounds include their lack of cross-resistance with ATP-based kinase inhibitors and their structural diversity, which enhances the probability that selective inhibitors will be found.

Previous studies demonstrated that the tyrphostin AG957 (Fig. 1) inhibits p210bcr/abl kinase activity in immune complex kinase assays (34). AG957 also inhibited p210bcr/abl autophosphorylation and macromolecular synthesis in K562 cells (34), a p210bcr/abl-positive cell line derived from a patient with blast crisis CML (reviewed in Ref. 35). Additional studies suggested that AG957 acts through a mechanism that involves stabilization of covalent high molecular weight complexes containing p210bcr/abl and its signaling adaptor molecules Shc and Grb2 (36).

Although these studies identified AG957 as a bcr/abl-directed agent, several issues remained unresolved. First, it was unclear whether AG957 is cytostatic or cytotoxic. Two recent studies suggested that AG957 inhibits p210bcr/abl under conditions that are not toxic to K562 cells (37) or CML marrow progenitors (38). These results were in sharp contrast to the reported induction of apoptosis in cells treated with bcr/abl antisense oligonucleotides (39) or CEP57148B (40, 41). Second, if AG957 was cytotoxic, the mechanism was unexplained. None of the previous studies describing cytotoxicity of bcr/abl-directed agents evaluated the cell death pathways activated by these agents. Third, it was unclear whether AG957 selectively affected bcr/abl-transformed cells. Bhatia et al. (38) reported that colony-forming cells from CML patients and normal controls were inhibited equally by a 1-h exposure to high AG957 concentrations. Moreover, Losiewicz et al. (42) reported recently that AG957 inhibits T-cell receptor, ligation-associated c-CBL phosphorylation and proliferation in Jurkat T-cell leukemia cells. Because AG957 mimics the peptide substrate(s) of p210bcr/abl and c-CBL is a substrate of this kinase (43, 44), the effects on Jurkat cells most likely resulted from inhibition of other kinases that phosphorylate c-CBL. Nonetheless, these observations raised the possibility that AG957 might not display useful selectivity for CML cells. Accordingly, the present studies were performed to determine the fate of AG957-treated K562 cells and assess the selectivity of AG957 for CML cells compared with normal myeloid progenitors. A preliminary account of this work was presented in abstract form (45).

MATERIALS AND METHODS

Materials. AG957 and its analogues were synthesized by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment and Diagnosis, National Cancer Institute (Bethesda, MD) using procedures that will be described elsewhere. Recombinant human erythropoietin, G-CSF and GM-CSF from R & D Systems (Minneapolis, MN) were reconstituted as 10× stocks in PBS containing 0.1% (w/v) BSA and frozen in small aliquots at −70°C. Bacto agar and Sea Plaque agar for colony-forming assays were from Difco (Detroit, MI) and FMC (Rockland, ME), respectively. Antibodies to phosphotyrosine and c-abl were purchased from Upstate Biotechnology (Lake Placid, NY) and Oncogene Research (Cambridge, MA), respectively. SCH66336 was kindly provided by Dr. W. R. Bishop (Schering-Plough Research Institute, Kenilworth, NJ). Wortmannin and PD98059 were from Alexis (San Diego, CA). Antibodies against the neoeptope at the COOH terminus of the large subunit of active caspase-9 were generated by injecting rabbits with the synthetic peptide CPEPD coupled to keyhole limpet hemocyanin and characterized as reported recently (46). All other materials were obtained as described previously (20).

Screening Assays for Inhibition of Cell Proliferation and p210bcr/abl Kinase Activity. The ability of a series of AG957 analogues to inhibit K562 cell proliferation was evaluated using a 6-day continuous exposure to drug, followed by measurement of MTT reduction (34). It is important to note that this assay does not distinguish between cytostatic and cytotoxic effects in continuously proliferating cultures. Immune complex kinase assays of p210bcr/abl autokinase activity were performed as described previously (36).

Culture of K562 Cells. K562 cells were purchased from American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 containing 5% (v/v) FBS, 100 units/ml penicillin G, 100 μg/ml streptomycin, and 2 mM glutamine (medium A) at densities of <1×10⁶ cells/ml to insure logarithmic growth. To assess the effect of various agents on colony formation, aliquots containing ~0.5×10⁵ cells/ml were incubated for 24 h with increasing concentrations of AG957, NSC 676448, NSC 680410, SCH66336, wortmannin, or PD98059 (added from 1000-fold concentrated stocks in DMSO or ethanol) in medium A, sedimented at 100× g for 5 min, diluted, and plated in
gridded 35-mm plates in the medium of Pike and Robinson (47) containing 0.3% (w/v) Bacto agar. After incubation for 10–14 days at 37°C, colonies containing ≥50 cells were counted on an inverted phase contrast microscope. Untreated K562 cells had a plating efficiency of ~40% under these conditions.

To examine the effect of higher NSC 680410 concentrations on proliferative ability, aliquots containing 5 × 10⁶ log phase K562 cells in 10 ml of medium A were exposed to the indicated drug concentration for 24 h, sedimented at 100 × g for 5 min, washed once with medium A, and incubated in 50 ml of drug-free medium A for the duration of the experiment. The number of nonapoptotic cells was determined at various intervals. To provide a standard curve for cell survival, aliquots containing 10¹–10⁷ untreated K562 cells were cultured in 50 ml of medium A and examined in parallel. The number of days required for drug-treated samples to reach a density of ~1 × 10⁶/ml was compared with the number of days required for various aliquots of untreated cells to reach the same density.

Evaluation of Biochemical Changes in Drug-treated K562 Cells. Cells were incubated for 8 h with increasing concentrations of AG957 or analogues, sedimented at 200 × g for 10 min, washed in serum-free RPMI 1640 containing 10 mM HEPES (pH 7.4 at 21°C), and lysed in 6 M guanidine hydrochloride under reducing conditions (48). Alternatively, cells were incubated with 29 μM AG957 for the indicated length of time and lysed under identical conditions. After samples were prepared for SDS-PAGE (48, 49), aliquots containing 50 μg of protein [quantitated by the bicinchoninic acid method (50)] were separated on gels containing 5–15% polyacrylamide gradients and fractionated as described previously (20). Alternatively, AG957-treated cells were washed, lysed, and fractionated as described previously (20). Aliquots containing 50 μg of cytosolic (280,000 × g supernatant) protein were assayed for cleavage of DEVD-AFC as described (49).

For morphological analysis, AG957-treated cells were fixed in 3:1 methanol:acetic acid, stained with 1 μg/ml Hoechst 33258 in 50% (v/v) glycerol, and examined under epi-illumination using a Zeiss Axioplan microscope equipped with a N.A. 1.40 63× objective, a 365-nm excitation filter, and a 420-nm emission filter. Three hundred to 600 cells/sample were scored for apoptotic changes (peripheral chromatin condensation or nuclear fragmentation).

Transfection. Log phase K562 cells were transfected with 40 μg of cDNA encoding GFP-dn caspase-9 (51), GFP-dn FADD (52), or GFP alone. Transfections were performed using a T840 square wave electroporator (BTX, San Diego, CA) delivering a 310-V pulse for 10 ms. After a 24-h incubation in medium A, 30–35% of the cells displayed GFP fluorescence. The brightest 10–12% of the total cell population was isolated by flow cytometry; incubated with diluted, 29 μM AG957, or 68 μM etoposide in medium A for 24 h; washed; incubated in drug-free medium A for 24 h; fixed; and evaluated for apoptotic morphological changes as described above.

Immunoprecipitation. Cells treated with AG957 for the indicated length of time (1 × 10⁷/aliquot) were washed twice with ice-cold PBS and resuspended in 1 ml of ice-cold lysis buffer consisting of 150 mM NaCl, 50 mM HEPES (pH 7.5), 5 mM MgSO₄, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM sodium PPi, 100 mM sodium fluoride, 10% (v/v) glycerol, 1% (w/v) thioglycollate, and 1% (w/v) Triton X-100 supplemented immediately before use with 1 mM α-phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. All further steps were performed at 4°C. After a 15-min incubation, samples were sedimented at 12,000 × g for 5 min. Supernatants were reacted overnight with 5 μg of monoclonal anti-c-abl antibody, diluted with 30 μl of preswelled protein A-Sepharose beads, and incubated for an additional 2 h with gentle agitation. The beads were sedimented at 3200 × g for 2 min, washed four times with 1-ml aliquots of wash buffer [150 mM NaCl, 20 mM HEPES (pH 7.5), 1 mM sodium orthovanadate, 10% (v/v) glycerol, 0.1% (w/v) Triton X-100, and 1% (w/v) thioglycollate], and eluted by heating for 20 min at 65°C with 50 μl of SDS sample buffer consisting of 4 M urea, 2% (w/v) SDS, 62.5 mM Tris-HCl (pH 6.8 at 21°C), 1 mM EDTA, and 5% (v/v) β-mercaptoethanol. One-third of each immunoprecipitate was subjected to electrophoresis and immunoblotting as described above.

Hematopoietic Colony-forming Assays. Clinical samples were studied under the aegis of protocols approved by the Institutional Review Board of the Mayo Clinic in accordance with the policies of the United States Department of Health and Human Services. To compare the effect of AG957 on CML and normal progenitors, 10 ml of peripheral blood were drawn from 11 normal volunteers and 11 consenting patients with chronic phase CML using EDTA as an anticoagulant. Because cells from one patient and the corresponding normal volunteer failed to form colonies in these assays, results obtained with the remaining 10 pairs of samples are presented. In a few of the experiments, some progenitors were not assayed because of limited monoclonal cell recovery.

The 10 CML patients providing samples were previously untreated (7 patients) or had previously received hydroxyurea and IFN (3 patients) but were off treatment for a minimum of 6 weeks at the time samples were obtained. Blood samples diluted with PBS were fractionated on Ficoll-Hypaque step gradients (d = 1.079 and 1.119 g/cm³) as described (53). Cells collected from the upper interface were diluted with Iscove’s modified Dulbecco’s medium containing 20% (v/v) FBS (medium B). Cells were sedimented at 200 × g for 10 min and resuspended in medium B. To assay progenitors, aliquots were cultured as follows. For CFU-E, 4 × 10⁵ cells in medium B modified to contain 30% FBS, 1% (w/v) BSA, 0.1 μM β-mercaptoethanol, 2 units/ml erythropoietin, and 0.3% (w/v) Sea Plaque Agar were cultured for 7–10 days; for CFU-G, 1 × 10⁶ cells in medium B containing 50 ng/ml G-CSF and 0.3% (w/v) Sea Plaque Agar were cultured for 7–10 days; and for CFU-GM, 2.5 × 10⁵ cells in medium B containing 25 ng/ml GM-CSF and 0.3% (w/v) Sea Plaque agar were cultured for 14 days. Replicate aliquots containing 1.25, 2.5, 3.75, 5.0, and 6.25 μg/ml (4.6, 9.1, 13.7, 18.2, and 22.8 μM) AG957 were simultaneously plated to determine the effect on progenitor cell proliferation. At the indicated times, plates were inspected on an inverted phase contrast microscope, and colonies containing ≥32 cells were counted. Colony numbers in the drug-treated plates were divided by colony numbers in the diluent-treated cultures to determine relative colony formation. From the linear portion of each dose-response curve, the
IC$_{50}$ was estimated by linear interpolation. For each of the types of progenitor cell assays, the distributions of IC$_{50}$s for controls and CML patients were compared using a two-sided Mann-Whitney-Wilcoxon U test (54).

To examine the effect of AG957 analogues, peripheral blood mononuclear cells from three CML patients (two previously untreated, one previously treated) and three normal controls were plated with increasing concentrations of AG957 and its analogues under conditions that allowed proliferation of CFU-G. Colonies were enumerated as described above.

RESULTS

AG957 Down-Regulates p210$^{bcr/abl}$ and Induces Apoptosis in K562 Cells. Treatment of continuously proliferating K562 cells with AG957 for 6 days decreased the number of viable cells detectable by MTT assays (Fig. 1 and Ref. 34). Efforts to determine whether this reflected a cytostatic or cytotoxic effect formed the starting point for the present study.

To address this issue, the biochemical consequences of AG957 treatment were examined at earlier time points. In initial experiments, p210$^{bcr/abl}$ was immunoprecipitated from cytosol of K562 cells using an anti-abl antibody. Consistent with previous findings (36), tyrosine phosphorylation of p210$^{bcr/abl}$ (Fig. 2A, upper panel) was below the limit of detection within 1 h of AG957 treatment. Reaction of a duplicate membrane with anti-abl antibody (Fig. 2A, lower panel) revealed that levels of p210$^{bcr/abl}$ diminished in AG957-treated cells concomitant with the appearance of higher molecular weight species.

Because a major fraction of the p210$^{bcr/abl}$ kinase present in K562 cells is tightly associated with cytoskeletal elements (55), the preceding experiment detected only a portion of the total cellular p210$^{bcr/abl}$ pool. Whole cell lysates were prepared under denaturing conditions and subjected to immunoblotting with the same antibodies. Results of this analysis (Fig. 2B) were notable in two respects: (a) high molecular weight complexes containing p210$^{bcr/abl}$ were difficult to discern in whole-cell lysates prepared from AG957-treated cells (Fig. 2B, second panel); (b) the time course for down-regulation of the total cellular pool of p210$^{bcr/abl}$ was slower than the down-regulation of the Triton X-100 soluble pool. Decreased tyrosine phosphorylation of p210$^{bcr/abl}$ kinase was evident within 1 h (Fig. 2B, upper panel), but the magnitude of this effect was less dramatic in whole-cell lysates than in cytosol (Fig. 2, compare A and B, upper panels). By 2 h after addition of AG957, decreased levels of the bcr/abl polypeptide itself were also evident in the whole-cell lysates (Fig. 2B, second panel), but the effect was again less dramatic. The longer time period required for the changes in Fig. 2B to become as large as those in Fig. 2A appears to reflect the slower loss of p210$^{bcr/abl}$ from the cytoskeletal compartment. Nonetheless, the whole cell changes paralleled the changes observed in the cytosolic pool of p210$^{bcr/abl}$.

This down-regulation of bcr/abl was followed by a series of additional biochemical changes. The first of these was a change in the tyrosine phosphorylation of other cellular polypeptides. A number of species, including the $M_1$ 130,000 cytoskeleton protein paxillin, the $M_1$ 120,000 signaling intermediate c-CBL, the $M_1$ 62,000 docking protein p62$^{Dok}$, and the $M_1$ 39,000 adaptor protein CrkL, are directly phosphorylated by p210$^{bcr/abl}$ (33, 44, 56), thereby producing extremely strong signals for phosphory-

5 Unpublished observations.
Blotting with anti-phosphotyrosine antibodies revealed decreased tyrosine phosphorylation of several cellular polypeptides between 2 and 8 h after addition of AG957 (Fig. 2B, top panel), suggesting that inhibition of p210 bcr/abl kinase activity was followed by turnover of phosphate on downstream signal transducers. At later time points, apoptotic changes were also observed. By 8 h, caspase(s) capable of hydrolyzing the fluorogenic substrate DEVD-AFC were detectable in cytosol from AG957-treated cells (Fig. 3A). PARP cleavage, which reflects caspase activation in situ (57, 58), was likewise present by 8 h (Fig. 3A, inset). DNA degradation also occurred, as evidenced by the detection of cells with subdiploid DNA content after fixation in ethanol and staining with propidium iodide (Fig. 3B). Consistent with these biochemical changes, AG957-treated cells displayed apoptotic morphological changes, including chromatin condensation and nuclear fragmentation (Fig. 3C). The number of cells displaying these morphological changes continued to increase for at least 24 h after removal of AG957 (Fig. 3D), indicating that continued presence of AG957 was not required once this agent set into motion biochemical changes that culminated in apoptosis.
AG957 Triggers the Cytochrome c/Apaf-1/Caspase-9 Pathway in K562 Cells. Previous studies (reviewed in Refs. 58 and 59; see also Refs. 20, 21, 49, 60, and 61) have demonstrated that a variety of chemotherapeutic agents trigger apoptosis in susceptible cells by inducing mitochondrial release of cytochrome c, which interacts with the cytosolic docking protein Apaf-1, thereby facilitating activation of procaspase-9 and subsequent proteolytic processing of procaspase-3 and procaspase-7. Alternatively, several studies have suggested that activation of a death receptor pathway might be involved in drug-induced apoptosis. In particular, treatment of certain cell lines with chemotherapeutic agents such as doxorubicin, etoposide, methotrexate, bleomycin, and 5-fluorouracil has been observed to enhance expression of CD95 ligand (62–66), which

Fig. 4 Evaluation of the apoptotic pathways activated by AG957 in K562 cells. A, K562 cells were incubated with the indicated concentration of AG957 for 24 h in the absence or presence of 1 μg/ml ZB4, an antibody that blocks CD95 ligation. At the completion of the incubation, the cells were sedimented and resuspended in drug-free medium ± ZB4 for an additional 24 h, fixed, stained with Hoechst 33258, and examined for apoptotic changes by fluorescence microscopy. Inset, Jurkat cells treated with 20 ng/ml CH-11 cross-linking anti-CD95 antibody for 24 h in the absence or presence of 1 μg/ml ZB4. B and C, 24 h after transfection with the indicated plasmid, GFP-expressing K562 cells were collected by flow cytometry; treated for 24 h with 29 μM AG957, 68 μM etoposide, or diluent; washed; incubated in drug-free medium for 24 h; fixed; and examined for apoptotic morphological changes as illustrated in Fig. 3C. D, after K562 cells were treated with 29 μM AG957 for the indicated period of time, cytosol was prepared and subjected to immunoblotting with anti-cytochrome c (α-cyt c) antibodies or (as a loading control) anti-glutathione S-transferase (α-GST)p antiserum. Cytochrome c is detectable in cytosol 8 h after the addition of AG957 but not in control cells. E, whole-cell lysates prepared from cultures treated with 29 μM AG957 for the indicated length of time were subjected to SDS-PAGE followed by immunoblotting with antisera that recognizes the abundant Mr 38,000 nucleolar protein B23 was performed to confirm equal loading and transfer of the samples.
bounds to the cell surface receptor CD95 (67, 68), causing recruitment of the intracellular adaptor molecules FADD and FLASH, binding and activation of procaspase-8, and subsequent proteolytic activation of caspase-3 and caspase-7. It has been proposed that this alternative pathway plays a major role in chemotherapy-induced apoptosis, although results obtained in mice containing targeted deletions of procaspase-8 (69) or FADD (70) clearly indicate that at least some drugs can induce apoptosis independent of this pathway.

Several different experiments were performed to determine which of these two prototypic pathways is activated by AG957. First, cells were treated with AG957 in the presence of ZB4, an antibody that inhibits CD95 ligation under a variety of circumstances (71–73). Although this antibody readily inhibited apoptosis induced in Jurkat T-cell leukemia cells by the cross-linking of anti-CD95 antibody CH-11 (Fig. 4A, inset), it had no effect on AG957-induced apoptosis in K562 cells (Fig. 4A), suggesting that the CD95 pathway is not involved in the latter process. Consistent with this conclusion, transfection with cDNA encoding dn FADD (52), a truncated adapter protein that inhibits lethal signaling from all previously characterized death receptors (74), likewise failed to block AG957-induced apoptosis in K562 cells (Fig. 4B).

To determine whether the cytochrome c/Apaf-1/caspase-9 pathway was required for AG957-induced apoptosis, cells were transfected with cDNA encoding dn caspase-9 (51) prior to AG957 treatment. In contrast to dn FADD, dn caspase-9 inhibited AG957-induced apoptosis (Fig. 4C). In additional experiments with untransfected cells, mitochondrial release of cytochrome c and activation of caspase-9 were examined by immunoblotting. Within 8 h after the addition of AG957, cytochrome c was detectable in cytosol (Fig. 4D). Moreover, a recently described antiserum that selectively recognizes the active forms of caspase-9 (46) detected increased levels of active caspase-9 species in AG957-treated cells by 8 h (Fig. 4E). Collectively, these results suggest that AG957 is triggering apoptosis through the cytochrome c/Apaf-1/caspase-9 pathway rather than a death receptor/FADD/caspase-8 pathway.

**Effect of AG957 on Colony-forming Ability of K562 Cells.** In anticipation of assessing the effect of AG957 on CML hematopoietic precursors, K562 cells were incubated for 24 h with increasing concentrations of AG957, washed, and plated in soft agar. Results of this experiment indicated that 6 ± 2 μM AG957 (mean ± SD, n = 4) inhibited the formation of K562 colonies by 50% (Fig. 5A). Interestingly, inhibitors of downstream signaling molecules, including the protein farnesyl transferase inhibitor SCH66336 (75), the PI3k inhibitor wortmannin (76, 77), and the MEK1 inhibitor PD98059 (78), had minimal effects on the same cells in clonogenic assays (Fig. 5, B–D). These observations suggest that inhibition of p210^bcr/abl kinase is singularly effective at inhibiting proliferation of these cells.

**AG957 Selectively Inhibits Myeloid Progenitors from CML Patients.** As indicated in the “Introduction,” AG957 was recently observed to inhibit T-cell receptor, ligation-induced proliferation of Jurkat cells (42). Additional experiments indicated that high concentrations of AG957 inhibited colony formation by HL-60 acute myeloid leukemia cells.6 These observations raised the concern that AG957 might not exhibit selectivity for CML cells. To address this issue, circulating hematopoietic progenitor cells from CML patients and normal controls were simultaneously examined for the ability to form colonies in the presence of AG957. Because of limited cell recovery in some samples, not all hematopoietic progenitor assays were performed on all samples. Results of these assays are illustrated in Fig. 6, A–C and summarized in Fig. 6D.

AG957 selectively inhibited CFU-G from CML patients as compared with concurrently examined normal donors (Fig. 6, A–D). The median IC_{50} in the CML samples was 7.3 μM (range, 1.0–18 μM; n = 9), whereas all eight normal marrow samples had an IC_{50} in excess of 20 μM. Analysis using the Mann-Whitney-Wilcoxon U test revealed that IC_{50}s in the CML specimens were significantly lower than in normal CFU-G (U’ = 72; P < 0.001).

Likewise, AG957 displayed selectivity for CFU-GM from CML patients (Fig. 6, B and D). The median IC_{50} in the CML samples was 5.3 μM (range, 1.5 to >20 μM; n = 10), whereas it was ≥18 μM in seven of nine normal specimens. Once again,

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6 P. A. Svingen and S. H. Kaufmann, unpublished observations.
statistical analysis revealed that IC₅₀ s were significantly lower in the CML specimens (U₉₅ 71.5; P < 0.05). Erythroid progenitors were decidedly less sensitive to the inhibitory effects of AG957 as compared with myeloid precursors (Fig. 6, C and D). The median IC₅₀ was 15.5 μM (range, 4 to >18 μM; n = 9) in CFU-E from CML samples and ≥20 μM in CFU-E from normal samples. There was again a trend toward lower IC₅₀ s in the CML specimens, but this did not reach statistical significance.

**NSC 680410 Demonstrates Enhanced Potency.** While the preceding experiments were in progress, a series of AG957 analogues was synthesized and evaluated for biological activity. To determine whether the results described above reflected AG957-induced changes in p210bcr/abl, we examined the effects of AG957 analogues that displayed widely disparate effects on p210bcr/abl kinase activity and K562 cell proliferation in screening assays. In particular, the dimethoxy analogue NSC 676448 was inactive as a bcr/abl kinase inhibitor and notably less potent than AG957 in MTT assays (Fig. 1). In contrast, the adamantyl ester NSC 680410 was more potent than AG957 in the MTT assays, although it was less potent than AG957 as a kinase inhibitor in vitro.

The comparative effects of these compounds on p210bcr/abl levels in K562 cells were evaluated by immunoblotting (Fig. 7A). In contrast to AG957, which induced a decrease in bcr/abl polypeptide levels at concentrations as low as 7 μM (Fig. 7A, Lane 2), incubation for 8 h with the dimethoxy analogue NSC 676448 at 53 μM had no effect on p210bcr/abl content (Fig. 7A, Lane 8). NSC 680410, on the other hand, was at least 3-fold more potent than AG957 at inducing down-regulation of p210bcr/abl in intact cells (Fig. 7A, compare Lanes 2 and 11).

The effects of the three analogues on K562 colony formation (Fig. 7B) paralleled their effects on p210bcr/abl levels in intact cells. In particular, NSC 676448 was almost without effect in colony-forming assays, whereas NSC 680410 was 3.8 ± 2.2-fold (n = 4) more potent than AG957. In further studies designed to examine antiproliferative effects at higher concentrations, NSC 680410 prevented K562 cell outgrowth without any evidence of a plateau in the dose-response curve over at least five logs of cell killing (Fig. 7C). Additional

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7 E. Sausville, manuscript in preparation.
experiments indicated that NSC 680410 was likewise a more potent inducer of apoptosis in K562 cells (Fig. 7B, inset).

In a final series of assays, the effects of the same agents on formation of CML CFU-G were examined (Fig. 8). Once again, relative potencies NSC 680410, AG957, and NSC 676448 (Fig. 8A) paralleled their effects on p210<sup>bcr/abl</sup> levels (Fig. 7A), with NSC 680410 being more potent than AG957 and NSC 676448 being much less potent. In addition, NSC 680410 was found to be more selective (Fig. 8B). In particular, this agent was slightly stimulatory to normal CFU-G at concentrations that decreased CML CFU-G by at least a log (Fig. 8B).

DISCUSSION

The present study examined the effects of AG957 and related tyrphostins on the p210<sup>bcr/abl</sup>-positive K562 cell line and on hematological progenitors in vitro. Interest in AG957 stems from recent reports that this agent is a selective inhibitor of p210<sup>bcr/abl</sup> signaling (34) that acts by a novel mechanism (36). Results of the present study not only demonstrate that AG957 induces apoptosis in K562 cells, but also indicate that AG957 shows selectivity for CML myeloid progenitors in vitro. NSC 680410, the adamantyl ester of AG957, appears to be even more potent and selective. When considered in light of the unique mechanism of action of these agents, these observations have several potentially important implications.

Previous studies have demonstrated that apoptosis is inhibited (15, 17, 18, 57, 79) or delayed (20–22, 80) in K562 cells subjected to a variety of prosapoptotic stimuli. Upon treatment with high doses of etoposide or cisplatin, for example, K562 cells do not develop apoptotic changes for 2–4 days, respec-
Effect of AG957 Analogues on CML

AG957 induces a sequence of biochemical changes that culminates in apoptotic events within 8 h. Inhibition of p210(bcr/abl) kinase activity is rapidly followed by down-regulation of p210(bcr/abl) levels and inhibition of the tyrosine phosphorylation of presumed downstream targets (Fig. 2B). These events are then followed by release of cytochrome c from mitochondria to cytosol (Fig. 4D), activation of caspase-9 (Fig. 4E) and caspase-3 (not shown), cleavage of caspase substrates (Fig. 3A), DNA fragmentation (Fig. 3B), and development of apoptotic morphological changes (Fig. 3C). Because AG957 down-regulates p210(bcr/abl) directly rather than inhibiting its synthesis, these events proceed more rapidly than bcr/abl antisense oligonucleotide-induced apoptosis (39). The mechanism by which these changes are induced is presently under investigation. Preliminary results indicate that AG957 induces apoptosis without changing the levels or subcellular distribution of Bcl-2, Bcl-xL, Bax, Bak, or Bad and without altering the phosphorylation state of procaspase-9. Although the mechanism of cytochrome c release after AG957 treatment remains to be determined, these observations nonetheless demonstrate that bcr/abl inhibition is accompanied by decreased tyrosine phosphorylation of cellular polypeptides, followed by caspase-9 activation and cell death.

To confirm that the effects of AG957 are mediated through p210(bcr/abl), we examined two closely related analogues, NSC 676448 and NSC 680410. NSC 676448, which had no effect on p210(bcr/abl) levels (Fig. 7A), was much less toxic (Fig. 7B). In contrast, NSC 680410 was 3- to 4-fold more potent than AG957 in down-regulating p210(bcr/abl) (Fig. 7A) and inhibiting colony formation (Fig. 7B), although it was a less potent inhibitor of bcr/abl kinase under cell-free conditions (Fig. 1). These results might be explained by an enhanced ability of the more lipophilic adamantyl ester to enter intact cells. Although additional experiments are required to confirm this explanation, these present observations indicate that down-regulation of p210(bcr/abl) levels in intact cells, rather than inhibition of kinase activity under cell-free conditions, is a better predictor of antiproliferative potency within this class of compounds.

Additional studies (not shown) examined further structural requirements for down-regulation of p210(bcr/abl) and induction of cytotoxicity. Replacement of the two hydroxyl groups in AG957 with carbonyl groups resulted in a quinone (NSC 676537) that exhibited poor inhibition of p210(bcr/abl) in immune complex kinase assays but caused down-regulation of p210(bcr/abl) in intact cells at extracellular concentrations that were only 2-fold higher than AG957. The quinone moiety of this analogue was, of course, capable of reduction to yield AG957 in the reducing environment of the cell, although we cannot rule out the possibility that other mechanisms (e.g., oxidative stress attributable to the quinone ring system) might also contribute to the cytotoxicity of NSC 676537.

In subsequent experiments, the potential selectivity of AG957 and its analogues was examined by comparing their effects in committed hematopoietic progenitors from CML patients and normal volunteers. Because myeloid progenitors are relatively rare (<1% of circulating mononuclear cells), it was not possible to evaluate the effect of AG957 using the biochemical and morphological assays applied to cell lines. Instead, colony-forming assays were used to assess the proliferation of these rare progenitors in the absence and presence of AG957 (Fig. 6). These assays demonstrated that CFU-G from all nine CML patients were more sensitive than the eight normal controls. Likewise, CFU-GM from 9 of 10 CML patients were more sensitive than CFU-GM from seven of nine normal patients. Moreover, when AG957, NSC 676448, and NSC 680410 were compared, the potency of these compounds in clinical samples (Fig. 8A) paralleled their ability to down-regulate p210(bcr/abl) and kill K562 cells (Fig. 7, A and B). Of particular interest were the effects of NSC 680410, which had no detectable inhibitory effect on normal CFU-G at concentrations that inhibited CML CFU-G by 90% (Fig. 8B). In further studies, NSC 680410 not only displayed selectivity for bcr/abl-transfected murine 32D leukemia cells when compared with the parental line9 but also

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9 B. Mow and S. H. Kaufmann, unpublished observations.
displayed superior activity against CML cell lines in vivo in the hollow fiber assay (81). These studies do not, of course, formally evaluate the specificity of AG957 or NSC 680410 for p210(BCR/ABL) vs. various other tyrosine kinases. We note, however, that the selectivity of these compounds for CML progenitors in the colony-forming assays (Figs. 6 and 8) implies selectivity for p210(BCR/ABL) relative to the kinases involved in G-CSF- or GM-CSF-induced proliferation in normal cells.

After the present studies were completed (45), Carlo-Stella et al. (82) reported that AG957 selectively inhibited growth of multilineage colony-forming units, erythroid burst-forming units, CFU-GM, and long-term culture-initiating cells from patients with CML. Our observations complement and extend these results by demonstrating for the first time that down-regulation of p210(BCR/ABL) activates the cytochrome c/Apaf-1/caspase-9 pathway, by using structural analogues to provide evidence that the effects of AG957 are mediated through p210(BCR/ABL) and by identifying NSC 680410 as a more potent and selective agent that might be worthy of further investigation. At the present time, therapeutic options for patients with CML remain unsatisfactory. Agents such as hydroxyurea control the leukocytosis associated with chronic phase CML but do not delay the progression to accelerated phase or blast crisis (2, 83). IFN-α delays progression of CML in patients in whom it produces major cytogenetic responses (2, 3, 83, 84), but only a small fraction of all IFN-treated patients have major cytogenetic responses. Allogeneic bone marrow transplantation can be curative in a high percentage of patients with chronic phase CML, but fewer than 30% of CML patients are candidates for this therapy, even when matched unrelated donors are considered (reviewed in Refs. 2, 3, 83, and 84). Finally, high-dose chemotherapy, followed by autologous stem cell rescue, results in 5-year survival of 55–80% when performed during the chronic phase of CML, but disease-free survival after autologous stem cell rescue is poor (reviewed in Ref. 85). A number of observations, including cell marking studies (86), have suggested that bcr/abl-positive cells in the graft contribute to relapse after autologous stem cell rescue. As a result, various strategies for removing CML cells from autografts have been evaluated (85, 87). NSC 680410 has several characteristics that recommend its further evaluation as a potential agent for bcr/abl-directed ex vivo purging, including selectivity for CML-expressing hematopoietic cells, noncompetitive inhibition of bcr/abl-initiated signaling, and relatively rapid induction of cytotoxicity. Additional preclinical studies to evaluate the potential usefulness of this compound as a potential ex vivo purging agent, e.g., time course studies and examination of its effect on more primitive hematopoietic precursors such as marrow repopulating cells, appear to be warranted.

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Effects of the bcr/abl Kinase Inhibitors AG957 and NSC 680410 on Chronic Myelogenous Leukemia Cells \textit{in Vitro}

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