Novel compounds with antiproliferative activity against imatinib-resistant cell lines

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Abstract

Chronic myelogenous leukemia is caused by the *Bcr-Abl* hybrid gene that encodes the p210Bcr-Abl chimeric oncoprotein. Although it reduces the total body burden of leukemia cells, the use of imatinib mesylate as a single agent may be accompanied by the evolution of resistance due mainly to the acquisition of point mutations. Imatinib has been combined with drugs that inhibit both the active and the inactive states of the p210Bcr-Abl kinase. These combinations have reduced but not completely eliminated the rate at which point mutations are acquired in the p210Bcr-Abl kinase. Thus, it is important to identify additional new inhibitors of the p210Bcr-Abl kinase. One possible method to prevent evolution of resistance is to simultaneously use multiple kinase inhibitors each with a different mechanism of action. To identify such a new class of inhibitors that could suppress the growth of chronic myelogenous leukemia cells and prevent the evolution of cells that are resistant to imatinib, we screened two low-complexity libraries of compounds based on planar and linear scaffolds. These libraries were screened using a cell-based assay for molecules that suppress p210Bcr-Abl–dependent cell growth. The application of this method resulted in the isolation of two new classes of drugs, both of which inhibited imatinib-resistant cells in the low micromolar range. Some of these drugs were potent inhibitors not only of Abl tyrosine kinase but also of the Src, Lyn, and Fyn tyrosine kinases. [Mol Cancer Ther 2007;6(2):655–66]

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Introduction

Chronic myelogenous leukemia (CML) is caused by the t(9;22) reciprocal chromosomal translocation that results in the formation of *Bcr-Abl* gene. The protein product of this chimeric gene is the tyrosine kinase p210Bcr-Abl fusion protein that is responsible for the clinical phenotype of CML. This oncoprotein kinase is constitutively active and is located in the cytoplasm. The augmented tyrosine kinase activity of p210Bcr-Abl produces phosphorylation of many downstream substrates.

In the absence of therapy, the genetic instability conferred on CML cells by the p210Bcr-Abl kinase leads to continual evolution of the phenotype of the CML cells from an indolent disorder associated with an elevated circulating myeloid cell mass to a fulminant acute leukemic disorder in which patients die of bleeding and infection (1). Allogeneic bone marrow transplantation was the first curative therapy for CML, which resulted in an overall survival of 50% to 80% of the patients so treated (2). Offsetting this benefit was the transplant-related mortality from acute or chronic complications. IFN resulted in cytogenetic remissions in 20% of low Sokal index patients but at a considerable cost in the quality of life for those individuals so treated (3).

Imatinib, a specific inhibitor of the *Bcr-Abl* tyrosine protein kinase, has revolutionized the therapy of CML because it selectively suppresses the number of leukemia cells without significant toxicity to normal hematopoietic cells (4–6). Offsetting this enormous benefit is the evolution of resistance in chronic- and advanced-phase patients (7, 8). Overexpression of p210Bcr-Abl protein, overexpression of multidrug resistance 1 P-glycoprotein, overexpression of other kinases, such as the Src-related kinases Lyn and Hck, and acquisition of point mutations in the *Bcr-Abl* gene have been shown to contribute to the evolution of clinical resistance to imatinib (7–9). Like the p210Bcr-Abl kinase, Lyn kinase is involved in the response of hematopoietic cells to exposure to DNA-damaging agents, such as ionizing radiation (10).

Several families of compounds have been reported to suppress CML cells following the first reports of imatinib activity (4–11). We investigated synthetic variations of chemical functionalities based on linear or planar scaffolds to identify new compounds that inhibited the p210Bcr-Abl–dependent proliferation of cell lines at low nanomolar concentrations in a highly selective fashion and that suppressed the growth of imatinib-resistant cells *in vitro*

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(12, 13). Two types of scaffold molecules were evaluated in this study, acetylenes, representing a linear scaffold, and furans, representing a planar scaffold, from which chemical functionalities could be projected in a diversity-based approach. In addition, the acetylenes are also precursors of the furan scaffold molecules, which facilitated a 2-fold investigation of the molecules from our combinatorial synthetic strategy (12–14). The results reported in this article show that both the furans and the acetylene scaffolds can be used to generate highly selective inhibitors of CML cells, some of which suppress the growth of imatinib-resistant cell lines.

Materials and Methods

Reagents and Compounds

Murine interleukin-3 (IL-3) culture supplement was provided by BD Biosciences (Bedford, MA). [γ-^{32}P]ATP was obtained from ICN (MP Biomedicals, Salon, OH). Src(p60c-src) and Lyn(p56) partially purified recombinant enzymes and their corresponding buffers and substrates were obtained from Upstate Biotechnology (Lake Placid, NY). Recombinant v-Abl protein tyrosine kinase and reaction buffer were purchased from Calbiochem (EMD Biosciences, San Diego, CA) and New England Biolabs (Ipswich, MA). In the first year of the research, the synthetic Abl peptide substrate EAIYAAPFAAKKK was synthesized at the Yale Keck facility from the sequence provided by Calbiochem. In later years, this peptide was purchased from New England Biolabs. Imatinib mesylate (Gleevec) is commercially available from Novartis (Cambridge, MA).

The acetylenes and furans were synthesized in the laboratory of David J. Austin in the Department of Chemistry at Yale University (New Haven, CT). All compounds underwent high-pressure liquid chromatography purification and mass spectrometry to determine the structural integrity of each compound both at the time of isolation and in a time frame that was proximate to the biological analysis. The acetylenes were created in one step by the coupling of a propiolate anion and isocyanate. These acetylenes were then used as a precursor in the synthesis of the furans.

The furan synthesis was based on early research in the Austin laboratory, which was centered on the concept of adapting the 1,3-dipolar cycloaddition reaction between a diazo ketone and assymmetrically substituted acetylenes using a rhodium(II)-mediated catalysis (13). This synthetic path was part of a larger strategy to create a planar system for presentation of a wide variety of functional groups for molecular recognition. Previous work in the Austin laboratory had suggested that a priority for inhibitory activity of the Bcr-Abl kinase was to clear the region of space on the amide side of the furan so that the molecule could interact with the Bcr-Abl kinase pocket. Although this interaction of these compounds with the kinase was conjecture, the Austin laboratory data on the furans suggested that the absence of steric hindrance on the amide side of the furan would increase the inhibition of the p210Bcr-Abl–dependent growth of cell lines in the low- and high-density proliferation assays.

Cell Lines

Generation of the 32Dtetp210Bcr-Abl Cell Line. The 32D cell line was initially obtained from Joel Greenberg at the University of Pittsburgh School of Medicine (Pittsburgh, PA). The introduction of a 'Tet-off' tetracycline-inducible *Bcr-Abl* transcription unit into the 32D myeloid cell line (which is IL-3 dependent for growth *in vitro*) generated a cell line that was IL-3 independent when Bcr-Abl was expressed in the absence of tetracycline. This cell line was designated 32Dtetp210Bcr-Abl (15, 16).

Imatinib-Resistant Cell Lines. The imatinib mesylate–resistant cell lines Baf/BCR-ABL-r and LAMA84-r and their imatinib-sensitive parental cell lines Baf/BCR-ABL-s and LAMA84-s were derived as described previously (17, 18).

Proliferation Assays

Low-Density Cell Proliferation Assay of 32Dtetp210Bcr-Abl and 32D Cell Lines in the Presence and Absence of Test Drugs. Between 50 and 100 32D and 32Dtetp210Bcr-Abl cells in medium without serum were exposed to the test compound for 15 min. An equal volume of 20% serum-supplemented tissue culture medium was then added, and the cells were inoculated in 32 wells of a 96-well plate for a total final volume of 100 μL/well. The end point of the assay was the percentage of the 32 wells filled with live cells after 7 days of incubation at 37°C.

During the growth assays, 32D cells were cultured in the presence of IL-3 and in the absence of tetracycline, whereas the 32Dtetp210Bcr-Abl cell line was grown in the absence of both tetracycline and IL-3. Each experiment was done once. However, a total of 32 wells was monitored for cell growth for each drug. Because each well contained between 50 to 100 cells, the addition of a drug totally suppressed all cell growth or a pellet developed in a well filled with viable cells. The formation of the pellet was the end point of the assay.

If a compound suppressed the growth of 32Dtetp210Bcr-Abl cell line without affecting the growth of the parental 32D cell line, then the compound was considered to be selectively inhibitory for p210Bcr-Abl–dependent growth. Compounds that would be bound to serum proteins due to low solubility in aqueous solvents could be studied because they were solubilized in 0.1% ethanol and added to the cells initially in the absence of serum. Finally, we selected the end point of inhibition of p210Bcr-Abl–dependent proliferation of 32Dtetp210Bcr-Abl cells to be able to identify inhibitors of the p210Bcr-Abl kinase as well as inhibitors of its downstream targets on which the continued expansion of the 32Dtetp210Bcr-Abl cells depends.

3-(4,5-Dimethylthiazol-2-yl)-5-(3-Carboxymethoxyphenyl)-2-(4-Sulfophenyl)-2H-Tetrazolium High-Density Cell Proliferation Assay. Cell proliferation assays of the imatinib-sensitive and imatinib-resistant cell lines were done in triplicate using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS; CellTiter 96 Aqueous, Promega, Madison, WI), which measures the numbers of viable cells. Between 2×10^3 and 2×10^4 imatinib-resistant cells were washed twice in RF-10 and plated in quadruplicate in the wells of a 96-well

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microtiter plate in 100 μL of RF-10 medium supplemented with various doses of test compounds. Controls using the same concentration of imatinib without cells were set up in parallel. The plate was then incubated for 72 h at 37°C. Two hours after MTS addition, the plate was read in a microplate autoreader at a wavelength of 490 nm. Results were expressed as the mean absorbance of the four-well set of each compound dose. All experiments were repeated at least thrice.

Growth Assay Conducted by the National Cancer Institute of the NIH. A panel of human tumor cell lines was screened by the NIH for suppression by AC22 and AC19. A 96-well format was used with cell densities ranging from 5,000 to 40,000 per well. The test drugs were dissolved in DMSO at the stated concentrations and added to the cell cultures. Afterwards, the cell growth was measured by absorbance measurements.

Gel Electrophoresis Assay of Kinase Activity

Compounds identified in the cell density proliferation assays were also tested for inhibition of the v-Abl and Src protein kinases in cell-free in-gel kinase assays. These assays have been carried out by phosphorylating a peptide substrate with [γ-^{32}P]ATP followed by separation of the ^{32}P-peptide product from the unreacted [γ-^{32}P]ATP. The kinase reaction mixture contained the given enzyme with the addition of testing compounds at varying concentrations. After incubation in enzyme buffer for 2 h at 0°C on ice, [γ-^{32}P]ATP, ATP, the protein or peptide substrate were added. The reaction mixture was then incubated at 30°C for 10 min at a final volume of 25 μL/sample. The reaction was terminated by the addition of Tris-tricine sample buffer (Bio-Rad, Hercules, CA) and boiled for 5 min in tightly capped tubes. The reaction products were separated by electrophoresis on Tris-tricine 16.5% gel (Bio-Rad), and the ^{32}P-labeled protein or peptides were visualized by autoradiography. Each assay contained an untreated control, in which a volume of diethylpyrocarbonate-treated water was added to replace the volume of the compound added in the treated samples.

Cell-Free Kinase Inhibition Assay

A 96-well plate assay has been carried out with the v-Abl–dependent kinase colorimetric reaction. Plates were precoated with a random polymer substrate containing multiple tyrosine residues, which may be phosphorylated by protein tyrosine kinases in the sample being assayed. The detector antibody is a purified horseradish peroxidase–conjugated, mouse monoclonal antibody that recognizes phosphotyrosyl residues. The v-Abl kinase inhibitory activity of a compound was detected by a decrease in the intensity of the colorimetric reaction. The results were assayed by a plate reader. The compound was added in triplicate, and concentration curves for each compound were carried out at four concentrations: 10 μmol/L, 1 μmol/L, 100 nmol/L, and 10 nmol/L. The analysis of the untreated control samples was carried out with the addition of diethylpyrocarbonate-treated water. The negative controls were studied without substrate and without compounds and without kinase and without compounds.

Cell-Free Inhibition of a Kinase Panel by K1P and AC22

A panel of 60 kinases (Upstate Biotechnology) was studied for cell-free inhibition to characterize the targets of the acetylene compounds K1P and AC22.

Results

Synthesis of Acetylenes and Furans

Our initial hypothesis was that a planar molecule, such as a furan, could inhibit the p210Bcr-Abl tyrosine-specific protein kinase. This was based on the fact that tyrosine kinases are susceptible to inhibition in the ATP pocket by heterocyclic molecules that possess a flat architecture that can reach within the ATP pocket yet do not disturb the conformational change in the kinase domain as it closes down on the binding molecule, in this case our inhibitor. By flanking this flat, planar scaffold with a wide range of chemical species, we endeavored to discover a p210Bcr-Abl kinase inhibitory molecule that was predicted to contain one or more monomeric functional units.

Early research centered on the concept of adapting the 1,3-dipolar cycloaddition reaction to combinatorial chemistry to create diverse libraries of molecules that could be screened for biological activity (12–14). The initial hypothesis was that the 1,3-dipolar cycloaddition of diazo ketones with activated acetylenes followed by heat induced cycloreversion (see Fig. 1A for this reaction) yielded a planar molecule known as a furan. This could serve as the core of a molecular scaffold from which a diverse array of chemical functionalities could be displayed, thereby generating a low-complexity library of chemicals to be tested as inhibitors of the 32Dtetp210Bcr-Abl cell line. We intended to screen both the acetylene precursors and the furan compounds for their ability to inhibit the 32Dtetp210Bcr-Abl cell line. We therefore synthesized a series of furan molecules from a series of acetylene precursor compounds.

The current lead acetylene molecules, K1P, AC19, and AC22, were selected from a study involving the acetylenic structures shown in Fig. 1B and represent a diverse array of chemical functionality and scaffold manipulation. The inhibitory activity of each chemical for the growth of the 32Dtetp210Bcr-Abl cell line in the low-density cell assay is shown in Table 1.

Relationship of Size of Functional Groups Adjacent to the Acetylenic Ester and Growth Inhibition of the 32Dtetp210Bcr-Abl Cells in the Low-Density Cell Proliferation Assay

Testing of a series of acetylenic scaffolds with different substituent chemical functionalities allowed us to determine the best positions on the linear acetylene scaffold for growth inhibition of the 32Dtetp210Bcr-Abl cell line. As shown in Fig. 2A, both K1P (Fig. 2A, *1*) and AC1 (Fig. 2A, *2*) inhibited the growth of the 32Dtetp210Bcr-Abl cell line to similar degrees in the low-density cell assay despite the difference in the size of the alkyl groups on their ester moieties. K1P contains a methyl ester, whereas AC1 contains a tertiary butyl ester. This suggested that the size of the alkyl groups on the ester portion of the acetylenes

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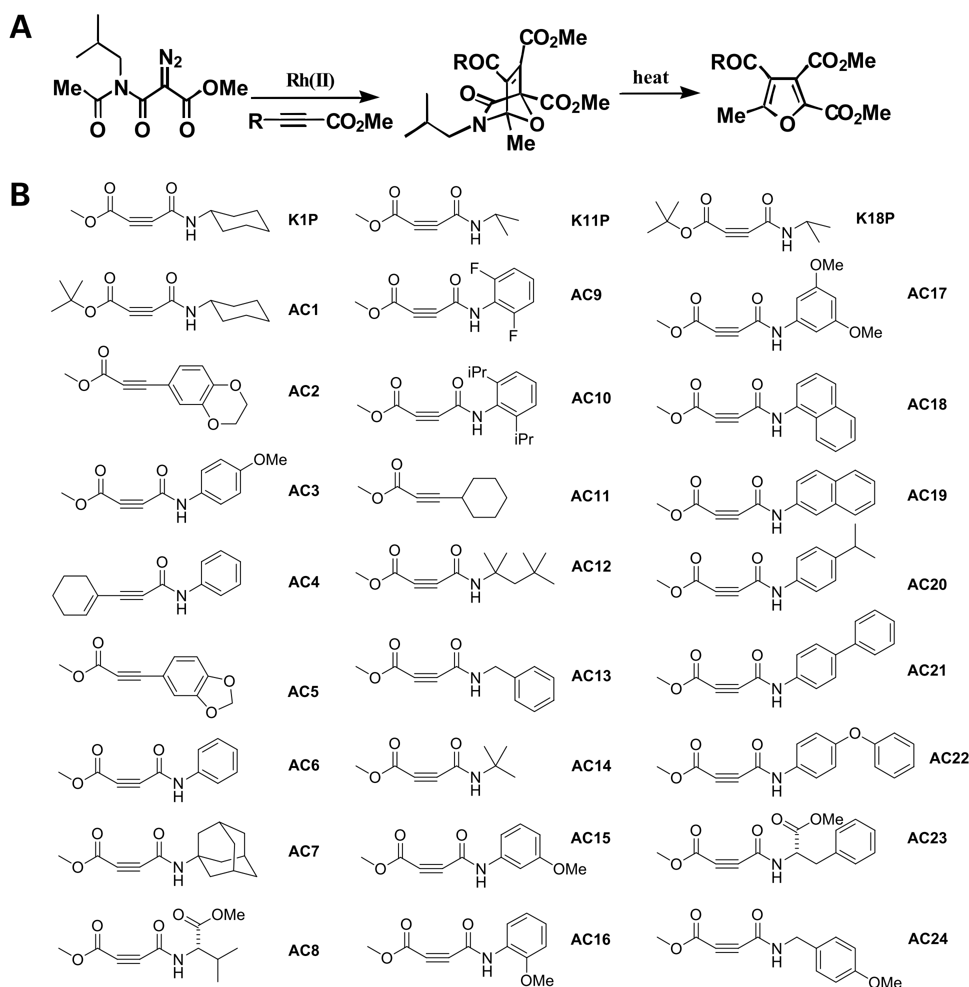

Figure 1. **A,** cycloaddition reactions for synthesis of furans from acetylenes. A rhodium catalyst is used to generate the furan planar scaffold from the acetylene linear scaffold. **B,** library of acetylenes. These compounds were manually synthesized for the analysis of the relationship between substitution of the various sites on the acetylenes and the suppression of p210Bcr-Abl–dependent growth.

was not a crucial part of the scaffold in terms of suppressing the growth of the 32Dtetp210Bcr-Abl cell line. AC1 was much more selectively inhibitory for p210Bcr-Abl–dependent growth than was K1P.

Relationship of Size of Groups on the Acetylenic Amide and Growth Inhibition of 32Dtetp210Bcr-Abl Cells in the Low-Density Cell Proliferation Assay

K1P is inhibitory for the growth of the 32Dtetp210Bcr-Abl cell line in the low-density cell assay, whereas K11P is not (see Table 1). K1P has a cyclohexane on the amine, whereas K11P has a less bulky group at that site (see Fig. 1B). These data suggest that the size of the alkyl groups on the amide may affect the level of inhibitory activity of the acetylenes for the growth of the 32Dtetp210Bcr-Abl cell line.

Importance of the Acetylenic Amide or the Ester Groups for Inhibition of the Growth of the 32Dtetp210 Bcr-Abl Cell Line in the Low-Density Cell Proliferation Assay

We next evaluated the importance of the acetylenic ester or amide groups for growth suppression of the 32Dtetp210Bcr-Abl cell line. K1P (see Fig. 2B, *1*) suppressed 32Dtetp210Bcr-Abl cell growth, whereas AC4 (see Fig. 2B, *2*), which lacks an ester but contains a similar amide group, did not. Conversely, AC2, which contains the ester group but not the amide, was found to be suppressive of the growth of Bcr-Abl–expressing cells in the same assay. Therefore, we can conclude that the ester carbonyl is essential for suppressing the growth of the 32Dtetp210Bcr-Abl cell line. As described in the preceding section, the size of the acetylenic ester substituent itself is less important. In contrast, the size of the group on the acetylenic amide is very important for a compound to inhibit the growth of the 32Dtetp210Bcr-Abl cell line in the low-density cell assay.

Inhibitory Effect of the Acetylenic Compounds K1P, AC19, and AC22 on the Growth of the 32Dtetp210Bcr-Abl Cell Line in the Low-Density Cell Proliferation Assay

Based on the experimental results shown in Figs. 1B and 2A and B and Table 1, three of the acetylenes were chosen for in-depth study: K1P, AC19, and AC22. Pilot experiments (data not shown) had established that 1 μmol/L AC19 suppressed both the proliferation of the parental 32D cell line (in the presence of IL-3) and the 32Dtetp210Bcr-Abl cells (in the absence of IL-3). As shown in Fig. 2C, AC19 at

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100 nmol/L is almost totally inhibitory of the growth of the 32Dtetp210Bcr-Abl cell line in the absence of IL-3. In contrast, AC19 does not suppress the growth of the 32D cell line in the presence of IL-3. Thus, AC19 is selectively inhibitory of the cell line expression of the *Bcr-Abl* gene.

As shown in Fig. 2C, AC22 at 100 nmol/L inhibited the growth not only of the 32Dtetp210Bcr-Abl cell line but also of the parental 32D cells (i.e., its toxicity was nonselective). K1P inhibits the growth of the 32Dtetp210Bcr-Abl cell line at 100 nmol/L as well, with less toxicity to the 32D cell line in the presence of IL-3. In addition, it is clear that neither the K1P nor the AC19 nor the AC22 has a significant inhibitory effect on the growth of the 32Dtetp210Bcr-Abl cell line at the 10 nmol/L concentration when either compound is used alone in the low-density cell growth inhibition assay.

Interaction of the Acetylenes and Imatinib in Suppressing the Growth of the 32Dtetp210Bcr-Abl Cell Line

We then studied the effect of AC19, AC22, and K1P when combined with imatinib on the growth of the 32Dtetp210Bcr-Abl cell line in the low-density cell assay. As shown in Fig. 2C, the combination of AC19 and imatinib (STI571) at a concentration of 10 nmol/L of each drug inhibited the growth of the 32Dtetp210Bcr-Abl cell line by 15%, which is less than the sum of the inhibitory effects of both drugs (25% suppression) when they are used separately.

In contrast, when AC22 and imatinib were combined at a 10 nmol/L concentration of each drug (see Fig. 2C), the growth inhibition of 32Dtetp210Bcr-Abl cells (80% suppression) was greater than the sum of the growth inhibition seen when each of the drugs was used alone (25% suppression by imatinib and 10% by AC22 when used alone). A similar enhanced inhibitory effect on the growth of the 32Dtetp210Bcr-Abl cell line is seen when K1P and imatinib are used together at 10 nmol/L each: at least 45% inhibition is seen for the combination, whereas at the same individual concentration imatinib alone induces only 25% suppression of cell growth and no inhibition is observed for K1P alone.

Table 1. Inhibition of growth of 32D and 32Dtetp210Bcr-Abl by acetylenes in low-density cell proliferation assay

Compound	Tested in serum	Tested serum-free	MIC	Selectivity
AC1	+*	−†	100 nmol/L	Selective
AC2	+	−	10 nmol/L	Selective
AC3	+	+*	10 nmol/L‡	Selective
AC4	+	−	Undetectable	
AC5	−†	+	Not done	
AC6	−	+	100 nmol/L	Selective
AC7	−	+	Not done	
AC8	−	+	100 nmol/L	Selective
AC9	−	+	Not done	
AC10	−	+	100 nmol/L	Not selective
AC11	−	+	Not done	
AC12	−	+	100 nmol/L	Not selective
AC13	+	+	100 nmol/L	Not selective
AC14 (K13P)	−	−	100 nmol/L	Selective
AC15	−	+	100 nmol/L	Not selective
AC16	−	+	Not done	
AC17	−	+	1 μmol/L	Not selective
AC18	−	+	100 nmol/L	Selective
AC19	−	+	100 nmol/L	Selective
AC20	+	−	100 nmol/L	Selective
AC21	−	+	100 nmol/L	Selective
AC22	+	+	100 nmol/L§	Not selective
AC23	+	−	1 μmol/L	Not selective
AC24	−	+	10 nmol/L	Not selective
K1P	+	+	100 nmol/L‡	Selective
K11P	+	−	Undetectable	
K18P	+	−	100 nmol/L	Selective
Imatinib	−	+	100 nmol/L	Selective

NOTE: Selectivity is defined by inhibition of the 32Dtetp210Bcr-Abl cell line in the absence of IL-3, which is twice the level of inhibition seen with the 32D cell line in the presence of IL-3.

Abbreviation: MIC, minimal inhibitory concentration.

*The test was carried out without serum.

†The test was carried out with serum.

‡The minimal inhibitory concentration was carried with serum.

§The minimal inhibitory concentration was carried without serum.

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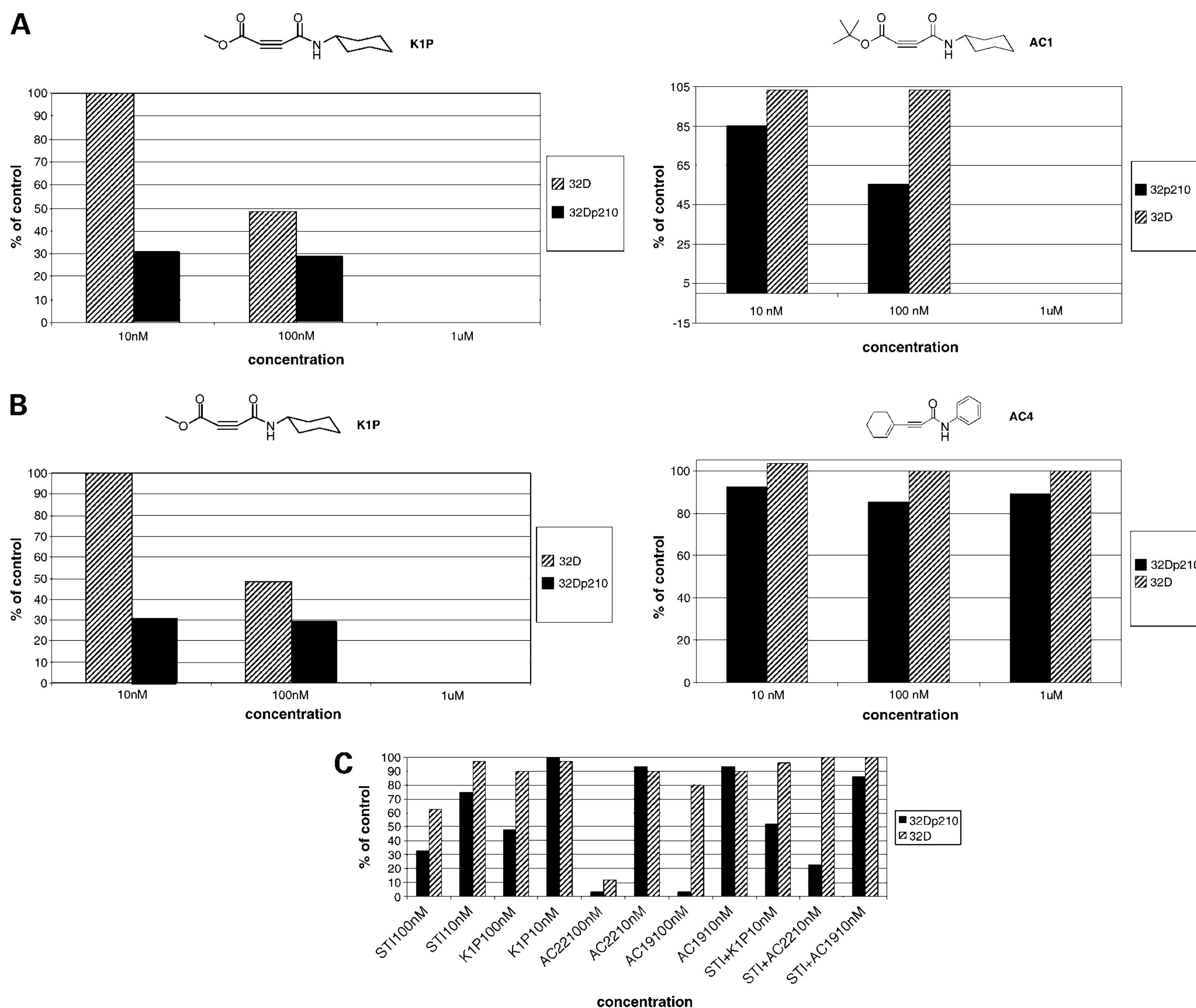

Figure 2. A, effect of substitutions on the inhibitory activity of the acetylenic ester on the growth of the 32Dtetp210Bcr-Abl cell line in the low-density cell proliferation assay. Comparison of the inhibitory effect of K1P (*left*) with AC1 (*right*) acetylenic compounds on the growth of the 32Dtetp210Bcr-Abl cell line was carried out in the low-density cell proliferation assay. 32D is the parental cell line that requires IL-3 for *in vitro* growth. 32Dp210 is the 32Dtetp210Bcr-Abl cell line derived from 32D that is independent of IL-3 *in vitro*. **B,** effect of substitutions on the inhibitory activity of the acetylenic amide on the growth of the 32Dtetp210Bcr-Abl cell line in the low-density cell proliferation assay. Comparison of the inhibitory effect of K1P (*left*) with AC4 (*right*) acetylenic compounds on the growth of the 32Dtetp210Bcr-Abl cell line was carried out in the low-density cell proliferation assay. 32D is the parental cell line that requires IL-3 for *in vitro* growth. 32Dp210 is the 32Dtetp210Bcr-Abl cell line derived from 32D that is independent of IL-3 *in vitro*. **C,** comparison of the suppression of growth of the 32Dtetp210Bcr-Abl cell line by the acetylenes AC19, AC22, and K1P with imatinib mesylate in the low-density cell proliferation assay. The suppressive effect of imatinib mesylate with the acetylenes AC19, AC22, and K1P on the growth of 32Dtetp201Bcr-Abl and 32D in the low-density cell assay was carried out. The acetylenes were tested alone and in combination with imatinib mesylate. 32D is the parental cell line that requires IL-3 for *in vitro* growth. 32Dp210 is the 32Dtetp210Bcr-Abl cell line derived from 32D that is independent of IL-3 *in vitro*.

Effect of K1P, AC19, and AC22 on the Growth of Imatinib-Resistant Cell Lines in the High-Density Cell Proliferation Assay (MTS)

The results with the 32Dtetp210Bcr-Abl cell line in the low-density cell assay suggested that at least some of the acetylenes (AC22 and K1P) might suppress the growth of imatinib-resistant cells. Therefore, we added each of the acetylene drugs (AC19, AC22, and K1P) to cultures (one drug per well) of the Baf/*BCR-ABL*-r and LAMA84-r cell lines, which are resistant to 1 μmol/L imatinib. These cell lines (17, 18) are grown continuously in 1 μmol/L imatinib to maintain the imatinib-resistant phenotype. Parental imatinib-sensitive variants of these cell lines (Baf/*BCR-ABL*-s and LAMA84-s) were maintained in parallel cultures without imatinib to be used as controls.

As shown in Fig. 3 and Table 2, at 1 μmol/L, K1P, AC22, and AC19, respectively, suppress 45%, 65%, and 40% of the growth of the Baf/*BCR-ABL*-r cell line in which the

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mechanism of drug resistance is overexpression of *Bcr-Abl* gene. The ratio of the growth of the Baf/*BCR-ABL*-r cell line (see Fig. 3A) to that of Baf/*BCR-ABL*-s cell line (see Fig. 3B) in the presence of 1 μmol/L imatinib was 2.4. In contrast, the ratios of the growth of the Baf/*BCR-ABL*-r cell line (Fig. 3A) to that of the Baf/*BCR-ABL*-s line (Fig. 3B) in the presence of 1 μmol/L of K1P, AC19, and AC22 were 1.2, 2.2, and 1.3, respectively. This suggested that the level of resistance of the Baf/*BCR-ABL*-r cell line to the K1P, AC19, and AC22 acetylenic drugs was less than to imatinib.

When the inhibitory effect of each acetylenic compound was compared with imatinib at 1 μmol/L, the ratios of the growth of Baf/*BCR-ABL*-r cells in the presence of imatinib divided by their growth in the presence of K1P, AC19, or AC22 were 1.8, 1.5, and 2.5, respectively. This suggests that the acetylenic compounds are more inhibitory to the Baf/*BCR-ABL*-r cell line than is imatinib.

We also tested the effect of K1P, AC19, and AC22 on the growth of the LAMA84-s imatinib-sensitive cell line (see Fig. 3F and Table 2) and the LAMA84-r imatinib-resistant cell line (see Fig. 3E and Table 2), in which the mechanisms of imatinib resistance are overexpression of *Bcr-Abl* and *multidrug resistance 1* genes. The ratio of the growth of the LAMA84-r cell line (Fig. 3E) to that of the LAMA84-s cell line (Fig. 3F) in the presence of 1 μmol/L imatinib was 4.1. In contrast, the ratios of the growth of the LAMA84-r cell line to that of LAMA84-s in the presence of 1 μmol/L of K1P, AC19, and AC22 were 2.8, 2.8, and 2.7, respectively. Although these data suggest that the

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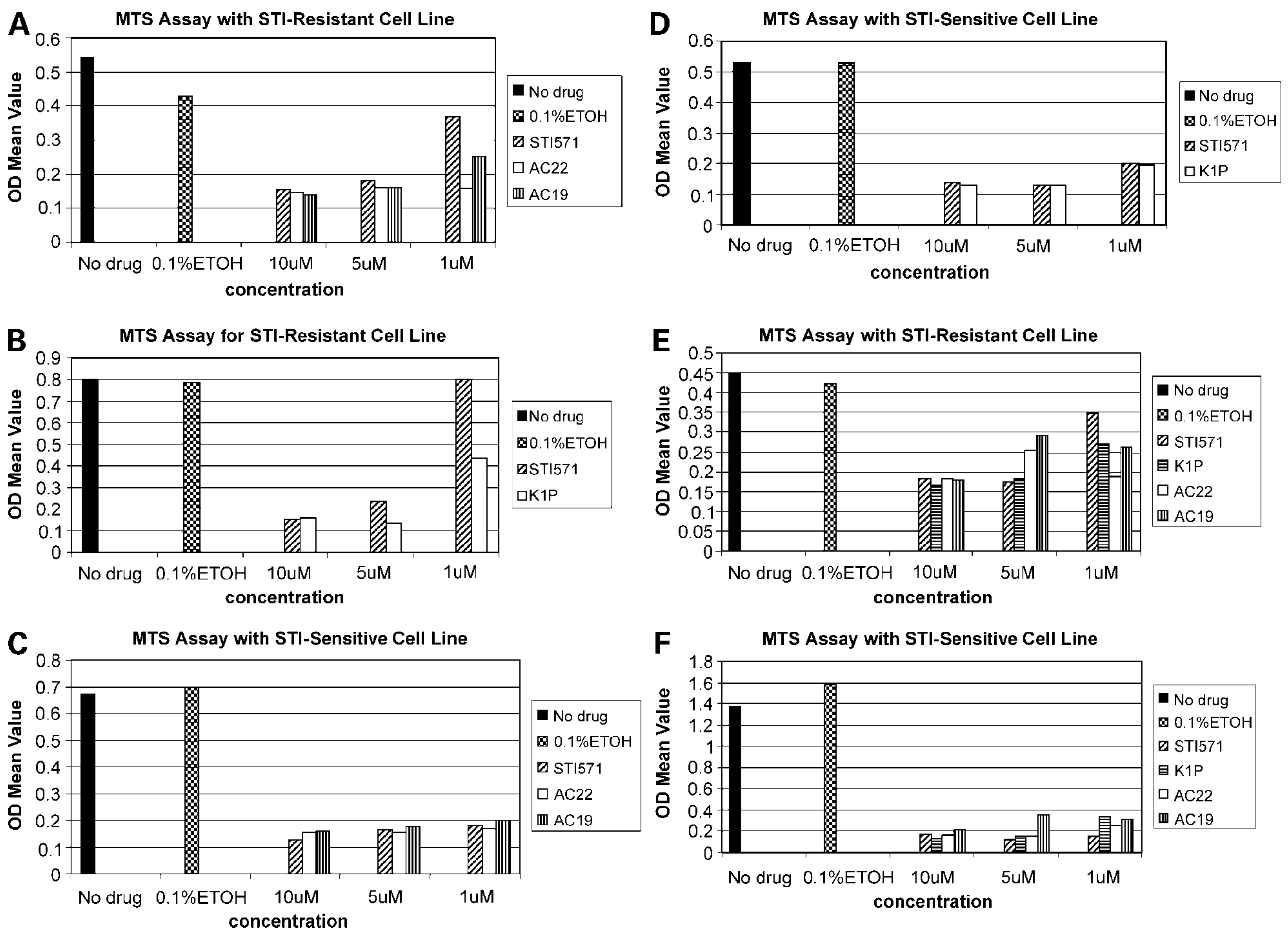

Figure 3. Effect of K1P, AC19, and AC22 acetylenes on the growth of imatinib-resistant and imatinib-sensitive cell lines as assessed by the high-density MTS cell proliferation assay. **A,** Baf/*BCR-ABL*-r comparison of the inhibitory effect of AC19 and AC22 with STI571 (imatinib) on Baf/*BCR-ABL*-r. **B,** comparison of the inhibitory effect of K1P with STI571 (imatinib) on Baf/*BCR-ABL*-r. **C,** comparison of the inhibitory effect of AC19 and AC22 with STI571 (imatinib) on Baf/*BCR-ABL*-s. **D,** comparison of the inhibitory effect of K1P with STI571 (imatinib) on Baf/*BCR-ABL*-s. **E,** comparison of the inhibitory effect of K1P, AC19, and AC22 with STI571 (imatinib) on LAMA84-r. **F,** comparison of the inhibitory effect of K1P, AC19, and AC22 with STI571 (imatinib) on LAMA84-s. The growth of the Baf/*BCR-ABL*-r cells in 1 μmol/L STI571 (imatinib) was significantly greater than the growth in 1 μmol/L of AC19 and AC22 at the $P < 0.05$ level. The growth of the Baf/*BCR-ABL*-r cells in 1 μmol/L AC19 was significantly greater than in 1 μmol/L AC22 at the $P < 0.05$ level. The growth of the LAMA84-r cells in 1 μmol/L STI571 (imatinib) was greater than the growth in 1 μmol/L AC22 at the $P < 0.05$ level. Analysis was not conducted for the significance of differences at >1 μmol/L concentration.

Table 2. Effect of AC19, AC22, and K1P compared with STI571 (imatinib) on the growth of Baf/BCR-ABL-r and LAMA84-r

	Absorbance from MTS assay			Absorbance from MTS assay		
	Baf/BCR-ABL-r (Fig. 3A)			Baf/BCR-ABL-s (Fig. 3C)		
	10 μmol/L	5 μmol/L	1 μmol/L	10 μmol/L	5 μmol/L	1 μmol/L
AC19	0.138	0.163	0.270	0.155	0.179	0.157
	0.119	0.167	0.264	0.171	0.182	0.191
	0.145	0.166	0.255	0.162	0.183	0.212
	0.156	0.149	0.217	0.142	0.158	0.235
Mean AC19	0.140	0.163	0.252	0.158	0.176	0.199
AC22	0.142	0.163	0.159	0.145	0.141	0.163
	0.155	0.162	0.154	0.163	0.148	0.183
	0.144	0.161	0.158	0.158	0.157	0.153
	0.141	0.157	0.159	0.147	0.170	0.153
Mean AC22	0.146	0.161	0.158	0.153	0.154	0.166
STI571	0.164	0.196	0.398	0.121	0.169	0.199
	0.163	0.183	0.398	0.127	0.170	0.191
	0.168	0.183	0.411	0.129	0.164	0.186
	0.122	0.155	0.296	0.136	0.155	0.146
Mean STI571	0.154	0.179	0.389	0.128	0.165	0.181
	LAMA84-r (Fig. 3E)			LAMA84-s (Fig. 3F)		
	10 μmol/L	5 μmol/L	1 μmol/L	10 μmol/L	5 μmol/L	1 μmol/L
AC19	0.194	0.273	0.275	0.186	0.344	0.424
	0.184	0.343	0.256	0.227	0.368	0.296
	0.190	0.311	0.266	0.230	0.350	0.261
	0.147	0.241	0.252	0.213	0.357	0.318
Mean AC19	0.179	0.292	0.262	0.214	0.357	0.318
AC22	0.222	0.279	0.136	0.161	0.170	0.234
	0.218	0.265	0.212	0.159	0.151	0.284
	0.189	0.251	0.195	0.176	0.154	0.258
	0.100	0.218	0.207	0.177	0.157	0.263
Mean AC22	0.182	0.253	0.187	0.168	0.158	0.260
K1P	0.156	0.192	0.197	0.144	0.157	0.291
	0.168	0.208	0.302	0.117	0.158	0.416
	0.173	0.191	0.282	0.126	0.157	0.344
	0.166	0.137	0.300	0.152	0.136	0.291
Mean K1P	0.166	0.182	0.270	0.135	0.152	0.335
STI571	0.205	0.162	0.396	0.203	0.126	0.155
	0.205	0.176	0.389	0.245	0.127	0.157
	0.211	0.175	0.383	0.167	0.132	0.159
	0.106	0.179	0.219	0.093	0.089	0.140
Mean STI571	0.182	0.173	0.347	0.177	0.118	0.153

acetylenes are more suppressive of the growth of the imatinib-resistant LAMA84-r cell line than is imatinib, it is clear that the level of cross-resistance to the acetylenes in the LAMA84-r cell line is greater than that of the Baf/*BCR-ABL*-r line (see above ratios of 1.2, 2.2, and 1.3 for the Baf/BCR-ABL-r/Baf/BCR-ABL-s cell lines compared with the ratios of 2.8, 2.8, and 2.7 for the LAMA84-r/LAMA84-s cell lines).

When the inhibitory effect of the acetylenes at 1 μmol/L on the growth of the LAMA84-r cell line is compared with the growth-suppressive effect of imatinib at 1 μmol/L (as calculated by the ratio of the growth of the LAMA84-r cell line in 1 μmol/L imatinib/growth in each of the acetylenes), the K1P, AC19, and AC22 were 1.3, 1.2, and 1.4 times as potent, respectively, as imatinib. This result suggests that the suppressive effect of the acetylenic drugs in the Baf/BCR-ABL-r line (in which the ratios of the growth in imatinib/growth in the acetylene compounds K1P, AC19, and AC22 were 1.8, 1.5, and 2.5, respectively) was greater than in the LAMA84-r cell line (in which the ratios of compounds K1P, AC19, and AC22 were 1.3, 1.2, and 1.4, respectively).

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Discovery of Compounds Based on the Furan Scaffold, Which Are Selectively Suppressive of the Growth of 32Dtetp210Bcr-Abl Cells

In Fig. 4A, we present the structures of the furans that were synthesized by the reaction shown in Fig. 1A. The initial analysis suggested a role for the amide group in the suppression of the growth of the 32Dtetp210Bcr-Abl cell line. These data suggested that a bulky group in *cis* to the amide on the furan ring could reduce its inhibitory effect. A series of furans was then synthesized in which the steric effects of groups neighboring the amide were minimized (see Fig. 4A).

These compounds completely suppressed the growth of the 32Dtetp210Bcr-Abl cell line (see Fig. 4B) at nanomolar concentrations in the low-density cell proliferation assay. Furthermore, their effect at the 10 nmol/L level was almost totally selective for the Bcr-Abl–expressing cell line because no growth suppression was seen on the 32D cell line in the presence of IL-3.

In-Gel Kinase Cell-Free Assay of Inhibition of Src and v-Abl Kinase Proteins by the Acetylenes and Furans

The cell-free inhibitory effects of the acetylene and furan compounds on the Src protein kinase in an ''in-gel'' kinase assay are shown in Fig. 5A. The addition of the furan compounds A101 and A103 and the acetylene AC22 individually suppressed the Src protein kinase activity by 1.5- to 2-fold. These assays suggested that AC22 acetylenic compounds as well as the furan compounds A101 and A103 possess inhibitory activity against the Src protein kinase.

The A102, A103, A104, and A105 furan compounds produced at least a 5-fold decrease in v-Abl protein kinase activity, whereas A101 led to an ~2-fold decrease (Fig. 5B). These results suggest that the inhibitory effects of the furan compounds are greater on the v-Abl than on the Src protein kinase.

Inhibitory Effect of the Acetylene Compounds on the Growth of Leukemia/Lymphoma and Solid Tumor Cell Lines

K1P, AC19, and AC22 were sent to the National Cancer Institute for an additional screening of a panel of 60 established cell lines derived from patients with leukemia, lymphoma, or solid tumors. The data for cases in which the inhibitory effect on the cell lines is measurable in the leukemic panel are presented in Table 3. K1P and AC22 inhibited the growth of cell lines derived from myeloid and lymphoid acute and chronic leukemias, multiple myeloma, non–Hodgkin's lymphoma, as well as colon and renal cancer cell lines.

Inhibitory Effect of AC22 and K1P on a Panel of 60 Kinases

To formally test the hypothesis that the acetylenic compounds could be also acting at another target in addition to p210Bcr-Abl, AC22 and K1P were tested for their inhibitory activity on 60 kinases through a contract firm, Upstate Biotechnology. At 10 μmol/L, AC22 almost completely inhibited the activity of the Src-related protein kinase Lyn and K1P inhibited Fyn, another Src family kinase (see Table 4).

Discussion

Two groups of compounds have been identified, which inhibit proliferation of the 32Dtetp210Bcr-Abl cell line under conditions in which cell growth is dependent on the Bcr-Abl protein kinase, in the absence of added growth factors, such as IL-3. When the inhibitory effects of acetylene-derived lead drugs (AC22, AC19, and K1P) on the growth of the 32Dtetp210Bcr-Abl cell line were tested in the absence of serum in the low-density cell growth inhibition assay, significant inhibition was seen at the

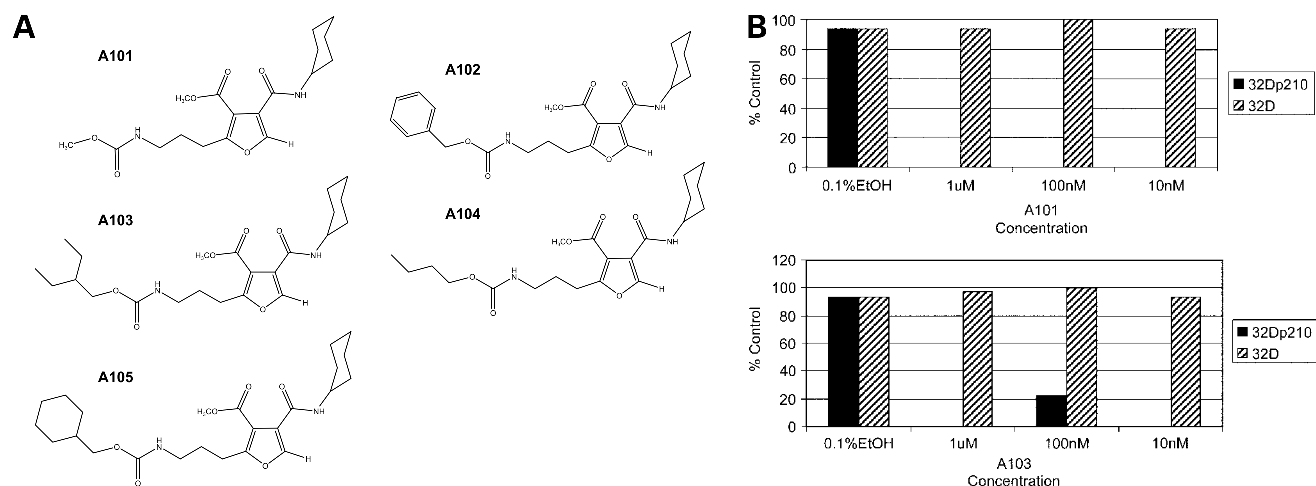

Figure 4. **A,** structure of the furans in which steric factors near the amide are minimized. Based on the early studies in which the role of the size of the chemical functionality in *cis* to the amide, a series of compounds was made in which only a hydrogen atom rather than a methyl group or larger in *cis* to the amide was created. Each of the compounds tested showed significant suppression of the growth in the low-density cell proliferation assay of the 32Dtetp210Bcr-Abl cell line at the 10 nmol/L level. As shown, the action of these drugs was almost totally selective for the p210Bcr-Abl – dependent growth. **B,** inhibitory effect of the furans on the growth of the 32Dtetp210Bcr-Abl cell line in the low-density cell proliferation assay. The low-density cell proliferation assay was used to study the suppression of the growth of the 32Dtetp210Bcr-Abl cell line following addition of the A101 and A103 furans.

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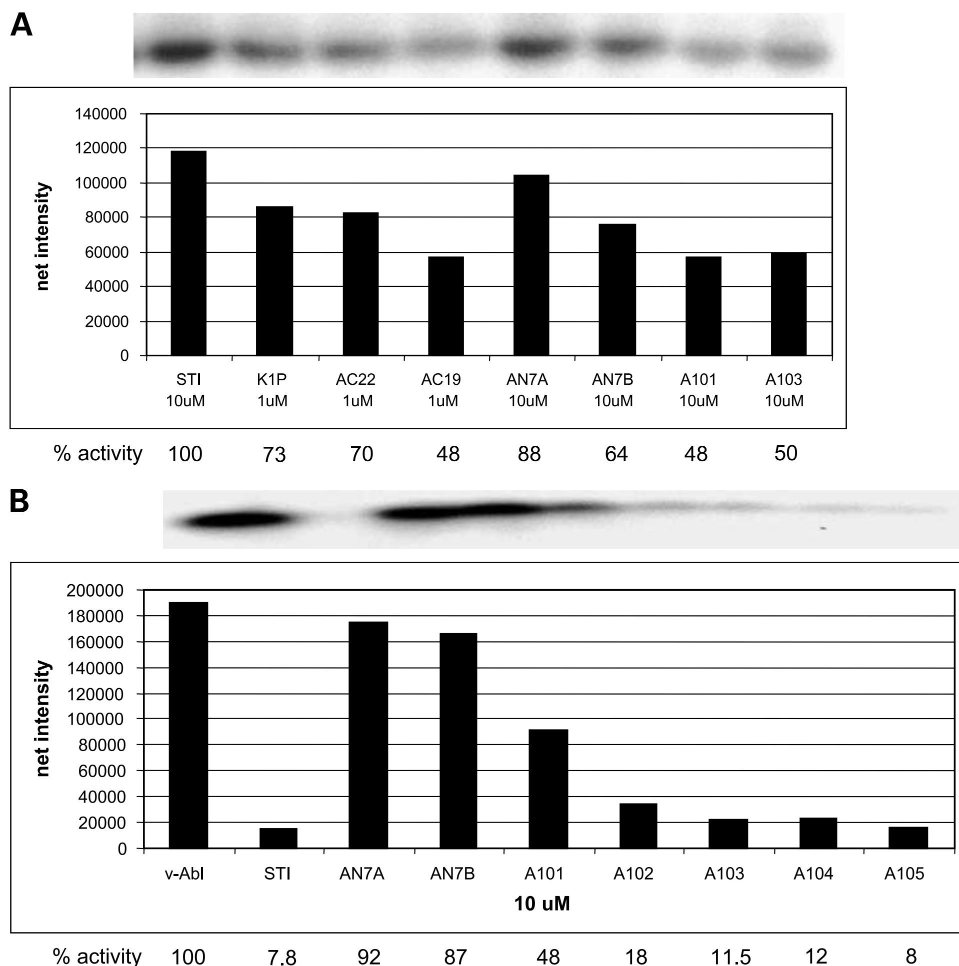

Figure 5. *In vitro* effect of furans and acetylenes on Src (**A**) and v-Abl (**B**) kinase activity in cell-free assay. The recombinant purified enzyme and its substrate following exposure to each of the test drugs were added in the presence of [γ-^{32}P]ATP to the sample well of a SDS-PAGE. Following the completion of the electrophoresis, the gel was autoradiographed with substrate for the Src kinase (**A**) and the v-Abl kinase (**B**). **A,** effects of the K1P, AC22, and AC19 acetylenic compounds as well as the furan compounds AN7A, AN7B, A101, and A103 on the Src kinase. **B,** effects of the furan compounds AN7A, AN7B, A101, A102, A103, A104, and A105 on the v-Abl kinase. *STI,* imatinib.

100 nmol/L level. When the same drugs were tested for suppression of growth of the 32Dtetp210Bcr-Abl cell line at high cell density (using MTS) in the presence of serum, the concentration of drugs required for significant growth inhibition is in the 1 μmol/L range.

Interestingly, when individual acetylene compounds AC22 and K1P are combined with imatinib in the absence of serum, they suppress the proliferation of the 32Dtetp210Bcr-Abl cells at the 10 nmol/L range. In addition, the inhibitory activity of the combination of AC22 or K1P with imatinib is extremely selective for p210Bcr-Abl–dependent growth because no inhibition is seen on the 32D cell line in the low-density cell proliferation assay. Similarly, the furan-derived compounds presented in Fig. 4 are also extremely selective and potent inhibitors of the growth of the 32Dtetp210Bcr-Abl cell line at the 10 nmol/L concentration level.

We also studied the effect of the acetylenes on the growth of two imatinib-resistant cell lines that overexpress Bcr-Abl. These compounds showed a higher inhibitory effect on the Baf/*BCR-ABL*-r than on the LAMA84-r cell line. This may be due to the fact that, in addition to Bcr-Abl overexpression, LAMA84-r, but not Baf/*BCR-ABL*-r, also overexpresses the *multidrug resistance 1* P-glycoprotein. Whether the acetylene compounds are substrates of this pump protein has not yet been proven. In future work, it will be also important to test the inhibitory effect of the furans and acetylenic compounds on cell lines in which the resistance to imatinib is due to the acquisition of point mutations in the p210Bcr-Abl kinase. This latter mechanism is the most common mechanism through which imatinib resistance is acquired in patients (7).

It is remarkable that two classes of such good compounds were identified from so few candidates. Our screening strategy was designed to identify compounds that would inhibit proliferation of the 32Dtetp210Bcr-Abl protein kinase-dependent cells at any point downstream of the complex pathways emanating from the action of the p210Bcr-Abl kinase. It is thus possible that this strategy increased the chances of finding compounds that were inhibitory to imatinib-resistant cell lines.

Imatinib was shown early on to suppress the level of the circulating myeloid cell mass in most patients with chronic-phase CML and in a lower fraction of patients with accelerated- or acute-phase CML. At least 24 different point mutations of the p210Bcr-Abl tyrosine-specific

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Table 3. Results from the National Cancer Institute screening of growth inhibition of a panel of leukemia and solid tumor cell lines with 1 μmol/L of test drug in a high-density cell proliferation assay

Cell line	% Cell growth inhibition by 1 μmol/L of test drug					
	From	K1P	AC22	AC19	K11P	Cell no.
HL-60 (TB)	PML	75	81	96	88	40,000
K-562	CML-EL	80	96	101	103	5,000
MOLT-4	ALL-T	50	78	104	66	30,000
RPMI-8226	MM*	79	77	80	84	20,000
CCRF-CEM	ALL-T	—	130	101	—	40,000
SR	NHL	—	65	96	—	20,000

Abbreviations: PML, promyelocytic leukemia; ALL, acute lymphocytic leukemia; MM, multiple myeloma; NHL, non–Hodgkin's lymphoma.
*Resistant to dexamethasone.

protein kinase were found to be associated with the acquisition of resistance to imatinib (19). In addition, other mechanisms of resistance were found other than point mutations, such as overexpression of the P-glycoprotein. The point mutations were found in a few patients with primary resistance to imatinib at diagnosis and in a much larger number of patients with acquired resistance at the time of emergence of resistance to imatinib in accelerated- and acute-phase patients.

Thus, a major priority was to find additional drugs that could be used to treat CML patients in whom resistance to imatinib was acquired or was present at diagnosis. Several drugs have been reported to suppress populations of CML cells that display resistance to imatinib (20–23). Both preclinical and clinical data are available on drugs that inhibit the p210Bcr-Abl kinase both in the activated and in the inactivated states (24). Clinical trial results of dasatinib (BMS 354825) have induced complete hematologic and cytogenetic responses in imatinib-resistant Philadelphia chromosome–positive leukemias (23). Unlike imatinib, this drug binds both the active and the inactive conformations of the p210Bcr-Abl protein (24).

A phase I trial showed that nilotinib (AMN107) induced hematologic responses in 13 of 33 and 9 of 33 cytogenetic responses in blast crisis patients (25). In accelerated-phase patients, 33 of 46 patients developed hematologic responses and 22 of 46 developed cytogenetic responses (25). Nilotinib is 10- to 50-fold more potent than imatinib in preclinical models (26, 27). A new non–ATP-competitive inhibitor (ON012380) has been shown to inhibit 100% of the mutations, which lead to imatinib-resistant CML cells, including the T3151 mutation, at the 10 nmol/L level (28). PD166326, which belongs to a family of pyridopyrimidines, inhibits *in vivo* growth of imatinib-resistant p210Bcr-Abl–positive cells in preclinical models at the nanomolar concentration (29). Eventually, such drugs may be combined at diagnosis with imatinib to reduce the probability of evolution of imatinib resistance, especially in patients with accelerated and acute phases of CML or in chronic-phase CML patients with adverse clinical features who are therefore considered to be at increased probability of evolution of resistance to imatinib.

There are several unique features of the drugs reported in this article that distinguish them from the other CML inhibitory drugs already reported (19–29), which suggest that they may play a unique clinical role: (*a*) the acetylene AC22 has been shown not only to suppress the growth of CML cells, which are resistant to imatinib, but also to inhibit p210Bcr-Abl–dependent cell growth at the nanomolar concentrations when combined with imatinib; (*b*) the level of inhibition of the combination of AC22 and imatinib is greater than the sum of the inhibitory effects of AC22 and imatinib when used separately; (*c*) the combination of AC22 and imatinib is totally noninhibitory to the non–p210Bcr-Abl-dependent cell growth at the 10 nmol/L level; (*d*) the acetylenes AC22 and K1P are inhibitory to imatinib-resistant cells; (*e*) the furans (e.g., A103 and A105) are inhibitory to p210Bcr-Abl–dependent cell growth at the 10 nmol/L concentration at which these drugs do not inhibit cell growth that is not p210Bcr-Abl dependent; and (*f*) the acetylene AC19 is inhibitory to p210Bcr-Abl–dependent cell growth at the 1 μmol/L level, at which level the drug is totally noninhibitory to the p210Bcr-Abl–independent cell growth.

The apparent synergy of AC22 with imatinib is unique among all of the classes of drugs tested thus far (19–30).

Table 4. Screening of a panel of 60 kinases for inhibition of enzymatic activity in the presence of test drug in cell-free assay

	% Inhibition at 10 μmol/L*	
	K1P	AC22
CaMKII	—	40
CaMKIV	—	64
CDK2/cyclinA	—	50
CK1	46	(37)
Fyn	49	(29)
IKKβ	—	45
Lyn	—	98
PKCy	—	61
PKCbII	(35)	48

*Data are presented only for kinases for which the inhibition activity is >45%.

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In addition, the selectivity of the furans A103 and A105 at the 1 nmol/L level seems to also be unique among all of the classes of drugs thus far developed. In addition, the experimental results suggest that these drugs may be useful for the treatment of patients in whom resistance to imatinib has already emerged.

Our studies showed that the acetylenic compounds K1P and AC22 also inhibit Src and the Src-related kinases Lyn and Fyn. This is a very interesting result in light of the recent report that the Lyn protein kinase is overexpressed in imatinib-resistant cell lines K-562-R (9). The Lyn kinase has been reported to be involved in the response of the cell to DNA-damaging agents, such as the Abl kinase (9, 10, 30). This suggests that these compounds might be acting at multiple different targets downstream of p210Bcr-Abl.

In addition, the use of a synthetic strategy to generate the low-complexity combinatorial libraries of compounds that maintains the chirality of the chemical functionalities on the two types of molecular scaffolds (linear-acetylenes or planar cyclic-furans) may have reduced the number of candidates needed to identify potent and selective inhibitors of the 32Dtetp210Bcr-Abl cell line.

Another remarkable feature that was observed among the acetylene-derived compounds from our libraries is that some of them suppress imatinib-resistant cell lines. Such compounds could conceivably be of value when used together with imatinib for the initial treatment of CML because the probability of the evolution of resistance to a combination might be lower than with single-agent therapy.

Further studies are required to characterize these compounds at the preclinical level before they can be considered for introduction into the clinic. The toxicity and bioavailability of each of these compounds are undergoing study in additional cell lines and, more importantly, in primary leukemic cells. Both the furans and the acetylenes have multiple sites at which additional functional groups can be added to increase bioavailability and that can improve the pharmacokinetics. It is envisaged that the ongoing systematic study of these compounds in animal models of Bcr-Abl–induced leukemia will help us optimize their pharmacology.

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