A Sequential Blockade Strategy for the Design of Combination Therapies to Overcome Oncogene Addiction in Chronic Myelogenous Leukemia

Rong Chen, Varsha Gandhi, and William Plunkett

Department of Experimental Therapeutics, University of Texas M.D. Anderson Cancer Center, Houston, Texas

Abstract

Some tumors are dependent on the continued activity of a single oncogene for maintenance of their malignant phenotype. The best-studied example is the Bcr-Abl fusion protein in chronic myelogenous leukemia (CML). Although the clinical success of the Abel kinase inhibitor imatinib against chronic-phase CML emphasizes the importance of developing therapeutic strategies aimed at this target, resistance to imatinib poses a major problem for the ultimate success of CML therapy by this agent. We hypothesized a sequential blockade strategy that is designed to decrease the expression of the Bcr-Abl protein, with the goal of complementing the action of imatinib on kinase activity. In this study, flavopiridol, an inhibitor of transcription, homoharringtonine (HHT), a protein synthesis inhibitor, and imatinib were used singly and in combination against the Bcr-Abl-positive human CML cell line K562. Flavopiridol alone inhibited phosphorylation of the RNA polymerase II COOH-terminal domain, specifically reduced RNA polymerase II–directed mRNA synthesis, and decreased the Bcr-Abl transcript levels. HHT inhibited protein synthesis and reduced the Bcr-Abl protein level. Imatinib directly inhibited the kinase activity of Bcr-Abl. The combinations of flavopiridol and HHT and flavopiridol and imatinib synergistically decreased clonogenicity as evaluated by the median-effect method. Greater synergy was observed when HHT and imatinib were given sequentially compared with simultaneous administration. Imatinib-resistant Ba/F3 cells that were transfected to express the E255K and T315I mutations of Bcr-Abl were not cross-resistant to flavopiridol and HHT. These results provided a rationale for the combination of inhibitors of transcription and/or translation with specific kinase inhibitors.

Introduction

Increased understanding of the molecular basis of cancer has suggested that, in some instances, the inappropriate expression of a single oncogene may confer stem cell–like properties. Typically, these tumorigenic characteristics are expressed as pleotropic effects on multiple pathways and networks, the stimulation of which enables prolonged cell survival and unlimited proliferative capacity. As individual tumor cells are dependent on the continued activity of specific activated or overexpressed oncogenes to maintain their malignant phenotype, they have been characterized as being “addicted” to the activity of the oncogene (1). Examples include Bcr-Abl in chronic phase of chronic myelogenous leukemia (CML; ref. 2), activated c-Kit in gastrointestinal stromal tumors (GIST), and platelet-derived growth factor receptor in myeloproliferative disorders (3). This has provided a rational basis for the development of therapeutics that are specifically directed at inhibiting the activity of the particular oncogene product. In such an approach, although the normal counterpart is also inhibited in normal tissue, the biological context of the dependency of the tumor on oncogene function provides a basis for the therapeutic index. Thus far, oncogenic kinases have been the most promising targets for such strategies.

The inhibition of the Bcr-Abl tyrosine kinase by imatinib is the most thoroughly studied and clinically successful example of targeted therapy. When evaluated in the clinic, CML in its early stage responds well to imatinib treatment. However, it is now established that resistance to imatinib therapy arises, most frequently associated with mutations in the kinase domain (4). A second generation of Bcr-Abl tyrosine kinase inhibitors with activity against some of the mutations conferring resistance to imatinib is under development (5, 6), raising the possibility of combinations of kinase inhibitors. Nevertheless, cross-resistance with imatinib remains for some mutations (5, 6). Thus, additional strategies must be considered for durable therapy of tumors that exhibit the oncogene addiction phenotype.

In considering rationales for combination treatment of oncogene-addicted tumors, the goal should be to minimize the oncoprotein activity. Therapeutic approaches that decrease expression of oncoproteins that are essential for tumorigenesis will complement the actions of specific enzyme inhibitors. Thus, a sequential blockade strategy that inhibited either oncogene transcription or its translation into protein would decrease the absolute amount of the oncoprotein. Such an approach would augment the effects of a kinase inhibitor and not be cross-resistant to cells that express a mutant kinase. Furthermore, in contrast to normal tissues, the fact that the target tumor is critically dependent on the oncogene activity would provide a biological context for a positive therapeutic index despite the lack of specificity of an inhibitor of transcription or translation.

We have used CML as a model system to evaluate the sequential blockade strategy on the Bcr-Abl oncogene. The transcription inhibitor flavopiridol and the inhibitor of translation homoharringtonine (HHT) have been used alone and in combination with imatinib in the CML cell line K562, which is dependent on Bcr-Abl for survival. Flavopiridol is a semisynthetic flavonoid derived from a plant from India. It was originally described as an inhibitor of cyclin-dependent kinases (CDK) due to its interaction with ATP-binding sites (7). In cycling cells, it blocks cell cycle progression at G1 and G2 interfaces by inhibiting CDK1, CDK2, CDK4, CDK6, and...
CDK7. However, the results of clinical trials of flavopiridol have been disappointing (8). Recently, flavopiridol has been recognized as the most potent inhibitor of the transcription elongation factor P-TEFb (CDK9/cyclin T; ref. 9), a protein kinase that phosphorylates the COOH-terminal domain (CTD) of RNA polymerase II, which is required for activation of transcriptional elongation. In indolent chronic lymphocytic leukemia (CLL), flavopiridol inhibited the transcription of the short-lived antiapoptotic proteins Mcl-1 and XIAP and induced apoptosis in vitro (10). This provided a proof of action of flavopiridol as a transcriptional inhibitor because most primary CLL cells are not actively cycling and therefore do not require the cell cycle–regulating CDKs. An ongoing clinical trial of flavopiridol has recently reported promising activity in refractory CLL (11).

HHT is a cephalotaxine ester derived from the evergreen tree Cephalotaxus harringtonia native to China (12). It inhibits protein synthesis, reportedly with actions on both the initiation and elongation phases of translation. The toxicity of HHT is proportional to protein synthesis inhibition. HHT has shown promising activity against leukemia in initial studies in China and in later studies in the United States (12). When administered as a continuous infusion to patients with CML in late chronic phase, HHT produced a complete hematologic remission in 72% of patients and a cyogenetic complete response rate of 32%. The response was further improved when HHT was administered in combination with cytarabine or IFN-α (12).

In the present study, flavopiridol inhibited RNA synthesis and decreased the Bcr-Abl transcript; HHT inhibited protein synthesis and decreased the Bcr-Abl protein; the two drugs together potentiated the action of imatinib and killed the K562 cells synergistically. These results validated the sequential blockade strategy to overcome oncogene addiction in this model of CML and provided a rationale for the combination of antagonists of transcription and/or translation with specific kinase inhibitors.

### Materials and Methods

#### Materials

HHT [cephalotaxine, 4-methyl-2-hydroxy-4-methylpentyl butanedioate (ester)] was purchased from LKT Laboratories, Inc. (St. Paul, MN). It was prepared as a 1 mmol/L stock solution in sterile PBS and stored at −20°C. Imatinib (Novartis, Basel, Switzerland) was prepared as a 10 mmol/L stock solution in DMSO and kept at −20°C. Flavopiridol was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). It was dissolved in DMSO at 10 mmol/L and stored at −70°C in small aliquots. [5,6-3H]uridine (50 Ci/mmol) was purchased from Moravek Biochemicals, Inc. (Brea, CA). [4,5-3H]leucine (170 Ci/mmol) was purchased from ICN Pharmaceuticals (Costa Mesa, CA). Puromycin, human serum albumin, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO).

**Cell lines.** K562, a Bcr-Abl-positive cell line (American Type Culture Collection, Manassas, VA), was maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen, Carlsbad, CA). Murine Ba/F3 cells transfected with vector (Ba/F3Vector), wild-type Bcr-Abl (Ba/F3p210E255K), and the Bcr-Abl mutants (Ba/F3p210E255K and Ba/F3p210E255K;257E) were kindly provided by Charles L. Sawyers (University of California, Los Angeles, CA). They were maintained in RPMI 1640 supplemented with 10% FBS. The vector-transfected cells were supplemented with 2 ng/mL murine interleukin-3 (IL-3; PeproTech, Rocky Hill, NJ) and 2 μmol/L puromycin. The Bcr-Abl-transfected cells were maintained in the absence of IL-3. They rely on the expression of Bcr-Abl for growth and survival.

**RNA and protein synthesis.** After incubation with drugs, K562 cells were labeled with [3H]uridine or [3H]leucine (1 μCi/mL) for 30 minutes to measure RNA and protein synthesis, respectively. The cells were collected, washed twice with 10 mL cold PBS, and then lysed with 1 mL 0.4 N perchloric acid. Following centrifugation, the pellet was washed with 1 mL 0.4 N perchloric acid and dissolved in 1 mL H2O with 50 μL of 10 N KOH overnight and radioactivity was determined by liquid scintillation counting. Control DPMs were 83,989 ± 27,111 and 11,503 ± 7,606 (mean ± SD) for RNA and protein synthesis, respectively.

**Growth inhibition.** K562 cells were seeded on 24-well plates at a concentration of 5 × 10^4/mL in the presence of increasing concentrations of drugs. Cell concentrations were measured after 72 hours by Cell and Particle Counter (Beckman Coulter, Inc., Fullerton, CA). Data were presented as percentage of control cell growth.

**Immunoblot analysis.** Cells lysates were prepared as described (10) and subjected to SDS-PAGE, electrophoresed into nitrocellulose membrane, and then incubated with primary antibodies for 3 hours followed by secondary antibodies conjugated to horseradish peroxidase (HRP) for 1 hour. Blots were visualized by enhanced chemiluminescence (Pierce, Rockford, IL). Antibodies for total RNA polymerase II (8WG16), phosphorylated CTD at Ser2 (H5) or Ser5 (H14) were purchased from Covance Research Products, Inc. (Berkeley, CA). The antibodies to c-Abi (24-11) and c-Myc (9E10) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Poly(ADP-ribose) polymerase (PARP) antibody was from PharMingen (San Diego, CA). Antibodies to signal transducers and activators of transcription 5 (STAT5), phosphorylated STAT5 at Tyr694/Tyr705, and CRKL were from Upstate (Waltham, MA). Antibodies to phosphorylated c-Abi at Tyr283 and phosphorylated CRKL at Tyr592 were purchased from Cell Signaling Technology (Beverly, MA). Anti-mouse IgG and anti-rabbit IgG HRP-conjugated antibodies were obtained from Amersham Biosciences (Piscataway, NJ).

**RNA isolation and real-time quantitative PCR.** Total cellular RNA was isolated by the RNeasy kit from Qiagen (Valencia, CA). Total RNA (20-50 ng) was used for the one-step real-time PCR as described previously (10). The relative gene expression was analyzed by the comparative Ct method using 18s rRNA as the endogenous control. The primers and probes for Bcr-Abl are as follows: forward primer, 5′-CGAGCGGCTTCTACGAG; reverse primer, 5′-ACAGCATTGCCGCTGACAT; and probe, 5′-FAM-CTGAGGCT-CAAGTCAGATGCTACTGG-TAMRA. All primers, probes, and reaction mix were purchased from Applied Biosystems (Foster City, CA).

**Nascent mRNA synthesis.** K562 cells were incubated with 1 μmol/L flavopiridol for 4 hours and pulsed labeled for 1 hour with [3H]uridine (1 μCi/mL) at 37°C. The polyadenylated mRNA was isolated using the Oligotex Direct mRNA Isolation kit (Qiagen), and the radioactivity was measured by scintillation counting. The RNA in the flow through was precipitated with equal volumes of 10% ice-cold trichloroacetic acid and collected on GF/A filters (Whatman, Inc., Clifton, NJ). The filters were then washed five times with 1 mL of 5% trichloroacetic acid, five times with 1 mL H2O, and twice with 1 mL of 95% ethanol, air dried, and transferred to scintillation vials for counting. The relative amount of RNA synthesis was determined by comparing the radioactivity of flavopiridol-treated samples with the untreated controls.

**Growth inhibition.** Cells were plated in triplicate at 5 × 10^4 per well on 96-well plate and exposed to increasing concentrations of each drug, and growth inhibition was measured by MTT assay at 48 hours. The doses that inhibited 50% proliferation (Dm) were analyzed by the median-effect method (CalcuSyn software, Biosoft, Ferguson, MO).

**Clonogenicity assays.** K562 cells in six-well plates were incubated with drugs for 24 or 48 hours and then washed twice with PBS and plated on six-well plates at 300 per well on Iscove’s medium containing 0.25% nutrient agar and 20% FBS. Colonies (aggregates composed of >50 cells) were counted after 10 to 14 days of culture at 37°C. The cloning efficiency of K562 cells was 55 ± 9%.

**Analysis of drug combinations.** The combination effects were assessed by clonogenicity assays after incubation of cells for 24 hours with each individual drug and in combination. The combinations were done either in a fixed molar ratio based on the Dm values (the dose required for 50% inhibition of clonogenic survival) of each single drug or in a matrix using five concentrations for each drug that achieve 10% to 50% inhibition alone. The effects of combinations were estimated using the CalcuSyn software, which was developed based on the median-effect method created by Chou et al. (13).

---

This text is a summary of a scientific research paper that discusses the effects of flavopiridol and HHT on the transcription and translation of Bcr-Abl transcripts in K562 cells. The study also examines the combination effects of these drugs on cell growth and clonogenicity. The authors used various techniques, including immunoblot analysis, RNA isolation, and real-time quantitative PCR, to assess the effects of the drugs on protein synthesis and mRNA synthesis. The paper highlights the potential of these drugs as therapeutic agents for chronic myeloid leukemia (CML) and other hematologic disorders.
Results

Flavopiridol inhibited RNA synthesis and reduced Bcr-Ab1 transcript and protein levels. RNA synthesis in K562 cells was inhibited by flavopiridol in a concentration- and time-dependent manner (Fig. 1A). The IC₅₀ for the inhibition of RNA synthesis estimated after 3 hours was 3.9 ± 0.5 μmol/L (mean ± SD; n = 3), RNA synthesis dropped to 80% of control in the first 30 minutes of incubation with flavopiridol and then decreased to ~60% in the following 6 hours during incubation with 1 μmol/L flavopiridol. Because the CTD is unique to RNA polymerase II, RNA polymerases I and III, which are responsible for the synthesis of ribosomal and low molecular weight RNA, may not be inhibited by flavopiridol. To clarify this issue, we measured the nascent mRNA synthesis after incubation with 1 μmol/L flavopiridol. After 4 hours, mRNA synthesis was inhibited by 51%, whereas the RNA that did not bind to the oligo(dT) column (including rRNA and tRNA) was inhibited only by 14% (data not shown). The inhibition of total RNA synthesis was 28%. Therefore, it seemed that flavopiridol selectively inhibited the mRNA synthesis that is directed by RNA polymerase II.

That flavopiridol was acting by affecting RNA polymerase II function was indicated by a decrease in phosphorylation on Ser² and Ser⁴ during incubation of K562 cells with flavopiridol (Fig. 1B). The most susceptible targets of RNA synthesis inhibitors are those mRNAs with short half-lives (16). Bcr-Ab1 transcript, which has a half-life of ~4 hours in K562 cells, is likely to be affected by this treatment. Indeed, Bcr-Ab1 mRNA was reduced to 50% after a 4-hour incubation with 1 μmol/L flavopiridol (Fig. 1C). Consequently, the Bcr-Ab1 protein decreased within 24 hours and continued to decline to 48 hours (Fig. 1B). Consistent with the short half-lives of the transcript (~0.5 hour; ref. 17) and protein (20-30 minutes; ref. 18), the mRNA of c-Myc (Fig. 1C) was reduced dramatically in 4 hours as was c-Myc protein, which was at the level of detection at 24 hours (Fig. 1B). However, the level of the more stable protein, β-actin, remained unchanged. The decrease of Bcr-Ab1 and c-Myc was accompanied by PARP cleavage by 24 hours, indicating induction of apoptosis (Fig. 1B). This action inhibited growth of K562 cells with an IC₅₀ of ~0.3 μmol/L after a 72-hour incubation (Supplementary Fig. S1A) and reduced clonogenicity (Fig. 1D) in a time-dependent manner. The IC₅₀ was 10 times less after a 48-hour incubation compared with 24 hours, consistent with a mechanism involving reduction of Bcr-Ab1 activity.

In addition to Bcr-Ab1, c-Myc gene amplification is associated with the progression of CML (19). To test directly whether decreasing Bcr-Ab1 expression is the dominant cause of toxicity in these Bcr-Ab1-dependent cells, we used siRNAs to specifically decrease transcripts of Bcr-Ab1 and c-Myc; the effects of these treatments on clonogenicity were then compared. Immunoblotting confirmed the decrease of Bcr-Ab1 and c-Myc protein levels by their specific siRNAs (Fig. 2A). However, only knocking down Bcr-Ab1 significantly decreased the clonogenic survival of the cells (35% of control), whereas the c-Myc knockdown cells retained the normal clonogenic survival (106%). The results of this comparison of these two oncogenes show the critical dependence of the cells on Bcr-Ab1 for survival and proliferation. Therefore, consistent with the oncogene addiction hypothesis, decreasing Bcr-Ab1 transcript levels by flavopiridol seems to be the major contributor to cell killing. This does not rule out the possibility that these agents also affect expression of yet other proteins that may support cell survival, albeit to a lesser extent.

When combined with imatinib, flavopiridol resulted in CI values <0.4, indicating strong synergy (Fig. 2B), consistent with the results reported by Yu et al. (20). Although down-regulation of anti-apoptotic proteins and activation of c-Jun NH²-terminal kinase may play a role, as suggested by Yu et al., the likely mechanism for

---

The combination of flavopiridol and imatinib. The cells were incubated 24 hours with densities relative to analyzed by immunoblotting, and results were expressed as the ratio of film density of Bcr-Abl and actin (23). To compare the potency of flavopiridol in human serum with FBS in cell culture, we determined the action of flavopiridol on decreasing the mRNA level of Bcr-Abl and c-Myc protein binding. However, 1 μmol/L flavopiridol in FBS and human serum were incubated with increasing concentrations of HHT for 24 hours and then pulse labeled with 1 μCi/mL [3H]leucine for 30 minutes. Points, mean percentage of protein synthesis compared with untreated controls of three independent experiments done in triplicate; bars, SE. B, concentration-dependent inhibition of protein synthesis by HHT. K562 cells were incubated with increasing concentrations of HHT for 24 hours and then pulse labeled with 1 μCi/mL [3H]leucine for 30 minutes. Points, mean percentage of protein synthesis compared with untreated controls of three independent experiments done in triplicate; bars, SE. C, Bcr-Abl protein decreased after incubation with HHT. K562 cells were incubated with 30, 100, or 300 nmol/L HHT or solvent for 24, 48, and 72 hours. Levels of Bcr-Abl and Abl protein were detected by immunoblot. Results were calculated as the mean percentage of radioactivity compared with controls of triplicate data; bars, SD. D, inhibition of clonogenicity by HHT was 168 nmol/L for 24 hours and 158 nmol/L for 48 hours in experiments done in triplicate.
Reduced the protein level of Bcr-Abl more than either drug alone. This combination was assessed by clonogenic assays after incubating K562 cells with HHT and imatinib together for 24 hours (C), or the cells were incubated for 24 hours with HHT and then for another 24 hours with imatinib (D). The combination was in a fixed molar ratio based on the Dm values of 168 and 158 nmol/L for HHT for 24 and 48 hours of incubation, respectively, and 7.7 μmol/L for imatinib. B. effect of the combination of HHT and imatinib on Bcr-Abl levels, its phosphorylation, and that of STAT5 in K562 cells. Cells were incubated 24 hours with 100 nmol/L HHT and then for another 24 hours with HHT together with 0.3 or 1 μmol/L imatinib. Results were expressed as the relative reduction in the proportion of phosphorylated Bcr-Abl (pBcr-Abl) and STAT5 (pSTAT5). C, synergistic combination of flavopiridol and HHT. The effect of the combinations was assessed by clonogenic assays 24 hours after the K562 cells were incubated with each individual drug or in combination. The combinations were in a fixed molar ratios based on the Dm values of 2.5 μmol/L flavopiridol and 168 nmol/L HHT. D, effect of the combination of flavopiridol and HHT on Bcr-Abl protein. The cells were incubated 24 hours with 0.3 μmol/L flavopiridol or 100 nmol/L HHT or in combination. The proteins were analyzed by immunoblotting. H, HHT.

Thus, arresting cells at the G1 or G2 phases by flavopiridol may have potentiated the cytotoxicity of HHT.

Imatinib inhibition of Bcr-Abl kinase. Inhibition of Bcr-Abl kinase activity was detected by immunoblotting using the antibody against the phosphorylated Tyr245 Abl, which is autophosphorylated by the activated Abl kinase. This treatment did not significantly affect the total protein level at 24 hours, although there was a decrease of total Bcr-Abl protein after a 48-hour incubation (Fig. 5A). Nevertheless, the kinase-specific activity, as indicated by the ratio of phosphorylated Bcr-Abl to total Bcr-Abl protein, decreased in response to imatinib in a time- and concentration-dependent manner. The phosphorylation of the downstream substrates of Bcr-Abl, STAT5 and CRKL, was also inhibited by imatinib, consistent with inhibition of the kinase activity (Fig. 5A).

In K562 cells, the IC50 for the inhibition of cell growth was 0.37 μmol/L at 72 hours (Supplementary Fig. S1C). The IC50 for the inhibition of clonogenicity was 7.7 μmol/L after 24 hours and 0.9 μmol/L for a 48-hour incubation (Supplementary Fig. S1D). Flavopiridol and HHT were strongly synergistic with imatinib in clonogenic analyses, indicated by CI values within the range of 0.2 to 0.4 (Fig. 5B). Immunoblots confirmed a substantial reduction of Bcr-Abl protein as well as kinase activity in the combination of the three drugs (Fig. 5C).

The DRI values, a measure of how much the dose of each drug in a synergistic combination may be reduced at a given effect level compared with the doses of each drug alone, were also calculated (Fig. 5D). To generate a 50% inhibition of clonogenicity, the required concentrations of flavopiridol and HHT were six to seven times lower when combined compared with their use alone and eight to nine times less than individual IC50s when both drugs were combined with imatinib. Viewed from the perspective of reducing the imatinib concentration, the combination with flavopiridol reduced the dose of imatinib by 12-fold and further by 19-fold when HHT was added in this combination. These results indicated that...
were incubated 24 hours with 1Cancer Research, flavopiridol, HHT, and imatinib on phosphorylation of Bcr-Abl and STAT5. Cells
Asterisk, values were calculated using the median-effect method (CalcuSyn software).
D, imatinib, or in combination. The proteins were analyzed by immunoblotting.

168 nmol/L for HHT, and 7.7Cancer Research mol/L for imatinib.

imatinib together for 24 hours. The combination was made in a fixed molar ratio based on the Dm values of 2.8
mol/L for flavopiridol, 300 nmol/L HHT, 0.3Cancer Research mol/L imatinib, or in combination. The proteins were analyzed by immunoblotting. D, DRI values for the combinations at 50% of inhibition on clonogenicity, DRI values were calculated using the median-effect method (CalcuSyn software). Asterisk, drugs were added simultaneously.

Flavopiridol and HHT greatly sensitized the cells to imatinib and complemented its function.

Ba/F3 cells dependent on imatinib-resistant Bcr-Abl were equally sensitive to flavopiridol and HHT. Transcription and translation inhibitors decrease the expression of Bcr-Abl, whereas imatinib directly inhibits its kinase activity. Therefore, cells resistant to imatinib because of Bcr-Abl kinase mutations should not be cross-resistant to either flavopiridol or HHT. To further investigate this issue, we compared the inhibition of proliferation by these drugs in the murine Ba/F3 cells selected for dependency on Bcr-Abl (Ba/F3p210Cancer Research wt) with isogenic lines in that had been transfected with clinically relevant mutations in the oncoprotein (Ba/F3p210T315I and Ba/F3p210E255K). Consistent with previous investigations (5), the lines exhibited varying resistance to imatinib that was related to the mutation status (Table 1). In contrast, these three cell lines were equally sensitive to flavopiridol and HHT. This was associated with a decrease in Bcr-Abl protein levels in transfected cell lines (data not shown). Thus, the action of flavopiridol and HHT was not affected by the mutation status in Bcr-Abl. These results provide a rationale for the combination of these agents in diseases expressing mutants of Bcr-Abl kinase that are resistant to imatinib. The vector-transfected cell line was also sensitive to flavopiridol and HHT probably because these cells are critically dependent on the IL-3-Janus-activated kinase (JAK) 1/2 pathway (26), which may also be affected by flavopiridol and HHT. In fact, 100 and 300 nmol/L HHT significantly decreased both JAK1 and JAK2 protein after 24 hours (Supplementary Fig. S2), consistent with a decrease in these signaling kinases as a basis for the sensitivity of these cells to HHT.

### Discussion

We hypothesized that inhibitors of transcription and translation that decrease the expression of an oncogenic kinase would complement the actions of specific kinase inhibitors. In tumors that are critically dependent on the sustained expression of such a kinase for survival, this combination strategy would induce cell death. Here, the Bcr-Abl-addicted CML cell line K562 was used as a model to evaluate this sequential blockade strategy. Our results showed that flavopiridol inhibited RNA polymerase II–mediated mRNA synthesis, reduced Bcr-Abl transcript and protein levels, and was synergistic with the Bcr-Abl kinase inhibitor imatinib. HHT inhibited protein synthesis and reduced the Bcr-Abl protein level. It was also synergistic with imatinib when imatinib was added following HHT. Flavopiridol and HHT together were strongly synergistic with imatinib, thus providing support for the sequential blockade approach to overcome oncogene addiction. This strategy to reduce oncogene activity is not confined to flavopiridol and HHT but rather may be extended to other agents that affect transcription and translation and approaches that target protein stability as well as other oncogenes. Optimal application of this strategy to other models may well require administration of agents in sequences that accommodate tumor- and drug-specific characteristics.

This sequential blockade strategy could be applied to other oncogenes, most effectively to those that have short turnover rates of the transcripts or proteins, in a biological context in which the tumor is strongly dependent on the oncogene expression for maintenance of its malignant phenotype. For example, the mutated

### Table 1. Dm values of the inhibition of cell proliferation of the Ba/F3 cells carrying the Bcr-Abl wild-type and mutations by MTT assay

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Flavopiridol (μmol/L)</th>
<th>HHT (nmol/L)</th>
<th>Imatinib (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ba/F3p210Cancer Research wt</td>
<td>0.22 ± 0.07</td>
<td>16.98 ± 5.50</td>
<td>0.38 ± 0.10</td>
</tr>
<tr>
<td>Ba/F3p210E255K</td>
<td>0.21 ± 0.01</td>
<td>16.86 ± 0.88</td>
<td>3.93 ± 1.38*</td>
</tr>
<tr>
<td>Ba/F3p210T315I</td>
<td>0.24 ± 0.07</td>
<td>13.52 ± 0.32*</td>
<td>18.27 ± 5.9*</td>
</tr>
<tr>
<td>Ba/F3Vector</td>
<td>0.27 ± 0.09</td>
<td>15.49 ± 0.41*</td>
<td>22.81 ± 0.02</td>
</tr>
</tbody>
</table>

NOTE: Drug concentrations that inhibit 50% of growth (Dm) were calculated by the median-effect method. The values are the mean ± SD of two independent experiments each done in triplicate. For data that did not qualify for the median-effect method (r < 0.9), IC50 was derived by the dose-response plot generated through the Prism software (GraphPad Software, San Diego, CA).

*One of the two values was derived from the dose-response plot.
c-Kit oncogene that results in the constitutive activation of this receptor tyrosine kinase and induces cell proliferation in GIST has a mRNA half-life of 2 to 3 hours (27). Activation of Src family kinases was found in imatinib-resistant cells (28). The mRNA half-life for Src is 2 hours, although that of the protein is ~20 hours (29). Mutation or amplification of c-Met seems to be an enabling event in many tumor types. The half-life of c-Met mRNA is <30 minutes and the protein half-life is ~5 hours (30). Application of this combination strategy is not limited to kinases; the genetic hallmark of Burkitt lymphoma is the chromosomal rearrangements of the c-MYC oncogene that leads to c-Myc overexpression and contributes to lymphomagenesis. c-Myc is a very labile oncoprotein with half-lives of <30 minutes for both mRNA (17) and protein (18). The antiapoptotic Bcl-2 family protein Mcl-1, which plays an important role in the maintenance of CLL and multiple myeloma, has a mRNA and protein half-life of 0.5 to 1 hour (16). Cyclin D1 expression is dysregulated by chromosomal translocation t(11;14)(q13;q32) in mantle cell lymphoma, a subtype of B-cell non–Hodgkin's lymphomas, and also plays an important pathogenic role in other tumors, such as breast cancer. The short half-lives of cyclin D1 mRNA (6 hour) and protein (30 minutes; ref. 31) suggest that it would be a susceptible target of transcriptional and translational inhibitors. Some short-lived mRNAs or proteins are characterized by sequence motifs that directed their rapid degradation, such as the adenylate uridylate-rich elements in the 3′-untranslated region of a transcript (32) and the PEST sequence (33) or the KFERQ motif (34) in proteins.

The sequential blockade strategy proposes that inhibition of each step in the production of an active oncoprotein would complement the activity of a specific inhibitor to reduce the tumorigenic activity. Transcription may be inhibited at several steps by agents that act by different mechanisms (35), either nonspecifically by flavopiridol, R-roscovitine (36), dactinomycin (37), and the nucleoside analogues fludarabine (38), cordycepin (39), and 8-chloroadenosine (40) or specifically by antisense oligodeoxynucleotides or siRNAs. Although the action of flavopiridol or R-roscovitine in proliferating cells may not be limited to transcriptional inhibition, as they also affect cell cycle progression by inhibiting the cell cycle–regulating CDKs. Nevertheless, flavopiridol reduced the Bcr-Abl mRNA and protein levels (Fig. 1B and C), clearly showing the inhibition of transcription. For agents that affect protein level, HHT inhibits elongation phase of translation by interfering with the peptide bond formation (12). Asparaginase depletes the supply of asparagine and leads to cell death in acute lymphocytic leukemia (41). The mammalian target of rapamycin (mTOR) facilitates the assembly of the translation preinitiation complex, mTOR inhibition by rapamycin or its analogues has been shown to decrease protein synthesis (42).

Although our current investigations have focused on inhibitors of aspects of transcription and translation, the processes of protein maturation and post-translational modification may be considered equally appropriate targets for such combination approaches. For instance, heat shock protein 90 (Hsp90) is a molecular chaperone required for the stability and function of several oncoproteins, such as Src, c-Raf, Bcr-Abl, Flt3, Akt, and Her2. The geldanamycins analogues 17-allylamino-demethoxygeldanamycin (43) and 17-(2-dimethylaminoethyl)amino-17-demethoxy-geldanamycin (44) inhibit Hsp90, causing destabilization of Hsp90 client proteins, which leads to their degradation (43). Promising use of Hsp90-active agents was reported with imatinib against CML cell lines (45) and with the Flt3 inhibitor PKC412 in acute myelogenous leukemia lines expressing the mutant protein (46). Post-translational modifications are essential for protein function. For example, membrane localization through the farnesyl group or geranylgeranyl group is critical for function of Ras oncoproteins (47). Inhibitors of the farnesyltransferase or geranylgeranylationtransferase are in clinical evaluation in Ras-associated cancers; these may be most effective in combination with specific inhibitors of kinases in pathways downstream of Ras.

The results of our investigations suggest that therapeutic strategies, which combine agents that reduce the expression of oncogenic kinases, may augment the activity of specific kinase inhibitors. We have not yet evaluated the efficacy of such approaches against quiescent leukemia-repopulating cells or with leukemias that have become independent of Bcr-Abl (48). Nevertheless, approaches that decrease protein level may bypass resistance that arises from mutations in the kinase domain or overexpression of the protein (4). This is consistent with emerging clinical observations. A preliminary report of a phase 1 clinical trial combining imatinib and flavopiridol showed complete hematologic remissions in CML, including patients previously treated with imatinib (49). In a phase 2 study, HHT was given s.c. to CML patients in late chronic phase after imatinib failure. All five evaluable patients achieved complete hematologic responses after a median of 4.5 courses of HHT. Two patients had mutations in the Abl kinase domain before therapy with HHT, and one of them achieved complete cytogenetic response (50). In addition, a phase 2 clinical trial combining HHT and imatinib in the treatment of advanced-stage CML seems well tolerated and has clinical activity in patients with CML in all phases, including in some who have failed prior therapy with imatinib and other tyrosine kinase inhibitors (51). Finally, a multicenter phase 2 clinical trial of HHT alone in CML patients with T315I mutations in Bcr-Abl has been initiated.

Acknowledgments

Received 4/17/2006; revised 8/25/2006; accepted 9/21/2006.

Grant support: National Cancer Institute, Department of Health and Human Services grant CA100632 and Cancer Center Support grant P30 CA16672.

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.

References


A Sequential Blockade Strategy for the Design of Combination Therapies to Overcome Oncogene Addiction in Chronic Myelogenous Leukemia

Rong Chen, Varsha Gandhi and William Plunkett