

In vitro studies of a FLT3 inhibitor combined with chemotherapy: sequence of administration is important to achieve synergistic cytotoxic effects

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Patients with acute myeloid leukemia (AML) harboring internal tandem duplication mutations of the FLT3 receptor (FLT3/ITD mutations) have a poor prognosis compared to patients lacking such mutations. Incorporation of FLT3 inhibitors into existing chemotherapeutic regimens has the potential to improve clinical outcomes in this high-risk group of patients. CEP-701, an indolocarbazole-derived selective FLT3 inhibitor, potently induces apoptosis in FLT3/ITD-expressing cell lines and primary leukemic blasts. We conducted a series of in vitro cytotoxicity experiments combining CEP-701 with che-

motherapy using the FLT3/ITD-expressing cell lines MV4-11 and BaF3/ITD as well as a primary blast sample from a patient with AML harboring a FLT3/ITD mutation. CEP-701 induced cytotoxicity in a synergistic fashion with cytarabine, daunorubicin, mitoxantrone, or etoposide if used simultaneously or immediately following exposure to the chemotherapeutic agent. In contrast, the combination of pretreatment with CEP-701 followed by chemotherapy was generally antagonistic, particularly with the more cell cycle-dependent agents such as cytarabine. This effect appears to be due to CEP-701

causing cell cycle arrest. We conclude that in FLT3/ITD-expressing leukemia cells, CEP-701 is synergistic with standard AML chemotherapeutic agents, but only if used simultaneously with or immediately following the chemotherapy. These results should be considered when designing trials combining chemotherapy with each of the FLT3 inhibitors currently in clinical development. (Blood. 2004; 104:1145-1150)

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Introduction

Less than half of all patients with non-M3 acute myeloid leukemia (AML) are cured using conventional chemotherapy.¹ Although intensive cytarabine-based regimens have the greatest chance of curing these aggressive hematologic malignancies, new treatments are urgently needed for the majority of patients whose disease is resistant to chemotherapy. FMS-like tyrosine kinase-3 (FLT3), a receptor tyrosine kinase with an important role in early hematopoiesis, is normally expressed along with CD34 in hematopoietic stem/progenitor cells.^{2,3} Most AML blasts also express this receptor, and activating mutations of FLT3 occur in approximately 30% of de novo AML.^{4,5} Most of these FLT3 mutations are internal tandem duplications (FLT3/ITD mutations) localized to the juxtamembrane domain in the cytoplasmic portion of the protein.^{6,7} A smaller number (7%) have point mutations localized to the activation loop within the kinase domain.^{8,9} Patients with AML harboring FLT3/ITD mutations are now generally recognized as having a significantly greater relapse rate and poorer overall survival than patients lacking such mutations.^{4,5} The prognostic significance of the kinase domain mutations is as yet unclear.

Several small-molecule FLT3 inhibitors have now been described in the literature.¹⁰⁻¹⁹ CEP-701, an indolocarbazole derivative, is a relatively selective FLT3 inhibitor with IC₅₀ (concentration that inhibits 50%) of 3 nM in in vitro kinase and cell-based FLT3 autophosphorylation assays.¹³ It is cytotoxic to FLT3/ITD-expressing cell lines in vitro and prolongs survival in a mouse

model of FLT3/ITD leukemia. In a phase 2 trial of patients with relapsed and refractory AML harboring FLT3-activating mutations, CEP-701 demonstrated biologic and clinical activity, with reductions in blast counts that correlated with successful, sustained inhibition of FLT3 activity in vivo.²⁰ The drug, given orally twice daily, caused only minimal side effects and was extremely well tolerated by patients. Unfortunately, the observed reductions in blast counts were of relatively brief duration, lasting only a few weeks to months. This was not surprising, given the nature of this disease, and given the fact that this was essentially monotherapy. By analogy, all-*trans*-retinoic acid (ATRA) alone does not cure acute promyelocytic leukemia (APL), and imatinib mesylate, a BCR/ABL tyrosine kinase inhibitor, induces only temporary responses in patients with chronic myelogenous leukemia (CML) with blast crisis.^{21,22} In addition, some patients with chronic-phase CML treated with imatinib mesylate develop resistance, most often by selection of cells carrying mutations of BCR/ABL that interfere with the binding of imatinib mesylate.²³ Because of these limitations, the next logical step is to try to combine the inhibitor with conventional chemotherapy in the hopes of improving the outcomes of patients with AML.

In this study, FLT3/ITD-expressing cell lines, as well as a primary leukemia sample from an AML patient harboring a FLT3/ITD mutation, were used to test combinations of CEP-701 with chemotherapy agents commonly used to treat AML. An

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important issue in combining FLT3 inhibitors with chemotherapy is the manner in which the agents are combined. We tested CEP-701/chemotherapy interactions in each of the 3 sequences: CEP-701 treatment before chemotherapy (“pretreatment”), CEP-701 treatment at the same time as chemotherapy (“simultaneous treatment”), and CEP-701 immediately following chemotherapy (“post-treatment”). Although none of these approaches can truly duplicate what occurs in an actual patient, we hoped that the data would provide guidelines for designing an effective combination regimen.

Materials and methods

Reagents

Cytarabine, daunorubicin, etoposide, and mitoxantrone were obtained from Sigma (St Louis, MO). Daunorubicin and etoposide were dissolved in dimethyl sulfoxide (DMSO) and stored frozen; cytarabine and mitoxantrone were dissolved in water and stored frozen. CEP-701, provided by Cephalon (West Chester, PA), was stored at -80°C as a 4-mM stock solution in DMSO and diluted as needed. Within any given experiment, DMSO concentration was maintained at a constant level in all samples, and in no case was it more than 0.1%. Ficoll-Hypaque was obtained from Amersham (Piscataway, NJ). Anti-FLT3 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antiphosphotyrosine antibody (4G10) was obtained from Upstate Biotechnology (Lake Placid, NY). All other reagents were from Sigma.

Cytotoxicity assays

Cytotoxicity was assessed using a dimethyl-thiazol diphenyl tetrazolium bromide (MTT) assay. In selected cases, we also used an annexin V-binding apoptosis assay to confirm that the cytotoxic effect observed (or lack thereof) was associated with an equivalent degree of apoptosis. MTT (Roche, Indianapolis, IN) and annexin V (PharMingen, San Diego, CA) assays were performed as described previously.¹²

Cells

Cell lines used for these studies were all cultured at 37°C with 5% CO_2 in RPMI medium with 20% fetal bovine serum (FBS) and penicillin/streptomycin (Invitrogen, Carlsbad, CA). The BaF3/ITD cell line was generated by transfecting the interleukin 3 (IL-3)-dependent murine pro-B lymphocyte line, Ba/F3, with an expression vector containing a FLT3/ITD

cDNA and has been described previously.^{13,24} MV4-11 is an AML cell line that expresses a naturally occurring 30 bp FLT3/ITD mutation in homozygous form.^{13,25} In addition, we used a primary AML sample, which was designated AML/ITD, obtained through a protocol approved by the institutional review board. The sample was obtained by Ficoll-purifying mononuclear cells from the peripheral blood of a newly diagnosed patient who presented with a white blood cell (WBC) count above $100 \times 10^9/\text{L}$ ($100\,000/\text{mm}^3$). The French-American-British classification was AML M0 and the cells expressed a 51-base pair (bp) FLT3/ITD mutation. The specimen was aliquoted and stored frozen for repeated use. Prior to each use, aliquots of these blasts were thawed rapidly into warm culture medium, incubated for 12 hours, and then recentrifuged over Ficoll to eliminate cells dying from the freeze-thaw process. Using this method we obtained blasts that maintained satisfactory viability (as determined by trypan blue exclusion) and responsiveness to cytotoxic agents in culture over 48 hours. Blasts incubated for longer periods of time appeared to become quiescent (ie, poor uptake of the MTT reagent but still excluding trypan blue). We were therefore able to use these blasts only in experiments completed in 48 hours or less. This precluded our use of these cells for those experiments in which cells were pretreated with CEP-701 because this required more than 72 hours of incubation.

Cell cycle analysis

Following drug exposure, cells were fixed in 70% cold methanol, washed in IFA buffer (150 mM NaCl, 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], pH 7.4, 4% FBS, 0.5% Tween-20) and resuspended in phosphate buffered saline (PBS) with 100 $\mu\text{g}/\text{mL}$ RNase (Roche). After incubation for 15 minutes at 37°C , propidium iodide (Sigma) in PBS was added to a final concentration of 25 $\mu\text{g}/\text{mL}$ and the cells were analyzed by flow cytometry.

FLT3 phosphorylation

FLT3 expression and phosphorylation status were analyzed by incubating cells for 1 hour with and without CEP-701 followed by cell lysis in detergent buffer, immunoprecipitation, gel electrophoresis, and immunoblotting with antiphosphotyrosine antibodies as described previously.¹³

Median effect analysis

To characterize the interactions between CEP-701 and chemotherapy, data were analyzed using the median effect method of Chou and Talalay.²⁶ In this method, for every combination of 2 agents tested, dose-response curves are generated for each agent individually, and these data are used to analyze the

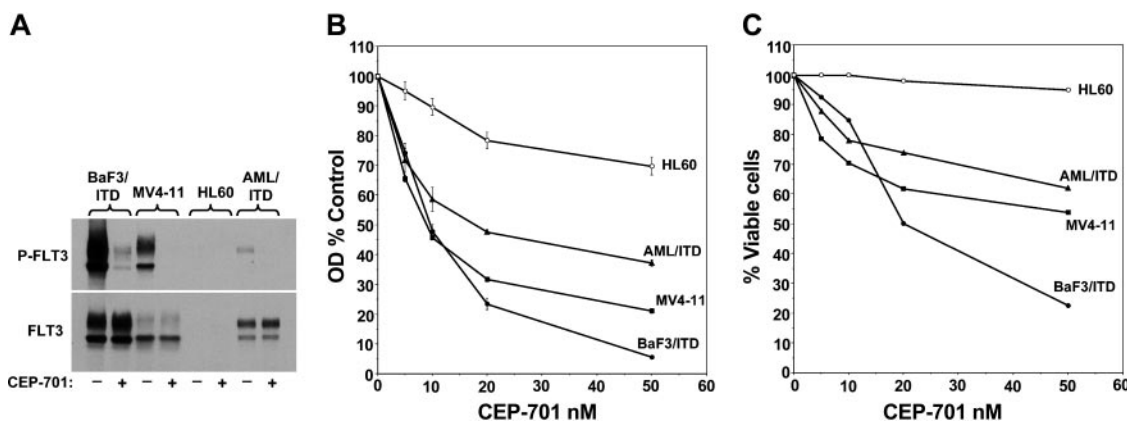


Figure 1. CEP-701 effects on FLT3/ITD-expressing cells. (A) Cells were incubated for 1 hour in culture medium with or without 50 nM CEP-701 and then lysed in detergent buffer. Then, 500 μg of this whole-cell extract was used for immunoprecipitation and immunoblotting as described in “Materials and methods.” The upper panel shows a blot probed with antiphosphotyrosine; the lower panel is the same blot stripped and reprobed with anti-FLT3. The blot reveals the characteristic appearance of FLT3, with a larger (160-kDa) mature, glycosylated form, and a smaller (130-kDa) immature form. (B) MTT assay of cells exposed for 48 hours to increasing doses of CEP-701. Each concentration point represents the mean of quadruplicate measurements of optical density (OD). The results are plotted as percent untreated control. This experiment was performed 3 times. The graph displays a representative result. Error bars represent the SD. (C) Annexin V apoptosis assay of cells exposed to CEP-701. The y-axis represents viable (ie, non-annexin V staining) cells plotted as percent untreated control. The concentration points represent a single measurement of annexin V binding. This experiment was repeated once with essentially identical results.

Table 1. Pretreatment with 10 nM CEP-701, followed by addition of chemotherapy

	Cytarabine		Daunorubicin		Etoposide		Mitoxantrone	
	IC ₅₀ , μM	CI	IC ₅₀ , nM	CI	IC ₅₀ , nM	CI	IC ₅₀ , nM	CI
MV4-11	3256	1.168	29	0.922	171	1.488	7	0.819
BaF3/ITD	2058	3.35	53	0.951	229	1.025	4	1.165
HL60	875	1.069	109	0.901	2396	0.922	47	0.977

IC₅₀ and CI values for the combination of 10 nM CEP-701 and chemotherapeutic agents at doses corresponding to the approximate IC₅₀. In this pretreatment scenario, triplicate samples of cells were exposed to CEP-701 for 24 hours and then transferred into medium containing both CEP-701 and the chemotherapeutic agent. MTT assays were then performed 48 hours later. Each CI value (generated by the Calcsyn program) was obtained by conducting dose-response experiments for CEP-701 and the indicated chemotherapy individually and in combination over a range of doses for both drugs. The values shown are representative of 3 different experiments.

results obtained from the combination treatment within the same experiment. A commonly used approach is to prepare a stock medium containing the 2 agents at a fixed ratio, and then perform serial dilutions of that original stock to test the drugs over a range of doses. This scenario mimics 2 drugs being given to a patient simultaneously, with both of them clearing from the plasma with roughly the same half-life. However, a patient treated with a FLT3 inhibitor would be maintained on a constant dose over a prolonged period of time. Therefore, we focused our efforts on studying the effects of combining a fixed dose of CEP-701 with varying doses of chemotherapy. Although a broad range (1-100 nM) of CEP-701 concentrations was used to generate the median effect analysis plots, of most interest were the interactions between chemotherapeutic agents and CEP-701 concentrations of 10 or 20 nM. These levels of CEP-701 in vitro result in FLT3 autophosphorylation inhibition to approximately 5% to 10% of baseline, which corresponds to the levels of FLT3 inhibition achieved in the recent clinical trial.²⁰ It should be noted that to achieve this degree of FLT3 inhibition in vivo, plasma levels of CEP-701 typically needed to be in the micromolar range because CEP-701 is so highly protein-bound. Therefore, the results reported here are primarily derived from combinations of a fixed concentration of CEP-701 (10 or 20 nM) and a concentration of chemotherapeutic agent as close as possible to the IC₅₀ value of the chemotherapeutic agent alone in these experiments, resulting in a nonfixed ratio of CEP-701 and chemotherapy.

For each CEP-701/chemotherapy drug combination experiment, a combination index (CI) number was generated using a commercially available software program (Calcsyn; Biosoft, Manchester, United Kingdom). For each experiment, linear regression analysis of the dose-response data was used to generate a median effect value Dm (corresponding to the IC₅₀) along with a slope *m* and linearity coefficient *r*. For all data reported here, *r* values were more than 0.95. The CI is calculated using the formula: $CI = (D)_1/(Dm)_1 + (D)_2/(Dm)_2 + (D)_1(D)_2/(Dm)_1(Dm)_2$.

This applies to 2 mutually nonexclusive drugs with independent modes of action, where (D)₁ and (D)₂, for example, represent CEP-701 and a chemotherapeutic agent, respectively, and (Dm) refers to the dose of each drug that, by itself, will generate a median effect (ie, the IC₅₀) from the data within that experiment. As indicated, we used a nonfixed ratio of the 2 drugs to better approximate what might occur in a patient treated with continuous CEP-701. Although quantifying the degree of synergy or antagonism is of uncertain validity, this method of analysis generally defines CI values of 0.9 to 1.1 as implying additivity, 0.3 to 0.9 as synergistic, less than 0.3 as strongly synergistic, 1.1 to 3.3 as antagonistic, and more than 3.3 as strongly antagonistic.²⁶

Results

Cell lines and primary samples treated with single-agent CEP-701

BaF3/ITD, MV4-11, and AML/ITD cells all express constitutively phosphorylated FLT3, albeit at widely differing levels (Figure 1A). MV4-11 cells represent an ideal model cell line for these studies because this line harbors a naturally occurring FLT3/ITD mutation.¹³ The BaF3/ITD cell line was generated by transfecting the IL-3-dependent murine lymphoid line Ba/F3 with a FLT3/ITD construct, as described.¹³ Expression of the constitutively activated FLT3 construct confers factor independence to the BaF3/ITD line.

Although it is obviously not an AML cell line, it does offer the advantage of studying cells that are transformed exclusively because of FLT3. HL60 cells were used as an FLT3⁻ control line. HL60 cells are AML-derived cells that express a low level of wild-type FLT3 detectable on immunoblot only with prolonged film exposure or by fluorescence-activated cell sorting (FACS) using an anti-CD135 antibody (data not shown).²⁷ We included such a “control” cell line to help identify non-FLT3-mediated drug interactions. Following a 1-hour exposure to 50 nM CEP-701, FLT3 autophosphorylation was inhibited in all 3 FLT3-expressing cell types. In MTT proliferation and annexin V-binding apoptosis assays, BaF3/ITD, MV4-11, and AML/ITD cells displayed dose-dependent cytotoxic responses to CEP-701 (Figures 1B-C). In contrast, HL60 cells showed no detectable FLT3 protein using even prolonged exposure of the immunoblot, and they demonstrated no apoptosis and only mildly inhibited proliferation in response to CEP-701 exposure (Figures 1A-C). These features made the use of HL60 cells an ideal control to identify chemotherapy/CEP-701 interactions that were not mediated through FLT3 inhibition.

CEP-701 combined with chemotherapy: pretreatment with CEP-701

The first combination we studied was the pretreatment of the leukemia cells with CEP-701 followed by the addition of individual chemotherapeutic agents. Cells were incubated with CEP-701 at various doses for 24 hours, then transferred into medium containing chemotherapy for an additional 48 hours. For any given sample, the CEP-701 concentration was maintained at a constant level throughout the 72-hour incubation. Under these conditions, 10 nM was the maximum dose of CEP-701 that could be meaningfully studied for the responsive cells because higher concentrations resulted in virtually complete cell death at 72 hours. Table 1 displays the CI values for 10 nM CEP-701 when combined with a dose of chemotherapy corresponding to the approximate

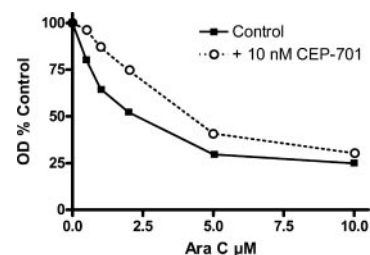


Figure 2. Pretreatment with CEP-701 followed by cytarabine. BaF3/ITD cells were exposed to increasing concentrations of CEP-701 for 24 hours, then transferred into medium containing increasing concentrations of cytarabine with continued exposure to CEP-701 for an additional 48 hours. Cytotoxicity was then assessed using the MTT assay. The graph shows a dose-response curve for cytarabine alone (solid line) and for cytarabine in the presence of 10 nM CEP-701 started 24 hours earlier (dashed line). The results are plotted as percent cytarabine-untreated control.

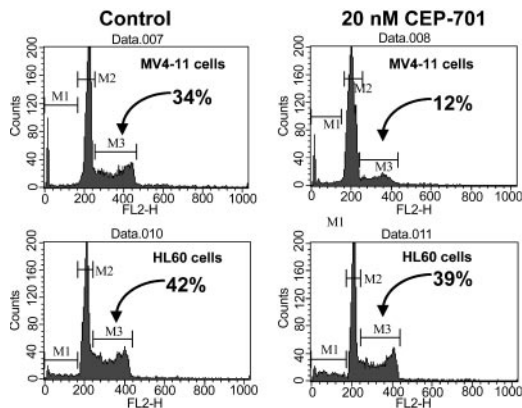


Figure 3. Cell cycle profile of cells treated with CEP-701. MV4-11 cells (upper panels) and HL60 cells (lower panels) were cultured in the absence (left panels) or presence (right panels) of 20 nM CEP-701 for 48 hours, then costained with annexin V and propidium iodide and analyzed by FACS. The graphs display propidium iodide (x-axis) staining of non-annexin V staining (ie, still viable) cells. The cells in G₂/S phase are designated in each graph with an arrow.

IC₅₀ value shown in the table. There was no evidence of any significant synergy with any of the combinations tested (except MV4-11 cells showing slight synergy with CEP-701/mitoxantrone). This pattern held true for lower doses of CEP-701, as well as for concentrations of chemotherapy corresponding to IC₇₅ values (data not shown). More important, there appeared to be antagonism with some of the combinations, most notably with cytarabine and etoposide. Graphic illustration of this is shown in Figure 2, in which the cytarabine dose-response curves of BaF3/ITD cells with and without 10 nM CEP-701 are plotted. At every concentration of cytarabine tested, the cytotoxic effect is less pronounced, and the IC₅₀ shifts from 2.1 μM to 4.5 μM in the presence of CEP-701. This result was highly reproducible and confirmed using an annexin V assay (CI = 1.798 for BaF3/ITD, 1.246 for MV4-11).

Examination of the CI values in Table 1 reveals that pretreatment of the FLT3-expressing cell lines (MV4-11 and BaF3/ITD) with CEP-701 followed by addition of daunorubicin or mitoxantrone led to additive cytotoxic effects, whereas pretreatment with CEP-701 appeared to antagonize the cytotoxic effects of cytarabine and etoposide. Neither synergy nor antagonism was observed between these agents in the HL60 cells, suggesting that the antagonistic interaction, when observed, was mediated through FLT3 inhibition. Because cytarabine in particular is highly cell cycle dependent, we investigated the possibility that the antagonism was due to CEP-701 influencing the cell cycle distribution of the FLT3/ITD cells. Figure 3 shows a cell cycle profile of MV4-11 and HL60 cells with and without CEP-701 treatment. CEP-701 exposure causes a decrease of S phase and G₂/M phase in both MV4-11 cells and BaF3/ITD cells (not shown), with corresponding increases in G₁. In contrast, the cell cycle distribution of HL60 cells was not affected. Because etoposide and cytarabine exert signifi-

cant effects during S phase, the relative decrease in S phase cells pretreated with CEP-701 may account for the observed antagonism between CEP-701 and the chemotherapies.

CEP-701 combined with chemotherapy: simultaneous treatment with CEP-701

The next sequence we tested was simultaneous exposure to CEP-701 and chemotherapy. Table 2 summarizes the CI values for simultaneous treatment of cells with 10 or 20 nM CEP-701 and chemotherapeutic agents at a concentration approximating the IC₅₀ value for the chemotherapeutic agent by itself in the same experiment. All FLT3-expressing cells (including the primary AML sample) displayed a synergistic cytotoxic response when exposed to the combinations of CEP-701 and chemotherapy. Virtually identical results were obtained from combinations doses of chemotherapy corresponding to the IC₇₅ (data not shown). In addition, we conducted a parallel experiment using the annexin V-binding apoptosis assay with MV4-11 cells exposed to cytarabine and CEP-701 and found a CI value of 0.587 for the dose of 20 nM CEP-701, confirming that the cytotoxic effect represented apoptosis.

As expected, only additive effects were seen in HL60 cells, with the interesting exception of daunorubicin. With this drug, the combination with CEP-701 was mildly synergistic, suggesting the presence of a non-FLT3-mediated interaction between the 2 agents. Because CEP-701 is highly protein-bound, we considered the possibility that the synergistic interaction we observed between daunorubicin and CEP-701 in HL60 cells was pharmacokinetic in nature. We hypothesized that competition between the 2 agents for protein-binding sites could lead to increased free levels of both, with resultant increased cytotoxicity. To test this hypothesis, we incubated BaF3/ITD cells in culture medium containing 10% FBS with a fixed concentration of CEP-701 (10 nM) plus increasing concentrations of daunorubicin, then lysed the cells, and immunoprecipitated FLT3. The results of this experiment, shown in Figure 4 (right panel), confirm that the presence of daunorubicin increases the free levels of CEP-701 in vitro, as measured by inhibition of FLT3 autophosphorylation. Daunorubicin alone showed no inhibition of FLT3 phosphorylation. We did not observe the effect when the experiment was performed using 20% FBS, nor was it observed with mitoxantrone, etoposide, or cytarabine using either 10% or 20% FBS. Although this immunoprecipitation assay does not duplicate the exact conditions of the cytotoxicity experiments shown in Table 2, the results suggest that at least part of the synergistic interaction between daunorubicin and CEP-701 may be related to this phenomenon. More importantly, these data highlight a theoretical potential for anthracycline-induced toxicity in patients treated with a combination of CEP-701 and daunorubicin. α₁-Acid glycoprotein (AAG) is the primary plasma-binding protein of both CEP-701 (Cephalon, unpublished data, December 2001) and daunorubicin.²⁸⁻³⁰ If CEP-701 displaced daunorubicin from its

Table 2. Simultaneous treatment with CEP-701 and chemotherapy

	Cytarabine			Daunorubicin			Etoposide			Mitoxantrone		
	IC ₅₀ , nM	CI, 10 nM	CI, 20 nM	IC ₅₀ , nM	CI, 10 nM	CI, 20 nM	IC ₅₀ , nM	CI, 10 nM	CI, 20 nM	IC ₅₀ , nM	CI, 10 nM	CI, 20 nM
MV4-11	7338	0.593	0.672	48	0.768	0.557	169	0.362	0.414	9	0.596	0.581
BaF3/ITD	6289	0.783	0.856	40	0.164	0.069	182	0.182	0.135	5	0.327	0.400
AML/ITD	3152	0.465	0.629	33	0.497	0.393	1514	0.761	0.765	14	0.508	0.590
HL60	734	0.980	1.046	56	0.895	0.754	1055	1.047	1.112	54	0.885	1.105

IC₅₀ and CI values for the combination of 10 or 20 nM CEP-701 and chemotherapeutic agents at doses corresponding to the approximate IC₅₀. In this simultaneous treatment scenario, triplicate samples of cells were simultaneously exposed to CEP-701 and the chemotherapeutic agent for 48 hours, and then MTT assays were performed. Each CI value was obtained by conducting dose-response experiments for CEP-701 and the indicated chemotherapy individually and in combination over a range of doses for both drugs. The values shown are representative of 3 different experiments.

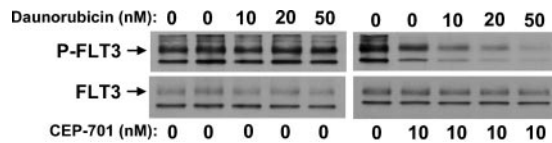


Figure 4. CEP-701–mediated FLT3 inhibition influenced by daunorubicin. BaF3/ITD cells were incubated in culture medium containing 10% FBS for 1 hour with the indicated concentrations of CEP-701 and daunorubicin. Cells were then lysed and FLT3 autophosphorylation was analyzed by immunoblotting as described in “Materials and methods.” The upper panel shows the blot probed with antiphosphotyrosine; the lower panel shows the same blot stripped and reprobed with anti-FLT3.

plasma-binding sites, this could result in increased free levels of the anthracycline.

Chemotherapy followed by exposure to CEP-701

The final sequence tested was treatment of cells with chemotherapy, followed by exposure to CEP-701. This sequence was designed to approximate the scenario of a patient starting continuous CEP-701 treatment only after the completion of induction chemotherapy. Cells were treated for 1 hour with daunorubicin, etoposide, or mitoxantrone, or for 48 hours with cytarabine, then washed and transferred into medium containing CEP-701. Cytotoxic effects were assessed 48 hours later. Table 3 summarizes the CI values for 10 and 20 nM CEP-701 combined with doses of chemotherapy corresponding to IC₅₀ levels under these conditions.

In general, median effect analysis of these combinations suggested synergistic interactions between CEP-701 and all 4 chemotherapeutic agents in all FLT3-expressing cells. A graphic view of this synergy is presented in Figure 5, which displays the raw optical density (OD) values from a representative experiment in which MV4-11 cells were exposed first to cytarabine and then to CEP-701. HL60 cells displayed no evidence of synergy in any of these experiments. To further validate these results, apoptosis assays were carried out with MV4-11 cells in this posttreatment scenario. As before, the results obtained using the annexin V assay were essentially the same as those obtained using the MTT assay. For example, using the annexin V assay, the CI value for 20 nM CEP-701 with mitoxantrone was 0.424.

Discussion

In this report, we established that CEP-701 can induce in vitro cytotoxic effects in FLT3/ITD-expressing cell lines and primary AML blasts in a manner that is synergistic with chemotherapeutic agents. With regard to the selection of chemotherapeutic agents tested, because in our planned clinical trial CEP-701 will be used in randomized fashion (eg, chemotherapy with or without CEP-701), the chemotherapy regimen needed to consist of agents that were generally acceptable for use as AML salvage therapy by them-

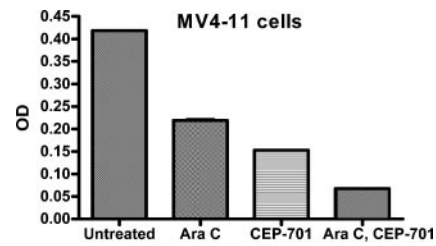


Figure 5. MV4-11 cells treated with cytarabine followed by CEP-701. Triplicate samples of cells were incubated for 48 hours in increasing concentrations of cytarabine and then washed and resuspended in increasing concentrations of CEP-701. After 48 additional hours in culture, an MTT assay was performed. The raw OD obtained from each treatment group is plotted on the y-axis. Error bars are included, but are too small to be seen in this graph.

selves. We therefore chose to study CEP-701 combined with cytarabine, daunorubicin, etoposide, and mitoxantrone. Although other agents have activity in AML, this selection represented some of the most commonly used drugs for this disease. The sequence with which the chemotherapy and CEP-701 are combined appears to be important because treatment of cells with CEP-701 prior to the addition of chemotherapy seemed to antagonize the cytotoxic effects of agents such as cytarabine and etoposide. Other FLT3 inhibitors have been shown to induce a cell cycle arrest, and this property probably accounts for the observed antagonism. In contrast, when CEP-701 was added simultaneously with or immediately following exposure of cells to chemotherapy, synergistic cytotoxicity was observed. Because constitutively activated FLT3 transduces signals that promote cell survival, it is possible that the synergy we observed was due to the withdrawal of these antiapoptotic signals in cells that had sustained DNA damage from chemotherapy. The results from the HL60 cell line are important in that they suggest that interactions between CEP-701 and chemotherapy are mediated primarily through inhibition of constitutively activated FLT3. We are interested in extending these studies to cells expressing wild-type FLT3 to see if combination therapy might yield antagonistic, additive, or synergistic effects.

We recognize that our in vitro model of combination therapy may not precisely mirror the conditions present in actual AML patients treated in vivo with such regimens. Such models as these, however, represent a means to generate hypotheses that can then be tested in a clinical setting. CEP-701 and PKC412 have already been shown to have clinical and biologic activity in patients with relapsed or refractory AML harboring FLT3-activating mutations.^{20,31} Therefore, the results from these in vitro combination studies should be applied to the designs for any new clinical combination trials. One limitation of these experiments is that MTT and apoptosis assays both examine the response of the “bulk” leukemic cells and not the much more rare leukemic stem cells responsible for propagating this disease. In future laboratory studies, we plan to use leukemia colony assays and animal models

Table 3. Treatment of cells with chemotherapy followed by treatment with CEP-701

	Cytarabine			Daunorubicin			Etoposide			Mitoxantrone		
	IC ₅₀ , nM	CI, 10 nM	CI, 20 nM	IC ₅₀ , nM	CI, 10 nM	CI, 20 nM	IC ₅₀ , nM	CI, 10 nM	CI, 20 nM	IC ₅₀ , nM	CI, 10 nM	CI, 20 nM
MV4-11	302	0.369	0.503	98	0.823	0.506	1 801	0.362	0.230	17	0.373	0.247
BaF3/ITD	1583	0.804	0.677	170	0.574	0.564	13 310	0.515	0.507	15	0.657	0.634
AML/ITD	ND	ND	ND	117	0.882	0.921	21 000	0.729	0.688	15	0.839	0.909
HL60	1325	1.068	1.074	366	0.928	1.021	14 470	0.927	0.946	119	1.120	0.937

Table shows IC₅₀ and CI values for the combination of 10 nM or 20 nM CEP-701 and chemotherapeutic agents at doses corresponding to the approximate IC₅₀. In this posttreatment scenario, cells were exposed to chemotherapy for 24 hours (cytarabine only), or 1 hour (daunorubicin, etoposide, or mitoxantrone), washed, and then transferred into medium containing CEP-701. MTT assays were then performed 48 hours later. Each CI value was obtained by conducting dose-response experiments for CEP-701 and the indicated chemotherapy individually and in combination over a range of doses for both drugs. ND indicates not determined.

of FLT3/ITD AML to characterize the effects of combinations and treatment sequences on the leukemic stem cells. The data generated from such studies can be used to refine effective FLT3 inhibitor/chemotherapy regimens identified in ongoing clinical trials. We will also need to explore any potential effects of CEP-701 on recovery of marrow from chemotherapy-induced aplasia.

Cytarabine is an important component of AML therapy, with most regimens requiring that the drug be administered over several days so as to capture a higher fraction of leukemia cells as they enter S phase (the phase during which they are most susceptible to the effects of cytarabine). CEP-701 causes almost immediate FLT3 inhibition when administered to patients with AML, and although we were unable to test cytarabine and pretreatment with CEP-701 in our primary blast sample in vitro, it is reasonable to conclude that at least some leukemia blasts in vivo might respond to FLT3 inhibition with cell cycle arrest. The therapeutic efficacy of cytarabine could thus potentially be blunted by FLT3 inhibition initiated either before or simultaneously with cytarabine. These concerns favor initial exploration of a treatment regimen using cytarabine followed by FLT3 inhibitors, a treatment sequence that yielded highly synergistic cytotoxic effects in these experiments. However, it would be inappropriate to definitively conclude from these in vitro studies that this would be the only effective treatment schedule. We are planning on further exploring these different treatment sequences in mouse models.

An equally important issue concerns the potential pharmacokinetic interaction between anthracyclines and CEP-701. The in vitro data suggest that CEP-701 and daunorubicin bind to the same

serum proteins in a competitive fashion. This interaction, if it occurs in human plasma, could potentially result in higher free levels of anthracyclines with subsequent increased toxicity to patients treated with both agents simultaneously. Because of this potential, and because of the observed synergistic cytotoxicity when daunorubicin or mitoxantrone was followed by CEP-701 treatment, the use of anthracycline treatment followed by FLT3 inhibitors is preferable.

Therefore, based on the clinical and correlative laboratory data from the initial trial of CEP-701 in patients with AML, along with the data from the current in vitro study, a new clinical trial has been designed. In this trial, the combination of mitoxantrone, etoposide, and cytarabine (MEC), followed by oral CEP-701 at a dose of 80 mg twice daily, will be tested in patients with relapsed AML harboring FLT3-activating mutations. This trial represents an important next step in the development of FLT3 inhibitors for clinical use, as well as an interesting test of the concept that leukemia treatment regimens incorporating targeted therapies can be successfully modeled preclinically.

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