Beneficial Effects of Combining Nilotinib and Imatinib in Preclinical Models of BCR/ABL+ Leukemias

Nilotinib and Imatinib Against Bcr-Abl+ Leukemia

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Andrew L. Kung assisted with interpretation of research reported in paper and analysis of the data
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Abstract

Drug resistance resulting from emergence of imatinib-resistant Bcr-Abl point mutations is a significant problem in advanced stage chronic myelogenous leukemia (CML). The Bcr-Abl inhibitor, nilotinib (AMN107), is significantly more potent against Bcr-Abl than imatinib, and is active against many imatinib-resistant Bcr-Abl mutants. Phase I/II clinical trials show that nilotinib can induce remissions in patients who have previously failed imatinib, indicating that sequential therapy with these two agents has clinical value. However, simultaneous, rather than sequential, administration of two BCR/ABL kinase inhibitors is attractive for many reasons, including the theoretical possibility that this could reduce emergence of drug-resistant clones. Here we show that exposure of a variety of BCR/ABL+ cell lines to imatinib and nilotinib results in additive or synergistic cytotoxicity, including testing of a large panel of cells expressing BCR/ABL point mutations causing resistance to imatinib in patients. Further, using a highly quantifiable bioluminescent *in vivo* model, drug combinations were at least additive in anti-leukemic activity, compared to each drug alone. These results suggest that despite binding to the same site in the same target kinase, the combination of imatinib and nilotinib is highly efficacious in these models, indicating that clinical testing of combinations of BCR/ABL kinase inhibitors is warranted.
Introduction

The BCR-ABL tyrosine kinase oncogene, which results from a reciprocal t(9;22) chromosome translocation in a hematopoietic stem cell\(^1\) causes CML and Philadelphia chromosome positive (Ph+) acute lymphoblastic leukemia (ALL). The 210 kDa Bcr-Abl protein is expressed in CML patients, and the p190 kDa Bcr-Abl protein occurs in Ph+ ALL patients and stems from a different breakpoint in the BCR gene\(^2,3\).

CML, which occurs with a frequency of about 1 in 100,000 people per year, when left untreated progresses in three phases: The initial chronic phase is a clonal myeloproliferative disorder that is marked by excessive production of mature granulocytes and immature myeloid cells in tissues including bone marrow, spleen, and peripheral blood. An intermediate accelerated phase, characterized by the appearance of undifferentiated blast cells in blood and bone marrow, is followed by a terminal blast-crisis phase, in which median survival is 18 weeks, and over 30% of the blood and bone marrow cells are blasts\(^4,5\).

Imatinib mesylate (Gleevec\textsuperscript{®}, STI571; Novartis Pharma AG) is an effective, frontline therapy for early, chronic phase CML, which acts by targeting the tyrosine kinase activity of Bcr-Abl\(^6,7\). Newly diagnosed patients treated for a median 19 months show an estimated 76% cytogenetic response (CCR) and 97% complete haematological response (CHR)\(^8\). However, after initially responding to treatment most ALL patients and many CML patients in the accelerated or blastic phases relapse under treatment within one year\(^9,10\). Resistance to imatinib also occurs in a small subset of early, chronic phase CML patients, with relapse occurring following months or years of treatment. Relapse is frequently due to point mutations in Bcr-Abl that reduce the binding affinity of imatinib to the protein, or occasionally with amplification of the BCR-ABL gene\(^11-18\).
The novel, selective Abl inhibitor, nilotinib (AMN107), was designed to interact with the ATP-binding site of Bcr-Abl with a higher affinity than imatinib. In addition to being significantly more potent compared to imatinib (IC50<30nM), nilotinib also maintains activity against most of the Bcr-Abl point mutants that confer imatinib resistance. The in vitro cellular efficacy of nilotinib translates into activity in vivo, as demonstrated by activity against a variety of imatinib-resistant Bcr-Abl point mutants in animal models of myeloproliferative disease. In phase I/II clinical trials, nilotinib is producing cytogenetic and hematologic responses in imatinib-refractory CML patients.

If nilotinib continues to show promise in further clinical trials, it could either be used as a single agent in patients that are refractory to imatinib, or it could be used in conjunction with imatinib to achieve a higher degree of patient responsiveness by suppressing the emergence of drug-resistant Bcr-Abl mutations. We have previously observed a synergistic interaction between nilotinib and imatinib in mouse hematopoietic cells expressing p210Bcr-Abl or p190Bcr-Abl. Here, we show positive cooperative effects of combinations of nilotinib with imatinib in a panel of imatinib-sensitive and imatinib-resistant Bcr-Abl expressing cells in vitro, which translate into additive effects in a mouse leukemia model.

Methods

Chemical compounds and biologic reagents

Nilotinib free-base (AMN107; NVP-AMN107-NX; 4-methyl-N-[3-(4-methyl-1H-imidazol-1-yl)-5-(trifluoromethyl) phenyl]-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-benzamide) and imatinib (Novartis Pharma AG) were dissolved in DMSO to make 10 mM stock solutions. Serial dilutions were made in DMSO to obtain final dilutions for cellular assays.
Antibodies

Anti-pTyr (clone 4G10; Upstate Biotechnology, NY) was used at 1:1000 for immunoblotting. The Abl antibody (C19; Santa Cruz Biotechnology, CA) was used at a dilution of 1:500 for immunoblotting. Monoclonal anti-beta-actin (Clone AC-15) was purchased from Sigma used at a dilution of 1:1000 for immunoblotting.

Cell lines and cell culture

The erythroleukemia cell line K562, derived from a patient in blast crisis CML, and the human CML cell line KU812 were purchased from American Type Culture Collection (Rockville, MD). Murine hematopoietic 32D cells were transduced with retrovirus to express p210 Bcr-Abl (32D.p210 cells)\(^2\); this cell line is rapidly lethal in syngeneic, non-immunosuppressed C3H mice. Imatinib-resistant BCR-ABL constructs (pCI-neo Mammalian Expression Vector; Promega (#E1841)) harboring the point mutations F317L, T315I, M351T and F486S, were stably transfected into Ba/F3 cells by electroporation. Similarly, 32D cells were transfected to express E255V Bcr-Abl. Cells were selected for neomycin resistance and IL3-independent growth. The E255K-Ba/F3 and Y253H-Ba/F3 cell lines were developed as previously described.\(^2\)

All cells were cultured in the presence of 5% CO2 at 37\(^\circ\)C, at a concentration of 5\(\times\)10\(^5\) cells/mL, in cellgro RPMI 1640 medium (Mediatech, Inc. Herndon, VA), supplemented with 10% fetal calf serum (FCS; Harlan Bioproducts, Indianapolis, IN), 1% glutamine, and penicillin/streptomycin. Media for cells expressing Bcr-Abl point mutants was supplemented with 1mg/mL G418.
Cell Viability, cell cycle and apoptosis analysis

The trypan blue exclusion assay has been previously described, and was used to determine proliferation of cells cultured in the presence and absence of nilotinib, imatinib, or a combination of the two agents. Cell viability is reported as percentage of control (untreated) cells. Apoptosis of drug-treated cells was measured using the Annexin-V-Fluos Staining Kit (Boehringer Mannheim, Indianapolis, IN), as previously described.24

Synergy Studies

For synergy studies, imatinib and nilotinib were added simultaneously at fixed ratios to imatinib-sensitive and imatinib-resistant Bcr-Abl expressing cells according to the method of Chou and Talalay.25 Cell viability was determined using the trypan blue exclusion assay. ED50 values were determined from the dose response curves using graphic extrapolation. Specifically, \((Y_2-Y_1)/(X_2-X_1)=(50-Y_1)(X_{50}-X_1)\), where \(X_{50}=X_1+((50-Y_1)(X_2-X_1)/(Y_2-Y_1))\) for linear X-axes and \(X_{50}=10^{(\text{LOG10}(C1)+(X-E1)*\text{LOG10}(C2)-\text{LOG10}(C1)/(E2-E1)}\) for logarithmic X-axes. For calculation of the combination index, the following formula was used: \((\text{ICXa in mix/ICXa alone})+(\text{ICXb in mix/ICXb alone})\). For the ICX value (nM), X is set to 25, 50, 75, or 90.

Immunoprecipitation

Protein lysis preparation and immunoprecipitation were carried out as previously described.24
**Bone Marrow Colony Assay and hematology profile assessment**

Normal murine bone marrow cells were flushed from the femur of a male NCR nude mouse. Normal human bone marrow cells were obtained from to-be-discarded bone marrow harvest collection bags, under a protocol approved by the Institutional Review Board. Cells were lysed in ammonium chloride buffer to remove erythrocytes and washed. Plates were prepared containing 60,000 mouse bone marrow cells each in mouse MethoCult (GF M3434, methylcellulose medium with recombinant cytokines, cat #03434, StemCell Technologies Inc., Vancouver, BC). Similarly, plates were prepared containing 60,000 CD34+ selected human bone marrow cells each in human MethoCult (GF H4434, “complete” methylcellulose medium containing recombinant cytokines, cat #04434, StemCell Technologies Inc., Vancouver, BC). These plates also contained varying concentrations of imatinib and nilotinib, respectively, in combination as compared to vehicle (DMSO) control. The plates containing murine and human bone marrow cells, respectively, +/- the drug combinations, were incubated at 37°C in 5% CO₂ for up to 12 days, at which time myeloid and erythroid colonies were counted on an inverted microscope.

The peripheral blood of five male NCR nude mice (2 NMP-PEG300 vehicle-treated mice, and 3 imatinib (75mg/kg)+nilotinib (20mg/kg)-treated mice) was obtained through tail bleeds. Blood samples were analyzed by the DF/HCC Research Pathology Core Facility (Boston, MA).

**Bioluminescent Bcr-Abl model of CML**

Cells were transduced with a retrovirus encoding firefly luciferase (MSCV-Luc), and selected with G418 at a concentration of 1mg/ml to produce the 32D.p210-luciferase (luc+) cell line. 32D.p210-luc+ cells free of *Mycoplasma* and viral contamination were washed once with
Hank’s Balanced Salt Solution (HBSS; Mediatech, Inc., VA), and resuspended in HBSS prior to administration to mice. Solutions of nilotinib were prepared just prior to administration, by dissolving 100 mg in 1.0 mL of NMP to give a clear solution and diluting with 9.0 mL PEG300.

Male NCR-nude mice (5-6 weeks of age; Taconic, NY) were sublethally irradiated with a single fraction of 300 rads, and approximately 3 h later, a total of 800,000 cells were administered by tail vein injection. Anesthesized mice were imaged and total body luminescence was measured as previously described. Baseline imaging two days after tumor cell inoculation was used to establish treatment cohorts with matched tumor burden. Cohorts of mice were treated with oral administration of vehicle (10% NMP-90% PEG300), osmotic pump administration of 75 mg/kg imatinib, oral administration of 20 mg/kg/day nilotinib (formulated as above), or a combination of imatinib (75 mg/kg; osmotic pump) and nilotinib (20 mg/kg; oral gavage). Due to the significantly shorter half-life of imatinib in mice as compared to humans, an alternative to continuous drug administration via the osmotic pump would entail twice daily I.P. administration of imatinib, which has proven in our hands to be inefficient in terms of achieving maximum efficacy in mice. Treatment with vehicle and nilotinib was carried out for a total of 8 days; osmotic pumps were loaded with enough imatinib to allow up to 8 full days of treatment. Images were taken on Day 2, 4, 5, and 7 post-IV injection of 32D.p210-luc+ cells. On Day 7 post-IV, mice had received a total of 5 days of treatment with vehicle, nilotinib alone, imatinib alone, or the combination of nilotinib and imatinib. At the planned end of this study (nine days following the final imaging day), any remaining mice were sacrificed, body and spleen weights were recorded, and tissues preserved in 10% formalin for histopathological analysis.

Additional *in vivo* imaging studies were performed that included a variety of combinations of doses of nilotinib and imatinib, each administered alone and in combination to
male NCR-nude mice (5-6 weeks of age; Taconic, NY). Drug formulations, treatments, and imaging were carried out as described above with some variations in experimental design (described in figure legend). Mice were administered the doses of nilotinib and imatinib, alone or in combination, at 20mg/kg + 50mg/kg, respectively; 15mg/kg + 50mg/kg, respectively; and 15mg/kg + 75mg/kg, respectively. Histopathological analysis was carried out as described above.

For all in vivo imaging studies, we estimated doses of nilotinib and imatinib, respectively, which would result in subcurative, partial suppression of tumor burden in mice with each agent used alone. 15-20mg/kg of nilotinib were determined to be ideal subcurative doses in vivo; 50-75mg/kg of imatinib were determined to be ideal subcurative doses as well in vivo. The partial inhibition of tumor burden by each agent observed in vivo is analogous to the partial inhibition of Bcr-Abl+ cell proliferation by each agent observed in vitro.

Results

Drug combination studies: imatinib and nilotinib against imatinib-sensitive, Bcr-Abl-expressing cell lines

Nilotinib was tested in combination with imatinib across a range of doses and against a panel of imatinib-sensitive Bcr-Abl expressing cells. Combination indices calculated for K562, 32D.p210, and KU812 cell lines treated with nilotinib alone, imatinib alone, and nilotinib combined with imatinib, suggest overall additive to slightly synergistic effects between the two Bcr-Abl inhibitors when combined, with no evidence for antagonism across a range of doses (Figure 1, Table I).

To determine if the combination of nilotinib plus imatinib is more toxic against normal bone marrow than individual drugs, we performed a mouse bone marrow colony assay,
measuring CFU-GM and BFU-E, using the same concentrations of imatinib and nilotinib as were used in combination in proliferation studies, and bone marrow cells obtained from the flushed femur of an untreated male NCR nude mouse. The highest concentration of imatinib (400 nM) combined with nilotinib (30 nM) appeared to lead to a delay in colony formation, since after 7 days the overall number of colonies was lower for this treatment group (supplementary data). However, by the 12th day of observation, colony numbers for the highest dose group were more similar to the other treatments, suggesting minimal toxicity of the combination of imatinib and nilotinib against mouse bone marrow (Figure 1D, upper panel). Similarly, a colony assay using CD34+ human bone marrow cells showed no decline in the number of colonies formed on imatinib+nilotinib-treated plates as compared to vehicle (DMSO)-treated plates (Figure 1, lower panel).

Drug combination studies: imatinib and nilotinib against imatinib-resistant, Bcr-Abl-expressing cell lines

A panel of imatinib-resistant Bcr-Abl expressing cell lines was tested with either Bcr-Abl inhibitor alone, or both combined. For the imatinib-resistant Bcr-Abl point mutants, F317L and M351T, results ranged from synergistic to additive effects across a range of doses (Figure 2, Table I). For the imatinib-resistant Bcr-Abl point mutant, F486S, results ranged from nearly additive effects to slight to moderate antagonism (Figure 2, Table I). Overall, results observed for both imatinib-sensitive and imatinib-resistant Bcr-Abl expressing cell lines suggest positive enhancement of the activity of nilotinib and imatinib, respectively, when the two inhibitors are combined.
Nilotinib+imatinib combination experiments were performed using Ba/F3 cells expressing the E255K and Y253H Bcr-Abl point mutants, both of which were identified as conferring resistance to nilotinib in a mutagenesis assay. A two-day treatment of E255K-Ba/F3 cells with nilotinib only, imatinib only, or a combination of the two agents showed an increase in cell killing when both drugs were used together (Figure 2D, Table I). This experiment suggests positive drug enhancement (effects that range from slight-to-moderate synergy to nearly additive) when nilotinib is combined with imatinib in the treatment of E255K-expressing cells, as compared to each drug alone. In contrast, for the Y253H mutant, it did not appear that the addition of nilotinib enhanced the effects of imatinib against this mutant (supplementary data).

The highly imatinib-resistant T315I-Bcr-Abl point mutant was investigated for responsiveness to the combination of nilotinib and imatinib (supplementary data). The addition of 10 µM imatinib, which does not significantly inhibit the growth of T315I-Bcr-Abl-expressing cells, to a range of concentrations of nilotinib, resulted in only a modest increase in inhibition of cellular proliferation (supplementary data). Inhibitory effects on cell growth seen with nilotinib and imatinib against the T315I mutant were far less pronounced than those seen for more imatinib-sensitive Bcr-Abl point mutants, such as F317L (supplementary data).

**Induction of apoptosis and inhibition of proliferation of non-mutated and imatinib-resistant Bcr-Abl-expressing cells by nilotinib and imatinib.**

Nilotinib was tested in combination with imatinib for induction of apoptosis at select concentrations. In imatinib-sensitive and imatinib-resistant Bcr-Abl expressing cell lines, the combination of nilotinib and imatinib resulted in a higher degree of induction of apoptosis and inhibition of cellular proliferation as compared to each inhibitor alone (Figures 3 and 4). A
modest increase in induction of apoptosis was observed for the nilotinib and imatinib combination in the K562 cell line (supplementary data).

**Inhibition of cellular tyrosine phosphorylation in Bcr-Abl expressing cells by imatinib and nilotinib, combined**

Treatment of 32D.p210-luc+ cells for two hours with a combination of both nilotinib (0.005 µM) and imatinib (0.1 µM) resulted in more pronounced inhibition of cellular tyrosine phosphorylation in these cells, as compared with either 0.005 µM nilotinib or 0.1 µM imatinib alone (Figure 5). In contrast, cells treated with either compound alone or in combination led to an apparent increase in Bcr-Abl expression (Figure 5). This is a previously observed phenomenon that may be related to Bcr-Abl protein stabilization due to drug-protein complex formation.

**In vivo effects of the combination of nilotinib and imatinib on Bcr-Abl-expressing cells in a murine leukemia model**

To directly assess the *in vivo* anti-tumor efficacy of nilotinib alone, imatinib alone, and the combination of nilotinib and imatinib, we utilized a mouse model of CML in which tumor burden was quantified by non-invasive imaging of the luminescent tumor cells (Figures 6 and 7). Murine 32D.p210 cells were engineered to stably express firefly luciferase, and NCR nude mice were then inoculated with these cells. Non-invasive imaging was used to serially assess tumor burden, and mice with established leukemia were divided into cohorts with similar tumor burden. Nilotinib was then administered via oral gavage, as was vehicle. Osmotic pumps were surgically implanted into mice receiving imatinib.

In the first series of studies, nilotinib was tested at a dose of 15 or 20mg/kg, respectively, alone or in combination with 50 mg/kg imatinib. In the first study, mice were administered
vehicle, nilotinib (15mg/kg), imatinib (50mg/kg), or a combination of both imatinib and nilotinib at their respective doses (Figure 6). The lowest tumor burden as assessed by bioluminescence was observed to be in the drug combination group on day 8 post-IV injection of 32D.p210-luc+ cells (Figure 6A and B). The Student t-test was used for statistical evaluation of this experiment and yielded $p \leq 0.0073$ (vehicle versus drug combination on day 8 post-IV injection); $p \leq 0.0098$ (vehicle versus nilotinib on Day 8 post-IV injection); and $p \leq 0.3194$ (vehicle versus imatinib on Day 8 post-IV injection). Lowest percent spleen weights were observed in mice treated with either nilotinib alone or both Bcr-Abl inhibitors together, as compared to vehicle and imatinib alone, following sacrifice 7 days after the last imaging day (Figure 6C). The Student t-test (type 2 analysis, 2 tails) was performed for statistical evaluation of these results and yielded $p \leq 0.0020$ (vehicle versus nilotinib); $p \leq 0.0004$ (vehicle versus drug combination); and $p \leq 0.0678$ (drug combination versus imatinib). Differences between percent spleen weights for vehicle versus imatinib, and drug combination versus nilotinib, were not statistically significant ($p \leq 0.2142$ and $p \leq 0.6312$, respectively).

In the second study, 20mg/kg of nilotinib was tested alone and in combination with 50 mg/kg imatinib (Figure 6). In mice treated for a total of eight days with vehicle, nilotinib (20mg/kg), imatinib (50mg/kg), or a combination of nilotinib and imatinib, lowest tumor burden as measured by bioluminescence was observed to be in the drug combination group (Figure 6D and E). Imatinib as a single agent at 50mg/kg did not display notable efficacy, whereas nilotinib treatment alone at 20mg/kg resulted in substantial tumor suppression in this study (Figure 6D and E).

Additional in vivo imaging experiments were carried out in which mice were first sublethally irradiated with 300 rads prior to tail vein injection of 32D.p210-luc+ cells in an
attempt to increase engraftment and aggressiveness of tumor burden. In the first of these studies, following 5 days of daily treatment, tumor burden remained lower in mice treated with the combination of imatinib (75mg/kg) and nilotinib (20mg/kg), as compared to mice treated with either vehicle, imatinib alone, or nilotinib alone (Figure 7A and B). Similarly, lower percent spleen weights were observed in mice treated with both Bcr-Abl inhibitors, as compared to vehicle and either agent alone, following sacrifice ten days after the last imaging day (Figure 7C). The Student t-test (type 2 analysis, 2 tails) was performed for statistical evaluation of these results and yielded $p \leq 0.0791$ (vehicle versus nilotinib); $p \leq 0.0740$ (vehicle versus imatinib); $p \leq 0.000004$ (vehicle versus drug combination); $p \leq 0.0952$ (drug combination versus imatinib); and $p \leq 0.0004$ (drug combination versus nilotinib). Histopathological analysis of mice used in this experiment showed an apparent absence of leukemic cells in the spleens of several mice treated with both nilotinib and imatinib. These results reflect the positive drug combination effects between nilotinib and imatinib observed in Bcr-Abl expressing cell lines tested in vitro. The Student t-test was used for statistical evaluation of this experiment and yielded $p<0.0264$ (vehicle versus drug combination on Day 7 post-IV injection); $p<0.0275$ (vehicle versus imatinib only on Day 7 post-IV injection); $p<0.0569$ (vehicle versus drug combination on Day 5 post-IV injection); $p<0.0419$ (vehicle versus imatinib only on Day 5 post-IV injection).

In another study involving sublethal irradiation of mice prior to cell injection, mice were treated for a total of five days with vehicle, nilotinib (15mg/kg), imatinib (75mg/kg), or nilotinib combined with imatinib at their respective doses (supplementary data). Again, the lowest tumor burden as assessed by bioluminescence was observed to be in the drug combination group (supplementary data).
The apparent lack of effect of nilotinib observed alone at 15-20mg/kg in experiments involving pre-irradiation may be due to the influence of sublethal irradiation of mice prior to cell injection, as a correlation was observed in all experiments between the potency of nilotinib as a single agent and the inclusion/exclusion of pre-irradiation. To investigate the effects of gamma irradiation prior to IV injection of 32D.p210-luc+ cells on leukemia growth and nilotinib responsiveness, we sublethally irradiated a group of four mice with a single fraction of 300 rads prior to being IV-injected with 600,000 32D.p210-luc+ cells. Another group of four mice were not irradiated prior to cell injection. Both groups were then treated for a total of 6 days with either vehicle or nilotinib (20mg/kg); final imaging was performed on post-injection Day 7. As shown in Figure 7D and E, there was greater nilotinib responsiveness and less variable final tumor burden within the treatment groups for mice that were not sublethally irradiated prior to cell injections, as compared to mice that were sublethally irradiated. Raw bioluminescence values shown in Figure 7F suggest higher tumor engraftment and aggressive tumor growth in mice that were sublethally irradiated prior to cell injection, as compared to mice that were not irradiated. These results suggest that the inclusion of irradiation may influence the aggressiveness and pattern of leukemia growth in mice, which may consequently influence measurement of anti-tumor effects of nilotinib as a single agent.

Histopathological study of vital organs did not suggest any evidence of gross organ toxicity in any of the imatinib-nilotinib combination studies performed \textit{in vivo}. We performed additional assays, including investigation of five athymic nude mice that were injected with 32D.p210-luc+ cells and treated for a total of 1 week with either vehicle (NMP-PEG300) or a combination of imatinib (75mg/kg)+nilotinib (20mg/kg). Two mice received vehicle via oral gavage, and 3 mice received imatinib administered via osmotic pump+nilotinib administered via
oral gavage. The hematology profiles between the two groups of mice were similar (supplementary data).

**Discussion**

Imatinib remains a highly effective, frontline therapy for CML. However, the discovery of Bcr-Abl point mutations that impede imatinib from effectively inhibiting the activity of Bcr-Abl led to the development of second generation Bcr-Abl inhibitors, including nilotinib and the dual Src/Abl inhibitor, dasatinib (BMS-354825). Nilotinib, a novel aminopyrimidine ATP-competitive inhibitor of Bcr-Abl, has been shown to be at least 20-fold more potent than imatinib in the killing of non-mutated Bcr-Abl-expressing cells, and has been demonstrated to inhibit the activity of over 30 mutant forms of Bcr-Abl occurring in imatinib-resistant patients.

Nilotinib is currently in Phase II clinical trials for imatinib-resistant CML. In recent phase I studies performed in parallel at three American and European centers that involved nilotinib treatment of mostly advanced-staged, imatinib-resistant CML patients, significant activity was observed.

Since it is expected that, similar to imatinib, resistance to the second generation inhibitors will also present a challenge in the treatment of CML and Ph+ ALL, mutagenesis screens designed to identify drug resistant Bcr-Abl point mutations have been carried out. Such assays have been performed in an attempt to predict resistance mechanisms for nilotinib and dasatinib, as well as to establish the resistance profiles of the available Bcr-Abl inhibitors. Although these studies did not consistently identify the same drug-resistant Bcr-Abl point mutations for imatinib, nilotinib, and dasatinib, it was clear from the results of these studies that all three compounds display different mutagenicity profiles.
A combination between any two of the three inhibitors might be expected to impart significant clinical benefit, as each agent could be effective in suppressing the emergence of Bcr-Abl point mutants conferring resistance to the other Bcr-Abl inhibitors. In the case of imatinib-resistant leukemia, because of the nature of resistance mechanisms, such as emergence of drug-resistant clones, it will likely be of significant clinical benefit to simultaneously administer more than one Bcr-Abl inhibitor to patients as a way to suppress the development of these drug-resistant mutants.

The distinct binding properties of imatinib and the second generation Bcr-Abl inhibitors add to the potential of combination therapy. Crystallographic structural studies show that one molecule of BCR/ABL could only bind one molecule of imatinib, nilotinib, or dasatinib at a time. Imatinib and nilotinib preferentially bind the kinase in its inactive conformation, while dasatinib can bind to either the active or inactive conformation.

Combinations of different Bcr-Abl inhibitors, including dasatinib and imatinib, have been effective in reducing the occurrence of drug-resistant mutants. Similarly, the combination of imatinib with the dual Scr/Abl inhibitors AP23848 and dasatinib, respectively, has shown promise in preclinical in vitro studies involving treatment of non-mutated Bcr-Abl-expressing cells, as well as imatinib-resistant Bcr-Abl point mutant-expressing cells. Imatinib combined with each Src/Abl inhibitor led to enhanced drug effects against non-mutated Bcr-Abl expressing cells and the imatinib-resistant Bcr-Abl point mutant, M351T, which has been found in patients and is located in the SH2 contact region of Bcr-Abl; however only slight enhancement of drug effects was observed against more highly imatinib-resistant Bcr-Abl point mutants like Y253H and E255K. The apparent lack of antagonism observed between imatinib and the two Src/Abl inhibitors, respectively, against these Bcr-Abl-expressing cell lines was similar to the overall
positive cooperation observed between imatinib and nilotinib against many of the imatinib-sensitive and resistant cell lines that we investigated in the present study. Here, the combination of nilotinib and imatinib yielded additive to synergistic effects against the F317L and M351T mutants across a wide range of concentrations, whereas the F486S mutant was inhibited in a nearly additive fashion by the two inhibitors across a more limited range of concentrations. Some enhancement of activity was also observed between nilotinib and imatinib against the highly imatinib-resistant point mutant, E255V.

Bcr-Abl point mutations occurring in the nucleotide-binding (P) loop of the Abl kinase domain, such as Y253H and E255K, confer imatinib resistance by disrupting the induced-fit binding of imatinib to Abl. We examined the combined effects of nilotinib and imatinib against the E255K and Y253H mutants, which in addition to being resistant to imatinib, were also uncovered in a random mutagenesis screen designed to identify nilotinib-resistant mutants. Whereas the combination of both Abl inhibitors was nearly additive to synergistic against the E255K mutant using concentrations of imatinib lower than or equal to 10 µM, the combination of the two agents using the same therapeutically relevant range of concentrations of imatinib did not display additive-to-synergistic effects against the Y253H mutant.

The T315I mutation, which is believed to cause resistance by sterically inhibiting imatinib, is located within the Abl kinase domain of Bcr-Abl, and is positioned at the periphery of the Abl nucleotide binding site where it forms a direct and critical contact point between Bcr-abl and imatinib. As has been observed in other studies investigating combination effects of different Bcr-Abl inhibitors, the combination of nilotinib and imatinib in the present study was ineffective against the highly imatinib-resistant T315I mutant. These results suggest that combination of nilotinib and imatinib may be most effective against Bcr-Abl point mutants that
respond to physiologically achievable levels of imatinib, and less effective against mutants like T315I and Y253H that confer high imatinib resistance.

The observed positive cooperativity between imatinib and dasatinib against Bcr-Abl-expressing cells could be explained in part because dasatinib adds the capability of targeting both the active and inactive conformations of Bcr-Abl, but since this is not true for nilotinib, other mechanisms are likely to apply. For example, recent studies suggest that synergy between nilotinib and imatinib might result from interactions with cell transporters, such as the multidrug efflux transporter ABCG2, which confers resistance to certain anticancer drugs38, or the organic cation transporter Oct-1, which is important for imatinib influx but not for the uptake of nilotinib39. Such differential cell transport mechanisms between nilotinib and imatinib could account for synergistic interactions between the two agents. It will be important to determine if one drug influences the intracellular concentration, the metabolism, or the affinity of the other drug in order to better understand these results.

While nilotinib could potentially be used as a single agent in selected patients intolerant or resistant to imatinib, alternatively, nilotinib and imatinib could be administered together to achieve higher patient responsiveness and potentially reduce the likelihood of emergence of some BCR/ABL mutations conferring resistance to imatinib. Combinations of BCR/ABL kinase inhibitors could theoretically be administered on either a sequential, rotating schedule, or administered simultaneously, provided the drugs did not interfere with each other.

References


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**Table I:** Combination indices calculated for dose-response curves shown in Figure 1 and Figure 2. These data are representative of two independent studies. *Missing IC25 values could not be calculated since the percentage of inhibition started at around 30% for imatinib alone and around 60% or 30% for the drug combination.
Figure Legends

Figure 1: Drug combination studies: imatinib and nilotinib against imatinib-sensitive, Bcr-Abl-expressing cell lines. Proliferation studies showing three-day treatments of K562 cells (A), 32D.p210 cells (B), and KU812 cells (C) with nilotinib, imatinib, or a combination of nilotinib and imatinib. (D) Normal murine bone marrow colony assay (upper panel). Colony count following 12 days of treatment. Normal human bone marrow colony assay (lower panel). Colony count following 19 days of treatment.

Figure 2: Drug combination studies: imatinib and nilotinib against imatinib-resistant, Bcr-Abl-expressing cell lines. Proliferation studies showing three-day treatments of F317L-Ba/F3 cells (A), M351T-Ba/F3 cells (B), and F486S-Ba/F3 cells (C) with nilotinib, imatinib, or a combination of nilotinib and imatinib. Proliferation study showing a two-day treatment of E255K-Ba/F3 cells (D) with nilotinib, imatinib, or a combination of nilotinib and imatinib.

Figure 3: Induction of apoptosis and inhibition of proliferation of non-mutated Bcr-Abl-expressing cells by nilotinib and imatinib.

(A) Effects of nilotinib and imatinib, alone and combined, on induction of apoptosis of non-mutated Bcr-Abl-expressing cells following two days of treatment.

(B) Corresponding effects of nilotinib and imatinib, alone and combined, on proliferation of non-mutated Bcr-Abl-expressing cells following two days of treatment (n=2 for 32D,p210, n=1 for KU812).
Figure 4: Induction of apoptosis and inhibition of proliferation of imatinib-resistant Bcr-Abl-expressing cells by nilotinib and imatinib.

(A) Effects of nilotinib and imatinib, alone and combined, on proliferation (left panel; n=2) and induction of apoptosis (middle and right panels; n=1) of imatinib-resistant Bcr-Abl-expressing cells: F317L.

(B) Effects of nilotinib and imatinib, alone and combined, on proliferation (left panel; n=1) and induction of apoptosis (middle and right panels; n=1) of imatinib-resistant Bcr-Abl-expressing cells: M351T.

(C) Effects of nilotinib and imatinib, alone and combined, on proliferation (left panel; n=2) and induction of apoptosis (middle and right panels; n=1) of imatinib-resistant Bcr-Abl-expressing cells: F486S.

(D) Effects of nilotinib and imatinib, alone and combined, on induction of apoptosis of imatinib-resistant Bcr-Abl-expressing cells: E255V (n=1).

Figure 5: Inhibition of cellular tyrosine phosphorylation in Bcr-Abl expressing cells by imatinib and nilotinib, combined.

Immunoblot showing inhibitory effects of nilotinib and imatinib (alone and combined) on total cellular tyrosine phosphorylation and Bcr-Abl expression in non-mutated Bcr-Abl-expressing cells. Immunoblot was hybridized with a beta-actin antibody as a loading control.

Figure 6: In vivo effects of the combination of nilotinib (15-20mg/kg) and imatinib (50mg/kg) on Bcr-Abl-expressing cells in a murine leukemia model.
(A) Effects of vehicle, nilotinib alone (15mg/kg), imatinib alone (50mg/kg), or a combination of nilotinib and imatinib on growth of 32D.p210-luc+ cells in NCR nude mice. Mice were IV injected via tail vein with 800,000 cells/mouse (with no prior sublethal irradiation). Images were taken on Day1 and Day8 post-IV injection. On Day 8 post-IV, mice had received a total of 7 days of treatment with vehicle or drug. Mice were sacrificed, weighed, and preserved for histopathological analysis approximately 7 days after the last imaging day. Photo images show bioluminescence as seen on the last imaging day.

(B) Bioluminescence values plotted as percent of baseline for mice treated with vehicle, nilotinib alone (15mg/kg), imatinib alone (50mg/kg), or a combination of nilotinib and imatinib. Vehicle (n=3), nilotinib alone (n=3), imatinib alone (n=3), nilotinib+imatinib (n=3).

(C) Percent spleen weights for 32D.p210-luciferase-injected NCR nude mice treated with nilotinib alone (15mg/kg), imatinib alone (50mg/kg), or a combination of nilotinib and imatinib.

(D) Effects of nilotinib (20mg/kg) and imatinib (50mg/kg), alone and combined, on growth of 32D.p210-luc+ cells in NCR nude mice. Mice for this experiment were injected with 32D.p210-luc+ cells via tail vein injection (with no prior sublethal irradiation). Images were taken on Day 1, Day 6, Day 8, and Day 9 post-IV injection. By Day 9 post-IV injection, mice had received a total of 8 days of treatment with vehicle or drug. Mice were sacrificed and preserved for histopathological analysis on Day 9 post-IV injection. Photo images show bioluminescence as seen on the last imaging day.

(E) Bioluminescence values are plotted as percent of baseline. Vehicle (n=2), nilotinib only (n=2), imatinib only (n=2), nilotinib+imatinib (n=2).
**Figure 7:** *In vivo* effects of the combination of nilotinib (20mg/kg) and imatinib (75mg/kg) on Bcr-Abl-expressing cells in a murine leukemia model. Influence of pre-irradiation on mouse responsivity to nilotinib.

(A) Effects of vehicle, nilotinib alone (20mg/kg), imatinib alone (75mg/kg), or a combination of nilotinib and imatinib on growth of 32D.p210-luc+ cells in NCR nude mice. Mice were sublethally irradiated with a single fraction of 300 rads followed by tail vein injection of 800,000 cells/mouse. Images were taken on Day2, Day 4, Day 5, and Day 7 post-IV injection. On Day 7 post-IV, mice had received a total of 5 days of treatment with vehicle or drug. Mice were sacrificed, weighed, and preserved for histopathological analysis approximately nine days after the last imaging day. Photo images show bioluminescence as seen on the last imaging day for representative mice.

(B) Bioluminescence values plotted as percent of baseline for mice treated with vehicle, nilotinib alone (20mg/kg), imatinib alone (75mg/kg), or a combination of nilotinib and imatinib. Vehicle (n=6), nilotinib alone (n=4), imatinib alone (n=6), nilotinib+imatinib (n=5).

(C) Percent spleen weights for 32D.p210-luciferase-injected NCR nude mice treated with nilotinib alone (20mg/kg), imatinib alone (75mg/kg), or a combination of nilotinib and imatinib.

(D) Investigation of effects of gamma irradiation prior to IV injection of 32D.p210-luc+ cells on leukemia growth and nilotinib responsiveness. Mice were divided up into two groups of four: One group was sublethally irradiated with a single fraction of 300 rads prior to being IV-injected with 600,000 32D.p210-luc+ cells. The other group was not irradiated prior to cell injection. Both groups were then treated for a total of 6 days with either vehicle or nilotinib (20mg/kg); final images were performed on post-injection Day 7. Photo images show bioluminescence as seen on the last imaging day.
(E) Bioluminescence values plotted as percent of baseline for mice treated with vehicle or nilotinib alone (20mg/kg). Vehicle (n=4), nilotinib alone (n=4).

(F) Raw bioluminescence values.
Figure 1

A. K562

B. 32D.p210

C. KUB12

D. Colony Number

Cell Number (% of Control)

[Drug, nM]

niotinib
imatinib
Combination

niotinib alone
imatinib alone
niotinib+imatinib

niotinib 0 1.875 3.75 7.5 15 30 600
imatinib 0 37.5 75 150 300 600
Combination 0 1.875+3.75 7.5+15+30+600

niotinib 0 25 50 100 200 400
imatinib 0 25 50 100 200 400
Combination 0 1.875+3.75+7.5+15+30+400

niotinib 0 20 40 80 160 320
imatinib 0 25 50 100 200 400
Combination 0 1.25+2.5+5+10+20+320

niotinib 0 1.875 3.75 7.5 15 30 600
imatinib 0 25 50 100 200 400
Combination 0 1.875+3.75+7.5+15+30+400

niotinib 0 25+50+100+200+400
imatinib 0 25+50+100+200+400
[nilotinib, nM]+[imatinib, nM]

Colony Number

[Imatinib, nM]+[nilotinib, nM]
Figure 2
Figure 3
Figure 5
Figure 6
Figure 7

A. For each group of mice, images show representative samples of tumors after treatment with different drugs:
- Vehicle
- imatinib (75 mg/kg)
- nilotinib (20 mg/kg)
- nilotinib + imatinib

B. Graph showing Tumor Burden (% baseline) over Days post-IV injection for each treatment group:
- Vehicle
- Nilotinib (20 mg/kg)
- Nilotinib (75 mg/kg)
- Nilotinib + Imatinib

C. Bar chart showing Percentage of Tumor burden for each treatment group:
- Vehicle
- Nilotinib (20 mg/kg)
- Nilotinib (75 mg/kg)
- Nilotinib + Imatinib

D. Graph showing the effect of Gamma irradiation and No irradiation on Tumor Burden (% baseline) over Days post-IV injection:
- Gamma irradiation
- No irradiation

E. Graph showing Total Flux (photons/sec) over Days post-IV injection for each treatment group:
- Vehicle + gamma irradiation
- Nilotinib (20mg/kg) + gamma irradiation
- Nilotinib (20mg/kg) (no irradiation)
- Nilotinib (20mg/kg) (gamma irradiation)
- Vehicle (no irradiation)
Beneficial effects of combining nilotinib and imatinib in preclinical models of BCR/ABL+ leukemias

Ellen L. Weisberg, Laurie Catley, Renee D. Wright, Daisy Moreno, Lolita Banerji, Arghya Ray, Paul W. Manley, Juergen Mestan, Doriano Fabbro, Jingrui Jiang, Elizabeth Hall-Meyers, Linda Callahan, Jamie L. DellaGatta, Andrew L. Kung and James D. Griffin