Anti-Leukemic Effects of the Novel, Mutant FLT3 inhibitor, NVP-AST487: Effects on PKC412-Sensitive and –Resistant FLT3-Expressing Cells

Anti-Leukemic Effects of NVP-AST487

Ellen Weisberg1*, Johannes Roesel2*, Guido Bold2, Pascal Furet2, Jingrui Jiang1, Jan Cools3, Renee D. Wright4, Erik Nelson,1 Rosemary Barrett,1 Arghya Ray1, Daisy Moreno5, Elizabeth Hall-Meyers5, Richard Stone1,6, Ilene Galinsky1, Edward Fox7, Gary Gilliland8, John F. Daley1, Suzan Lazo-Kallanian1, Andrew L. Kung4, and James D. Griffin1,9

1Department of Medical Oncology/Hematologic Neoplasia, Dana Farber Cancer Institute, Boston, Massachusetts
2Novartis Pharma AG, Basel, Switzerland
3Department of Molecular and Developmental Genetics, Flanders Interuniversity Institute for Biotechnology (VIB), University of Leuven, Belgium
4Department of Pediatric Oncology, Dana Farber Cancer Institute and Children’s Hospital, Boston, Massachusetts
5Animal Resources Facility, Dana Farber Cancer Institute, Boston, Massachusetts
6Department of Medicine, Harvard Medical School, Department of Medicine, Brigham and Women’s Hospital, Boston, Massachusetts
7Molecular Diagnostics Laboratory, Dana Farber Cancer Institute, Boston, Massachusetts
8Brigham and Women's Hospital, Boston, Massachusetts
9Corresponding author:
James D. Griffin, M.D.
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Abstract

An attractive target for therapeutic intervention is constitutively-activated, mutant FLT3, which is expressed in a subpopulation of AML patients and is generally a poor prognostic indicator in patients under the age of 65. PKC412 is one of several mutant FLT3 inhibitors that is undergoing clinical testing, and which is currently in late-stage clinical trials. However, the discovery of drug-resistant leukemic blast cells in PKC412-treated AML patients has prompted the search for novel, structurally diverse FLT3 inhibitors that could be alternatively used to override drug resistance. Here, we report the potent and selective antiproliferative effects of the novel mutant FLT3 inhibitor, NVP-AST487, on primary patient cells and cell lines expressing FLT3-ITD or FLT3 kinase domain point mutants. NVP-AST487, which selectively targets mutant FLT3 protein kinase activity, is also shown to override PKC412 resistance in vitro, and has significant anti-leukemic activity in an in vivo model of FLT3-ITD-positive leukemia. Finally, the combination of NVP-AST487 with standard chemotherapeutic agents leads to enhanced inhibition of proliferation of mutant FLT3-expressing cells. Thus, we present a novel class of FLT3 inhibitors that displays high selectivity and potency toward FLT3 as a molecular target, and which could potentially be used to override drug-resistance in AML.

Introduction

Acute myelocytic leukemia (AML) is a malignant disorder of hematopoietic cells with an incidence of around 10,000 new cases per year in the U.S. The main features of AML are excessive proliferation of myeloid precursor cells and a block of cellular differentiation. The aberrant survival advantage of leukemic cells leads to infiltration of bone marrow and peripheral blood with immature leukemic myeloblasts resulting in bone marrow failure and such symptoms as anemia, bleeding and
infection. Age, history of myelodysplasia, cytogenetics, and MDR1 expression are major prognostic
determinants.5

Current therapies for AML often fail because of treatment-induced mortality or drug resistance2. The use of conventional chemotherapeutic agents alone is associated with a high risk of relapse, but a low treatment-induced mortality3. Allogeneic transplantation (alloBMT), a standard approach for the treatment of adults with AML, has a lower risk of relapse but a high treatment-induced mortality3. AlloBMT results in 25-30% ten-year survival for young patients, however the outcome is poor for patients near the age of 60, and since the median age of AML patients is 64 years, the impact of current therapy on the majority of patients with this disease is small4.

The class III receptor tyrosine kinase, FLT3 (Fms-Like Tyrosine kinase-3; STK-1, human Stem Cell Tyrosine Kinase-1; or FLK-2, Fetal Liver Kinase-2)5, is constitutively activated by mutations occurring in approximately 30% of AML patients and is regarded as an attractive target for therapy. The most common type of FLT3 mutation thus far identified is internal tandem duplications in the juxtamembrane (JM) domain (FLT3-ITD)6, observed in approximately 20-25% of AML patients, but <5% of patients with myelodysplastic syndrome (MDS)6,7,8,9,10,11. Another type of FLT3 mutation is point mutations within the "activation loop" of the kinase12, which are believed to change the conformation of the domain, causing it to adopt an "activated" configuration. This mutation occurs in approximately 7% of AML cases, most with a missense mutation in the aspartic acid residue at position 835. Less commonly, other point mutations in the kinase domain have been reported, including N841I13 and Y842C14.

FLT3-ITD is associated with decreased survival, while the prognostic impact of the D835Y mutation is less clear. Expression of each of these constitutively activated mutants in cells enhances viability, confers growth-factor independent growth, and increases FLT3 autophosphorylation and
tyrosine phosphorylation of other signaling factors\textsuperscript{11,15}. In addition, the transplantation of murine bone marrow cells infected with a retrovirus expressing a FLT3-ITD mutant leads to the development of a rapidly lethal myeloproliferative disease in mice\textsuperscript{16}.

Several inhibitors of mutant FLT3 have been developed and are being tested as a novel therapeutic approach for AML, based on the prevalence of mutant forms of FLT3 in AML patients, and the demonstrated enhancement of cellular proliferation, viability, and tyrosine phosphorylation by mutant FLT3. We have previously described the inhibitory effects of the protein tyrosine kinase inhibitor PKC412 on mutant FLT3-expressing cells \textit{in vitro} and \textit{in vivo}\textsuperscript{17}. Up to now, none of these inhibitors has achieved sustained cytogenic responses as a single agent in AML patients and combination therapy has emerged as the currently preferred therapeutic strategy. However, the detection of drug-resistant leukemic blast cells in PKC412-treated AML patients has prompted us to search for novel, structurally diverse FLT3 inhibitors. These are expected to prevent development of drug-resistance if applied in combination with anti-leukemic agents.

We report here initial characterization of NVP-AST487, a potent and selective inhibitor of mutant FLT3 protein kinase activity. We demonstrate that this compound selectively induces cell cycle arrest and apoptosis of leukemic cells harboring mutant FLT3 with a potency approximately 50X higher than that of the FLT3 inhibitor PKC412, with no apparent effect on cells expressing wild-type FLT3. Furthermore, we show that NVP-AST487 actively inhibits proliferation of patient blasts harboring the FLT3-ITD mutation and PKC412-resistant isoforms of FLT3-ITD. We also show that NVP-AST487 significantly extends the survival of mice with FLT3-ITD-induced leukemia. These results support the notion that FLT3 is a promising therapeutic target for AML, and demonstrates the emergence of a novel class of FLT3 inhibitors that display high selectivity and strong efficacy toward FLT3 as a molecular target.
**Materials and Methods**

**Cell lines and cell culture**

The IL-3-dependent murine hematopoietic cell line Ba/F3 was transduced with either FLT3-ITD or FLT3-D835Y-containing MSCV retroviruses harboring a neomycin selectable marker, and selected for resistance to neomycin\(^{16}\). Mutant FLT3-transduced cells were selected for growth in G418 (1mg/ml) PKC412-resistant Ba/F3 cell lines expressing FLT3 harboring mutations in the ATP-binding pocket were developed as described previously\(^{18}\). All cell lines were cultured with 5% CO\(_2\) at 37\(^\circ\)C, at a concentration of 2X10\(^5\) to 5X10\(^5\) in RPMI (Mediatech, Inc., Herndon, VA) with 10% fetal calf serum and supplemented with 1% glutamine. Untransfected parental Ba/F3 cells were similarly cultured with 15% WEHI-conditioned medium as a source of IL-3. Mutant FLT3-expressing cells were cultured in media supplemented with G418 (1mg/ml).

**Chemical compounds and biologic reagents**

NVP-AST487 and PKC412 were synthesized by Novartis Pharma AG, Basel, Switzerland, and were dissolved in DMSO to obtain 10 mM stock solutions. Serial dilutions were then made, to obtain final dilutions for cellular assays with a final concentration of DMSO not exceeding 0.1%. Cytosine β-D-arabinofuranoside (Ara-c) (C1768) and doxorubicin hydrochloride (D1515) were purchased from Sigma-Aldrich (St. Louis, MO).

**Cell viability, cell cycle and apoptosis analysis**

The trypan blue exclusion assay has been previously described\(^{17}\), and was used to determine proliferation of cells cultured in the presence and absence of NVP-AST487. Cell viability is reported as
percentage of control (untreated) cells, and data are presented as the average of two independent experiments, except where indicated. Error bars represent the standard error of the mean for each data point. Apoptosis of drug-treated cells was measured using the Annexin-V-Fluos Staining Kit (Boehringer Mannheim, Indianapolis, IN), as previously described\textsuperscript{17}. Cell cycle analysis was performed as previously described\textsuperscript{17}.

\textit{Antibodies}

Anti-p-Tyr (clone 4G10, Upstate Biotechnology, NY) was used at 1:1000 for immunoblotting. Anti-FLT3/Flk-2 (C-20, Santa Cruz Biotechnology, Inc., CA) was used at 1:200 for immunoblotting. Anti-p-STAT5 (#9359, Cell Signaling Technology, MA) and STAT5 (sc-835, Santa Cruz Biotechnology, Inc. CA) were used at 1:10,000 for immunoblotting.

\textit{Immunoprecipitation}

Protein lysis preparation and immunoprecipitation and immunoblotting were carried out as previously described\textsuperscript{17}.

\textit{Drug combination studies}

For drug combination studies, NVP-AST487 and Ara-C, doxorubicin, or PKC412 were added simultaneously at fixed ratios to FLT3-ITD-Ba/F3 cells. Cell viability was determined using the trypan blue exclusion assay, and expressed as the function of growth affected (FA) drug-treated versus control cells; data were analyzed by CalcuSyn software (Biosoft, Ferguson, MO and Cambridge, UK), using the Chou-Talalay method\textsuperscript{19}. The combination index=$[D_1][D_2]/[D_3]$, where $[D_1]$ and $[D_2]$ are the concentrations required by each drug in combination to achieve the same effect as concentrations $[D_3]$.\textsuperscript{19}
and \([D_{x}]_2\) of each drug alone. Values less than one indicate synergy, whereas values greater than one indicate antagonism.

**Bone Marrow Colony Assay**

Normal human bone marrow cells were obtained from to-be-discarded bone marrow harvest collection bags, under approval of the Institutional Review Board. Cells were lysed in ammonium chloride buffer to remove erythrocytes and washed. Mononuclear cells were isolated from normal bone marrow by density gradient centrifugation through Ficoll-Plaque Plus (Amersham Pharmacia Biotech AB, Uppsala, Sweden) at 2000 rpm for 30 minutes, followed by two washes in 1X PBS. Normal human bone marrow was analyzed in a colony assay: plates of \(5 \times 10^4\) cells in “complete” methylcellulose medium containing recombinant cytokines (contents: fetal bovine serum, rh SCF, rh GM-CSF, rh IL-3, Bovine Serum Albumin, methylcellulose in Iscove’s MDM, 2-Mercaptoethanol, rh Erythropoietin, L-Glutamine) (MethoCult GFH4434, StemCell Technologies, Inc., Vancouver, BC) were prepared. The plates also contained NVP-AST487 at the indicated concentrations. The plates were incubated at 37°C in 5% CO₂ for > 1 week, and then myeloid and erythroid colonies (early progenitors with erythroid and myeloid components: CFU-GM, CFU-E, BFU-E, and CFU-GEMM) were counted on an inverted microscope.

**AML patient cells**

Frozen vials of peripheral blood and bone marrow samples from AML patients identified as harboring non-mutated FLT3 or the FLT3-ITD mutation (supplementary data) were thawed prior to
isolation of mononuclear cells (obtained as above, using Ficoll-Plaque Plus). Mononuclear cells were then tested in liquid culture (Iscove’s MDM, supplemented with 20% FCS) in the presence of different concentrations of NVP-AST487. All blood and bone marrow samples from AML patients were obtained under approval of the Dana Farber Cancer Institute Institutional Review Board.

Mouse studies

The FLT3-ITD-Ba/F3 cell line used in animal studies was free of Mycoplasma contamination and viral contamination. Cells were washed once with 1X Hank's balanced salt solution (HBSS) (Mediatech, Inc., Herndon, VA, USA), and then resuspended in 1X HBSS prior to administration to animals. Female nude mice (Nu/Nu) (Charles River Laboratories, Massachusetts), initially weighing 12-15 g and 6 weeks of age at delivery, were administered cell suspensions containing 1X10^6 FLT3-ITD-Ba/F3 cells via tail vein injection. After 3 days, 3 groups of eight FLT3-ITD-Ba/F3-injected mice were treated by gavage with either vehicle (10% N-Methylpyrrolidinone (NMP)-90% polyethyleneglycol (PEG300)) or NVP-AST487 (30mg/kg/day or 50mg/kg/day) once daily for 21 days. At the planned end of the study, body and spleen weights were recorded, and tissues were preserved in 10% formalin for histopathological analysis. Studies involving mice were performed with ACUC protocols at Dana Farber Cancer Institute.

Survival was measured as time from cell injection to morbidity, according to Institute protocols, at which time mice were sacrificed. All starting animals were included in the statistical analysis. Survival analysis was performed using the method of Kaplan and Meier with statistical significance assessed using the log rank test.

For the in vivo bioluminescence assay, FLT3-ITD-Ba/F3-luc+ cells free of Mycoplasma and viral contamination were resuspended in Hank’s Balanced Salt Solution (HBSS; Mediatech,
Inc., VA) prior to IV administration to mice. PKC412, synthesized by Novartis Pharma AG, Basel, Switzerland, was supplied as a microemulsion preconcentrate (5% w/v) and diluted with water to achieve the desired final concentrations. Diluted solutions were stored at 4°C until used for gavage treatment of mice. NVP-AST487 was prepared as described above.

Male nude mice (Nu/Nu) (Charles River Laboratories, Massachusetts), 6 weeks of age at delivery, were administered a total of 800,000 FLT3-ITD-Ba/F3-luc+ cells by tail vein injection. Mice were imaged and total body luminescence quantified as previously described. Baseline imaging one day after tumor cell inoculation was used to establish treatment cohorts with matched tumor burden. Cohorts of mice were treated with oral administration of vehicle, 50 or 100 mg/kg/day PKC412 (formulated as above), or 50mg/kg NVP-AST487 (formulated as above). Imaging was performed 6 days post-IV injection of cells. Mice were then sacrificed, and tissues were preserved in 10% formalin for histopathological analysis.

**Determination of drug concentration in mouse plasma**

Female OF1 mice (n=4) received a single oral dose of 15 mg/kg formulated in 10 % N-methyl pyrrolidone/90 % PEG200 (v/v). At the allotted times mice were sacrificed and the plasma concentration of compound determined by reversed-phase HPLC/MS-MS analysis.

**Results**

*NVP-AST487 is a potent FLT3 inhibitor*

NVP-AST487 (structure, Figure 1A) was tested in biochemical assays for inhibition of FLT3 kinase activity. The Ki was determined to be 0.12 μM (supplementary data). Besides FLT3, NVP-AST487 inhibits RET, KDR, c-KIT and c-ABL kinase with IC50 values below 1 μM.
Inhibition of cellular proliferation of mutant-FLT3-expressing cell lines and AML patient cells by NVP-AST487

Treatment of FLT3-ITD-Ba/F3 cells and D835Y-Ba/F3 cells with NVP-AST487 potently inhibited cellular proliferation (IC50 < 0.005 μM) (Figure 1B and 1C). Supplementation of culture media with WEHI, used as a source of IL-3, led to rescue of the cells, suggesting that NVP-AST487 selectively inhibits FLT3-ITD, and has no effect on IL-3 signaling. The antiproliferative activity of NVP-AST487 was not blunted by addition of human serum (supplementary data). Cells expressing the novel point mutant FLT3-N841I also showed sensitivity to NVP-AST487 (supplementary data).

Parental Ba/F3 cells were not affected by up to 0.1 μM NVP-AST487 (Figure 1B). Similarly, the results of a CFU-GM colony formation assay showed no toxicity of human bone marrow progenitor cells at concentrations up to 0.1 μM NVP-AST487 (Figure 1D).

Several AML patient samples, characterized as harboring the FLT3-ITD mutation (supplementary data), were treated for three days with NVP-AST487 in parallel with FLT3-ITD-Ba/F3 cells (as a control) (Figure 2). NVP-AST487 treatment of FLT3-ITD-Ba/F3 cells with 0.01μM NVP-AST487 resulted in complete cell killing, compared to approximately 50% killing of AML patient samples at the same concentration (Figure 2A). PKC412 and NVP-AST487 were equipotent in efficacy against AML patient samples harboring mutant FLT3 and wild-type FLT3 (Figure 2B-E and supplementary data).

Inhibition of cellular proliferation of PKC412-sensitive and -resistant mutant FLT3-expressing cells by NVP-AST487
NVP-AST487 was tested in parallel with PKC412 against FLT3-ITD-Ba/F3 cells, and was found to exhibit significantly higher potency (10-50 fold) in inhibiting this cell line (Figure 3A). Similarly, as compared with PKC412, NVP-AST487 was approximately 100-fold more potent in inhibiting proliferation of PKC412-resistant FLT3-ITD-Ba/F3 cells that were previously generated\textsuperscript{17} by culturing FLT3-ITD-Ba/F3 cells in the presence of gradually increasing concentrations of PKC412 over a period of time (Figure 3B).

A panel of Ba/F3-derived cell lines that express FLT3-ITD harboring point mutations in the ATP-binding pocket of FLT3 were treated with either PKC412 or NVP-AST487; these mutations were previously shown to confer resistance to PKC412\textsuperscript{18}. There was an approximately 10-fold difference in potency of PKC412 against Ba/F3 cells expressing the FLT3-ITD mutation, as compared with the majority of Ba/F3 cells harboring mutations in the ATP-binding pocket of FLT3 (Figure 3C). A similar shift in potency was observed in NVP-AST487-treated cells (Figure 3D). However, already 0.01 μM NVP-AST487 proved cytotoxic for the majority of mutant FLT3-expressing cells, whereas 0.1 μM PKC412 were necessary for killing the majority of mutant FLT3-expressing cells (Figure 3C and Figure 3D). These results demonstrate an overall high potency of NVP-AST487 toward PKC412-resistant and non-resistant, mutant FLT3-expressing cells.

\textit{Induction of apoptosis and inhibition of cell cycle progression of mutant FLT3-expressing cells by NVP-AST487}

A dose-dependent increase of apoptotic cells was observed in FLT-ITD-Ba/F3 cells cultured in the presence of NVP-AST487 (at concentrations up to 0.1 μM), (Figure 4A). Viability of cells cultured in the presence of the inhibitor in media supplemented with IL-3 was preserved following three days of
treatment (Figure 4A). Induction of apoptosis was similarly observed in D835Y-Ba/F3 cells treated for three days in the presence of NVP-AST487 at concentrations of 0.01μM and 0.1μM (Figure 4B). There was no apparent induction of apoptosis of parental Ba/F3 cells cultured with IL-3 in the presence of NVP-AST487 for the same length of time (Figure 4C).

Treatment of FLT3-ITD-Ba/F3 cells with 0.01 μM NVP-AST487 for 36 hours resulted in G1 arrest of cells (Figure 4D). This suggests that the mechanism whereby these inhibitors inhibit cellular proliferation is via inhibition of cell cycle progression as well as induction of programmed cell death.

**Inhibition of autophosphorylation of FLT3 in mutant-FLT3-expressing cells**

Treatment of FLT3-ITD-Ba/F3 cells with 0.01 μM NVP-AST487 inhibited autophosphorylation of mutant FLT3 in these cells, with no apparent reduction in levels of the FLT3 protein (Figure 5A). Similar results were obtained with FLT3-N841I-Ba/F3 cells treated with 0.1 μM inhibitor (Figure 5B). A downstream effector of FLT3, STAT5, was also inhibited by both NVP-AST487 and PKC412 at 10 nM (Figure 5C and D). These results suggest that FLT3 kinase is a target of NVP-AST487 and inhibition of mutant FLT3 kinase activity leads to loss of growth factor independence and consequent cell death.

**Plasma concentrations of NVP-AST487 in mice after oral administration**

The bioavailability of NVP-AST487 was analyzed in mice. Mice received a single oral dose of 15 mg/kg NVP-AST487 formulated in 10% NMP-90% PEG300 by gavage. Plasma concentrations peaked after 1 hr (Cmax 0.5 uM) and exceeded 0.1 uM for 6 hrs (supplementary data). C24hr was found to be >20 nM (supplementary data). Therefore, NVP-AST487 is orally bioavailable and reaches plasma concentrations expected to antiproliferative based on cellular experiments.
Efficacy of NVP-AST487 in mice bearing mutant FLT3-expressing cells

The ability of the inhibitor, NVP-AST487, to inhibit proliferation of mutant FLT3-expressing cells in vivo was investigated using athymic nude mice that had been inoculated with FLT3-ITD-Ba/F3 cells via tail vein injection. Mice were orally administered vehicle (10% NMP-90% PEG300), 30mg/kg NVP-AST487 (“low dose” NVP-AST487) or 50mg/kg of NVP-AST487 (“high dose” NVP-AST487) for a total of 21 days by gavage. Drug was not administered on weekends. All vehicle-treated mice died after 24 days following initial injection of the FLT3-ITD-Ba/F3 cells, whereas the majority of NVP-AST487-treated mice (at both doses) survived up to Day 29 (Figure 6A). Median survival for vehicle control mice was 20 days; median for low and high dose mice was 30 days. The survival was different among the three groups, p < 0.0001. Vehicle control mice died sooner than the low dose-treated mice (p < 0.0001) and sooner than the high dose-treated mice (p=0.005). There was no significant difference in survival between the low and high dose mice, p=0.70.

At necropsy, all mice showed enlarged spleens, indicative of disease progression (supplementary data). The p-value for differences in total mouse weight was 0.52; for spleen it was 0.37. Overall, these results demonstrate that NVP-AST487 prolongs the lifespan of mice harboring the FLT3-ITD mutation.

To confirm the in vivo anti-tumor efficacy of NVP-AST487 versus PKC412 as observed in the first in vivo study, we tested a mouse model of acute leukemia in which tumor burden was quantified by non-invasive imaging of luminescent tumor cells (Figure 6B and C). NCr nude mice were inoculated with FLT3-ITD-Ba/F3 cells engineered to stably express firefly luciferase. Non-invasive imaging was used to assess tumor burden, and mice with established leukemia were divided into cohorts with similar tumor burden. NVP-AST487 (50mg/kg, 1X daily) and PKC412 (100mg/kg, 1X daily) were then administered via oral gavage, as was vehicle. Both
NVP-AST487 and PKC412 suppressed leukemia burden in mice, as compared to vehicle-treated controls (Figure 6B and C).

**NVP-AST487 positively combines with Ara-c, doxorubicin, and PKC412**

NVP-AST487 was tested in combination with Ara-C, doxorubicin, and PKC412 against FLT3-ITD-Ba/F3 cells. CalcuSyn analysis of the combined effects of Ara-C and NVP-AST487 (Figure 7A) suggested slight to moderate synergism. CalcuSyn analysis of a second, independent analysis of the combination of Ara-C and NVP-AST487 (supplementary data) suggested effects that ranged from nearly additive to moderately synergistic. CalcuSyn analysis of the combined effects of doxorubicin and NVP-AST487 (Figure 7B) suggested slight synergy between the two agents. CalcuSyn analysis of the combined effects of PKC412 and NVP-AST487 (Figure 7C) suggested effects that ranged from slight antagonism to slight synergism between the two agents.

NVP-AST487 was also tested for synergy with Ara-c and doxorubicin by administering the agents sequentially, in comparison to simultaneous treatment. The simultaneous administration of NVP-AST487 with Ara-C or doxorubicin led to the strongest positive combination effect, which was similar to and comparable with sequential administration of Ara-c or doxorubicin 24 hr prior to administration of NVP-AST487 (supplementary data). However, the administration of NVP-AST487 24 hr prior to either Ara-c or doxorubicin resulted in a slightly weaker positive combination effect as compared to the other regimens (supplementary data).

**Discussion**

Constitutively activating mutations of FLT3 occur in a subset of AML patients and are associated with a poor prognosis. Because FLT3 is an attractive molecular target for the treatment of
AML, a variety of inhibitors of the FLT3 tyrosine kinase have been developed and are currently being investigated in early phase clinical trials involving patients with AML refractory to standard chemotherapy.

The N-benzoylstaurosporine, PKC412 (Novartis Pharma AG), is an orally bioavailable inhibitor of FLT3, PDGFR-beta, c-KIT, and c-FMS that induces cell cycle arrest and apoptosis of mutant-FLT3-expressing cells via direct inhibition of FLT3. Results of a Phase II clinical trial testing PKC412 as a single agent showed the drug to be generally well tolerated, demonstrated inhibition of patient FLT3 phosphorylation and a decrease in peripheral blast counts in 35% of treated relapsed/refractory AML patients with a median response duration of 13 weeks; PKC412 induces a hematological response rate in advanced AML patients comparable to what is observed in CML blast crisis patients treated with imatinib. The indolinone SU5416 (SuGen), which also inhibits c-KIT, VEGFR1/2, and the SCF receptor, was demonstrated in a multicenter Phase II clinical trial to inhibit phosphorylation of FLT3 in patients with refractory AML; partial responses were observed in a subset of patients lasting from 1-5 months. In another multicenter phase II study, SU5416 as a single agent had modest clinical activity in refractory AML patients, with overall median survival of 12 weeks and grade 3 or 4 drug-related toxicities believed to be due to drug formulation. A phase II hematological malignancy trial in the US showed inhibition of FLT3 phosphorylation in SU5416-treated refractory AML patients, although the vast majority of patients did not show a clinical response. Treatment of mutant FLT3-positive AML patients in Phase I clinical trials with the orally administered indolinone SU11248 (SuGen) was demonstrated to have anti-FLT3 activity in patients and resulted in morphologic or partial responses of short duration. The novel, orally-administered, FLT3 inhibitor, CEP-701 (Cephalon), an indolocarbazole derivative, shows effectiveness against FLT3-ITD-expressing AML cells, and induces clinical responses of short duration in a Phase I/2 clinical trial in patients with relapsed or refractory...
Complete inhibition of FLT3 autophosphorylation was observed in several patients with no accompanying clinical response. Several other patients showed a decrease in peripheral blood leukemic blasts to <5%; 1 patient showed a decrease in bone marrow blasts to a similar extent that was still apparent after 1 month. Also in early clinical trials involving patients with relapsed or refractory AML is the piperazonyl quinazonline MLN518 (CT53518; Millennium). Recent reports of new FLT3 inhibitors in preclinical development include Ki23819, which has been shown to be effective against FLT3-ITD-expressing human cell lines.

However, the FLT3 inhibitors tested thus far generally induce partial and transient responses in patients when used as single agents, suggesting a need for development of newer, possibly more efficacious/less toxic inhibitors of FLT3 that can be used effectively as single agents. It also suggests a need to investigate the potential of these compounds in combination with other therapeutics already in clinical use.

In our report, we present preclinical data for NVP-AST487 that show high potency and selectivity toward mutant FLT3 as a target, as evidenced by inhibition of cellular proliferation of FLT3-ITD- and D835Y-expressing Ba/F3 cells with an IC50 < 0.005 μM, induction of apoptosis of mutant FLT3-expressing cells and inhibition of cell cycle progression, and inhibition of FLT3 autophosphorylation in these cells. In addition, NVP-AST487 was observed to significantly reduce leukemia burden and prolong the survival of mice harboring mutant FLT3. Furthermore, NVP-AST487 was shown to potently kill a panel of PKC412-resistant Ba/F3 cell lines expressing FLT3 harboring mutations in the ATP-binding pocket. This observation is clinically relevant, as resistance to PKC412 in patients has been attributed to pre-existing or acquired mutations in the kinase domain of FLT3.

A model of NVP-AST487 bound to the FLT3 kinase domain was created to rationalize our observations (supplementary data). The kinase was modeled in the inactive “DFG out”
conformation based on a crystal structure of the Abl kinase in complex with a similar inhibitor. According to the model, as in the case of PKC412, the side chains of residues A627 and N676 do not have any contact with NVP-AST487. However, contrary to PKC412, NVP-AST487 does not make extensive contacts with residues G697 and F691, and mutations of these residues are not expected to dramatically alter the interaction of the inhibitor with the binding site. The observation that all the mutations investigated uniformly reduce the inhibitory activity of NVP-AST487 by approximately one order of magnitude, together with the previous modeling considerations, suggest an indirect effect of these mutations on the binding of the inhibitor by destabilization of the “DFG out” conformation.

As treatment with FLT3 inhibitors as single agents has demonstrated limited efficacy in refractory AML patients, clinical outcome can potentially be improved by incorporation of novel FLT3 inhibitors into standard chemotherapeutic regimens. Various FLT3 inhibitors have been tested with standard AML chemotherapy drugs as a way to assess the overall efficacy of combined therapies. SU11248 was found, when combined with cytarabine or daunorubicin to exhibit additive-to-synergistic inhibitory effects on mutant FLT3-ITD-expressing cells. The FLT3 inhibitor, CEP-701, was demonstrated to be synergistic with cytarabine, daunorubicin, mitoxantrone, and etoposide, respectively when administered simultaneously with the chemotherapy agents or immediately following their administration.

The combination of NVP-AST487 with Ara-C, doxorubicin, or PKC412 generally resulted in additive to synergistic effects. This suggests that FLT3 inhibitors like NVP-AST487 could potentially be used in combination with standard chemotherapeutic agents currently in use for AML, and that the addition of such potent inhibitors of FLT3 to AML chemotherapy regimens could potentially result in improved treatment results.
The poor clinical outcome of standard chemotherapeutic agents presently in use for AML, and the limited utility of more promising approaches such as alloBMT, point to a need for development of novel therapeutic strategies that could translate into higher overall drug responsiveness and a lower incidence of relapse. One approach is the use of two different FLT3 inhibitors that could potentially be used together if the mechanism whereby cells develop resistance to each is different. The development of novel agents such as NVP-AST487, with unique structures conferring higher potency and selectivity toward FLT3 as a target, represents a step toward overcoming some of the existing challenges and obstacles in the therapy of AML. It will be of great interest to determine whether or not this emerging new class of compounds has a beneficial therapeutic effect in AML patients.
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Editorial Note: Authors’ Contributions:

Ellen Weisberg is responsible for generation of research findings reported in paper (design/performance of in vitro and in vivo imaging experiments), integrity and analysis of the data, writing of the manuscript

Johannes Roesel is responsible for generation of research findings reported in paper (design/performance of in vitro experiments), integrity and analysis of the data, writing of the manuscript

Guido Bold assisted with preclinical characterization of NVP-AST487

Pascal Furet assisted with preclinical characterization of NVP-AST487

Jingrui Jiang assisted with technical aspects of the manuscript (specifically, proliferation studies and immunoblotting associated with the N841I FLT3 mutant)

Jan Cools developed the PKC412-resistant mutant FLT3-expressing cells screened with NVP-AST487

Renee D. Wright assisted with technical aspects of in vivo imaging experiments

Erik Nelson assisted with STAT5 immunoblotting

Rosemary Barrett assisted with STAT5 immunoblotting

Arghya Ray assisted with histopathological analysis of mice used in in vivo imaging experiments, and assisted with testing of AML patient samples

Daisy Moreno assisted with technical aspects of in vivo imaging experiments (specifically, gavage)

Elizabeth Hall-Meyers assisted with technical aspects of in vivo imaging experiments (specifically, gavage)

Richard Stone assisted with patient sample acquisition and analysis.

Ilene Galinsky assisted with patient sample acquisition and analysis.

Edward Fox assisted with patient sample analysis.

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

John F. Daley assisted with flow cytometry

Suzan Lazo-Kallanian assisted with flow cytometry

Gary Gilliland assisted with the development and provision of the PKC412-resistant mutant FLT3-expressing cells that were screened with NVP-AST487.

James D. Griffin is responsible for conception of research reported in paper, integrity and analysis of the data

Conflict of Interest Disclosure

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**Figure Legends**

Figure 1. Inhibition of cellular proliferation of mutant-FLT3-expressing cell lines by NVP-AST487

(A) Structure of PKC412 and NVP-AST487.

(B) Three-day treatment of FLT3-ITD-Ba/F3 cells, in the presence and absence of IL-3, with NVP-AST487.

(C) Three-day treatment of FLT3-D835Y-Ba/F3 cells, in the presence and absence of IL-3, with NVP-AST487.

(D) Colony assay formation of human bone marrow progenitor cells in the presence of increasing concentrations of NVP-AST487.

Figure 2. Inhibition of cellular proliferation of mutant-FLT3-expressing AML patient cells by NVP-AST487.
(A) Three-day treatment of AML FLT3-ITD-expressing patient cells versus FLT3-ITD-Ba/F3 cells with NVP-AST487. Experiments were performed 1X for each AML patient sample. Cell viability was determined by the Trypan blue exclusion.

(B) Treatment of an AML peripheral blood sample with NVP-AST487 and PKC412, respectively. This sample tested positive for harboring both the FLT3-ITD mutation and the D835Y mutation. Cell viability was determined by Trypan blue exclusion.

(C-E) Treatment of AML bone marrow samples with NVP-AST487 and PKC412, respectively. These samples tested negative for harboring the FLT3 mutation. Cell viability was determined by Trypan blue exclusion.

Figure 3. Inhibition of cellular proliferation of PKC412-sensitive and -resistant mutant FLT3-expressing cells by NVP-AST487.

(A) Three-day treatment of FLT3-ITD- Ba/F3 cells with NVP-AST487 or PKC412.

(B) Three-day treatment of PKC412-resistant FLT3-ITD-Ba/F3 cells with NVP-AST487 or PKC412 (n=1 for 487 treatment; n=2 for PKC412 treatment).

(C) Two-day treatment of FLT3-ITD-Ba/F3 cells and PKC412-resistant, mutant FLT3-expressing Ba/F3 cells with PKC412 (n=2).

(D) Two-day treatment of FLT3-ITD-Ba/F3 cells and PKC412-resistant, mutant FLT3-expressing Ba/F3 cells with NVP-AST487 (n=2).

Figure 4. Induction of apoptosis and inhibition of cell cycle progression of mutant FLT3-expressing cells by NVP-AST487. Effects of NVP-AST487 on viability of (A) FLT3-ITD-Ba/F3 cells,
(B) FLT3-D835Y-Ba/F3 cells, and (C) parental Ba/F3 cells following 3 days of treatment. Effects of NVP-AST487 on cell cycle progression of FLT3-ITD-Ba/F3 cells (D) following 36 hours of treatment.

Figure 5. Inhibition of autophosphorylation of FLT3 and phosphorylation of STAT5 in mutant-FLT3-expressing cells.

(A) I.P./Western: Treatment of FLT3-ITD-Ba/F3 cells for 15 minutes with NVP-AST487 at 0.01μM. A vertical line has been inserted to indicate a repositioned gel lane.

(B) I.P./Western: Treatment of FLT3-N841I-Ba/F3 cells for 15 minutes with 0.01 or 0.1 μM NVP-AST487.

(C) Immunoblot: Treatment of FLT3-ITD-Ba/F3 cells for 2 hr with NVP-AST487 at 0-1000 nM.

(D) Immunoblot: Treatment of FLT3-ITD-Ba/F3 cells for 2 hr with PKC412 at 0-1000 nM.

Figure 6. Efficacy of NVP-AST487 in mice bearing mutant FLT3-expressing cells. (A) Time to onset of morbidity in mutant FLT3-Ba/F3-harboring athymic nude mice, treated with vehicle or NVP-AST487 (30mg/kg, or “low dose”) or NVP-AST487 (50mg/kg, or “high dose”).

(B) Mouse photos show bioluminescence of mice following 6 days post-IV injection. B708, C711, and D718 are NMP/PEG300 vehicle controls for NVP-AST487-treated mice. B709, D720, and E725 are vehicle controls for PKC412-treated mice.

(C, D) Bioluminescence values graphed for treatment groups.

Figure 7. NVP-AST487 positively combines with Ara-c, doxorubicin, and PKC412.
(A) Proliferation study investigating the combination of NVP-AST487 and Ara-c. Results shown here are representative of two independent studies. Calcusyn analysis suggests slight to moderate synergism between the two agents (ED25: 0.84008; ED50: 0.77655; ED75: 0.73874; ED90: 0.72264).

(B) Proliferation study investigating the combination of NVP-AST487 and doxorubicin, performed once. Calcusyn analysis suggests slight synergy between the two agents (ED25: 0.88419; ED50: 0.87274; ED75: 0.86262; ED90: 0.85377).

(C) Proliferation study investigating the combination of NVP-AST487 and PKC412, performed once. Calcusyn analysis suggests effects that ranged from slight antagonism to slight synergism between the two agents (ED25: 0.88582; ED50: 0.95322; ED75: 1.02619; ED90: 1.10522).
Figure 1

(A) Structures of PKC412 and NVP-AST487.

(B) Cell number (% of control) as a function of [NVP-AST487, μM] for FLT3-ITD-Ba/F3 (-IL3) and FLT3-ITD-Ba/F3 (+IL3) cells.

(C) Cell number (% of control) as a function of [NVP-AST487, μM] for FLT3-D835Y-Ba/F3 (-IL3) and FLT3-D835Y-Ba/F3 (+IL3) cells, as well as Parental Ba/F3.

(D) Colony number as a function of [NVP-AST487, μM].
Figure 2

A

AML Patient#1
AML Patient#2
AML Patient#3
AML Patient#4
AML Patient#5
Ba/F3-Flt3-ITD

[NVP-AST487, μM]

Cell Number (% of Control)

0 0.01 0.1 1

B

AML #6

Cell Number (% of Control)

AST-487
PKC-412

[Inhibitor, μM]

0 0.001 0.01 0.1 1

C

AML #7

Cell Number (% of Control)

AST-487
PKC-412

[Inhibitor, μM]

0 0.001 0.01 0.1 1

D

AML #8

Cell Number (% of Control)

AST-487
PKC-412

[Inhibitor, μM]

0 0.001 0.01 0.1 1

E

AML #9

Cell Number (% of Control)

AST-487
PKC-412

[Inhibitor, μM]

0 0.001 0.01 0.1 1

For personal use only.
Figure 3

A. FLT3-ITD-Ba/F3

B. PKC412-Resistant FLT3-ITD-Ba/F3

C. [PKC412, μM]

D. [NVP-AST487, μM]
Figure 4

A. FLT3-ITD-Ba/F3

B. D835Y-Ba/F3

C. Parental Ba/F3

D. FLT3-ITD-Ba/F3
Figure 5

(A) FLT3-ITD-Ba/F3

I.P.: FLT3

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<tr>
<td>176.5</td>
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(B) FLT3-N841I-Ba/F3

I.P.: FLT3

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(C) W.B.: Phospho-STAT5

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(D) W.B.: Phospho-STAT5

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Figure 5

Phospho-STAT5

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Figure 6

A

B

Vehicle Control

Low Dose 487

High Dose 487

Days

Probability

0 0.2 0.4 0.6 0.8 1.0

0 10 20 30 40

Vehicle (micro-emulsion)

PKC412 (100 mg/kg)

AST487 (50 mg/kg)

C

D

Vehicle

PKC412 [100 mg/kg]

NVP-AST487 [50 mg/kg]

Figure 6
Figure 7
Anti-leukemic effects of the novel, mutant FLT3 inhibitor, NVP-AST487: Effects on PKC412-sensitive and -resistant FLT3-expressing cells


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