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Study of Two Tyrosine Kinase Inhibitors on Growth and Signal Transduction in Polycythemia Vera

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Abstract

Objective—An activating somatic mutation of Janus kinase 2 V617F (*JAK2*^{V617F}) is present in most polycythemia vera (PV) patients. We studied efficacy of 2 potent tyrosine kinase inhibitors (TKI), AEE788 and AMN107 in vitro on cells bearing this mutation.

Patients and Methods—We employed reporter cells expressing wild-type *JAK2* and mutant *JAK2*^{V617F}, human erythroleukemic cells (HEL) carrying *JAK2*^{V617F} to study the efficacy of these TKIs by cell proliferation assay, annexin V/PI staining, and on relevant cell-signaling and apoptotic events. These data were compared to *ex vivo* expanded native human erythroid PV progenitor cells grown in liquid cultures.

Results—AEE788 showed a time and dose dependent growth inhibitory effect that was greater in FDCP cells expressing *JAK2*^{V617F} and HEL cells than in cells expressing wild-type *JAK2*. AEE788 caused dephosphorylation of Akt^(S243) and STAT5^(Y694) proteins, increase in annexin V binding and caspase-3 cleavage, suggesting induction of apoptosis. We also observed AEE788 mediated decrease in Hsp70 and Hsp90 anti-apoptotic proteins. Similarly, native PV erythroid progenitors showed more sensitivity to AEE788 than normal erythroid progenitors. AEE788 also exerted dose-dependent inhibition of PV-specific erythroid colonies. Nilotinib (AMN107) however, lacked specificity and required high (>8μM) concentrations to inhibit growth of *JAK2*^{V617F} carrying cells.

Conclusion—Our data suggest that AEE788 exerts its apoptotic activity via down-regulation of proliferative and anti-apoptotic regulatory proteins. To our knowledge, this is the first report demonstrating effect of AEE788 on PV erythroid progenitors. Differential effects on PV and normal progenitor cells suggest AEE788 has potential in the treatment of PV and other *JAK2*^{V617F} positive hematologic malignancies.

Introduction

Polycythemia vera (PV) is an acquired clonal stem cell myeloproliferative disorder characterized by cytokine independence/hypersensitivity. Accumulation of erythrocytes is a hallmark of PV, while increase in the platelets, neutrophils, basophils and eosinophils is variable (1,2). PV is the most common primary polycythemia. It is often held that PV is a rare disorder, when in fact, with a prevalence of ~2.8/10⁵ persons (3) it is more common than chronic

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myelogenous leukemia (CML). Currently no therapies of PV are satisfying. Phlebotomy is the cornerstone of PV therapy, but this intervention does not diminish the high risk of thromboembolic complications and leukemic transformation. The therapy with radioactive phosphorus, chlorambucil, and other alkylating chemotherapeutic agents' increase incidence of acute leukemia and myelodysplastic syndrome, as well as other malignancies but decreases thrombotic complications (1,2). Hydroxyurea therapy decreases thromboembolic complications and has no measurable effect on increase risk of leukemia, while aspirin has only a modest effect on decrease of thrombotic complications. Clearly more specific therapies are needed.

A clonal somatic mutation in the pseudo-kinase domain of the Janus kinase 2 (JAK2) protein substituting valine at position 617 with phenylalanine (V617F) was reported in most PV patients and also in other myeloproliferative disorders (MPD). *JAK2*^{V617F} mutation causes constitutive activation of the JAK2/STAT5 pathway in PV patients (4–7). A growing number of anti-cancer therapeutic modalities are based on inhibition of de-regulated tyrosine kinases (8). Imatinib is a strong inhibitor of Bcr-Abl kinase with an impressive therapeutic efficacy in CML (9). Imatinib also inhibits other tyrosine kinases such as c-kit, and PDGF (9). We showed that imatinib concentrations achievable *in vivo* have moderate desirable therapeutic effects in a limited number of PV patients (10,11). In addition to imatinib, a novel TKI aminopyrimidine inhibitor was recently developed - AMN107 (nilotinib) as a potent alternate Abl inhibitor with activity against many imatinib-resistant Bcr-Abl kinase mutants (12,13). Another TKI inhibitor, AEE788, a member of the 7H-pyrrolo [2, 3] class of pyrimidines – is a novel orally available specific inhibitor of EGFR and VEGFR (14). Here we report the study of AMN107 and AEE788 in cells bearing wild and *JAK2*^{V617F} mutation.

Materials and Methods

Reagents

AEE788 and AMN107 were a kind gift from Novartis Pharma, (Basel, Switzerland). Histopaque, MTT, propidium iodide, RNAase A, insulin growth factor 1 (IGF-1) and set of protease inhibitors were purchased from Sigma Chemical Co (St. Louis, MO). AnnexinV was obtained from Biovision, (Mountain View, CA). Cytokine cocktail (CC110:100X stock containing 10µg/ml each of fetal liver tyrosine kinase 3 ligand, recombinant human (rh) thrombopoietin and rh stem cell factor), Stem Factor Cell medium and methylcellulose medium (free of erythropoietin) were purchased from Stem Cell Technologies (Vancouver, Canada). Erythropoietin (40,000units/ml, Epogen) was purchased from Amgen (Thousand Oaks, CA). RPMI 1640 medium was obtained from Invitrogen (Carlsbad, CA), protein A Sepharose from Santa Cruz (Santa Cruz, CA) and fetal bovine serum from Hyclone, (Logan, UT). Protein estimation was performed using Bradford reagent from BioRad (Hercules, CA). ATP viability bioluminescent assay kit, passive lysis buffer was obtained from Promega (Madison, WI). Restore western blot stripping buffer was purchased from Pierce Biotechnology (Rockford, IL).

Antibodies for immunoblot analysis

Antibodies to heat shock proteins (HSP) 70, 90, and grp78 were purchased from StressGen (now Nventa, Ann Arbor, MI). Antiphospho-STAT5 (Y694), antiphospho-AKT (Ser 463) and caspase 3 (that recognize full-length and cleaved product) antibodies were purchased from Cell Signaling (Beverly, MA). Antibodies against total STAT5, Bclxl were purchased from Santa Cruz (Santa Cruz, CA) and cleaved caspase3 as well as β-actin antibodies from Sigma (St. Louis, MO).

Cell-lines and culture conditions

Mouse reporter FDCP cells previously transfected with mouse erythropoietin receptor cDNA (FDCP-*EpoR*) that expressed either the wild-type JAK2 (FDCP JAK2) or FDCP JAK2^{V617F} obtained by transduction with a retroviral vector containing JAK2 V617F cDNA display cytokine hypersensitivity and were provided by Dr. Vainchenker (4). Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100µg/ml each of penicillin, streptomycin and amphoterecin B obtained from Sigma (St. Louis MO) and WEHI cell supernatant as the source of interleukin-3. Cells were maintained at $\sim 0.5 \times 10^6$ cells/ml. Human erythroleukemic cells 92.1.7 (HEL); that naturally express the mutant JAK2^{V617F} protein, Jurkat T-leukemic cells (ATCC, Manassas, VA) and NB4 acute promyelocytic leukemic cells (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH DMSZ, Braunschweig, Germany) were grown in the above medium without WEHI cell supernatant.

MTT assay

MTT proliferation assay was performed using standard procedures and as described previously (11) using mouse FDCP-*EpoR* or HEL cells at a concentration of $\sim 0.5 \times 10^5$ cells/ml and incubated with various drug concentrations for 24–48h, followed by addition of MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide drug. After 2–4h, the cells in the 96 well plates were centrifuged at 1000 rpm in Jouan G412 centrifuge. We employed the Beckman Biomek 1000 automated lab workstation BMN 803 robotic system (Beckman Coulter, Fullerton, CA) to remove the supernatant and add 150µl of dimethyl sulfoxide to each well to dissolve the MTT crystals. The absorbance was determined at 570nm using Tecan Spectrofluor Plus (Vienna, Virginia) 96 well plate reader. An IC₅₀ (in some experiments, IC₂₅) value is defined as the concentration of the drug required to obtain 50% inhibition (or 25% inhibition for an IC₂₅ value) in cell growth.

Synergy or additive effect of the drugs was examined by performing growth inhibition assays as described above, by combining the two drugs in varying proportions.

ATP bioluminescent viability assay

Human erythroid progenitors do not metabolize MTT efficiently. We have therefore optimized measurement of growth inhibition of the native human erythroid progenitors using a sensitive ATP bioluminescent viability assay. Briefly, *ex vivo* expanded erythroid progenitors (1×10^4 cells/ml) at the beginning of the S3 step of expansion (15) were incubated in a 100µl volume with varying drug concentrations for 48h. Post incubation, 100µl of the ATP bioluminescent reagent was added. The plates were read for emission of luminescence using BioTek luminometer (BioTek Instruments Winooski, Vermont). The decrease in the relative luminescent units (RLU) between untreated and drug-treated cells was used to calculate the percent growth inhibition.

AnnexinV/Propidium iodide staining

HEL and native erythroid progenitor cells were treated with the drug for 0–16h. Cells were washed with ice-cold PBS and incubated in a binding buffer (125mM NaCl, 25mM Hepes-Cl, pH7.5 and 1% bovine serum albumin) with annexinV/propidium iodide (1µg/0.2µg/ml) in a 100µl volume for 30 minutes in dark on ice, then 0.5ml of the binding buffer was added and cells were analyzed by flow cytometry using a Becton Dickinson FACS instrument. Specific gates were chosen based on staining the cells with annexin V or propidium iodide alone. Analysis was performed using Cellquest™ acquisition software (BD, San Jose, CA). The upper right quadrant (annexinV/PI positive) cells were used for measurement of apoptotic cells.

Ex vivo expansion of erythroid progenitors

Whole blood was obtained from consenting PV patients using an approved IRB protocol. Peripheral blood mononuclear cells (MNC) were isolated using histopaque density gradient and standard protocols. Expansion of progenitor cells from the mononuclear cells (15) was done in three steps using modification of a previously published protocol (16). The expanded progenitor cells (5×10^5) were stained with phycoerythrin (PE)-conjugated anti-CD235A (glycophorin) and fluorescein isothiocyanate (FITC)-conjugated anti-human-CD71 (transferrin receptor) monoclonal antibodies. Analysis of the harvested cells was performed on a Becton Dickinson FACS instrument using Beckman-Coulter System II analysis software (17); the majority of cells used in the experiments belonged to R4 fraction (early basophilic erythroblasts).

Assay of PV erythroid colonies

PV erythroid colonies were grown as described (18) with following modifications: Fresh peripheral blood from PV patients was used to isolate the MNC by ficoll hypaque gradient. 0.5×10^5 MNC suspended in IMDM medium were mixed in 5ml of pre-warmed methylcellulose medium containing 0.03 units of erythropoietin/ml, 100 μ g/ml each of penicillin and streptomycin, and 0–6 μ M of AEE788 drug. The 5ml methyl-cellulose medium was distributed in two 35mm dishes with 18 gauge needle attached to 3ml syringe. The dishes were kept in 100mm plate and incubated for 18 days for BFU-E colony formation. An empty 35mm open plate containing sterile water prevented drying of the medium. Colonies were photographed at 40 $\times$ magnification using a Nikon Eclipse TE 300 microscope (Melville, NY). Images were captured using CoolSNAPTM CCD camera and software provided by the manufacturer (RS Photometrics, Tucson, AZ) and scored as described (18).

Treatment of cells

We first established that the tested agents had no change in their activities in a RPMI medium containing 1% FBS medium chosen for the treatment of the reporter FDCP and HEL cells with studied agents.

AEE788 and AMN107 studies—Cells were treated with studied TKI for 0–24h followed by stimulation with 7.5U/ml of erythropoietin for 4h. The early expanded erythroid progenitors (in their S3 stage of expansion (15)) were treated with TKIs for 0–24h as described in the Result section. Post-treatment, cells were used for FACS analysis or lysed in a lysis buffer containing protease inhibitor cocktail and phosphatase inhibitors (50 μ M sodium ortho-vanadate and 100 μ M sodium fluoride) for signal transduction analyses. Protein was estimated using Bradford method and Western analysis was performed as described (19). The results shown are from three-four independent experiments.

Statistical analysis

Statistical significance between normal and PV samples or between untreated and drug-treated samples was performed using paired Students' *t*-test. P value of less than 0.05 was used to determine biological significance.

Results

AEE788 inhibits preferentially cells expressing JAK^{V617F}

A 24h incubation of mouse FDCP reporter cells carrying *JAK2*^{V617F} with AEE788 was inhibited at an IC₅₀ of 0.6 μ M while FDCP cells expressing wild-type *JAK2* showed an IC₅₀ of 1.2 μ M. AEE788 inhibited the HEL cells with an IC₅₀ of 1.2 μ M after 24h of incubation (Fig 1A). When cells were exposed to AEE788 for 48h, there was a decrease in the IC₅₀ of FDCP

JAK2^{V617F} cells to 0.4μM and HEL cells to 0.75μM. FDCP *JAK2* cells; however, displayed increased resistance during 48h of incubation with an IC₅₀ of ~2μM (Fig 1B). AnnexinV/PI staining of HEL cells treated with 0–2μM AEE788 for 16h showed about two-fold increased apoptosis (Fig. 1C), supporting the observed growth inhibitory activity of AEE788.

Growth inhibition of *JAK2*, V617F and HEL cells by AMN107

Since imatinib is reported to have the therapeutic benefit of in some PV patients (10,11,20), we also tested AMN107 a more potent TKI than imatinib (12). An IC₅₀ of 14μM was observed in FDCP *JAK2*^{V617F} after 24–48hrs of incubation with AMN107 (Fig. 2A and B) while FDCP *JAK2* cells had 25–40% cell death at 14μM AMN107 during 24–48h of treatment. HEL cells had an IC₅₀ of 6–8μM during 24–48h of treatment (Fig. 2A and B). AnnexinV/PI staining of HEL cells treated with AMN107 (0–4μM) for 16h showed 1.6-fold increase in apoptotic cells (Fig. 3C).

Since AMN107 lacked specificity and potency to selectively inhibit FDCP *JAK2*^{V617F} cells compared to AEE788, further studies were concentrated on understanding AEE788 mediated inhibition of *JAK2*^{V617F} bearing cells.

Effect of AEE788 on proliferation and apoptosis of erythroid progenitors

The erythroid cell progenitors expanded from 4 normal and 8 PV patients were incubated with 0–1.6μM of AEE788 for 48h. Native PV cells showed 40–60% decrease in the proliferation compared to ~10–15% decrease in normal progenitors ($P < 0.025$, Fig. 3A). These concentrations are comparable with the inhibitory concentration observed for FDCP *JAK2*^{V617F} and HEL cells (Fig. 1B). All 8PV patients carried the *JAK2*^{V617F} mutation (15). PV sample #2-5 carried ~15–30% of mutant *JAK2* T-allele (encoding *JAK2*^{V617F}) burden whereas PV sample #9-13 had ~65–90% of mutant T-allele frequency mutation (15). AEE788 mediated growth inhibition of PV erythroid cells showed modest dependence on their percent *JAK2* allele status (~44% versus 60% of mean growth inhibition for PV cells expressing less than 30 and over 60% *JAK2* T-allele, respectively, at 1.6μM AEE788; $P < 0.05$, Inset table of Fig. 3A). AnnexinV/PI staining of normal and PV erythroid progenitors treated with 0–2μM AEE788 for 16h indicated a concentration dependent increase in apoptotic cells with minimal effect on normal erythroid progenitors (Fig. 3B).

AEE788 inhibits PV endogenous erythroid colony formation

PV is characterized by increased sensitivity of the committed erythroid progenitors (BFU-E and CFU-E) to erythropoietin (21–23) and they form colonies at 0 and 30 mU of erythropoietin. The erythroid colonies in the presence of 30 mU of erythropoietin grown at 3 and 6μM AEE788 had a significant decrease in numbers (30–77%, $P < 0.05$ and 0.01, respectively), as well as in their size and morphology (Fig. 4), (normal erythroid progenitors do not form BFU-E and CFU-E at 30 mU of erythropoietin).

AEE788 alters cell signaling and apoptotic pathways

To elucidate the molecular basis of action of AEE788, we examined the phosphorylation status of the STAT5 protein, a down-stream target of *JAK2* kinase (24). One μM AEE788 treatment for 24h caused a significant dephosphorylation of the STAT5 transcription factor in FDCP *JAK2*^{V617F} and the HEL cells, with no effect on FDCP *JAK2* (Fig. 5A, panel 1). Total STAT5 protein was unaltered in all the cells (Fig. 5A, panel 2). Inactivation of STAT5 caused concomitant decrease in one of its downstream anti-apoptotic targets, Bclxl (25) in FDCP *JAK2*^{V617F} cells (panel 3). Caspase3 cleavage was evident in FDCP *JAK2*^{V617F} treated with AEE788 (Fig 5A, panel 4).

Next, we studied AEE788 mediated time dependent changes in HEL cells. AEE788 is known to target PI3K/Akt pathway (26). About 1 μ M AEE788 treatment caused time dependent decrease in basal AKT phosphorylation (at serine 463) starting as early as 2h (Fig. 5B, panel 5). De-phosphorylation of STAT5 (at tyrosine 694) was evident between 2 and 4h of AEE788 treatment (Fig 5B, panel 6). Hsp70 chaperone protein markedly decreased post 4h of AEE788 treatment (Fig 5B, panel 7). Down-regulation of the proliferative (Akt) and anti-apoptotic (Bclx1) cellular signaling molecules by AEE788 caused a time dependent increase in cleaved/activated caspase 3 (Fig 5B, panel 9).

The observed AEE788 mediated decrease in Hsp70 in HEL cells was also evident in FDCP *JAK2^{V617F}* (Fig. 5C, panel 10). Hsp90 also showed a marked decrease in HEL cells and a marginal decrease in FDCP *JAK2^{V617F}* cells (panel 11) with no apparent change in either of these chaperone proteins in cells carrying FDCP *JAK2* (Fig. 5C, panel 10,11).

AEE788 activity on reporter and established erythroleukemic cells prompted us to examine if the drug acts similarly in native PV erythroid progenitors. We observed a significant decrease in chaperone proteins, Hsp70 (Fig. 5C, panel 13) and Hsp90 (panel 14) upon 24h of AEE788 treatment in PV erythroid progenitors. No apparent change in normal erythroid progenitors was observed.

AEE788 treatment also caused a decrease in the phospho-STAT5 (tyrosine 694) levels in PV erythroid progenitors (panel 15).

Synergy of AEE788 and AMN107

AMN107 and AEE788 target different tyrosine kinases (12–14). We therefore examined if combining the two drugs will have synergic or additive activity in inhibition of cells expressing *JAK2^{V617F}*. Treatment of FDCP *JAK2^{V617F}* cells with a combination of 4 μ M AMN107 and 0.1 μ M AEE788 showed a marked increase in growth inhibition (Fig 6, 85%) compared to growth inhibition obtained with single drug agents (Fig 1A and 2A). In contrast, FDCP *JAK2* and HEL cells had 40 and 45% growth inhibition, respectively (Fig. 6).

Discussion

A somatic point mutation, *V617F* in the auto-inhibitory domain of JAK2 is found in most PV patients (3–6). This mutation constitutively activates Jak2 kinase and hyper-phosphorylates STAT5, similarly to that observed in other hematological and solid tumor malignancies (7). Imatinib, a TKI that inhibits Bcr-Abl, revolutionized the treatment of CML. Similar TKI for *JAK2^{V617F}* is needed for therapy of PV and other *JAK2^{V617F}* positive myeloproliferative disorders (8,9).

When imatinib resistant CML cases were reported, AMN107 (nilotinib) a potent alternate Abl inhibitor, with activity against many imatinib-resistant BCR-ABL kinase domain was developed (12,13). AMN107, a novel aminopyrimidine TKI has more than 20 fold higher activity in inhibiting Bcr-Abl kinase than imatinib (13). AMN107 also inhibits myeloid proliferation driven by TEL-PDGFR β and FIP1L1-PDGFR α (27) and was also shown to inhibit the c-Kit receptor kinase at pharmacologically achievable concentrations (28). Relatively modest responses of PV patients to imatinib have been reported (29–31). We have also demonstrated moderate efficacy of imatinib in vivo and in vitro, in PV erythroid progenitors via its coordinated inhibition of cKIT, JAK2 and cellular metabolism (10,11), albeit at markedly higher inhibitory levels than for Bcr-Abl. Therefore, we examined the effect of AMN107 on reporter *JAK2^{V617F}* cells and HEL cells. Our studies showed AMN107 to be less effective than imatinib (11) on cells bearing *JAK2^{V617F}* (Fig 2). To our knowledge, this is the

first report demonstrating only minimal efficacy of AMN107 growth inhibitory activity in cells bearing *JAK2*^{V617F} mutation (32).

In contrast, AEE788 TKI, that inhibit EGFR, VEGFR, (14) MAPK and Akt kinases (26) displayed marked growth inhibition of cells bearing the *JAK2*^{V617F} (Fig 1, 3 and 4). Unlike AMN107, the aminopyrimidine drug AEE788, showed only limited cytotoxicity against reporter and native human erythroid cells carrying wild-type *JAK2* (Fig 1 and 3). Furthermore, AEE788 did not inhibit the growth of the two leukemic cells demonstrating its specificity (Fig. 7) for erythroid cells expressing the mutant *JAK2*^{V617F}.

Novel therapies that inhibit a single molecular target may slow tumor progression, but malignancies are generally not dependent on a single signal transduction pathway/event. Recently metronomic chemotherapy (continuous or frequent treatment with low doses of anticancer drugs, often given with other methods of therapy) with taxanes in combination with AEE788 showed additive effect and prolongation of survival (33). Similarly, studies combining AEE788 with RAD001, an inhibitor of mTOR suggest that simultaneous inhibition of growth factor receptor and mTOR pathways offer increased benefit in glioma therapy (34). Our preliminary data corroborates these reports; growth inhibitory activity of AEE788 at 0.1 μ M causes ~15% cell-death of *JAK2*^{V617F} cells (Fig. 1B) against *JAK2*^{V617F} while AMN107 at 0.4 μ M causes ~20% cell death (Fig 2B). When these two drugs were used in combination at the above dosages, over 85% cell growth inhibition of cells expressing the *JAK2*^{V617F} was attained (Fig. 6). These results demonstrate the potential use of AEE788 in combinatorial therapy for PV and other *JAK2*^{V617F} malignancies.

Evidence now suggests that VEGF has a major role in the development and progression of hematologic malignancies (35,36). Therefore, several therapeutic strategies blocking VEGF or VEGF-induced signaling are currently being investigated for the treatment of neoplastic diseases. In line with these reports, we have observed a significant inhibitory effect of AEE788 on PV erythroid colony formation (Fig. 4). Since AEE788 has antiangiogenic activity via inhibition of VEGFR1 receptor (14) and since VEGF plays a major role in the development and progression of hematologic malignancies (35,36) it remains to be explored if the potential therapeutic benefit of AEE788 may be exerted via VEGF signaling.

Recent evidence suggests PI3K/Akt (Protein kinase B) activation promotes cell proliferation and survival in several malignancies by inhibiting apoptosis (37). We show here that AEE788 down-regulates both these crucial signal transduction pathways, by dephosphorylation of STAT5 and Akt proteins in cells expressing the mutant *JAK2*^{V617F} (Fig. 5) causing programmed cell-death.

Amongst the known mechanisms of acquired resistance to apoptosis, over-expression of heat shock proteins (38) is associated with drug resistance and poor prognosis (36). Hsp70 negatively interferes with caspase-dependent and caspase-independent processes of apoptosis (38). Down-regulation of HSPs has been shown to overcome apoptotic resistance (19,40). We show AEE788 mediates selective down-regulation of both Hsp70 and 90 proteins in cells expressing mutant *JAK2*^{V617F} (Fig. 5). No change in an endoplasmic reticulum grp78 chaperone protein was observed with AEE788 treatment (not shown). To our knowledge, this is the first report demonstrating down-regulation of HSPs by a tyrosine kinase inhibitor. It remains to be shown if the down-regulation of several signal transduction pathways including Akt, and STAT5 or other unknown targets lead to this decrease; or if this is an independent mechanism by which AEE788 contributes towards apoptosis of PV cells.

Since the *JAK2*^{V617F} mutation does not appear to be a disease initiating defect (15,42) but a somatic mutation that correlates with many complications of PV, the clinical benefits of its selective inhibition remain to be established.

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C: Effect of AEE788 on apoptosis of HEL cells

Figure 1. Effect of AEE788 on cell proliferation

JAK2, *V617F* and HEL cells were treated with AEE788 from 0–3.2 μ M for 24h (A) or 48h (B). After incubation with the drug, MTT dye (A&B) was added and cells prepared for measurement of cell cytotoxicity as described in methods. A representative result from three experiments has been shown. (C) HEL cells were treated with 0–2 μ M AEE788 for 16h, harvested and prepared for annexinV/PI staining as described in methods. The percent annexinV/PI positive (apoptotic population) is shown at the bottom.

C: Effect of AMN107 on apoptosis of HEL cells

Figure 2. Effect of AMN107 on cell proliferation

JAK2, *V617F* and HEL cells were treated with AMN107 from 0–16 μM for 24h (A) or 48h (B). Post incubation, MTT (A&B) was added to the cell suspension and prepared for measurement of cytotoxic assay as described in methods. A representative result from three experiments has been shown. (C) HEL cells were treated with 0–4 μM AMN107 for 16h, harvested and prepared for annexinV/PI staining as described earlier. The percent annexinV/PI positive (apoptotic population) is shown at the bottom.

A: Proliferation assay

B: AnnexinV/PI staining

Figure 3. Effect of AEE788 on proliferation and apoptosis of erythroid progenitors
(A) *Ex vivo* expansion of native (normal and PV) erythroid progenitors were performed as described previously (11,15). Erythroid cells were treated with 0–1.6 μ M of AEE788 for 48h. An ATP bioluminescent assay was employed to measure the live cells. The relative luminescent units (RLU) obtained from untreated cells was used to measure percent death upon drug treatment. PV erythroid progenitors (PV#2-5 have less than 30% *JAK2 T-allele* while PV#9-13 carry over 60% of *JAK2*^{V617F} mutation as described earlier (15). Con 1-4 represents erythroid progenitors from normal healthy individuals. The inset table shows percent *JAK2 T-allele* frequency in PV erythroid progenitors # 2-13 measured by using allele specific real time PCR (42). PV sample numbers 2-5 carried 13–30% and PV sample numbers 9-13 carried 66–91% *JAK2 T-allele* burden. Growth inhibition of PV cells at 1.6 μ M AEE788 was used to estimate the average difference of inhibition between these two groups (**P*<0.05). (B) Annexin V/PI staining of erythroid progenitors treated with AEE788 from 0–2 μ M for 16h was performed as

described. The diagram represents AEE788 mediated apoptotic events monitored by specifically gating the live cell population. Percent apoptosis is shown at the bottom.

Figure 4. Effect of AEE788 on colony formation

About 0.5×10^5 mononuclear cells isolated from peripheral blood of four PV patients were used to examine the effect of AEE788 on PV colony formation. At day 18, colonies were counted using a magnifying mirror. Colonies were photographed using phase contrast microscope with 40 $\times$ magnification as described in methods. The figure shows few representative colonies. The table summarizes the number of colonies obtained from MNC of four PV patients upon treatment with 0–6 μ M AEE788. * $P < 0.05$ and ** $P < 0.01$.

Figure 5. Effect of AEE788 on signal transducing and apoptotic proteins

(A): *JAK2*, *V617F* and HEL cells were treated with 0–1 μM of AEE788 for 16h followed by incubation with 7.5 units/ml of erythropoietin for 4h. Cell lysates were prepared for Western analysis. Ae denotes AEE788, X denotes a control drug, a natural pro-apoptotic triterpenoid electrophile (41) in testing, panel 1 (STAT5^{Y694}), panel 2 (total STAT5 protein), panel 3 (Bclxl), panel 4 (caspase 3). (B) HEL cells were treated with 1 μM AEE788 for 0–24h. Cellular proteins were analyzed by Western analysis for: panel 5 (Akt^{Ser463}), panel 6 (STAT5^{Y694}), panel 7 (Hsp70), panel 8 (Bclxl and cleaved caspase 3) and panel 9 (β-actin). (C) *JAK2* and *V617F* cells were treated with 1 μM AEE788 for 16h. Cellular proteins were examined for changes in Hsp70 (panel 10) and Hsp90 (panel 11). A non-specific band has been shown to monitor loading pattern (panel 12). Erythroid progenitor cells from normal and PV patients were treated with 0–2 μM AEE788 for 24h. Cellular proteins were resolved on SDS PAGE to examine Hsp70 (panel 13), Hsp90 (panel 14) and STAT5^{Y694} (panel 15). A non-specific band has been shown to monitor loading pattern (panel 16). NOR represents erythroid progenitors from normal healthy individual and PV represents a PV patient, respectively. – and + indicate presence and absence of the drug treatment.

Figure 6. Synergy of AEE788 and AMN107

JAK2, *V617F* and HEL cells were treated with AMN107 and AEE788 drugs at a ratio of 2 +0.1μM and 4+0.1μM for 48h. MTT assay was performed as described earlier. Results represent the mean from triplicate values and of two independent experiments.

Figure 7. Effect of AEE788 leukemic cell lines

NB4 and Jurkat T-cells were treated with 0–32μM of AEE788 for 72h. Post-treatment, cells were analyzed for cytotoxic effect of the drugs by MTT assay as described in the Methods section. The data shown is a best representation of three separate experiments.