BPR1J373, an Oral Multiple Tyrosine Kinase Inhibitor, Targets c-KIT for the Treatment of c-KIT-Driven Myeloid Leukemia

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Abstract

Acute myelogenous leukemia (AML) carrying t(8;21)(q22;q22) or inv(16)/t(16;16)(p13;q22) is classified as core binding factor (CBF)-AML and accounts for approximately 15% of AML. c-KIT mutation can be detected in 17%–46% of CBF-AML and is associated with poor prognosis. c-KIT mutation is a crucial hit and cooperates with AML1-ETO resulting from t(8;21)(q22;q22) to cause overt AML. Tyrosine kinase inhibitors (TKI) targeting c-KIT, such as imatinib, has been used successfully to treat c-KIT-driven gastrointestinal stromal tumors. However, the effect of TKI on c-KIT-driven leukemia, including CBF-AML and systemic mastocytosis (SM), has not been satisfactory. BPR1J373 is a 5-phenylthiazol-2-ylamine-pyrimidine derivative targeting multiple tyrosine kinases. It was shown to inhibit cell proliferation and induce apoptosis in AML cells with constitutively activated c-KIT via inhibiting c-KIT phosphorylation and its downstream signals. The compound induced apoptosis by the mitochondrial intrinsic pathway through upregulation of proapoptotic proteins Bax and Bak and caspase 8 and 9 activation in c-KIT mutant Kasumi-1 cells. Furthermore, it induced cell-cycle arrest via targeting aurora kinase B in c-KIT wild-type KG-1 cells. The antitumor response of BPR1J373 was also shown in subcutaneously grafted SCID mice. BPR1J373 was shown to effectively suppress c-KIT phosphorylation of D816V mutation by treating c-KIT–null COS-1 cells transfected with c-KIT D816V mutant plasmid. In conclusion, BPR1J373 inhibits cell proliferation of c-KIT–driven AML cells via induction of apoptosis and cell-cycle arrest. It is also effective for multiple drug-resistant c-KIT D816V mutation. BPR1J373 deserves further development for clinical use in c-KIT–driven myeloid leukemia. Mol Cancer Ther; 15(10): 2323–33. ©2016 AACR.

Introduction

Cytogenetic and molecular aberrations provide important insights for the pathogenesis and diagnosis of acute myelogenous leukemia (AML). Moreover, they may predict the clinical outcome and determine the treatment strategies for the patients (1, 2). AML carrying t(8;21)(q22;q22) and inv(16)/t(16;16)(p13;q22) are classified as core binding factor (CBF) AML. CBF-AML is associated with the French-American-British (FAB) AML subtype M2 and monocytic and eosinophilic differentiation (M4Eo) and associated with the French-American-British (FAB) AML subtype M2 and monocytic and eosinophilic differentiation (M4Eo) and accounts for approximately 15% of AML (3, 4). Patients with CBF-AML have a better prognosis than those with normal karyotype or other chromosomal abnormalities (5). c-KIT mutation was detected in 17%–46% of CBF-AML (3, 4, 6–8). Exon 8 and exon 17 were the most frequent mutation sites of c-KIT and CBF-AML patients with c-KIT mutation, particularly D816V, had higher relapse rate and shorter event-free survival/overall survival than those without c-KIT mutation (3, 6, 7). c-KIT encodes a 145-kilodalton type III tyrosine kinase that is structurally related to platelet-derived growth factor receptor, kinase insert domain receptor (KDR or VEGFR-2), and the Fms-like tyrosine kinase receptor (FLT3). c-KIT was shown to be the key driver in gastrointestinal stromal tumor (GIST) and is essential for the growth and survival of GIST. Around 85% of GIST patients had gain-of-function mutation in c-KIT with a primary mutation sites in exon 11 and exon 9 and secondary mutations in exons13/14 and exon17. Preclinical and clinical data indicated that imatinib effectively suppressed the GIST cell line and significantly prolonged survival for c-KIT–mutated GIST patients such that imatinib has been approved as a first-line treatment for advanced GIST (9). c-KIT mutation is a crucial hit and cooperates with AML1-ETO resulting from t(8;21)(q22;q22) to cause overt AML and distinct classes of c-KIT mutation demonstrated their ability to promote RUNX1-ETO–associated AML in the mouse model (10–12). On the basis of the molecular

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aberration, tyrosine kinase inhibitors (TKI), including imatinib, dasatinib, and sunitinib, have been demonstrated to exert growth inhibition and induce apoptosis in leukemia cells carrying c-KIT mutations (13–15) and imatinib has been used for treatment of c-KIT mutation–positive systemic mastocytosis (SM) and CBF-AML (16–18). However, imatinib was not effective for those with D816V mutation–positive SM and CBF-AML patients (16–18).

BPR1J373, a 5-phenylthiazol-2-ylamine-pyrimidine derivative, is a multitargeted kinase inhibitor. The preliminary kinase profiling indicated that BPR1J373 potently inhibited FLT3, c-KIT, VEGFR1-3, Aurora A, PDGFR, RET, and SRC with IC50 values of <100 nmol/L. The chemical structure of BPR1J373 is shown in Supplementary Fig. S1. In this study, we evaluated the efficacy of BPR1J373 in inhibiting cell proliferation and inducing apoptosis of both c-KIT mutated and wild-type leukemia cell lines via inhibition of c-KIT tyrosine phosphorylation. The antitumor effect of BPR1J373 was also demonstrated by Kasumi-1 xenograft mouse model. The promising data suggest that BPR1J373 is a potential target agent for the treatment of c-KIT–driven myeloid leukemia, particularly c-KIT–mutated leukemia.

Materials and Methods
Cell lines and reagents
THP-1, U937, KG-1, K562, and Kasumi-1 were purchased from Bioresource Collection and Research Center (BCRC), Food Industry Research and Development Institute, Hsinchu, Taiwan around 5 years ago. We used the stocked cells for this study. The 5 cell lines we used were sent to Food Industry Research and Development Institute for analysis of STR-DNA profiles 2 months ago. All 5 cell lines we used have the same STR PCR DNA profile as those in BCRC. HMC1.2 was provided by Dr. Butterfield, Mayo Clinic (Rochester, MN) in March, 2014. We did direct sequencing for HMC1.2 cells 2 months ago and confirmed that there is heterozygous mutation at V560G and D816V of c-KIT. All the cell lines were cultured with RPMI-1640 plus 10% hyclone FBS and streptomycin/glutamine in 37°C incubator with 5% CO2. COS-1 cells were obtained from Dr. Shih (Neng-Yao Laboratory, National Health Research Institutes) and they were cultured in DMEM supplemented with 10% FBS. COS-1 was confirmed to be c-KIT null by RT-PCR 2 months ago. Mcl-1, Bcl-2, p16, p53, and GAPDH were purchased from Santa Cruz Biotechnology. The primary antibodies, Akt, p-Akt, Bax, Bid, Bad, PARP, caspase-9, 9H13K, p-p85, p-MEK, p-MAPK, Aurora A, Aurora B, p-Aurora A, p-Aurora B, p-Histone H3 (ser10), cyclin D1, and p-mTOR were purchased from Cell Signaling Technology. P-c-KIT was purchased from Invitrogen. c-KIT was purchased from DAKO. P27 was purchased from BD transduction. Pan-caspase inhibitor, Z-VAD-FMK, was purchased from Promega.

Cell proliferation assay (WST1 assay)
Triplicated samples of 4 × 104 leukemia cells (3 × 104 for K562) were incubated with serial 2-fold dilution of BPR1J373 from 1 μmol/L. The cultures were kept for 72 hours at 37°C in 5% CO2. Then, 10 μL of Premixed WST-1 Cell Proliferation Reagent (Clontech) was added to each well and incubation was continued for further 1 hour. The absorbance was measured at 450 nm using MS-5 multilabel counter.

Apoptosis assay
Kasumi-1 and KG-1 cells were cultured in 6-multiwell plates and treated with vehicle, imatinib 1 μmol/L, and BPR1J373 1 μmol/L for 2, 8, 12, 24, 48, and 72 hours. Treated cells were collected, and labeled with annexin V-FITC and propidium iodide, followed by analysis on BD FACSCalibur (Becton Dickinson).

Cell-cycle analysis
Kasumi-1 cells were incubated for 12, 24, 36, 48, 60, and 72 hours and treated with or without BPR1J373. Each condition was triplicated. Cells were collected, centrifuged, and washed with PBS. Then the cells were fixed with 70% ethanol and stored in −20°C overnight. After that, cells were washed with PBS twice and re-suspended in 1 mL of PBS containing 20 μg/mL of propidium iodide (Sigma) and 0.2 mg/mL of RNaseA (Sigma) for 1 hour at room temperature (RT) in dark. The stained cells were measured for cell cycle by using a FACS Calibur machine, and the data were analyzed by WinMDI 2.9 software. Data were represented in bar graph with mean ± SE.

Immunofluorescent staining
KG-1 cells were treated with or without 150μmol/L of BPR1J373 for indicated durations. The cells were cytospined at 500 rpm for 5 minutes and fixed with 4% paraformaldehyde for 15 minutes at RT and 0.1% Triton X-100 for another 10 minutes. Then the cells were blocked in 5% BSA in PBS for 30 minutes at RT followed by incubation in primary antibody (monoclonal anti-α-tubulin, Sigma; 1:2,000) overnight at 4°C, and secondary antibody (chicken anti-mouse, invitrogen; 1:250) 1 hour at RT. DNA was labeled with DAPI at a final concentration of 100 ng/mL for 15 minutes.

Western blot analysis
The whole-cell lysates were extracted with cell lysis buffer (Celllytic M mammalian cell lysis/extraction solution, sigma C2978) and electrophoresed on SDS-PAGE. They were transferred to a polyvinylidene difluoride membrane (Bio-Rad) and blocked with 5% BSA in PBST for 1 hour. The membrane was probed overnight at 4°C with primary antibody (monoclonal anti-α-tubulin, Sigma; 1:2,000) and incubated with secondary antibody. The immunocomplexes were detected with the Enhanced Chemiluminescence Detection Kit (PerkinElmer Western Lightning Plus-ECL).

In vivo study
SCID (6- to 8-weeks-old) male mice (CB17/Icr-Pkd+/–; lcr/lcr/CrlBlc) were obtained from the National Laboratory Animal Center (Tainan, Taiwan) and housed under specific pathogen-free conditions according to the guidelines of the Animal Care Committee at the National Health Research Institutes, Taiwan. Kasumi-1 cells (5 × 106/mouse) were injected subcutaneously into mice. The mice were treated with BPR1J373, imatinib, doxorubicin/cytarabine, and vehicle control after tumor growth to around 200 mm3. BPR1J373 and imatinib were dissolved in distilled water containing 20% of 2-Hydroxypropyl-β-cyclodextrin for oral feeding. Doxorubicin and cytarabine was dissolved in PBS and injected into the mice via intraperitoneal route. Tumor volume was measured for up to 30 days by the following formula: length (mm) × width2 (mm2) × (π/6; ref. 19).
c-KIT mutant transfection

The plasmid containing c-KIT D816V mutation was produced as described previously (20). COS-1 cells were cultured in 6-cm plate overnight and added with vector (pcDNA3.1) only, or vector with wild type and mutant c-KIT with Lipofectamine 2000 (Invitrogen, 11668-019) according to the manufacturer's protocol. The cells were cultured for 24 hours and then treated with or without drugs (imatinib, sunitinib, nilotinib, and BPR1J373) for indicated durations. The cell lysates in each condition were collected for Western blot analysis.

Results
BPR1J373 inhibits cell proliferation of myeloid leukemia cell lines and primary leukemia cells with constitutively activated c-KIT

A series of leukemia cell lines, including THP-1, U937, KG-1, K562, and Kasumi-1, were treated with BPR1J373 and tested with WST1 assay to screen for the inhibitory effect of BPR1J373 against cell proliferation. BPR1J373 was able to inhibit the cell proliferation of KG-1 and K562 with IC50 of 45 and 49 nmol/L, respectively, at 72 hours. Kasumi-1 cells were very sensitive to BPR1J373 with a very low IC50 of 4.4 and 4.2 nmol/L at 48 and 72 hours, respectively. On the other hand, BPR1J373 was not effective to inhibit the proliferation of THP-1 and U937 cells (Supplementary Fig. S2). Kasumi-1 is a t(8;21) AML cell line carrying c-KIT mutation on codon 822 (N822K). K562 is an erythroleukemia cell line, KG-1 is a myeloid leukemia cell line carrying t(9;22) translocation with wild type c-KIT expression, and K562 is an AML cell line bearing wild-type c-KIT. We evaluated the expression of c-KIT and phosphorylated c-KIT for all 5 cell lines and found that phosphorylated c-KIT was present in KG-1, K562 and Kasumi-1 cells whereas constitutively c-KIT phosphorylation was not seen in THP-1 and U937 cells (Fig. 1A). We treated KG-1, Kasumi-1, and K562 cells with 1 μmol/L of BPR1J373 for 2 hours and the phosphorylated c-KIT of all three cell lines was significantly suppressed (Fig. 1A), which suggested that BPR1J373 is effective to inhibit the proliferation of leukemia cell lines with constitutively activated c-KIT, particularly c-KIT–mutated leukemia. In addition, we treated primary leukemia cells obtained from the bone marrow of AML patients with indicated dose of BPR1J373 for 24, 48, and 72 hours. BPR1J373 inhibited the cell proliferation and suppressed the c-KIT phosphorylation of the primary leukemia cells of two patients with constitutively activated c-KIT expression as shown in Fig. 1B. Figure 1B also shows the induction of apoptosis of primary leukemia cells by BPR1J373 with the presence of PARP cleavage and increased proportion of annexin V–positive cells after exposing the primary leukemia cells of patient 2 and patient 4 to BPR1J373.

BPR1J373 induces apoptosis by mitochondrial intrinsic pathway through upregulation of proapoptotic proteins Bax and Bak and caspase 8 and 9 activation in Kasumi-1 cells

To evaluate whether apoptosis was induced by BPR1J373 for leukemia cells, Kasumi-1 and KG-1 cells were treated with 1 μmol/L of BPR1J373 and imatinib for various duration and flow cytometry for annexin V and propidium iodine staining positive cells were measured. Increase of annexin V–positive cells was noted for both cells in a time-dependent manner (Supplementary Fig. S3). Kasumi-1 cells were treated with various doses of BPR1J373 for 24 hours. The phosphorylation of c-KIT was suppressed significantly by BPR1J373 at 5 nmol/L accompanied with cleavages of PARP, caspase-8 and caspase-9 (Fig. 1C). Cleavage of PARP and caspase-8 was also present in KG-1 and K562 cells treated with BPR1J373 (Supplementary Fig. S4). We further checked the Bcl2-family proteins involved in mitochondrial pathway–induced apoptosis for Kasumi-1 cells treated with 1 μmol/L of BPR1J373 by Western blot analysis. The expressions of proapoptotic proteins, Bax and Bak, were significantly increased 8 hours after BPR1J373 treatment. The antiapoptotic proteins, including Bad, McI1, and Bcl2, were not affected (Fig. 1D). Upregulation of the mRNA expression of BAX and BAK was also noted in Kasumi-1 cells 8 hours after treatment with BPR1J373 (Fig. 1E). The cleavage of PARP, caspase-8, and caspase-9 and the upregulation of Bax and Bak in Kasumi-1 cells treated with BPR1J373 could be reversed by addition of pan-caspase inhibitor, Z-VAD-FMK, as shown in Fig. 1F. The result demonstrates that BPR1J373 induces apoptosis by activating mitochondrial intrinsic pathway in c-KIT–driven AML cells.

BPR1J373 inhibits cell proliferation and induces apoptosis in Kasumi-1 cells via inhibiting c-KIT phosphorylation and its downstream signals

We treated Kasumi-1 cells with 1 μmol/L of BPR1J373 and imatinib for 2, 8, and 24 hours to evaluate the biological effect of BPR1J373 and imatinib on Kasumi-1 cells. The expression of c-KIT was not decreased by both drugs. The phosphorylated form of c-KIT was completely suppressed by BPR1J373 whereas it was suppressed by imatinib at 2 hours but recovered at 8 hours after imatinib treatment. The downstream signatures of c-KIT, including PI3K/Akt/mTOR, and MAPK/MEK pathways, were affected by BPR1J373 but not imatinib. Western blot analysis for Kasumi-1 treated with BPR1J373 and imatinib showed that phosphorylated p85, Akt (ser473), and mTOR were suppressed by BPR1J373 accompanying suppressed c-KIT phosphorylation whereas recovery of these markers were seen for cells treated with imatinib as recovery in c-KIT phosphorylation (Fig. 2A). Similarily, the phosphorylation of MEK and MAPK in Kasumi-1 cells were also suppressed by BPR1J373, and they recovered 8 hours after treatment of imatinib. These results demonstrated that BPR1J373 is effective to inhibit cell proliferation and overcome imatinib-resistant mechanism for c-KIT–mutated Kasumi-1 cells via targeting c-KIT. Downregulation of phosphorylated c-KIT and its downstream signatures were also seen for KG-1 cells treated with BPR1J373 (Fig. 2B).

BPR1J373 induces cell-cycle arrest for c-KIT–driven leukemia cells via targeting different signals

Because downstream signals of c-KIT were associated with cell cycle, we evaluated the effect of BPR1J373 on cell-cycle progression in Kasumi-1, KG-1, and K562 cells. Figure 3A shows that increase of G1 proportion and decrease of S phase by time when Kasumi-1 cells were treated with BPR1J373 at 2-folds concentration of IC50 (10 nmol/L). The cell-cycle inhibitor p27 and p16 of Kasumi-1 cells were upregulated by BPR1J373 as shown in Fig. 3B. High dose of BPR1J373 (1 μmol/L) induced initial G2–M arrest at 12 and 24 hours and increase of subG1 population time dependently for Kasumi-1 cells (Supplementary Fig. S5A). When KG-1 cells were treated with 50 nmol/L of BPR1J373, 1X IC50, G1 arrest was noted in cell-cycle analysis similar to the pattern seen in Kasumi-1 cells.

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BPR1J373 suppressed c-KIT phosphorylation and induced apoptosis of AML cell lines and primary AML cells with constitutively activated c-KIT. A, the top figure shows the expression of c-KIT and phosphorylated c-KIT in the 5 AML cell lines. The bottom figure shows BPR1J373 suppressed c-KIT phosphorylation of K562, KG-1, and Kasumi-1 cells. B, the constitutive expression of c-KIT phosphorylation was noted in primary bone marrow mononuclear cells of two AML patients, patient 2 and patient 4 (top left and top right). The primary leukemia cells were treated with BPR1J373 with indicated dose and durations. The survival rate of primary leukemia cells was significantly decreased by BPR1J373 (top left and right). BPR1J373 induced apoptosis of the leukemia cells of both patients, which was demonstrated by the presence of PARP cleavage in both patients (top right) and increased percentage of annexin V-positive cells in patient 4 (bottom left). The c-KIT phosphorylation in the primary leukemia cells of both patients was significantly suppressed by BPR1J373 (top right). C, significant suppression of c-KIT phosphorylation and presence of cleavage of PARP, caspase-8, and caspase-9 were seen in Kasumi-1 cells treated with BPR1J373 for 24 hours as at low as 5 nmol/L. D, Western blot analysis of proapoptotic and antiapoptotic proteins of Kasumi-1 cells treated with 1 μmol/L of BPR1J373 for 2, 8, and 24 hours. E, Western blot analysis of PARP, caspase 8, caspase 9, Bad, and Bak of Kasumi-1 cells treated with 1 μmol/L of BPR1J373, 50 μmol/L of Z-VAD-FMK alone, or combination of both drugs for 24 hours.

(Supplementary Fig. S5B). For K562 cells, the proportions of G1 and S phase were increased but G2-M phase was decreased by 50 nmol/L (1IC50) of BPR1J373 time dependently (Supplementary Fig. S5C). High dose of BPR1J373 (1 μmol/L) induced a decrease in the proportion of G1, S phase and an increase in the proportion of sub-G1 and G2-M phase of K562 cells (Supplementary Fig. S5D). Interestingly, 150 nmol/L of BPR1J373 induced significant G2-M arrest on cell cycle of
KG-1 cells as seen in K562 cells (Fig. 3A). Meanwhile, the proportion of 4N cells, polyploidy, in KG-1 was increased by BPR1J373 in a time-dependent manner and that was not present in K562 (Fig. 4A). The cell-cycle inhibitor p27 of KG-1 cells was upregulated by BPR1J373 as shown in Fig. 3B. The upregulation of cell-cycle inhibitors by BPR1J373 in Kasumi-1 and KG-1 cells was independent of p53 as p53 was present in Kasumi-1 but not in KG-1. We evaluated the morphology of KG-1 cells by Liu’s stain. KG-1 cells treated with 150 nmol/L of BPR1J373 for 72 hours showed prominent enlargement of cell size and increased nucleus formation without separation of cytoplasm compared with KG-1 cells not treated with BPR1J373 (Fig. 4B). Immunofluorescent staining for α-tubulin and DAPI showed abnormal polymerization of cellular microtubules of KG-1 cells after treatment with BPR1J373 (Fig. 4C). The polyploidy cells were significantly increased from 4% at baseline to 13% and 31.5% after 72 and 120 hours treatment with BPR1J373. The patterns of mitotic phase in KG-1 cells with or without exposure to BPR1J373 are shown in Fig. 4C. The induction of G2-M arrest in KG-1 cells by BPR1J373 was demonstrated by the presence of multipolar spindles. We evaluated the inhibitory effect of BPR1J373 on aurora kinase activity in KG-1 cells based on the development of G2-M arrest and dysregulation of spindle formation by BPR1J373, which is shown in Fig. 4D. The expression of aurora kinase B and its phosphorylated form in KG-1 cells was decreased by BPR1J373 treatment for 1, 3, and 5 days. The inhibition of aurora kinase B was also demonstrated by the decrease in its substrate, phosphorylation of histone H3 on the serine 10 residue, and induction of hypophosphorylated Rb, which suggested that induction of cell-cycle arrest and postmitotic endoreduplication, endomitosis, in KG-1 cells with higher dose of BPR1J373 were through targeting aurora kinase B. Taken together, BPR1J373 inhibited the cell proliferation of KG-1 cells not only through the induction of apoptosis but also endomitosis by targeting c-KIT and aurora kinase B.

BPR1J373 effectively suppressed Kasumi-1 cells in xenograft mouse model

Kasumi-1 cells were subcutaneously grafted into SCID mice, which were treated with 2 weeks of BPR1J373 with 50 mg/kg day 1 to day 5 with 2 days off per week per oral route after tumor inoculation. Imatinib with 100 mg/kg in the same schedule and route as BPR1J373 and chemotherapy with doxorubicin 3 mg/kg day 1 to day 3 and cytarabin 100 mg/kg day 1 to day 5 via intraperitoneal route were given to the mice as two referent groups. One group was given 10 mg/kg of vehicle as control. The bodyweight and tumor volume of each mouse were measured twice per week after drug prescription. There were 6 mice in each group. Three mice treated with chemotherapy died at day 5, so cytarabin in day 5 was not given to the other three and another 6 mice were treated with half dose of chemotherapy in the same schedule later. The mice in BPR1J373, imatinib, half dose of chemotherapy, and control group were all alive after the completion of treatment. Figure 5A shows that BPR1J373 induced significant tumor regression with the mean tumor size of 203.6 ± 9.0 mm³ at baseline reduced to 27.8 ± 27.8 mm³ in day 5 and kept at 28.5 ± 28.5 mm³ in day 12. The tumor regrew gradually after withdrawal of BPR1J373. Interestingly, the tumors in 5 mice treated with BPR1J373 disappeared and become unmeasurable at day 5 and only one had mild tumor regression as shown in Fig. 5B. The average tumor size of the mice treated with imatinib was similar to that in the control group. The tumors were not significantly suppressed by doxorubicin 3 mg/kg and cytarabin 100 mg/kg and all the mice died at day 8. The body weights of mice in control, imatinib and BPR1J373 groups were not reduced during treatment period whereas chemotherapy induced severe body weight loss of the treated mice (Supplementary Fig. S6A). Figure 5C shows that half dose of chemotherapy induced regression of tumors with a less extent than BPR1J373. Among the 6 mice in the chemotherapy group, the mean tumor size was reduced gradually from 206.8 ± 23.5 mm³ at baseline to the nadir of 95.1 ± 24.0 mm³ in day 21 and then increased.

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gradually. Only the tumor of one mouse disappeared on day 14 (Fig. 5D). Around 5% of body weight loss was noted in these mice during drug administration but recovered later (Supplementary Fig. S6B). The toxicities of BPR1J373 to non-grafted mice were evaluated for the body weight and mortality of ICR mice (non-grafted mice) fed with BPR1J373 at the same schedule for Kasumi-1 xenograft mice for 2 weeks. A maximum weight loss of 2% was observed at day 12 and no mortality was found for the ICR mice fed with BPR1J373. The results demonstrated that BPR1J373 induced more potent antitumor effect than imatinib and chemotherapy for Kasumi-1 xenograft mice.

BPR1J373 is effective to suppress c-KIT phosphorylation of D816V mutation

Because c-KIT D816V mutation is a frequent mutation in SM and CBF-AML and is resistant to most TKIs used in the treatment of leukemia, we evaluated the response of cells with this mutation to BPR1J373. We transfected the plasmid carrying c-KIT D816V mutation into c-KIT null COS-1 cells and treated the cells with 1 μmol/L of BPR1J373, imatinib, sunitinib, and nilotinib. Figure 6A shows that c-KIT phosphorylation in COS-1 cells carrying c-KIT D816V mutation was successfully suppressed by BPR1J373 in a time-dependent manner whereas that was not suppressed by imatinib, sunitinib, or nilotinib at all by 24 hours. We measured the inhibitory effect of BPR1J373 on the cell proliferation of HMC-1.2, which is a cell line derived from mast cell leukemia and carries c-KIT V560G and D816V mutations (21, 22). The IC_{50} value of HMC-1.2 in response to BPR1J373 was 385 nmol/L whereas to Midostaurin, a PKC inhibitor, was 630 nmol/L. The IC_{50} of HMC-1.2 in response to other TKIs targeting c-KIT, including imatinib, dasatinib, sunitinib, nilotinib, and regorafenib, were more than 2,000 nmol/L. Figure 6B shows that BPR1J373 inhibited c-KIT phosphorylation of HMC-1.2 in a dose- and time-dependent manner. Compared with the other TKIs, the inhibitory effect on c-KIT phosphorylation was only seen in BPR1J373–treated cells but not in the others, which is shown in Fig. 6C. When compared with Midostaurin, BPR1J373 had better effect in suppressing phosphorylation of c-KIT and its downstream signals, including Akt, mTOR, p70S6, MEK, and MAPK, and inducing the apoptosis of HMC-1.2 cells as shown in Fig. 6D. Figure 6E shows that 1 μmol/L of BPR1J373 induced a decreased proportion in G1 and increased the percentage of HMC-1.2 cells in subG1, S, and G2-M phases time dependently. The pattern of cell-cycle change to HMC1.2 cells induced by Midostaurin is similar to that by BPR1J373.

Discussion

This study demonstrated that BPR1J373 was effective to induce the apoptosis and cell-cycle arrest of c-KIT–driven myeloid leukemia by targeting c-KIT and aurora kinase B. The antitumor effect of BPR1J373 was also demonstrated in Kasumi-1 xenograft...
mouse model and primary AML cells with constitutively activated c-KIT. c-KIT was detected in most AML blasts. The use of TKI in c-KIT–positive AML showed variable results. Clinical trials evaluating monotherapy with TKIs, including imatinib and SU5416, in elder or refractory c-KIT–positive AML patients revealed low response rate (23, 24). Phase II trial using combination of imatinib and low dose Ara-C for elder patients with c-KIT–positive AML and high-risk myelodysplastic syndrome (MDS) patients achieved a low hematologic response rate of 11% (25). However, imatinib plus re-induction therapy with mitoxantrone, etoposide, and cytarabine achieved a complete response rate of 62% with relapse-free survival and OS of 6.8 and 8.3 m, respectively, for c-KIT–positive relapsed/refractory AML patients in phase I/II study (26). Although CBF-AML patients had better outcome among AML patients, the patients with c-KIT mutations had higher relapse rate and worse OS than those with wild-type c-KIT after standard chemotherapy (27). The result of imatinib in the treatment of CBF-AML patients with c-KIT mutations from few case reports was disappointing (6). However, the initial result of the CALGB 10801 study adding dasatinib to standard induction and consolidation therapy for newly diagnosed CBF-AML patients showed promising data. A complete remission rate of 92% (54/59 patients) was reported but the correlation between outcome and c-KIT mutation was not reported yet (28). In addition to c-KIT–positive or c-KIT mutant AML, SM is another disease associated with constitutively activated c-KIT.

**Figure 4.**
BPR1J373 induced polyploidy and post-mitotic endoreduplication in KG-1 cells. A, the proportion of >4N cells in KG-1 cells was increased by 150 nmol/L of BPR1J373 time-dependently. B, the Liu's stain of KG-1 cells treated without and with 150 nmol/L of BPR1J373 for 72 hours. The cells exposed to BPR1J373 showed enlargement in size and multi-nucleus formation without separation of cytoplasm (endomitosis). C, immunofluorescent staining of α-tubulin in KG-1 cells with or without exposure to 150 nmol/L of BPR1J373 for 3 days. The cells not exposed to BPR1J373 had fewer mitotic figures and the tubulin formations were seen in figures in the upper three rows. KG-1 cells treated with BPR1J373 showed significantly increased multipolar spindle formations in the cells. D, the status of aurora kinase A and aurora kinase B in KG-1 cells treated with BPR1J373. Aurora kinase A was not affected. The total and phosphorylated forms of aurora kinase B were suppressed by BPR1J373 accompanied with suppression of phosphorylated histone H3 at serine 10 residue and phosphorylated Rb.
Approximately half of pediatric mastocytosis cases had c-KIT mutation at exon 17 and the other cases had mutations at exon 8 or 9 (29). According to the analysis by Garcia-Montero and colleagues (30) 93% of 113 adult SM patients had c-KIT D816V mutation in their mast cells whereas 40% of them had D816V mutation in bone marrow cells other than mast cells. On the basis of the molecular pathogenesis of SM, imatinib was approved for use in aggressive SM without D816V mutation (31). Imatinib was shown not to be a good candidate to treat SM due to ineffectiveness to inhibit constitutive activation mutations of c-KIT at D816V (32). It was demonstrated in the phase II trial using imatinib as therapy for patient with SM that only one of 20 patients was responsive to imatinib and the patient was negative for D816V mutation (33). The other TKIs targeting c-KIT showed variable response to SM in clinical trials. Nilotinib was not effective for SM. Dasatinib and Midostaurin achieved an

Figure 5.

The tumor sizes of mice by time after starting treatment. A, tumor sizes of mice treated with vehicle, imatinib, chemotherapy, and BPR1J373. Imatinib and BPR1J373 were given at indicated dose from day 1 to day 5 with 2 days off for 2 weeks per oral route. Chemotherapeutic agents included doxorubicin and cytarabine. Doxorubicin 3 mg/kg was given from day 1 to day 3 and cytarabine 100 mg/kg was given from day 1 to day 5 via intraperitoneal route. The value of tumor size is represented in average ± SE. Significant shrinkage of tumors was noted in the mice treated with BPR1J373 when compared with the tumor size of mice treated with imatinib and vehicle (Wilcoxon rank sum test, BPR1J373 versus imatinib, P = 0.014; BPR1J373 versus control, P = 0.026; BPR1J373 versus chemotherapy, P = 0.026 at day 5. BPR1J373 versus imatinib, P = 0.017; BPR1J373 vs control, P = 0.03 at day 15). The tumor size of mice treated with chemotherapy was not significantly reduced and all the mice died at day 8. B, tumor size of each mouse in BPR1J373 group. Five mice had tumor size of 0 at day 5 and kept tumor unmeasurable at day 12. C, tumor size of the mice treated with half dose of chemotherapy and the control group. Significant tumor shrinkage was also noted in the mice treated with chemotherapy in the second week when compared with the control group (Wilcoxon rank sum test: chemotherapy vs. control, P = 0.06 at day 10 and P = 0.06 at day 17). D, tumor size of each mouse in half dose of chemotherapy group. Five mice had tumor regressed to around 50% at day 21 and then tumor regrew. Only one mouse had tumor size reduced to 0 at day 14 and regrew to 205 mm^3 at day 24.
BPR1J373 Targets c-KIT for c-KIT-Driven Myeloid Leukemia

**Figure 6.**
The expression status of c-KIT in c-KIT D816V mutant transfected COS-1 cells and HMC-1.2 cells by various TKIs and the cell-cycle analysis for HMC-1.2 cells treated with BPR1J373 and midostaurin. **A,** the expression of total and phosphorylated form of c-KIT in COS-1 cells. COS-1 cells were transfected with c-KIT D816V mutation and treated with 1 μmol/L of BPR1J373, imatinib, sunitinib, and nilotinib for 2, 4, 8, 16, and 24 hours. The left lane is COS-1 cells transfected with empty vector. The second lane is COS-1 cells transfected with c-KIT D816V mutation not treated with TKIs. **B,** the expression of c-KIT and phosphorylated c-KIT in HMC-1.2 cells treated with various doses of BPR1J373 for 24 hours (left) and 1 μmol/L of BPR1J373 for 2, 24, 48, and 72 hours (right). **C,** the expression of c-KIT and phosphorylated c-KIT of HMC-1.2 cells treated with 1 μmol/L of BPR1J373, imatinib, dasatinib, nilotinib, sunitinib, and regorafenib for 24 hours. The c-KIT phosphorylation of HMC-1.2 cells was only suppressed by BPR1J373. **D,** the expression of c-KIT, phosphorylated c-KIT, PARP, and the phosphorylation of downstream signals for c-KIT, including mTOR, p70S6, Akt, MEK, and MAPK in HMC-1.2 cells treated with or without 0.5 and 1 μmol/L of BPR1J373 and Midostaurin for 24 hours. **E,** cell-cycle analysis of HMC-1.2 cells treated with 1 μmol/L of BPR1J373 or Midostaurin for 0, 12, 24, 36, 48, 60, and 72 hours. A decrease in G1 and increase in subG1, S, and G2–M phase was observed in HMC-1.2 cells treated with BPR1J373 and Midostaurin with a similar pattern whereas the increase in G2–M phase was more prominent in HMC-1.2 cells treated with Midostaurin.

overall response rate of 33% and 69%, respectively, for SM patients in the phase II studies (31). c-KIT D816V (exon17) mutation was also commonly detected in CBF-AML patients and patients with exon17 mutation confer poorer prognosis than patients with wild-type or exon 8 mutation (27). Most TKIs are not able to inhibit constitutive activating mutations of c-KIT at codon 816. The BPR1J373 exerted potent effect on the inhibition of c-KIT activation in wild type, KG-1, K562, and c-KIT–mutated myeloid leukemia cell lines, Kasumi-1. The potent antitumor response was also demonstrated in Kasumi-1 xenograft mice and showed better response than imatinib and conventional chemotherapy. Furthermore, BPR1J373 was shown to be more potent than imatinib, sunitinib, and nilotinib to inhibit c-KIT phosphorylation in COS-1 cells transfected with c-KIT D816V mutation.

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In conclusion, BPR1J373 inhibited cell proliferation of c-KIT–driven AML cells via induction of apoptosis and cell-cycle arrest. It also induced polyploidization of leukemia cells via inhibition of aurora kinase B. This compound deserves further development for the clinical use in c-KIT–driven AML, SM, and the potential application in megakaryocytic disorders, MDS and PMF.

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No potential conflicts of interest were disclosed.

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References


Driven Myeloid Leukemia−c-KIT for the Treatment of BPR1J373, an Oral Multiple Tyrosine Kinase Inhibitor, Targets c-KIT for the Treatment of c-KIT–Driven Myeloid Leukemia

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