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BPR1J373, a novel multitargeted kinase inhibitor, effectively suppresses the growth of gastrointestinal stromal tumor

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Gastrointestinal stromal tumor (GIST) is a type of *KIT*-driven cancer. *KIT* gene mutations are found in approximately 80% of GISTs, and most of these mutations occur in exon 9 and exon 11. Imatinib has been successfully used as a first-line treatment for advanced GIST, with a significant improvement in progression-free survival (PFS) and overall survival. However, disease progression might develop due to primary or secondary resistance to imatinib. Sunitinib and regorafenib have been approved as second- and third-line treatments for advanced GIST patients, with median PFS values of 6.8 and 4.8 months, respectively. However, these relatively modest improvements in PFS underscore the need for more effective KIT inhibitors. BPR1J373 is a multitargeted kinase inhibitor that has been shown to inhibit the proliferation of *KIT*-driven acute myeloid leukemia cells in vitro and in vivo. In this study, we found that BPR1J373 inhibited proliferation and induced apoptosis by targeting KIT in GIST cells with *KIT* gene mutations. BPR1J373 also induced cell cycle arrest and senescent change in *KIT*-mutant GIST48 cells, probably by targeting aurora kinase A. In the *KIT*-null COS-1 cell-based system, BPR1J373 effectively inhibited KIT with single or double mutations of *KIT* developed in GIST. The antiproliferative effect was also consistently evident in GIST430 tumor-grafted mice. The results suggest that BPR1J373 could be a potential anticancer drug for GIST and deserves further investigation for clinical applications.

KEYWORDS
Aurora kinase A, BPR1J373, gastrointestinal stromal tumor, KIT, multitargeted kinase inhibitor

1 | INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are neoplasms arising from mesenchymal tissue of the gastrointestinal tract and are thought to originate from progenitors of the interstitial cells of Cajal. The stomach and small intestine are the most common sites of GISTs, and approximately 60% of GISTs can be cured by surgery. However, approximately 40% of patients initially diagnosed with localized GISTs develop metastasis.[1] Gastrointestinal stromal tumors are a type of *KIT*-driven cancer, which was found by Hirota et al[2] in 1998 to harbor mutations in the *KIT* oncogene. Gastrointestinal stromal tumors are usually unresponsive to conventional chemotherapy. Imatinib, which targets platelet-derived growth factor receptor (PDGFR) and Bcr-Abl,[3,4] was shown to induce a good response in chronic myeloid leukemia and Philadelphia chromosome-positive acute lymphoblastic leukemia patients in phase I/II studies and was approved as a first-line treatment for both diseases.[5-8] In 2000, Heinrich et al[9] showed that this drug could inhibit the phosphorylation of KIT in *KIT*-mutated HMC-1 cells and effectively inhibit the proliferation of HMC-1 cells. Imatinib was also shown to induce a notably good response in an advanced GIST patient who had failed multiple treatments.[10] In addition, imatinib treatment achieved a disease control rate of 81.6% for advanced GIST, leading to significant improvement in progression-free survival (PFS) and overall survival (OS) in the initial phase II study.[11] Therefore, imatinib has become the first-line treatment for advanced GIST with a long-term median OS of 51-57 months in phase II and phase III trials.[12,13]

Disease progression of GIST might develop further due to either primary or secondary resistance to imatinib.[14,15] *KIT* mutations are found in approximately 80% of GISTs, and mutations in exons 9 and 11 are the most frequent at diagnosis.[16,17] Patients with exon 9 mutations have a lower response rate to imatinib and inferior PFS and OS when compared with patients with primary exon 11 mutations.[16-20] Secondary mutations of *KIT* occur in exons 13, 14, 17, or 18, which might indicate a clonal selection after long-term imatinib therapy, and develop more frequently in patients with primary exon 11 mutations.[21-23] For imatinib-resistant GIST patients, sunitinib, a multitargeted tyrosine kinase inhibitor (TKI), was shown to improve PFS and OS and is approved as a second-line treatment for advanced GIST patients after treatment failure or intolerance to imatinib.[24] Considering the genotype of GIST and response to sunitinib, patients who harbor primary exon 9 mutations and WT *KIT* have longer median PFS and OS than patients with exon 11 mutations. Moreover, sunitinib showed poorer response in patients with secondary mutations in exons 17/18 than in exons 13/14.[24] In addition, regorafenib was approved in 2013 as the third-line treatment for GIST patients who failed to respond, or were intolerant to, treatment with imatinib and sunitinib, according to results from a phase III study.[25] The median PFS was 4.8 months compared with 0.9 month for the placebo control group. Therefore, for refractory GIST, development and identification of novel agents are mandatory.

BPR1J373, a 5-phenylthiazol-2-ylamine-pyriminide derivative, is a multitargeted kinase inhibitor with potent inhibitory activity against fms like tyrosine kinase 3, KIT, vascular endothelial growth factor receptor (VEGFR), Aurora A, Aurora B, PDGFRα, PDGFRβ, reannanged during transfection, and sarcoma in preliminary kinase profiling. The structure of BPR1J373 was shown previously.[26] The antikinase profile of BPR1J373 is presented in Figure S1. BPR1J373 has been shown to inhibit proliferation of *KIT*-driven acute myeloid leukemia cells in vitro and in vivo.[26] In this study, we report the effectiveness and mechanisms by which BPR1J373 inhibits the proliferation of *KIT*-mutant GIST cells in vitro and in vivo.

2 | MATERIALS AND METHODS

2.1 | Cell lines and reagents

The GIST cell lines GIST882, GIST48, GIST430, GIST-T1, GIST62, GIST48B, and COS-1 were selected for the current study. All cells were cultured in incubators maintained at 37°C and 5% CO_2. GIST882 and GIST62 were cultured in RPMI-1640 supplemented with 20% FBS. GIST-T1 was cultured in RPMI-1640 supplemented with 10% FBS. GIST48 and GIST48B were cultured in F10 supplemented with 20% FBS, 0.5% Mito ± serum extender (355006; BD Biosciences, San Jose, CA, USA) and 1% pituitary extract bovine (354123; BD Biosciences). GIST430 was cultured in Iscove's modified Dulbecco's medium supplemented with 20% FBS. COS-1 was cultured in DMEM supplemented with 10% FBS. GIST882, GIST430, GIST48, GIST62, and GIST48B cells were gifts from Dr. Jonathan A. Fletcher (Harvard Medical School, Boston, MA, USA) in 2010. COS-1 cells were obtained from Dr. Neng-Yao Shih (National Health Research Institutes [NHRI]) (Tainan, Taiwan) in 2013. GIST-T1 cells were obtained from Dr. Yan-Shen Shan (National Cheng Kung University Hospital, Tainan, Taiwan) in 2018. Because no database is available for checking cell line authentication, we used direct sequencing in December 2016 for GIST882, GIST430, and GIST48 cells and confirmed that a homozygous mutation of K642E exists for GIST882, an exon 11 in-frame deletion and a heterozygous secondary exon 13 missense mutation (V654A) exist for GIST430, and a homozygous exon 11 missense mutation (V560D) and a heterozygous secondary exon 17 mutation (D820A) exist for GIST48 cells.

GIST62, GIST48B, and COS-1 cells were confirmed to be *KIT*-null by RT-PCR in January 2017 and March 2016. In July 2018, GIST-T1 was confirmed by direct sequencing to contain an exon 11 (V560-Y579) deletion. BPR1J373 was supplied by Dr. Weir-Torn Jiaang (Institute of Biotechnology and Pharmaceutical Research, NHRI). Sunitinib, imatinib, nilotinib, and regorafenib were purchased from Selleckchem (Houston, TX, USA).

2.2 | Apoptosis assay

Gastrointestinal stromal tumor cell lines GIST882, GIST430, and GIST48 were cultured in 6-well plates and treated with or without 2× IC_{50} and 1 μmol/L BPR1J373. The cells were collected at prespecified times, stained with annexin V-phycoerythrin and propidium iodide, and analyzed by BD FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA).

2.3 | Cell proliferation assay

Gastrointestinal stromal tumor cells (4×10^4) were treated with different dosages of BPR1J373. The treated GIST882 cells were incubated for 144 h, and GIST48, GIST430, and GIST-T1 cells were incubated for 120 hours at 37°C in 5% CO_2. Cell proliferation was determined by incubating the cells with methylene blue (Clontech, Mountain View, CA, USA) for 1 hour. The absorbance was measured at 450 nm using the SpectraMax M5 microplate reader (Molecular Devices, San Jose, CA, USA).

2.4 | Western blot analysis

The GIST cells were treated with 1 μmol/L imatinib, sunitinib, nilotinib, regorafenib, and BPR1J373 at prespecified time points to evaluate the time effect. The GIST cells were treated with different doses of BPR1J373 for 24 hours to evaluate the dose effect. Cell lysates were extracted with CelLytic M mammalian cell lysis/extraction solution purchased from Sigma (St. Louis, MO, USA). The cell lysates were resolved in SDS-PAGE gel and transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA). Non-specific binding was blocked using 5% BSA/PBST for 1 h, and the gels were washed four times with 0.1% Tween-20/PBS, followed by incubation with primary antibodies overnight at 4°C. The immunocomplexes were detected by probing with anti-mouse or anti-rabbit IgG conjugated with HRP and visualized using an Enhanced Chemiluminescence detection kit (Western Lightning Plus-ECL; PerkinElmer, Waltham, MA, USA). The primary antibodies PI3K, AKT, p-AKT, poly(ADP-ribose) polymerase (PARP), Puma, Bax, Bad, Bak, p21, Aurora A, p-Aurora A, Aurora B, p-Aurora ABC, p-Rb, p-MEK, and p-MAPK were purchased from Cell Signaling (Danvers, MA, USA). Antibodies against Bcl-2, BcL-xL, p53, p16, and GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, TX, USA). Antibody against p-KIT was purchased from Invitrogen (Frederick, MD, USA). P27 was purchased from BD Transduction Laboratories (BD Biosciences). KIT was purchased from Dako (Carpinteria, CA, USA).

2.5 | Cell cycle analysis

Cells were treated with or without BPR1J373, sunitinib, nilotinib, or regorafenib and harvested at the designed time courses. The harvested cells were washed twice with PBS and fixed overnight with 70% cold ethanol. The cells were collected by centrifugation at 5000 *g* for 5 minutes, incubated with 0.2 mg/mL RNase A (Sigma) for 1 hour, and stained with 20 μg/mL propidium iodide (Sigma) at room temperature. The stained cells were measured using a FACSCalibur machine, and the data were analyzed with the WinMDI 2.9 software (Purdue University Cytometry Laboratories, West Lafayette, IN, USA). Data represent the mean ± SE of triplicate experiments.

2.6 | β-Galactosidase staining

Amounts of 3×10^5 GIST48 cells were cultured with or without 10 nmol/L, 100 nmol/L, or 1 μmol/L BPR1J373. The cells in each condition were harvested 96 hours later and washed twice with 1× PBS. The cells were transferred to a slide by cytospinning at 500 rpm for 5 minutes and fixed with 0.5% glutaraldehyde for 10 minutes at room temperature. The slides were washed twice with 1× PBS and stained with β-galactosidase solution (Invitrogen, Foster City, CA, USA) at 37°C in the dark overnight. The slides were washed twice with 1× PBS for further evaluation.

2.7 | *KIT*-mutant transfections

Plasmids containing *KIT* mutations were produced as described previously.[27] COS-1 cells were cultured in a 6-cm plate overnight and added with vector (pcDNA3.1) only, or vector with WT or mutant *KIT* with Lipofectamine 2000 (11668-019; Invitrogen) according to the manufacturer's protocol. The cells were cultured for 24 hours and treated with or without drugs (imatinib, sunitinib, nilotinib, regorafenib, and BPR1J373) for the indicated time. The cell lysates in each condition were collected for western blot analysis.

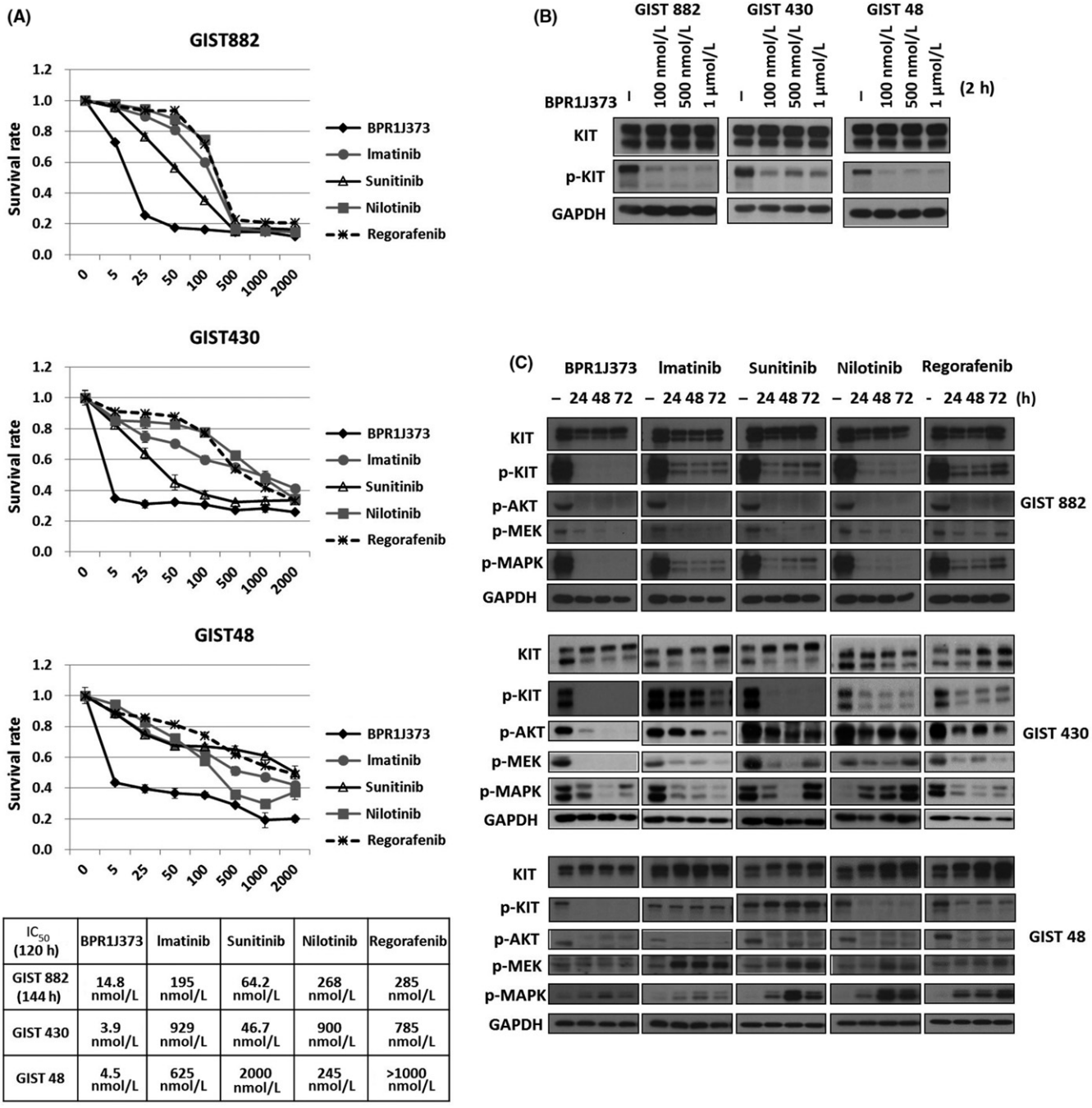
2.8 | Animal study

NOD-SCID male mice (6-8 weeks old) 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 NHRI. GIST430 cells (2×10^7/mouse) were injected s.c. into the mice. The mice were treated with a vehicle control, sunitinib 40 mg/kg, regorafenib 30 mg/kg, or BPR1J373 50 mg/kg from day 1 to day 5 with 2 days of rest for 2 weeks after tumor growth to 50-100 mm^3. The doses of sunitinib and regorafenib were chosen according to efficacy studies in mouse xenograft models.[28-32] The maximum tolerated dose of BPR1J373 was 75 mg/kg, and we chose 50 mg/kg in this study for safety considerations. The drugs were dissolved in 20% (2-hydroxypropyl)-β-cyclodextrin (Sigma) for oral feeding. The tumor volume was measured three times per week for up to 16 days using the following formula: length (mm) × width^2 (mm^2) × (π/6).[33] Body weight was also measured at the same time as the tumor measurement.

3 | RESULTS

3.1 | BPR1J373 inhibited the proliferation of *KIT*-mutant GIST cells

Gastrointestinal stromal tumor cell lines, including KIT-dependent GIST882, GIST48, and GIST430 and KIT-null GIST48B and GIST62, were treated with BPR1J373 and screened for the inhibitory effect of BPR1J373 on cell proliferation using methylene blue staining. The BPR1J373 IC_{50} values for 2× doubling time were 14.8, 3.9, 4.5, >2000, and >2000 nmol/L in GIST882, GIST430, GIST48, GIST62, and GIST48B cell lines, respectively. BPR1J373 did not suppress cell proliferation in the KIT-null GIST cell lines. Compared with imatinib, sunitinib, nilotinib, and regorafenib, the IC_{50} values of BPR1J373 that suppress cell proliferation in the three *KIT*-mutant GIST cell lines are much lower (Figure 1A). Among the drugs for comparison with



IC_{50} (120 h)	BPR1J373	Imatinib	Sunitinib	Nilotinib	Regorafenib
GIST 882 (144 h)	14.8 nmol/L	195 nmol/L	64.2 nmol/L	268 nmol/L	285 nmol/L
GIST 430	3.9 nmol/L	929 nmol/L	46.7 nmol/L	900 nmol/L	785 nmol/L
GIST 48	4.5 nmol/L	625 nmol/L	2000 nmol/L	245 nmol/L	>1000 nmol/L

FIGURE 1 Survival rate and KIT activation status of gastrointestinal stromal tumor (GIST) cell lines treated with 5 types of tyrosine kinase inhibitors (TKIs): BPR1J373, imatinib, sunitinib, nilotinib, and regorafenib. A, Survival rate and IC_{50} values of *KIT*-mutant GIST cell lines treated with indicated doses of the 5 TKIs. B, Dose effect of BPR1J373 on inhibition of phosphorylation of KIT to *KIT*-mutant GIST cell lines. C, Time effect of 1 μmol/L of the 5 TKIs to inhibit KIT activation and the downstream signals of KIT to *KIT*-mutant GIST cell lines

BPR1J373, imatinib was relatively active against GIST882, sunitinib was active against GIST882 and GIST430, and nilotinib had the greatest activity against GIST48. Regorafenib was less effective than sunitinib and nilotinib in suppressing the growth of GIST430 and GIST48, respectively. Another imatinib-sensitive GIST cell line, GIST-T1, was also evaluated for an antiproliferative effect by the above agents. BPR1J373, sunitinib, and nilotinib exerted the best antiproliferative effects, as shown in Figure S2A.

3.2 | BPR1J373 effectively suppressed KIT phosphorylation and downstream signaling in GIST cell lines

Because *KIT* is a driving oncogene responsible for the cell growth of GIST cells with *KIT* mutations, activation of KIT was determined by its phosphorylation status in GIST882, GIST430, and GIST48 cells. Western blot analysis revealed that phosphorylation of KIT in the *KIT*-mutated GIST cells was dramatically decreased by 100 nmol/L BPR1J373 within 2 hours (Figure 1B). BPR1J373 exerted the most potent effect on inhibiting KIT activation and the downstream signals of KIT in GIST882 cells; this effect was better than those of imatinib, sunitinib, nilotinib, and regorafenib (Figure 1C). The potency of KIT inhibition in GIST430 and GIST48 was observed in the treatment with sunitinib and nilotinib, respectively. In contrast, BPR1J373 displayed no differential potency among these cells and remarkably downregulated KIT activation and its driven signaling molecules such as AKT, MEK, and MAPK (Figure 1C). Furthermore, BPR1J373, as well as imatinib, sunitinib, nilotinib, and regorafenib, exerted a potent effect in suppressing KIT activation and its downstream signaling molecules of imatinib-sensitive GIST-T1 cells (Figure S2B,C).

3.3 | BPR1J373 differentially induced cell apoptosis of *KIT*-mutated GIST cells

To investigate the mechanisms of the inhibitory effect of BPR1J373 on GIST cells, we evaluated the molecular markers of apoptosis. Cleavage of PARP was evident in the three KIT-dependent GIST cell lines after 48 hours of exposure to BPR1J373 (Figure 2A). We further evaluated the effect of BPR1J373 on mitochondria-associated apparatuses that regulate cell survival and apoptosis. BPR1J373 had a profound effect on the upregulation of pro-apoptotic molecules Bax and Puma in GIST48 and GIST430 cells. BPR1J373 also induced the upregulation of Bax and Bak in GIST882 cells. No change in pro-survival molecules Bcl2 and BcL-xL was noted for GIST430 cells, whereas BPR1J373 significantly suppressed the expression of Bcl2 in GIST48 cells, as shown in

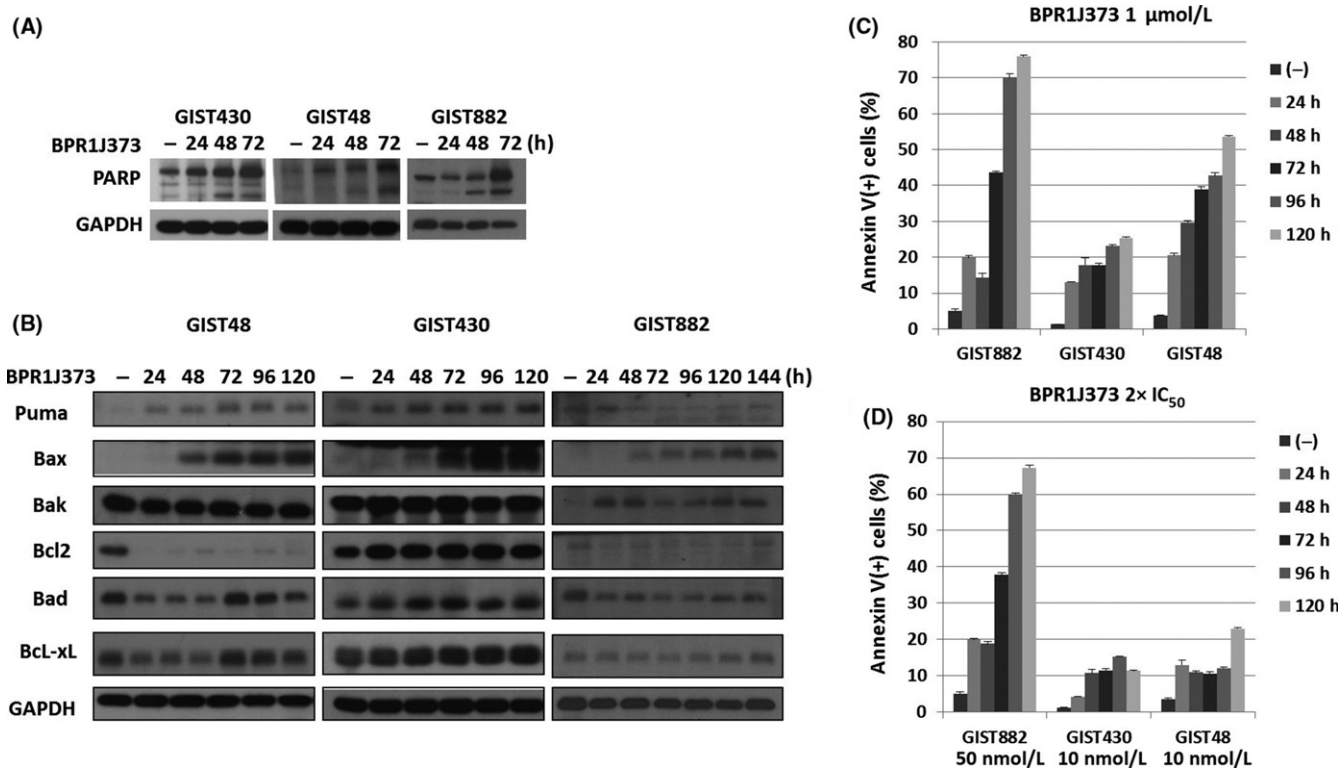


FIGURE 2 BPR1J373 induced apoptosis of *KIT*-mutant gastrointestinal stromal tumor (GIST) cell lines differentially. A, Presence of poly (ADP-ribose) polymerase (PARP) cleavage in *KIT*-mutant GIST cells by BPR1J373 in a time-dependent manner. B, Changes of pro-apoptotic and anti-apoptotic proteins in *KIT*-mutant GIST cells due to BPR1J373. C, Differential increase of annexin-V-positive cells in *KIT*-mutant GIST cells treated with 1 μmol/L BPR1J373 over time. D, Differential increase of annexin-V-positive cells in *KIT*-mutant GIST cells treated with 2× IC_{50} of BPR1J373 over time. –, baseline

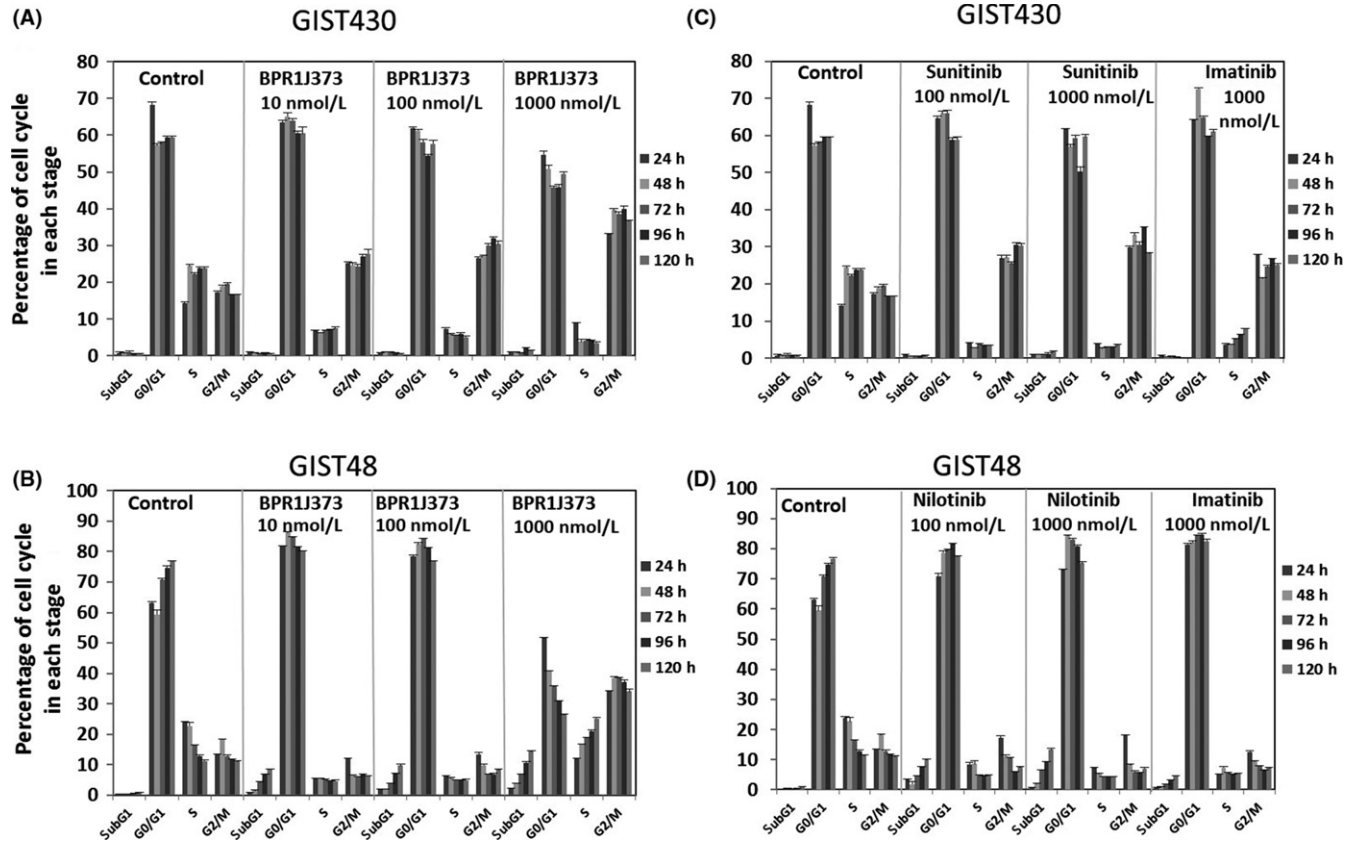


FIGURE 3 BPR1J373 induced cell cycle arrest in gastrointestinal stromal tumor (GIST)430 and GIST48 cells. A, Cell cycle analysis for GIST430 cells treated with indicated doses of BPR1J373 over time. B, Cell cycle analysis for GIST48 cells treated with BPR1J373 in indicated doses over time. C, Cell cycle analysis of GIST430 cells treated with indicated doses of sunitinib and imatinib over time. D, Cell cycle analysis of GIST48 cells treated with indicated doses of nilotinib and imatinib over time

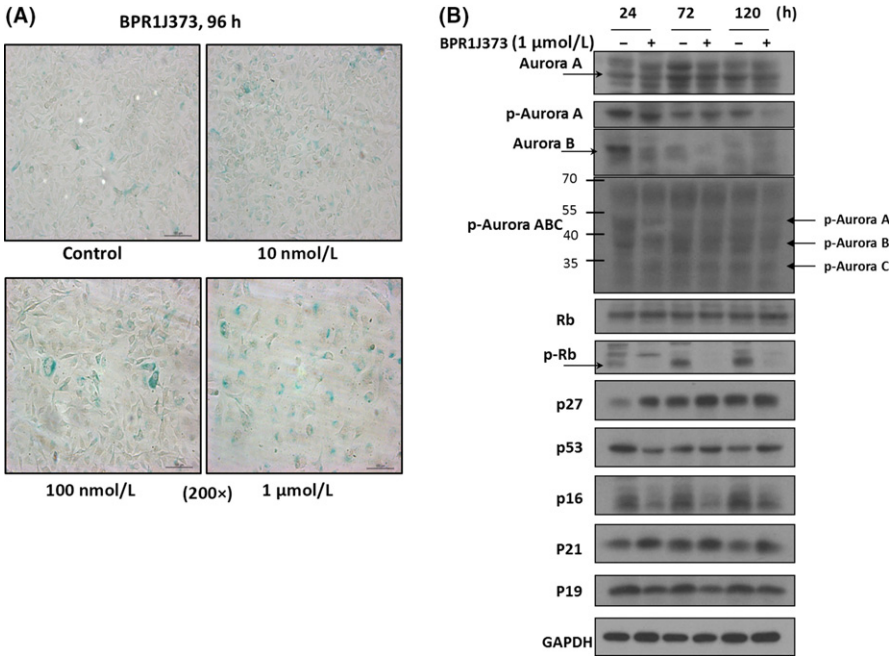


FIGURE 4 BPR1J373 induced senescent change of gastrointestinal stromal tumor (GIST)48 cells by targeting of Aurora kinase A. A, β-Galactosidase staining of GIST48 cells treated with the indicated dose of BPR1J373 for 96 hours. B, Protein expression of total and phosphorylated (p-) forms of Aurora A and B, and the cell cycle-associated proteins in GIST48 cells treated with or without 1 μmol/L BPR1J373 for indicated durations

Figure 2B. The expression of Bcl2 in GIST882 was suppressed by BPR1J373. Furthermore, the apoptotic effect of BPR1J373 in GIST cells was also determined by annexin-V/propidium iodide staining. The annexin-V-positive cells increased gradually in response to increased exposure time of GIST882, GIST48, and GIST430 to BPR1J373 at a concentration of 1 μmol/L (Figure 2C). More than 70% of GIST882 cells

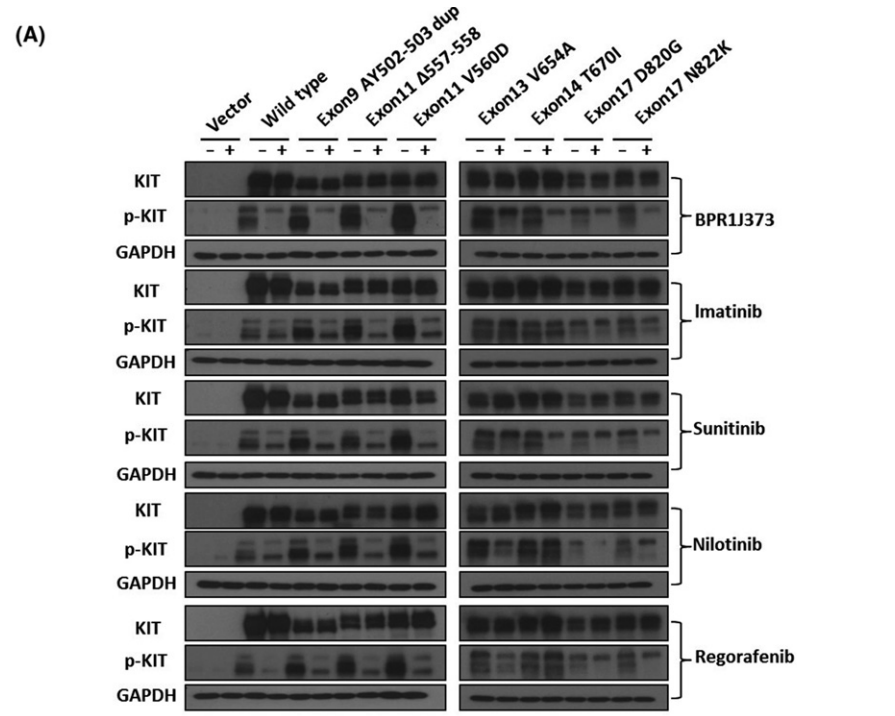


FIGURE 5 BPR1J373 suppressed KIT phosphorylation of *KIT*-mutant transfected COS-1 gastrointestinal stromal tumor cells. A, Inhibitory effect of 1 μmol/L of the 5 tyrosine kinase inhibitors (TKIs) on phosphorylation of KIT in COS-1 cells transfected with *KIT* single mutation plasmids. B, Inhibitory effect of the 5 TKIs on phosphorylation of KIT in COS-1 cells transfected with *KIT* double mutation plasmids

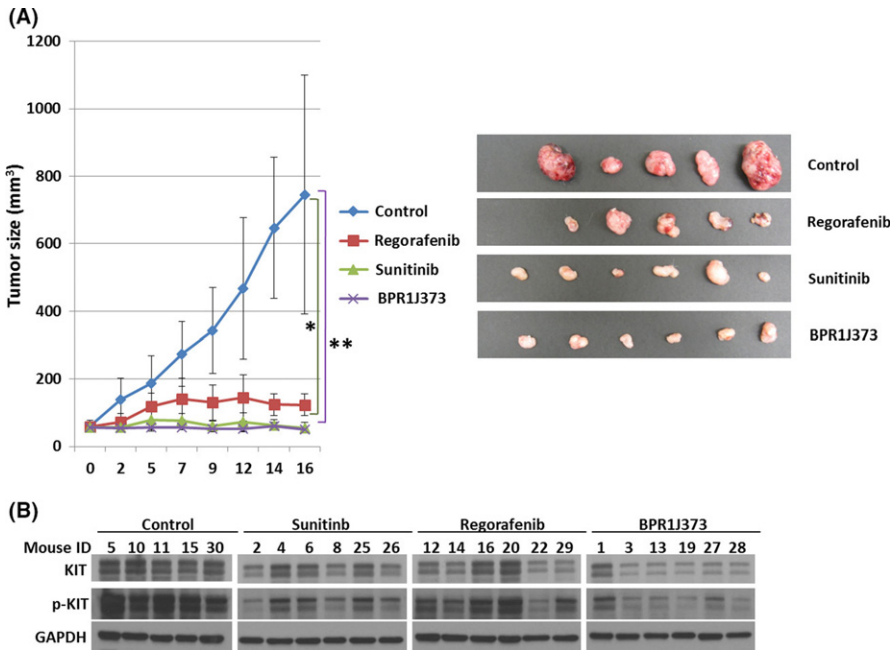


FIGURE 6 BPR1J373 suppresses tumor growth of GIST430 xenograft. A, Tumor growth curves and pictures of tumors of GIST430 xenograft mice treated with vehicle control, sunitinib, regorafenib, and BPR1J373. *Control vs sunitinib, $P = 0.03$ (Wilcoxon rank sum test); **Control vs BPR1J373, $P = 0.02$ (Wilcoxon rank sum test). B, KIT and phosphorylated (p-)KIT expression of tumors in GIST430 xenograft mice treated with vehicle control, sunitinib, regorafenib, or BPR1J373

were annexin-V-positive after 96 hours of exposure to BPR1J373. The cells were treated with their corresponding double IC_{50} doses (2× IC_{50}) of BPR1J373. Intriguingly, the apoptotic event for GIST882 cells obviously remained in a time-dependent manner but was less significant for GIST48 and GIST430 cells (Figure 2D). This result indicates that BPR1J373 inhibited cell proliferation of GIST882 cells mainly through

induction of apoptosis. However, the percentage of annexin-V-positive cells was much lower than the inhibitory proportion of BPR1J373-treated GIST48 and GIST430 cells (Figure 1A). This result suggests that antiproliferation of GIST48 and GIST430 by BPR1J373 might be mediated by both apoptotic- and non-apoptotic-dependent mechanisms.

3.4 | Cell cycle arrest and cellular senescence caused by BPR1J373

To investigate the potential non-apoptotic events in GIST48 and GIST430 cells, the cell cycle was further analyzed after BPR1J373 treatment at the indicated dose and time points. Compared with the control, a decrease in S phase and increase in G_2/M phase were noted in GIST430 cells after exposure to 10 nmol/L, 100 nmol/L, and 1 μmol/L BPR1J373, as shown in Figure 3A. For GIST48 cells, treatment with 10 nmol/L and 100 nmol/L BPR1J373 apparently caused a decrease in both S and G_2/M phases but gradually increased the sub-G_1 or G_0/G_1 phase. Intriguingly, 1 μmol/L BPR1J373 caused a marked decrease in G_0/G_1 phase but simultaneously increased the S and G_2/M cell populations in a time-dependent manner (Figure 3B). The results in Figure 3C show that alteration of the cell-cycle subpopulations induced by imatinib or sunitinib displayed a similar pattern comparable to that caused by 10 nmol/L and 100 nmol/L BPR1J373 for GIST430 cells. Neither 1 μmol/L imatinib nor nilotinib induced S or G_2/M arrest in GIST48 cells, but 1 μmol/L BPR1J373 did (Figure 3D). Regorafenib did not induce significant cell cycle changes in GIST430 cells and induced only a mild increase in subG_1 and a mild decrease in S and G_2/M proportions of GIST48 cells (Figure S3).

Because cell cycle arrest at the G_2/M phase of GIST430 and GIST48 cells was noted with BPR1J373 treatment, we undertook β-galactosidase staining to evaluate whether senescent change was induced by BPR1J373. Figure 4A shows increased numbers and density of β-galactosidase-positive GIST48 cells after treatment with 10 nmol/L, 100 nmol/L, and 1 μmol/L BPR1J373 for 96 hours, whereas these responses were not convincingly observed in GIST430 cells. Upregulation of senescence-associated genes, including *HP1γ*, *p19*, *p53*, and *HMGA1*, further supported the induction of senescent formation in GIST48 cells treated with BPR1J373, particularly at a dose of 1 μmol/L (Figure S4). Hypophosphorylation of Rb protein and increased expression of p21 and p27 were found in GIST48 treated with BPR1J373 (Figure 4B). In addition, because BPR1J373 was shown to have an inhibitory effect on the activation of Aurora kinase, we examined the expression of Aurora kinase A and B and found that the phosphorylation of Aurora kinase A could be suppressed by BPR1J373 in GIST48 cells (Figure 4B). Thus, data suggest that BPR1J373-induced senescence in GIST48 cells might be mediated by the inactivation of Aurora kinase A activity.

3.5 | BPR1J373 suppressed KIT activation of COS-1 cells with *KIT*-mutant genes

To determine whether BPR1J373 suppresses KIT phosphorylation for other *KIT* mutations common in GIST, we transfected COS-1 cells using plasmids with various *KIT* mutant genes, including single (AY502-503 duplication in exon 9, Δ557-558 deletion in exon 11, V560D in exon 11, V654A in exon 13, T670I in exon 14, and D820G, N822K, and D816V in exon 17) and double mutations (Δ557-558 in exon 11/V654A in exon 13, Δ557-558 in exon 11/T670I in exon 14, Δ557-558 in exon 11/D820G in exon 17, and Δ557-558 in exon 11/N822K in exon 17). COS-1 cells transfected with the *KIT* mutants were treated with 1 μmol/L BPR1J373, imatinib, sunitinib, nilotinib, or regorafenib for 30 minutes. All 5 drugs were able to suppress KIT phosphorylation of COS-1 cells with individual single mutations in exon 9 and 11 with BPR1J373 as the most potent. Imatinib, sunitinib, nilotinib, and regorafenib differentially suppressed KIT phosphorylation in COS-1 cells with single mutations in exons 13, 14, and 17. Compared with the 4 drugs, BPR1J373 exerted the most potent effect on suppression of KIT phosphorylation of these COS-1 transfectants, as shown in Figure 5A. Furthermore, BPR1J373 also showed a remarkable potency in the suppression of KIT activation in COS-1 transfectants with all 4 double mutations, whereas imatinib, sunitinib, nilotinib, and regorafenib showed variable effects on suppression of KIT phosphorylation (Figure 5B). For D816V in exon 17, which is resistant to imatinib, sunitinib, and nilotinib, BPR1J373 still suppressed KIT phosphorylation in a time-dependent manner, whereas the other 3 tyrosine kinase inhibitors did not, as shown previously.[26]

3.6 | Tumor growth suppressed by BPR1J373 in a xenograft model

To test the therapeutic effect of BPR1J373 in vivo, GIST430 cells were s.c. grafted into NOD-SCID mice. After tumor inoculation, the mice were orally treated with BPR1J373 (50 mg/kg), sunitinib (40 mg/kg), regorafenib (30 mg/kg), or vehicle control from day 1 to day 5 per week for 2 consecutive weeks. The body weight and tumor volume of each mouse were measured the day after the drugs were given, and the mice were killed after completion of treatment. There were 5 mice in the control group and 6 mice each in the BPR1J373, sunitinib, and regorafenib groups. All mice were alive before completion of the experiment except for 1 mouse in the regorafenib group that died at day 14. BPR1J373 at the designed dose caused a mild body weight loss compared with control mice (mean ± SE, 30.74 ± 0.34 g). Body weights were not significantly different among mice treated with the 3 drugs (28.82 ± 0.52 g in the regorafenib group; 29.1 ± 1.04 g in the sunitinib group; 27.8 ± 0.30 g in the BPR1J373 group). The body weight changes of the mice in the treatment period are shown in Figure S5. Although these 3 drugs could suppress the tumor growth compared with the control, only sunitinib (P = 0.03, Wilcoxon rank sum test) and BPR1J373 (P = 0.02, Wilcoxon rank sum test) showed statistically significant antitumor effects. The antitumor effect of BPR1J373 was greater than that of regorafenib, although not statistically significant (P = 0.15, Wilcoxon rank sum test). The tumor sizes of mice in the BPR1J373 and sunitinib groups were comparable (P = 0.59, Wilcoxon rank sum test; Figure 6A). In addition, decreased

expression levels of KIT and KIT phosphorylation were observed in mice treated with BPR1J373, sunitinib, or regorafenib compared with those in the controls (Figure 6B). Taken together, all data indicated that BPR1J373 was more effective in suppressing tumor growth of GIST430 cells and showed a non-inferior effect compared with sunitinib in vivo.

Collectively, these studies indicate that BPR1J373 induced cell apoptosis or cell cycle arrest/senescence in *KIT*-mutated GIST cells by targeting KIT and likely Aurora kinase A. The antiproliferative effect shown in tumor-grafted mice suggests that BPR1J373 could be a potential anticancer drug for GIST, therefore, further investigation for its clinical applications is warranted.

4 | DISCUSSION

KIT is the major target for current GIST treatments. Imatinib targets KIT and its downstream signals, thereby inhibiting GIST cell proliferation and inducing apoptosis. Resistance to imatinib is an inevitable consequence of most advanced GIST patients, and the mechanisms of resistance to imatinib have been widely studied. *KIT* mutations (including primary and secondary mutations), drug metabolism, gene amplification of *KIT* or *PDGFRA*, activation of alternate growth pathways, and loss of *KIT* can all contribute to resistance to imatinib.[1,14] The differential inhibitory effect on KIT phosphorylation of commercially available TKIs, including imatinib, sunitinib, nilotinib, dasatinib, and sorafenib, was evident in the in vitro KIT-expressing COS-1 cell-based system.[34] No single drug showed a potent effect targeting all of the common *KIT* mutations in GIST, including single and double mutations. This result corresponds to the variable responses to these TKIs among advanced GIST patients. In the present study, we revealed the potent antiproliferative effect of BPR1J373 in suppressing the growth of both imatinib-sensitive and imatinib-resistant GIST cell lines. Furthermore, the inhibition of KIT phosphorylation by BPR1J373 in a COS-1 cell-based system was superior to that obtained with other available TKIs for various *KIT* mutations, including *KIT D816V* mutation, which is resistant to all available TKIs. In addition to KIT, other targets were shown to contribute to the survival of GIST. Sunitinib was shown to be effective for GIST by targeting KIT and VEGFR.[28] In an in vitro study, sorafenib was shown to be a potential active agent for GIST based on its potent suppression of mutant KIT and PDGFRA.[35] Abrogation of the protective effect of heat shock protein 90 in preventing KIT proteasome-mediated degradation is another approach for targeting imatinib-resistant KIT-positive GIST and has been evaluated in clinical trials. Monoclonal antibodies targeting PDGFRA and VEGFR, mTOR inhibitors, and PI3K/AKT inhibitors have also been studied to treat GIST in phase II or III studies in patients with imatinib-resistant GIST.[1,15,36,37] The efficacy of these agents remains to be determined. In our study, BPR1J373 induced apoptosis in imatinib-sensitive and imatinib-resistant GIST cells via targeting KIT. Moreover, compared with sunitinib, nilotinib, and regorafenib, BPR1J373 exerted a more potent inhibitory effect on KIT phosphorylation and its downstream signals AKT and MEK in GIST882, GIST48 and GIST430 cells. For imatinib-resistant GIST430 and GIST48 cells, the antiproliferative effect of BPR1J373 is thought to occur through targeting of KIT-induced apoptosis and other mechanisms. BPR1J373 induced up to 30% and 50% of apoptotic cells in GIST430 and GIST48 cells, respectively, as shown in Figure 2. However, the survived GIST430 and GIST48 cells after BPR1J373 treatment were only approximately 20% of the untreated cells, as shown in Figure 1. Therefore, the antiproliferative effect of GIST430 and GIST48 by BPR1J373 is suspected to be caused by the synergistic effect of apoptosis and cell cycle arrest, as shown in Figures 2 and 3.

In this study, we found that BPR1J373 evoked senescence and G_2/M arrest in imatinib-resistant GIST48 cells. BPR1J373 also induced G_2/M arrest in GIST430 cells. Yeh et al[38] showed that overexpression of Aurora kinase A has been associated with poor overall survival in GIST patients, and MLN8273, an Aurora kinase A inhibitor, was shown to induce apoptosis, G_2/M arrest and senescence in imatinib-resistant GIST cell lines. In our study, BPR1J373 induced cell cycle arrest and senescent change in GIST48 cells, which is consistent with the result reported by Yeh et al[38] with Aurora kinase A inhibitor MLN8273. In addition, the inhibitory effect on Aurora kinase A in GIST48 cells by BPR1J373 further supports the hypothesis that the antiproliferative effect of BPR1J373 in GIST48 cells occurs through targeting of KIT and Aurora kinase A. This result also suggests that the cell growth of GIST48 is dependent on Aurora kinase A activity. The antiproliferative effect of BPR1J373 in imatinib-sensitive GIST882 cells occurs mainly through induction of apoptosis because BPR1J373 induced more than 70% of annexin-V-positive GIST882 cells, either in low or high dose, as shown in Figure 2C,D. BPR1J373 also exerted an inhibitory effect on Aurora kinase A and B in GIST882 cells, as shown in Figure S6, which suggests that the induction of apoptosis in GIST882 might occur through targeting of KIT and possibly Aurora kinase A and B. We have previously shown that BPR1J373 was effective in suppressing phosphorylation of Aurora kinase B and inducing polyploidy of the acute myeloid leukemia cell line KG-1.[26] Although BPR1J373 has both anti-Aurora kinase A and B activity, its inhibition on Aurora kinase A or B might depend on the cellular contents of different cancer cell types.

In conclusion, BPR1J373, a multitargeted TKI, effectively inhibits the proliferation of *KIT*-mutant GIST cells by inducing cell apoptosis and cell cycle arrest/senescence by targeting KIT and Aurora kinase A. Compared with other TKIs, BPR1J373 exerts a more potent inhibitory effect on KIT. The antitumor effect of BPR1J373 was also evident in a GIST430 xenograft mouse model. Therefore, BPR1J373 is a potential anticancer drug for advanced GIST and deserves further investigation for clinical use.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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SUPPORTING INFORMATION

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