The Src-selective Kinase Inhibitor PP1 Also Inhibits Kit and Bcr-Abl Tyrosine Kinases*

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4-Amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]-pyrimidine (PP1) was identified as an Src-selective tyrosine kinase inhibitor and has been used extensively to investigate signaling pathways involving Src kinases, including events downstream of the stem cell factor (SCF) receptor c-Kit. While investigating the role of Src kinases in SCF signaling, we found that PP1 completely abrogated the proliferation of M07e cells in response to SCF. PP1 inhibited SCF-induced c-Kit autophosphorylation in intact cells and blocked the activation of mitogen-activated protein kinase and Akt. In vitro kinase assays using immunoprecipitated c-Kit confirmed direct inhibition by PP1. SCF-induced c-Kit phosphorylation was also inhibited by the related inhibitor 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]-pyrimidine (PP2) and by STI571 but not by the Src inhibitor SU6656. PP1 inhibited the activity of mutant constitutively active forms of c-Kit (D814V and D814Y) found in mast cell disorders, and triggered apoptosis in the rat basophilic leukemia cell line RBL-2H3 that expresses mutant c-Kit. In addition, PP1 (and PP2) inhibited the in vitro kinase activity and autophosphorylation in whole cells of p210 Bcr-Abl. PP1 reduced the constitutive activation of signal transducer and activators of transcription 5 and mitogen-activated protein kinase and triggered apoptosis in FDCP1 cells expressing Bcr-Abl. These results have implications for the use of PP1 in investigating intracellular signaling and suggest that PP1 or related compounds may be useful in the treatment of malignant diseases associated with dysregulated c-Kit or Abl tyrosine kinase activity.

The pyrazolo-pyrimidine compound PP1* was identified as a high potency inhibitor of Src tyrosine kinase family members that acts as a competitive inhibitor of ATP binding (1). PP1 has been used in a number of studies to evaluate the role of Src tyrosine kinases in cellular function (2–7). PP1 does not affect the activity of other non-receptor tyrosine kinases, such as Jak-2 and Zap-70 (1). Src kinases have been reported to be critically involved in signaling via the stem cell factor (SCF) receptor c-Kit; inhibition of Src kinases has been shown to abrogate SCF induced proliferation of hemopoietic (3) and small cell lung cancer cells (8) and to block internalization of c-Kit after ligand binding (4). These findings have relied largely on the use of PP1 as a Src-selective inhibitor. c-Kit is expressed on hemopoietic progenitors, mast cells, and germ cells (for review, see Refs. 9 and 10). Loss of either c-Kit or its ligand in mice results in impaired hemopoiesis (in particular anemia), abnormalities of mast cell development, and impaired melanogenesis and gametogenesis (11). c-Kit has been implicated in tumor cell development, including mast cell disorders, (12) acute myeloid leukemia, gastrointestinal stromal tumors (13), Ewing’s sarcoma, peripheral neuro-ectodermal tumors, malignant melanoma (11) and cancers of lung, ovary, and breast (14–16). In some instances, this is because of activating mutations in c-Kit (e.g. in mastoectyosis, acute myeloid leukemia, and gastrointestinal stromal tumors) (17); in others, the receptor is wild type but there is associated autocrine production of SCF (16).

The development of inhibitors of c-Kit-mediated signals would be useful to investigate the role of c-Kit in maintaining these tumors and could provide the basis for a potential therapeutic agent. In this study, we have examined the effects of PP1 on signaling via c-Kit. We find that PP1 and the related compound PP2 block SCF-induced proliferation and activation of the ERK mitogen-activated protein kinase and Akt. This is associated with an inhibition of c-Kit autophosphorylation both in intact cells and in in vitro kinase assays. PP1 decreases the activity of constitutively active c-Kit and triggers the apoptosis of mast cell leukemia cells expressing this mutant. PP1 also inhibits Bcr-Abl kinase activity and triggers apoptosis in Bcr-Abl-dependent cells. These results have implications for the use of PP1 and related compounds in the experimental investigation of cell signaling pathways and suggest that this family of molecules may be useful in the development of treatment for diseases with abnormal c-Kit signaling.

MATERIALS AND METHODS

Cell Culture—The RBL-2H3 cell line (18) (kindly supplied by S. Cockcroft, Dept. of Physiology, University College, London, UK) was maintained in Earle’s minimal essential medium supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS). M07e cells (originally described by Avanzi et al. (19)) were maintained in RPMI 1640 medium with 10% (v/v) FCS, supplemented with 20 ng/ml recombinant human GM-CSF and 20 ng/ml human IL-3 (Sandoz, Frimley Park, UK). FDCP1 cells expressing p210 Bcr-Abl were generated as described previously (20) and were maintained in Dulbecco’s modified Eagle’s medium with 10% FCS without additional growth factors. PP1 was from Affiniti (Exeter, UK), whereas PP2 and the inactive PP3 were from Calbiochem-Novabiochem Biosciences. Human SCF and thrombopoietin (Tpo) were...

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¶ The abbreviations used are: PP1, 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]-pyrimidine; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]-pyrimidine; PP3, 4-amino-7-phenylpyrazolo[3,4-d]-pyrimidine; SCF, stem cell factor; FCS, fetal calf serum; IL-3, interleukin-3; Tpo, thrombopoietin; MTS, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ERK, extracellular signal-regulated kinase; STAT, signal transducer and activator of transcription; PIPES, piperoxane-N,N′-bis(2-ethanesulfonic acid); GST, glutathione S-transferase; GM-CSF, granulocyte-macrophage colony-stimulating factor.
from PegroTech EC Ltd. (London, UK), and Escherichia coli-derived recombinant human GM-CSF was from Behringwerke-Hoechst (Marburg, Germany).

**Cell Proliferation Assay**—After overnight growth factor withdrawal in RPMI 1640 medium with 10% FCS, M07e cells were plated at 3 \times 10^5/ml in 12-well plates, and inhibitors were added. After 60 min of incubation, growth factors were added. Cells were then incubated at 37 °C for up to 72 h. Cell proliferation was measured using an MTS assay (CellTiter 96; Promega). The assay was performed according to manufacturer's instructions. Briefly, at specified time points, 100 μl of sample was added to 20 μl of MTS reagent in a 96-well plate and incubated at 37 °C for 2 h. Absorbance at 490 nm was measured using a 96-well plate reader. All measurements were performed in triplicate.

**Proliferation**—Briefly, cells were incubated at room temperature for 5 min in kinase buffer (20 mM HEPES, pH 7.4, 10 mM MgCl2, 10 μM CaCl2) with 40 μg/ml phosphatidic acid (Sigma-Aldrich) and 10 μg/ml Annexin-V-fluorescein isothiocyanate. Samples were then placed on ice and immediately analyzed for Annexin-V binding and propidium iodide uptake with flow cytometry (Epics Elite; Beckman Coulter).

**Plasmid Generation and 293T Cell Transfection**—Murine c-kit cDNA in pUC19 was cloned into the pCDNA3.1 vector and site-directed mutagenesis (Stratagene QuikChange kit) was used to generate D814V and D814Y mutants. Human embryonic kidney 293T cells were transfected by the calcium phosphate method using a Promega kit according to the manufacturer's instructions. Cells were transfected on 10-cm dishes with the respective constructs and replated into 6-well plates 24 h later. Incubation with PP1 at the indicated concentrations for 2 h was carried out 48 h after initial transfection, and cells were lysed as detailed below. Immunoprecipitation with goat anti-c-Kit antibody (Santa Cruz) was for 4 h; the last hour included protein G-Sepharose (Sigma).

**SDS-PAGE and Immunoblotting**—Cells were incubated overnight in the absence of growth factor before resuspension at 4 \times 10^5/ml in PBS with 5 mM glucose. The indicated concentrations of inhibitors were added, and cells were incubated for 60 min at 37 °C. M07e were then stimulated with 20 ng/ml human SCF for 10 min at 37 °C. Cells were pelleted by centrifugation and lysed in lysis buffer (50 mM HEPES, pH 7.5, 100 mM NaCl, 1% Triton X-100, 2 mM EDTA, 20 mM NaF, 1 mM NaVO4, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 10 μg/ml leupeptin, 1 mM Pefabloc, and 5 μM microcystin) for 10 min at 4 °C. Lysates were cleared of nuclear debris (centrifuged at 14,000 rpm for 5 min at 4 °C), added to sample buffer, and boiled for 5 min. Equal volumes of sample were resolved using SDS-PAGE and transferred to nitrocellulose membranes (Hybond-C extra; Amersham Biosciences). Activated proteins were detected using phosphospecific primary antibodies. The monoclonal anti-phosphotyrosine antibody 4G10 was from Upstate Biotechnology, and polyclonal antibodies raised against phosphorylated (active) ERK1+2 (T202/Y204), Akt (Y473), and STAT5 (Y694) were from New England Biolabs, as was the Akt antibody. Polyclonal ERK2, c-Kit, and monoclonal Abl antibodies were from Santa Cruz Biotechnology. Peroxidase conjugated secondary antibodies were from Amersham Biosciences. Phosphorylation was quantified using NIH Image software. c-Kit and Bcr-Abl Tyrosine Kinases

**RESULTS**

**The Src Selective Inhibitor PP1 Abolishes SCF-stimulated Proliferation**—SCF and GM-CSF, Tpo, and IL-3 stimulate proliferation of the human myeloid factor-dependent cell line M07e. It has previously been shown that of the Src family of tyrosine kinases, Lyn is present in M07e cells and that activation of this kinase can be detected after SCF stimulation (3, 21). To investigate the potential role of Src family kinases in the SCF-induced proliferation of M07e cells, we used PP1, a selective inhibitor of Src kinases.

M07e cells became quiescent without undergoing apoptosis after 24 h of factor withdrawal and remained so for 48–72 h in growth factor-free conditions (data not shown). Proliferation was measured using the colorimetric MTS assay over a 72-h period after factor re-addition. Fig. 1A shows that PP1 inhibited SCF-induced proliferation in a dose-dependent manner (IC50, 0.5–1 μM), whereas 2.5 μM PP1 completely prevented SCF-induced proliferation. PP2 had a similar effect (IC50 ~ 1 μM), whereas the inactive analogue PP3 had no significant effect on proliferation (Fig. 1B).

In contrast, PP1 had only a minor effect on thrombopoietin-induced proliferation of M07e cells (Fig. 1C), indicating that the effects of PP1 on SCF-stimulated proliferation were not caused by nonspecific toxicity.

**PP1 Inhibits SCF-induced Autophosphorylation of c-Kit in Intact Cells and c-Kit Tyrosine Kinase Activity in Vitro**—These effects of PP1 suggested a very significant role for Src family kinases in SCF-induced biological responses. However, these results could potentially come about from the direct inhibition of c-Kit by PP1 and not exclusively from inhibition of Src kinases. To investigate this possibility, M07e were incubated with or without PP1, stimulated with SCF, and cell extracts were assessed by immunoblotting with an anti-phosphotyrosine antibody. Fig. 2A shows that c-kit autophosphorylation was inhibited in a dose-dependent manner by PP1 and completely blocked at a concentration of 1 μM PP1.

These results suggested that PP1 could be inhibiting c-Kit kinase activity. To examine this further, unstimulated M07e were lysed and c-Kit immunoprecipitated, and in vitro autophosphorylation kinase assays were performed in the absence or presence of different PP1 concentrations. Fig. 2B shows that PP1 (and PP2) directly inhibit c-Kit kinase activity in vitro in a dose-dependent manner (PP1 IC50 ~ 75 nM).

**PP1 but Not the Unrelated Src Inhibitor SU6656 Inhibits c-Kit Autophosphorylation and Activation of Downstream Signaling Pathways**—To further exclude the possibility that the effects of PP1 on c-kit autophosphorylation were dependent on Src kinases, we used the unrelated Src inhibitor SU6656 (22, 23). This inhibits Src family kinases with IC50 values in the nanomolar range, with an IC50 for lyn kinase of 130 nM. Inhibition by SU6656 of lyn kinase activity was confirmed in an in vitro kinase assay using immunoprecipitated lyn from M07e cells with complete inhibition of autophosphorylation at 1 μM inhibitor (data not shown). SU6656 failed to inhibit c-kit in an in vitro kinase assay, whereas imatinib mesylate/STI571 (24, 25) was able to reduce activity (Fig. 2C). Fig. 3A shows that SU6656 is unable to inhibit c-kit autophosphorylation in whole cells even at concentrations of up to 10 μM. Preincubation with PP1 and STI571, but not SU6656, was also able to inhibit the activation of Akt and ERK1/2 in response to SCF (Fig. 3B). Inhibition of Akt and extracellular signal-regulated kinase activation was nearly complete at concentrations between 1 and 2.5 μM PP1. In contrast, PP1 at 20 μM did not affect the tyrosine phosphorylation of GST-Crk and c-kit as substrate. Phosphorylation of GST-Crk and c-kit was detected using autoradiography and quantified using NIH Image software.
phosphorylation of STAT5 in response to GM-CSF (Fig. 3C), in keeping with its reported lack of activity against Jak family kinases.

**PP1 Inhibits the Kinase Activity of Constitutively Active Mutants kit D814V and D814Y**—Several kinase inhibitors, including STI571 and SU9529, have been reported to potently inhibit the activity of wild-type c-Kit but to have low or no activity against enzymatic site point mutants (D814V and D814Y in murine or D816V and D816Y in human numbering) (26, 27). We investigated the effects of PP1 on wild-type and activated kit mutants by transfection of 293 cells. Incubation of cells expressing wild-type kit with PP1 confirmed the inhibition of SCF-induced kinase activity (Fig. 4A) with complete block at 0.2 μM PP1. PP1 potently inhibited the basal kit autophosphorylation in whole-cell assays of the D814V mutant (IC₅₀, 0.1 μM) and the D814Y mutant (IC₅₀, 0.2 μM) and markedly reduced the phosphorylation of multiple intracellular substrates at these concentrations (Fig. 4B). These results indicate that PP1 has significant activity against both wild-type c-Kit and enzymatic site point mutants found in mast cell disorders.

**PP1 Induces Apoptosis in RBL-2H3 Basophilic Leukemia Cells That Express a Constitutively Active Mutant c-Kit—**RBL-2H3 cells are a factor independent, rat basophilic leukemia cell line with a point mutation in c-Kit (D817Y), which renders it constitutively active (28). Cells in complete medium were incubated with increasing concentrations of PP1 and apoptosis was measured by annexin-V-fluorescein isothiocyanate binding using flow cytometry (Fig. 4C). Incubation with PP1 triggered apoptosis in these cells. PP2 had similar effects, whereas the inactive analogue PP3 had no effect on cell viability (data not shown).

**PP1 Also Inhibits Bcr-Abl Tyrosine Kinase Activity and Triggers Apoptosis in Bcr-Abl-transformed Cells**—The kinase inhibitor imatinib mesylate/STI571, currently being employed in the
treatment of chronic myelogenous leukemia, blocks the activity of Abl, Bcr-Abl, and c-Kit tyrosine kinases (25). Having detected the unexpected effects of PP1 on c-Kit, we investigated whether it could also inhibit Bcr-Abl kinase activity. FDCP1 cells were transfected with p210 Bcr-Abl and clonal stable cell lines isolated by standard techniques. Parental FDCP1 cells are normally dependent on IL-3, but the p210 Bcr-Abl/FDCP1 cells are factor-independent for survival and proliferation and can be cultured without IL-3 (20). Fig. 5 shows that incubation of p210 Bcr-Abl/FDCP1 with PP1 led to a dose-dependent reduction in Bcr-Abl autophosphorylation (Fig. 5A) and inhibited the activation of STAT5 and ERK1&2 (Fig. 5B). PP1 and PP2 directly inhibited Bcr-Abl kinase activity in an in vitro assay (Fig. 5C) with an IC_{50} for PP1 of 1 μM. A decrease in the number of viable cells as measured by an MTS assay (IC_{50}, 3 μM) (Fig. 5D) was also observed upon incubation of p210 Bcr-FDCP1 cells with PP1. Assessment of cell survival by flow cytometric assay of annexin-V binding showed that this was caused by rapid apoptosis (Fig. 5E) In contrast, survival of parental cells incubated with PP1 (in the presence of IL-3) was unaffected (data not shown).

**DISCUSSION**

PP1 was originally described as a selective, ATP-competitive inhibitor of Src family kinases (1) and has been widely used to...
investigate the contribution of Src kinases to a number of biological functions. In vitro kinase assays carried out by Hanke et al. (1) showed that Src family kinases were inhibited at low nanomolar concentrations and that Zap-70 and Jak-2 were not inhibited at concentrations up to 50 μM. Biological effects on intact T-cell function were seen with an IC50 around 0.5 μM — this higher concentration requirement may be caused by high intracellular levels of ATP compared with the low concentrations used in in vitro kinase assays. Since then, Waltenerberger and colleagues (29) have shown that PP1 can also directly inhibit the platelet-derived growth factor-receptor tyrosine kinase with an IC50 of 0.1 μM and an IC50 in intact cells of 0.6 μM; more recently, Carlomagno et al. have shown inhibition of Ret tyrosine kinase by PP1 (30). In this study, we investigated the effect of PP1 on signaling via the SCF receptor c-Kit. We found that PP1 abrogated the proliferative effects of SCF on M07e cells and that this was associated with blockade of downstream signaling pathways, including mitogen-activated protein kinase and Akt, and of receptor autophosphorylation. c-Kit autophosphorylation in intact cells was blocked with an IC50 of ~100 nM. In vitro kinase assays done with immunoprecipitated c-Kit and incubated with PP1 gave an IC50 of ~75 nM. This indicates that PP1 is a direct inhibitor of c-Kit. Bondzi and colleagues (31) have also shown that PP1, at a single concentration of 10 μM, could inhibit the in vitro kinase activity of c-Kit. We found that the closely related inhibitor PP2 is also an inhibitor of c-Kit. Src kinases have been postulated to play a significant role in signaling downstream from c-Kit; these conclusions have partly relied on the effects of PP1 and PP2 (3, 4, 8, 32) Our data suggest that, as with most inhibitor studies, corroborating evidence using other experimental techniques should be obtained where possible.

c-Kit plays a significant role in the pathophysiology of a number of human malignancies. Mutations have been identified in mast cell disorders, gastrointestinal stromal tumors, and acute myeloid leukemia, among others (13, 17, 33). These mutations take a number of forms; the majority have been shown to confer constitutive activation. The best characterized mutation is in the kinase domain at aspartate 816 (human numbering) (34, 35); in a recent series, this mutation was identified in all cases of adult sporadic cutaneous mastocytosis and in a significant proportion of pediatric cases (36). In addition, many tumor types, including ovarian, lung, and breast malignancies, express both c-Kit and SCF, indicating the pos-
sibility of autocrine growth stimulation (16). Therefore, there has been interest in developing kinase inhibitors that target c-Kit to increase the treatment options for patients with c-Kit abnormalities.

Investigation of a number of indolone compounds showed that several could inhibit wild type c-Kit but were generally inefficient at inhibiting the kinase activity of c-Kit with a mutation in the kinase domain (814 murine, 816 human, 817 rat); only SU6577 was effective at the high concentration of 40 μM (26). The Abl-selective tyrosine kinase inhibitor STI571 can inhibit wild-type c-Kit with an IC₅₀ around 0.1 μM (24) but has been reported not to inhibit the activity of 814/816 mutant c-Kit at concentrations up to 10 μM (27). We found that PP1 could potently inhibit the activation of D816V and D816Y mutant forms of c-Kit. PP1 triggered apoptosis in rat basophilic leukemia cells with the homologous D817Y mutation. These results indicate that further investigation of the effects of PP1 and related compounds on primary mastocytosis cells expressing the human counterpart of this mutation are required. The inhibitory effects of PP1 on additional kinases, such as Src, Abl, and the platelet-derived growth factor receptor have the potential to increase the toxic effects of PP1 on normal cells. Our data show a relative lack of effect of PP1 on thrombopoietin-induced proliferation, but this area requires further study with other growth factors and cell types. Using PP1 as a starting point, it may be possible to refine the development of an inhibitor that is more selective for c-Kit and its mutants.

We have also identified PP1 (and PP2) as direct inhibitors of the Bcr-Abl kinase. Inhibition of Bcr-Abl autophosphorylation was seen in intact cells with an IC₅₀ of ~1 μM, and apoptosis was induced in Bcr-Abl-transformed cells with an IC₅₀ of ~2 μM. This compares with biological effects of STI571 on Bcr-Abl-transformed cell lines that are seen at concentrations between 0.1 and 0.5 μM. STI571 is being investigated widely for its effects on Bcr-Abl in chronic myelogenous leukemia and acute lymphoblastic leukemia, its effects on c-Kit in a number of disorders, including mast cell neoplasms, and its effects on the platelet-derived growth factor receptor in malignant glioblastomas (37). It is interesting that PP1 is also able to inhibit each of these kinases. Resistance to STI571 may result from a number of factors, such as overexpression of Bcr-Abl (38, 39) tumor-specific selection of point mutations that are resistant to STI571 (40, 41), and binding to plasma proteins such as α1 acidic glycoprotein (42) (tumor nonspecific). It will be of interest to determine whether there is cross-resistance to PP1 in these different cases.

The data presented in this article extend the range of tyrosine kinases that are inhibited by PP1 from Src-family members (1), platelet-derived growth factor receptor-β (29), and Ret (30) to include c-Kit (and its mutants) and Bcr-Abl. Hanke et al. (29) showed no inhibition of Jak-2 and Zap-70 by PP1, and Waltenberger and colleagues (1, 29) found no effect of PP1 on the kinase activity of KDR/VEGFR-2, FGFR-1, and IGF-1R. This is conflicting evidence of the effects of PP1 on EGFR activity (1, 29). This indicates that although PP1 shows some selectivity, it can inhibit a significant number of tyrosine kinases. In our study we found that PP2 also inhibits c-Kit and Bcr-Abl; it is likely that this compound has an inhibitory profile similar to that of PP1.

Recent articles have shed some light on the structural basis of PP1 inhibition of Src kinases. A single residue, corresponding to threonine 338 in c-Src, largely controls the ability of PP1 to inhibit protein kinases with high potency (43). Mutation of this residue to, or the natural occurrence of, a larger amino acid (e.g. methionine or phenylalanine) is sufficient to confer resistance to PP1. The corresponding residue in the PP1-sensitive kinases platelet-derived growth factor receptor, c-Kit, and c-Abl is a threonine but in both Jak2 and Zap-70 is a methionine, which have both been shown to be resistant to PP1.

In conclusion, we have shown that, at concentrations routinely used by investigators to study the role of Src kinases in cell function, the Src-selective inhibitors PP1 and PP2 are also potent inhibitors of the c-Kit and the Bcr-Abl tyrosine kinases. In addition, PP1 is able to inhibit the activity of mutant forms of kit commonly found in mast cell disorders. These results have implications for the use of PP1 and PP2 for the investigation of intracellular signaling pathways and biological function and also indicate a potential role for PP1 or related compounds in the treatment of human disease.

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