# The associated pyrazolopyrimidines PP1 and PP2 inhibit protein tyrosine kinase 6 activity and suppress breast cancer cell proliferation

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**Abstract.** Protein tyrosine kinase (PTK)6, also known as breast tumor kinase, is a non-receptor tyrosine kinase. It is closely associated with, but evolutionarily distinct from, the Src family members. PTK6 has a role in proliferation, migration and invasion in various cancers, and therefore has been suggested as a potentially valuable therapeutic target. In an attempt to develop PTK6 inhibitors, chemicals known to inhibit various kinases were screened for their ability to inhibit PTK6. Pyrazolopyrimidine (PP)1, PP2 and a lymphocyte-specific protein tyrosine kinase inhibitor strongly inhibited the catalytic activity of PTK6 *in vitro*. These chemicals suppressed the phosphorylation of PTK6 substrate proteins, including signal transducer and activator of transcription 3, in human embryonic kidney (HEK) 293 cells expressing hyperactive PTK6. They also expressed selectivity towards PTK6 over other PTK members in HEK 293 cells. PP1 and PP2 specifically inhibited the PTK6-dependent proliferation of human breast carcinoma T-47D cells. PP1 and PP2 were more selective for PTK6 than for Src family kinases, and may be useful for the treatment of PTK6-positive malignant diseases such as breast cancer.

## Introduction

Protein tyrosine kinase (PTK)6, also known as breast tumor kinase, is a non-receptor type kinase that consists of an Src homology (SH)3 domain, an SH2 domain and a catalytic domain of tyrosine kinase (1,2). PTK6 is overexpressed in >60% of breast carcinoma tissue samples and in the majority of breast cancer cell lines (3). PTK6 expression is also increased in colon, head and neck, ovary, prostate, lung, bladder, bile duct, pancreas and gastric cancers, and in T- and B-cell lymphomas (4,5).

Expression of PTK6 enhances the proliferation of mammary epithelial and breast cancer cells (6). PTK6 also promotes cell migration and invasion (7). Sublocalization of PTK6 at the plasma membrane is important for its oncogenic potential (8). Activated PTK6 consistently accumulates at the plasma membrane in breast cancer cell lines and tissues (9). Although PTK6 was detected in the nucleus and cytoplasm of normal mammary gland epithelial cells, Tyr342 in the PTK6 activation loop was not phosphorylated, and thus, PTK6 was not active (9).

PTK6 promotes tumorigenicity by enhancing signaling pathways of receptor tyrosine kinases and is particularly well known for sensitizing epidermal growth factor receptor (EGFR) family members (10). Various downstream substrates and interacting proteins, including signal transducing adaptor protein-2, paxillin, Akt, p130 Crk-associated substrate, p190Rho GTPase-activating protein-A and ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 1, contribute to the oncogenic roles of PTK6 (11,12). Similar to other PTKs, mutations of PTK6 identified in different cancer types increase its kinase activity (13).

In view of its oncogenic activity and its presence in various carcinomas such as breast cancer, PTK6 is a potentially valuable therapeutic target for decelerating or arresting tumor growth (14). (E)-5-(Benzylideneamino)-1H-benzo[d] imidazol-2(3H)-one derivatives were previously developed as novel PTK6 inhibitors that exhibited little cytotoxicity, excellent inhibition *in vitro* and at the cellular level, and selectivity for PTK6 (15). Imidazo[1,2-a]pyrazin-8-amines and 4-anilino α-carbolines were also identified as PTK6-selective inhibitors that block its catalytic activity (16,17). In the present study, pyrazolopyrimidine (PP)1 [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] and a lymphocyte-specific protein tyrosine kinase (Lck) inhibitor [4-amino-5-(4-phenoxyphenyl)-7H-pyrrolo [3,2-d]pyrimidin-7-yl-cyclopentane] were screened as potent PTK6 inhibitors among the evaluated kinase inhibitors. The selectivity of these compounds for PTK6 and for other PTK family members was analyzed in HEK 293 cells, and

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it was then examined whether these compounds inhibited PTK6-dependent signaling processes and the proliferation of breast carcinoma T-47D cells.

## Materials and methods

*Chemicals*. PP1, PP2 and the aforementioned Lck inhibitor were purchased from Calbiochem (EMD Millipore, Billerica, MA, USA). Genistein was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

*Cell culture*. Human embryonic kidney (HEK) 293 cells and human breast cancer T-47D cells (both American Type Culture Collection, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; HyClone; GE Healthcare Life Sciences, Logan, UT, USA) at 37˚C in a $CO_2$ incubator with a humidified atmosphere of 5% $CO_2$ and 95% air.

*ELISA-based in vitro kinase assay for PTK6*. ELISA plates (96-well; Greiner Bio-One GmbH, Frickenhausen, Germany) were incubated with 100 $\mu$l of 0.1 mg/ml Poly (Glu, Tyr) (Glu:Tyr, 4:1; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) in PBS for 16 h at 37˚C, and then washed three times with PBS. The Poly (Glu, Tyr)-coated wells were blocked with 1% bovine serum albumin in PBS for 1 h at 37˚C, washed three times with PBS, and then incubated for 30 min at room temperature with 10 nM glutathione S-transferase-fused PTK6 catalytic domain (18) in 20 $\mu$l of kinase reaction buffer [20 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 1 mM $MnCl_2$ and 50 $\mu$M $Na_3VO_4$) in the presence of the chemical of interest (Table I) containing a final concentration of 1% dimethyl sulfoxide. The phosphorylation of tyrosine residues was initiated by addition of 300 $\mu$M adenosine triphosphate (ATP) to the reaction mixtures. The wells were washed three times with PBS after incubation for 20 min at room temperature. For the quantification of phosphorylated tyrosines, the wells were incubated with anti-phospho-tyrosine (4G10; 1:1,000; EMD Millipore) and horseradish peroxidase-conjugated anti-mouse immunoglobulin G (K0211589; 1:10,000; Koma Biotech, Seoul, Korea) antibodies for 1 h each at room temperature. The optical density was measured with a 3,3',5,5'-tetramethylbenzidine solution (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

*Western blot analysis*. HEK 293 cells expressing hyperactive PTK6 (Flag-PTK6-3PA/Y447F) (19), Src, Fyn, Lck, bone marrow tyrosine kinase gene on chromosome X (Bmx) or EGFR were treated with the indicated concentrations of compounds for 2 days. Western blot analysis and immunoprecipitation were performed as previously described (15). Immunoreactive proteins were visualized using anti-phospho-tyrosine (4G10; 1:1,000), anti-PTK6 (sc-1188; 1:2,000; Santa Cruz Biotechnology, Inc.), anti-phospho-signal transducer and activator of transcription (STAT)3 (sc-8059; 1:2,000; Santa Cruz Biotechnology, Inc.), anti-STAT3 (sc-7179; 1:2,000; Santa Cruz Biotechnology, Inc.) and anti-GAPDH (AbC-2003, 1:2,000; AbClon, Inc., Seoul, Korea) primary antibodies overnight at 4˚C, followed by incubation with a horseradish peroxidase-conjugated secondary antibody (K0211589 or K0211708; 1:10,000; Koma Biotech, Seoul, Republic of Korea) for 1 h at room temperature and an enhanced chemiluminescence detection kit (EMD Millipore). For the quantification of the phosphorylation levels in the cell lysates, chemiluminescence was detected using a LAS-3000 imaging system (Fujifilm, Tokyo, Japan) and analyzed using Multi Gauge version 2.2 software (Fujifilm). The half maximal inhibitory concentration ($IC_{50}$) at the cellular level was determined by quantifying the phosphorylation levels in the HEK 293 cell system. The reference level was the phosphorylation level of the chemical-free control, which was set at 100%.

*MTT assay*. Subconfluent empty vector-transfected and PTK6-knockdown T-47D cells were incubated for 4 days in DMEM-10% FBS containing various concentrations of the chemicals. Viable cells were measured using MTT assay, as previously described (15). The viability of chemical-free, vector-transfected T-47D cells was set at 100%.

*Statistical analysis*. All data were expressed as the mean ± standard deviation of three independent experiments. Statistical analysis was performed using Microsoft Excel (version, 2007; Microsoft Corporation, Redmond, WA, USA). The significant differences between the groups were assessed using a Student's t-test. $P>0.05$ was considered to indicate a statistically significant difference.

## Results

*PP1, PP2 and Lck inhibitor inhibit the catalytic activity of PTK6 in vitro*. Protein kinase inhibitors were analyzed for the inhibition of the PTK6 catalytic activity using an ELISA-based *in vitro* kinase assay system for PTK6. Among the tested kinase inhibitors, PP1, PP2 and the Lck inhibitor exhibited strong inhibition of PTK6 (Table I). The $IC_{50}$ values for PP1, PP2 and the Lck inhibitor were 230.0, 50.0 and 60.0 nM, respectively (Table II).

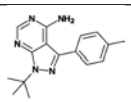
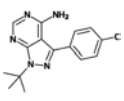
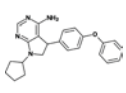
*PP1, PP2 and Lck inhibitor are highly selective for PTK6 at the cellular level*. PP1, PP2 and the aforementioned Lck inhibitor were developed as Src family kinase (SFK) inhibitors (20,21). The present study analyzed the selectivity of each inhibitor for the inhibition of several PTKs, including PTK6, SFK members (Src, Fyn and Lck), a non-receptor type PTK (Bmx) and a receptor type PTK (EGFR). HEK 293 cells expressing hyperactive PTK6, Src, Fyn, Lck, Bmx or EGFR were incubated in the presence of various concentrations of these inhibitors. Inhibition of each PTK activity at the cellular level was assessed for each inhibitor by measuring the decrease in tyrosine phosphorylation levels of cellular proteins via western blot analysis using an anti-phospho-tyrosine antibody. As expected, PP1, PP2 and the Lck inhibitor exhibited strong inhibition of SFK members. The $IC_{50}$ value of PP1 to Lck was 1.76 $\mu$M; the $IC_{50}$ value of PP2 to Lck was 4.36 $\mu$M; and the $IC_{50}$ values of the Lck inhibitor to Lck, Fyn and Src were 0.37, 1.22 and 3.46 $\mu$M, respectively (Table III). Unexpectedly, PP1, PP2 and the Lck inhibitor inhibited PTK6 to a greater degree than SFK members. The $IC_{50}$ values of PP1, PP2 and the Lck inhibitor to PTK6 were 2.5, 13.0 and 53.0 nM, respectively (Table III and Fig. 1, top panel).

Table I. PTK6 inhibitory activities of protein kinase inhibitors screened from a targeted kinase inhibitor.

| Chemical name | PubChem compound identifier | Molecular weight (Da) | Known target | *In vitro* $IC_{50}$ (nM)[a] |
|---|---|---|---|---|
| Genistein | 5280961 | 270.2 | PTKs | >10,000 |
| PP1 | 1400 | 281.4 | Src, Fyn | <300 |
| PP2 | 4878 | 301.8 | Lck, Fyn, Hck | <300 |
| Lck inhibitor | 6603792 | 370.5 | Lck, Src | <300 |
| LFM-A13 | 54676905 | 360.0 | Btk | >10,000 |
| JAK inhibitor I | 5494425 | 309.3 | JAK1- 3 | >10,000 |
| Syk inhibitor | 6419747 | 353.4 | Syk | >3,000 |
| Emodin | 3220 | 270.2 | Lck, ErbB2 | >10,000 |
| Erbstatin analog | 5353609 | 194.2 | EGFR, Abl | >300 |
| Lavendustin C | 3896 | 275.3 | EGFR, Src, CaMKII | >300 |
| Daphnetin | 5280569 | 178.1 | EGFR, PKA, PKC | >3,000 |
| PD 157432 | 10085225 | 283.3 | EGFR, ErbB2 | >10,000 |
| Flt-3 inhibitor | 1048845 | 360.4 | Flt-3 | >10,000 |
| cFMS inhibitor | 11617559 | 366.4 | FMS | >10,000 |
| GTP-14564 | 3385203 | 234.3 | FMS, Kit, Flt-3, PDGFRB | >10,000 |
| PDGFR inhibitor I | 5330535 | 276.3 | PDGFRB | >10,000 |
| Oxindole I | 5908088 | 210.2 | VEGFR1, PDGFRB | >10,000 |
| VEGFR2 inhibitor II | 23301538 | 343.2 | VEGFR2, PDGFRB | >3,000 |
| TrkA inhibitor | 5413390 | 315.4 | TrkA | >10,000 |
| Picropodophyllin | 72435 | 414.4 | IGF1R | >10,000 |
| JNK inhibitor II | 8515 | 220.2 | JNK1-3 | >10,000 |

[a]The $IC_{50}$ value was the concentration of each inhibitor required for 50% inhibition of the catalytic activity of purified PTK6 in the absence of inhibitors (dimethyl sulfoxide). Data were obtained from three independent experiments, each performed in duplicate. $IC_{50}$, half maximal inhibitory concentration; PTK, protein tyrosine kinase; PP, pyrazolopyrimidine; Lck, lymphocyte-specific protein tyrosine kinase; LFM-A13, leflunomide metabolite-analog A13; STAT, signal transducer and activator of transcription; JAK, Janus-associated kinase; Syk, spleen tyrosine kinase; PD, Parke-Davis; Flt, Fms-like tyrosine kinase; cFMS, cellular feline McDonough sarcoma; GTP, guanosine triphosphate; PDGFRB, platelet-derived growth factor receptor beta; VEGFR, vascular endothelial growth factor receptor; TrkA, tropomyosin receptor kinase A; JNK, c-Jun N-terminal kinase; Hck, hematopoietic cell kinase; Btk, Bruton's tyrosine kinase; EGFR, epidermal growth factor receptor; Abl, Abelson murine leukemia viral oncogene homolog 1; CaMKII, $Ca^{2+}$/calmodulin-dependent protein kinase II; PK, protein kinase; IGF1R, insulin-like growth factor 1 receptor.

Table II. Inhibition of the catalytic activity of purified PTK6 by selected kinase inhibitors *in vitro*.

| Chemical name | Structure | Molecular weight (Da) | *In vitro* $IC_{50}$ (nM)[a] |
|---|---|---|---|
| PP1 |  | 301.8 | 230±20 |
| PP2 |  | 317.4 | 50±10 |
| Lck inhibitor |  | 370.5 | 60±20 |

[a]The $IC_{50}$ value was the concentration of each inhibitor required for 50% inhibition of the catalytic activity of purified PTK6 in the absence of inhibitors (dimethyl sulfoxide). Data are expressed as the mean ± standard deviation values of three independent experiments, each performed in duplicate. $IC_{50}$, half maximal inhibitory concentration; PTK, protein tyrosine kinase; PP, pyrazolopyrimidine; Lck, lymphocyte-specific protein tyrosine kinase.

Table III. Selectivity of genistein, PP1, PP2 and a Lck inhibitor for various PTKs in the HEK 293 cell system.

| | $IC_{50}$ at the cellular level (nM)[a] | | | |
|---|---|---|---|---|
| PTKs | Genistein | PP1 | PP2 | Lck inhibitor |
| PTK6 | 43,180±1,340 | 2.5±0.3 | 13±2 | 53±7 |
| Src | >100,000 | 70,300±7,320 | 53,060±8,230 | 3,460±850 |
| Fyn | 70,640±8,520 | 13,630±3,160 | 35,980±1,030 | 1,220±50 |
| Lck | >100,000 | 1,760±250 | 4,360±730 | 370±40 |
| Bmx | 69,550±7,460 | 2,390±430 | 16,460±2,780 | 15,980±3,680 |
| EGFR | 24,830±1,590 | 21,840±1,140 | 56,800±3,920 | >10,000 |

[a]The $IC_{50}$ values were determined by quantifying the phosphorylation levels in HEK 293 cells expressing hyperactive PTK6, Src, Fyn, Lck, Bmx or EGFR. Data are expressed as the mean ± standard deviation values of three independent experiments. HEK, human embryonic kidney; $IC_{50}$, half maximal inhibitory concentration; PTK, protein tyrosine kinase; PP, pyrazolopyrimidine; Lck, lymphocyte-specific protein tyrosine kinase; EGFR, epidermal growth factor receptor; Bmx, bone marrow tyrosine kinase gene on chromosome X.

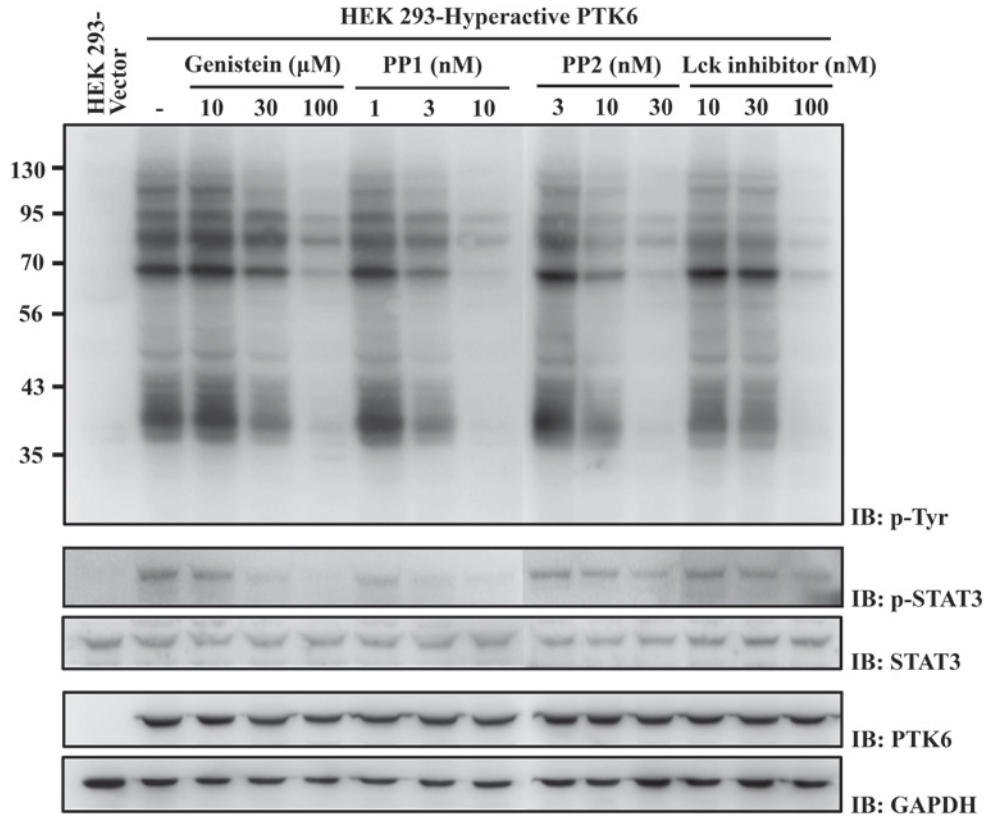



Figure 1. Effects of selected chemicals on the phosphorylation of cellular proteins and a specific PTK6 substrate, STAT3, in HEK 293 cells. HEK 293 cells stably expressing hyperactive PTK6 were incubated with the indicated concentrations of chemicals for 48 h. Cell lysates were analyzed by western blotting using anti-phospho-tyrosine (4G10), anti-phospho-STAT3, anti-STAT3, anti-PTK6 and anti-GAPDH antibodies. The numbers on the left of the top panel indicate apparent molecular weights in kDa. HEK, human embryonic kidney; PP, pyrazolopyrimidine; Lck, lymphocyte-specific protein tyrosine kinase; PTK, protein tyrosine kinase; STAT, signal transducer and activator of transcription; p-, phosphorylated.

This result suggested that PP1, PP2 and the Lck inhibitor were highly selective for PTK6 compared with other PTKs, including the SFK family members, at the cellular level.

*PP1, PP2 and Lck inhibitor inhibit the phosphorylation of PTK6 substrate proteins in HEK 293 cells*. To analyze whether PP1, PP2 and the Lck inhibitor block the PTK6-mediated signaling pathway, HEK 293 cells expressing hyperactive PTK6 were treated with these chemicals at concentrations i) lower than, ii) approximately the same as, and iii) higher than their $IC_{50}$ values. Phosphorylation of STAT3, which is a known specific substrate of PTK6 (22), was inhibited at

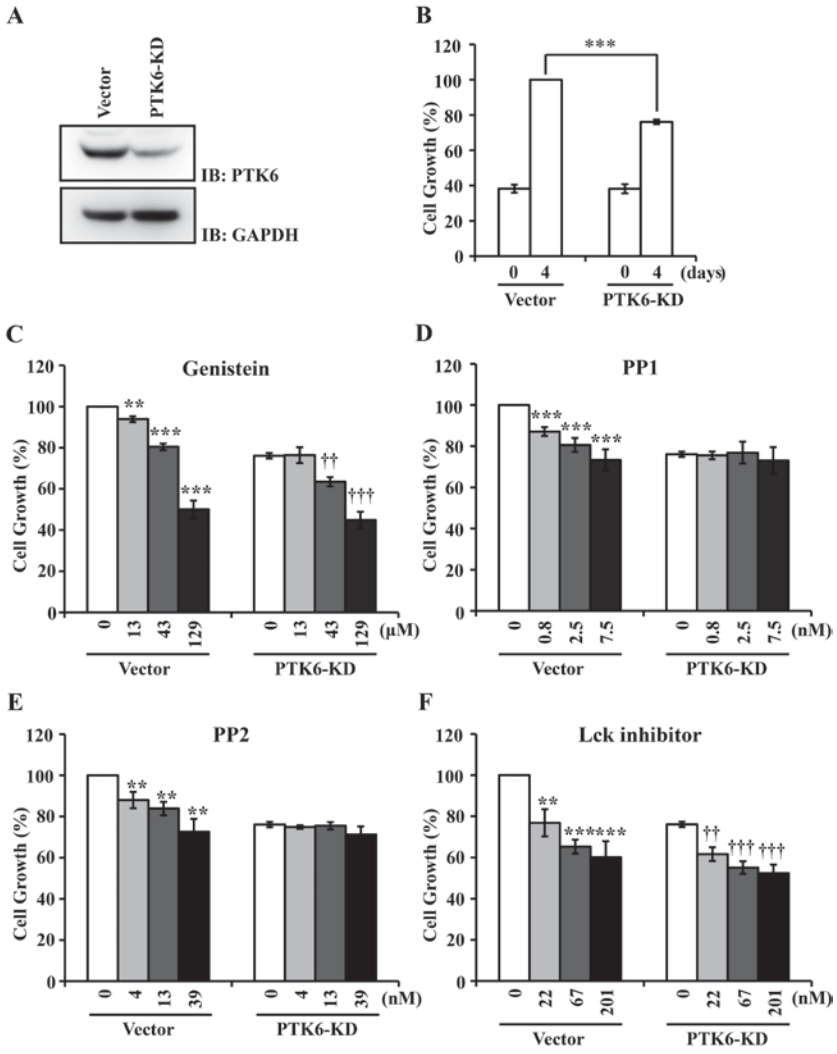



Figure 2. Effects of PTK6 inhibitors on the proliferation of breast cancer T-47D cells. Empty vector-transfected (Vector) and PTK6-knockdown (PTK6-KD) T-47D cells were generated by stable transfection of pLKO.1 and pLKO.1-small hairpin RNA PTK6, respectively (12). (A) Analysis of PTK6 expression levels by western blotting using an anti-PTK6 antibody. (B) Proliferation of Vector and PTK6-KD T-47D cells. (C) Effect of genistein on Vector and PTK6-KD T-47D cells. (D) Effect of PP1 on Vector and PTK6-KD T-47D cells. (E) Effect of PP2 on Vector and PTK6-KD T-47D cells. (F) Effect of a Lck inhibitor on Vector and PTK6-KD T-47D cells. Vector and PTK6-KD cells were incubated in complete medium containing the indicated concentrations of chemicals for 4 days and then subjected to MTT assay. All data are expressed as the mean ± standard deviation values of three independent experiments, each performed in duplicate. $^{**}$P<0.01, $^{***}$P<0.001 vs. Vector T-47D cells without inhibitors. $^{††}$P<0.01, $^{†††}$P<0.001 vs. PTK6-KD T-47D cells without inhibitors. PTK, protein tyrosine kinase; KD, knockdown; PP, pyrazolopyrimidine; Lck, lymphocyte-specific protein tyrosine kinase.

concentrations equal or greater than the $IC_{50}$ value of each chemical (Fig. 1, mid panel).

*PP1 and PP2 inhibit the PTK6-mediated proliferation of T-47D cells*. PTK6 is often expressed in breast cancer cell lines (3). Knockdown of PTK6 decreases the proliferation of breast cancer cells (14). Consistent with this observation, the silencing of PTK6 in breast carcinoma T-47D cells using a small hairpin RNA vector suppressed ~24% of cell proliferation, compared with vector transfection (Fig. 2A and B). PP1, PP2, the Lck inhibitor and genistein (used as a control) were applied to T-47D cells at concentrations of 0.33-, 1- and 3-fold their $IC_{50}$ values. PP1 and PP2 inhibited the proliferation of vector-transfected T-47D cells in a dose-dependent manner, but did not affect the proliferation of PTK6-knockdown T-47D cells at values of ≤3-fold their $IC_{50}$ values (Fig. 2D and E). However, the Lck inhibitor and genistein suppressed the proliferation of both vector-transfected and PTK6-knockdown T-47D cells (Fig. 2C and F). These results suggest that the Lck inhibitor is not specific for PTK6, which is similar to the general PTK inhibitor genistein (23).

## Discussion

In our study, PP1, PP2 and a Lck inhibitor were screened as potential inhibitors for PTK6 using an *in vitro* kinase assay. These chemicals were initially developed as ATP-competitive inhibitors of SFKs (21,22). PP1 inhibits Lck, Fyn and Src with $IC_{50}$ values of 5, 6 and 170 nM, respectively, as assessed with an *in vitro* kinase assay (20). PP2 inhibits Lck and Fyn with $IC_{50}$ values of 5 and 6 nM, respectively (20). The Lck inhibitor was developed as a derivative of PP1 and PP2, and inhibits Lck and Src with $IC_{50}$ values of <1 and 70 nM, respectively (21). These chemicals were widely used to investigate

the physiological roles for SFKs, but were not used in human clinical trials. PTK6 is evolutionarily distinct from, but still closely associated with, SFKs (2). Thus, it is expected that PP1, PP2 and the Lck inhibitor inhibit PTK6.

Although PP1 and PP2 are more selective for SFKs than the previous generation of PTK inhibitors (including herbimycin A and genistein), they can inhibit off-target kinases (including C-terminal Src kinase, ephrin type-A receptor 2, platelet-derived growth factor receptor, fibroblast growth factor receptor 1, p21 activated kinase, receptor-interacting protein 2, p38 and casein kinase 1δ) with sufficient potency (24-27). When the selectivity of these chemicals for various PTKs was analyzed in HEK 293 cells expressing one of the PTKs, they displayed high selectivity for PTK6 over various SFK members, including Src, Fyn, Lck, and other PTK family members such as Bmx and EGFR. In particular, PP1 and PP2 inhibited PTK6 activity at $IC_{50}$ values of 2.5 and 13.0 nM, respectively. Although they also inhibited the catalytic activities of other PTKs, including SFKs, their $IC_{50}$ values were mostly at micro-molar concentrations. Incubation of T-47D cells with 0.8-7.5 nM PP1 or 4.0-39.0 nM PP2 reduced the PTK6-dependent proliferation of T-47D cells without a decrease in the proliferation of PTK6-knockdown cells. The Lck inhibitor also exhibited inhibitory selectivity for PTK6, but reduced the proliferation of T-47D cells in a PTK6-independent manner. These results demonstrate that PP1 and PP2 reduce the PTK6-mediated signaling pathways and cell proliferation in PTK6-positive cells. Thus, it can be suggested that PP1 and PP2 could be applied as therapeutic agents in PTK6-positive malignant diseases.

Resistance to chemotherapy and molecularly targeted therapies is a major problem confronting current cancer research. The results of a recent study indicated that PTK6 confers resistance of breast cancer SUM102 cells to cetuximab, an EGFR-blocking antibody that is approved for the treatment of several types of human solid tumors (28). Knockdown of PTK6 sensitized the cells to cetuximab by inducing apoptosis. Assuming that PTK6 catalytic activity is essential for drug resistance (28), PTK6 inhibitors such as PP1 and PP2 may be useful for the treatment of chemotherapy-resistant cancer cells.

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