Targeting PIM2 by JP11646 results in significant antitumor effects in solid tumors

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Abstract. Proviral integration of Moloney virus 2 (PIM2) is a pro-survival factor of cancer cells and a possible therapeutic target in hematological malignancies. However, the attempts at inhibiting PIM2 have yielded underwhelming results in early clinical trials on hematological malignancies. Recently, a novel pan-PIM inhibitor, JP11646, was developed. The present study examined the utility of targeting PIM2 in multiple solid cancers and investigated the antitumor efficacy and the mechanisms of action of JP11646. When PIM2 expression was compared between normal and cancer tissues in publicly available datasets, PIM2 was found to be overexpressed in several types of solid cancers. PIM2 ectopic overexpression promoted tumor growth in *in vivo* xenograft breast cancer mouse models. The pan-PIM inhibitor, JP11646, suppressed *in vitro* cancer cell proliferation in a concentration-dependent manner in multiple types of cancers; a similar result was observed with siRNA-mediated PIM2 knockdown, as well as an increased in cell apoptosis. By contrast, another pan-PIM inhibitor, AZD1208, suppressed the expression of downstream PIM2 targets, but not PIM2 protein expression, corresponding to no apoptosis induction. As a mechanism of PIM2 protein degradation, it was found that the proteasome inhibitor, bortezomib, reversed the apoptosis induced by JP11646, suggesting that PIM2 degradation by JP11646 is proteasome-dependent. JP11646 exhibited significant anticancer efficacy with minimal toxicities at the examined doses and schedules in multiple *in vivo* mice xenograft solid cancer models. On the whole, the present study demonstrates that PIM2 promotes cancer progression in solid tumors. JP11646 induces apoptosis at least partly by PIM2 protein degradation and suppresses cancer cell proliferation *in vitro* and *in vivo*. JP11646 may thus be a possible treatment strategy for multiple types of solid cancers.

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Abbreviations: PIM2, proviral integration of Moloney virus 2; 4EBP1, 4E-binding protein 1; TSC2, tuberous sclerosis complex 2; BAD, BCL2-associated agonist of cell death; TCGA, The Cancer Genome Atlas; PDAC, pancreatic ductal adenocarcinoma; NSCLC, non-small cell lung cancer; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline

Key words: PIM2, JP11646, drug development, solid cancer, apoptosis, proteasome

Introduction

Proviral integration of Moloney virus 2 (PIM2) belongs to a family of serine/threonine kinases consisting of three members: PIM1, PIM2 and PIM3 (1). PIMs are highly conserved proteins (2), and play roles in various cellular processes, such as cell growth, proliferation, apoptosis and regulation of signal transduction (3). PIM2 is overexpressed in hematopoietic cancers (3), and is considered to be an oncogene involved in multiple signaling pathways (4). PIM2 can phosphorylate and activate substrates that control cancer progression and tumorigenesis (4). PIM2 inhibits apoptosis by phosphorylating downstream targets, including eukaryotic

translation initiation factor 4E-binding protein 1 (4EBP1), tuberous sclerosis complex 2 (TSC2), and BCL2-associated agonist of cell death (BAD) (5-8). In hematological malignancies, PIM2 promotes oncogenic progression as a pro-survival factor (9).

PIM2 has been demonstrated to be a possible therapeutic target in hematological malignancies (7,10-15), as the inhibition of PIM2 induces apoptosis and inhibits cancer cell proliferation *in vitro* and *in vivo* (7). Ongoing clinical trials for PIM2 inhibitors (https://clinicaltrials.gov/; Identifier: NCT01456689 and NCT01588548) (16) have yielded discouraging results with not sufficient efficacy or dose-limiting toxicity in hematological malignancies, and a phase 1 trial (NCT03715504) commended in April, 2019 in solid tumors. Previous studies reported that PIM2 also plays important roles in tumor progression, epithelial to mesenchymal transition, chemotherapy resistance (17), and aerobic glycolysis (18) in solid tumors. However, the importance and the strategy for targeting PIM2 in solid cancers has not been fully elucidated. Recently, it has been shown that a novel pan-PIM inhibitor, JP11646, demonstrates anticancer activity in multiple myeloma (7). The present study examined the utility of targeting PIM2 in multiple solid cancers, and investigated the antitumor efficacy and mechanisms of action of JP11646.

Materials and methods

Bioinformatics analysis. PIM2 expression was compared between tumors and normal tissues in The Cancer Genome Atlas (TCGA) dataset. Clinical and gene expression data from RNA sequence were downloaded through the cBioportal (19,20) website. The expression levels of PIMs were compared among the cell lines using the CCLE dataset (21,22).

Cells, cell culture and reagents. Human cancer cell lines, including pharyngeal carcinoma FaDu (HTB-43), ovarian cancer SK-OV-3 (HTB-77), breast cancer MDA-MB-231 (CRM-HTB-26) and BT549 (HTB-122), prostate adenocarcinoma PC-3 (CRL-1435), liver cancer HepG2 (HB-8065), pancreatic ductal adenocarcinoma (PDAC) MIAPaCa-2 (CRM-CRL-1420) and PANC-1 (CRL-1469), colorectal cancer HT-29 (HTB-38) and DLD-1 (CCL-221), and non-small cell lung cancer (NSCLC) H1650 (CRL-5883), H661 (HTB-183), H460 (HTB-177) and A549 (CCL-185) cell lines were obtained from ATCC. The FaDu, SK-OV-3, MDA-MB-231, PC-3, HepG2, MIAPaCa-2, PANC-1 and HT29 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco; Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Inc.); the BT549, DLD-1, H1650, H661, H460 and A549 cells were cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) with 10% FBS in a humidified incubator at 37˚C in 5% CO_2. All cell lines were used within 20 passages after revival. All cell lines were shown to be mycoplasma-free using the PlasmoTest kit (InVivoGen, Inc.). The MDA-MB-231 cells stably overexpressing PIM2 and controls were generated by the transfection of either 2 μg/ml PIM2-p3xFlag-CMV-14, which was kindly provided by Dr Jeremy Don (Bar-Ilan University, Ramat Gan, Israel) or empty vector (MilliporeSigma) using Lipofectamine LTX (Thermo Fisher Scientific, Inc.) into MDA-MB-231 cells. PIM2 stably overexpressing and control clones were selected with G418 for >2 weeks and used in further experiments. Human PIM2 specific siRNA (sense, 5'-ACCUUC UUCCCGACCCUCAtt-3' and antisense, 5'-UGAGGGUCG GGAAGAAGGUtt-3') or non-targeting siRNA (#4390843, Thermo Fisher Scientific, Inc.) was transfected into the BT549 cells at a final concentration of 30 nM using DharmaFECT 1 Transfection Agent (GE Dharmacon), according to the manufacturer's instructions. The cells transfected with the siRNA were collected at 72 h following transfection and changes in protein expression were examined using western blot analysis.

The novel pan-PIM inhibitor, JP11646, was obtained from Jasco Pharmaceuticals, LLC and its structure is illustrated in Fig. S1 (23,24). Another pan-PIM inhibitor (AZD1208) (25) and the proteasome inhibitor, bortezomib, were obtained from Selleck Chemicals.

Drug sensitivity assay in in vitro. A total of 5,000 cells were seeded per well of a 96-well plate and incubated overnight at 37˚C. Several concentrations of JP11646, ranging from 0.005 to 10 μM, were added to each well, and the cells were incubated for 72 h at 37˚C. Cell proliferation was measured using the CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega Corporation), according to the manufacturer's protocol. The half-maximal growth inhibitory (GI_{50}) values were calculated using non-linear regression.

Western blot analysis. The breast cancer cell lines, MDA-MB-231 and BT549, were cultured with either the vehicle (H_2O) or JP11646 (100 or 200 nM) for 24 h. The BT549 cells were treated with 1 μM AZD1208 for 24 h. The MDA-MB-231 cells were pre-treated with 1 nM Bortezomib for 12 h, and then treated with 200 nM JP11646 for 24 h. Cells were lysed using RIPA lysis buffer (Cell Signaling Technology, Inc.), and lysates were quantified using the Micro BCA Protein Assay kit (Thermo Fisher Scientific, Inc.) and equal amount of proteins were separated by electrophoresis using 4-12% gradient gel and transferred to a nitrocellulose membrane. The membranes were blocked with 5% milk for 1 h at room temperature, and then incubated with primary antibodies (PIM1; cat. no. 3247, 1:1,000, 34, 44 kDa, PIM2; cat. no. 4730, 1:1,000, 34,38,40 kDa, PIM3; cat. no. 4165, 1:1,000, 35 kDa, cleaved PARP; cat. no. 5625, 1:1,000, 89 kDa, 4EBP; cat. no. 9452, 1:1,000, 20 kDa, p-4EBP^{Ser65}; cat. no. 9451, 1:1,000, 20 kDa, TSC2; cat. no. 4308, 1:1,000, 200 kDa, or GAPDH; cat. no. 5174, 1:1,000, 37 kDa; all from Cell Signaling Technology, Inc. and p-TSC2^{Ser1798}; cat. no. sc-293149, 1:1,000, 200 kDa from Santa Cruz Biotechnology, Inc.) at 4˚C overnight. Bands were developed with HRP-labeled anti-rabbit secondary antibodies (1:2,500; cat. no. W4011, Promega Corporation) for 3 h at room temperature, followed by the Clarity Western ECL detection system (Bio-Rad Laboratories, Inc.). Chemiluminescence signals were acquired using a ChemiDoc MP imager (Bio-Rad Laboratories, Inc.).

Apoptosis assay. The breast cancer cell lines, MDA-MB-231 and BT549, were treated with either the vehicle (DMSO, MilliporeSigma), JP11646 (100 or 200 nM) or AZD1208 (1 μM) for 48 h. The cells were washed with phosphate-buffered saline (PBS) and suspended in Annexin V binding

buffer (BioLegend, Inc.), followed by stained with Annexin V (BioLegend, Inc.) and propidium iodide (MilliporeSigma). The apoptotic rate was analyzed using FACS LSRFortessa (BD Biosciences).

In vivo xenograft model. Approval from the Roswell Park Cancer Institution Animal Care and Use Committee was obtained for the experiments in the present study. The animals were accommodated at a constant temperature of 22˚C and 50-60% humidity with a 12-h light/dark cycle, and standard conditions with free access to food and water. A total of 114 CB17 SCID mice (female, 6-8 weeks-old, weighing 18-22 g) were purchased in-house from the Roswell Park Comprehensive Cancer Center. For the experiment with MDA-MB-231 over-expressing PIM2, $1x10^6$ cell suspensions in a mixture of 2 μl PBS and 18 μl Matrigel (Corning, Inc.) were injected into the chest mammary fat pads and tumor growth were observed for up to 28 days (2 groups; control and PIM2 overexpression, n=7 mice per group). For JP11646 treatment, cell suspensions ($1x10^6$ of MDA-MB-231, $3x10^6$ of MIAPaCa-2, $2x10^6$ of PANC-1, $5x10^6$ of HepG2, $5x10^6$ of A549, $5x10^6$ of HT29 and $5x10^6$ of H1650) in a mixture of 50 μl PBS and 50 μl Matrigel were injected subcutaneously into the mouse flanks, or in the case of MDA-MB-231 cells, into the abdomen mammary fat pads. When the average tumor sizes reached 100 mm^3, the mice were randomized and treated with the vehicle, standard care agent, or JP11646 (For MDA-MB-231: 2 groups; control and JP11646, for HepG2, MIAPaCa-2, PANC1, A549, H1650 and HT29: 3 groups; control, JP11646 and standard care agent, n=5 per group). JP11646 was prepared fresh (2.5 mg/ml) in a proprietary carrier solution of 30% modified β-cyclodextrin (Ligand Pharmaceuticals Inc.). The vehicle (proprietary carrier solution of 30% modified β-cyclodextrin) or 15 mg/kg JP11646 were administered by intraperitoneal injection continuously for 2 days a week. The details of standard care agents and the vehicles are summarized in Table SI. The control group received two different vehicles. Tumor size and mice conditions were monitored 2-3 times a week using calipers, and the tumor volume was estimated using the following equation: Volume=(length) x $(width)^2/2$. The humanitarian endpoints were set by the institutional IACUC, and the animals were monitored closely by independent veterinarian technicians who evaluated whether any endpoints had been reached. The maximum tumor dimension was set as one of the institutional endpoints which prevents the tumors from reaching >10% of the animal body weight, as weighing tumors is not practical as the experiment is proceeding. When the maximum tumor diameter reached 2 cm (maximum observed dimension and volume: MDA-MB-231, 20 mm and 3,062.5 mm^3; HepG2, 20.1 mm and 2,231.2 mm^3; MIAPaCa-2, 20.4 mm and 3,459.6 mm^3; PANC-1, 20.5 mm and 2,007.7 mm^3; A549, 21.6 mm and 3,179 mm^3; H1650, 20.8 mm and 2,306.4 mm^3; HT29, 20.4 mm and 2,844.7 mm^3), or other humanitarian endpoints were observed, such as weight loss (≥20%) or tumors with ulcers, the experiment was terminated and the animals were euthanized with CO_2 inhalation (30-70%) as per institutional guidelines for the humanitarian care of animals (MDA-MB-231, 24 days; HepG2, 23 days; MIAPaCa-2, 29 days; PANC-1, 40 days; A549, 29 days; H1650, 18 days; HT29, 12 days).

Statistical analysis. Data are presented as the mean ± standard error of the mean. Comparisons between two groups were performed using an unpaired Student's t-test, and those among more than two groups were performed using one-way ANOVA followed by Tukey's post hoc test. All statistical analyses were performed using GraphPad Prism7 (GraphPad Software, Inc.).

Results

PIM2 is overexpressed in various solid cancers. It was hypothesized that PIM2 is a factor in solid tumor progression. Thus, TCGA datasets were interrogated to compare the PIM2 mRNA levels between cancerous and normal tissues. PIM2 was overexpressed as compared to normal tissue in several types of solid cancers, including breast, esophageal, head and neck, renal clear cell carcinoma, lung adenocarcinoma and endometrial cancer (Fig. 1). On the other hand, its expression was lower in hepatocellular carcinoma and colorectal cancer, and the authors were not able to investigate its expression in PDAC as there was no normal tissue mRNA expression data of pancreatic cancer in TCGA cohort (Fig. 1). Thus, the present study investigated whether PIM2 plays a role in tumor growth and whether it is a potential therapeutic target in solid cancers.

PIM2 overexpression promotes tumor growth in vivo. To investigate whether PIM2 plays a role in promoting tumor growth in breast cancer, either empty or PIM2 inserted p3xFlag-CMV-14 were transfected and PIM2 overexpression was confirmed in the MDA-MB-231 breast cancer cells (Fig. 2A). Control or PIM2-overexpressing cells were injected into mice mammary fat pads. Tumor growth generated by PIM2-overexpressing cells occurred more rapidly compared with the controls (Fig. 2B and C), suggesting that PIM2 promotes the progression of MDA-MB-231 tumors.

Pan-PIM inhibitor, JP11646, inhibits cell growth in multiple solid cancers. Subsequently, the present study examined whether the novel pan-PIM inhibitor, JP11646, inhibits cell proliferation in solid cancers. Although the GI_{50} values varied among the cancer cell lines, JP11646 suppressed cancer cell proliferation in a concentration-dependent manner in all cell lines tested, including in head and neck cancer FaDu, ovarian cancer SK-OV-3, breast cancer MDA-MB-231 and BT549, prostate cancer PC-3, liver cancer HepG2, PDAC MIAPaCa-2 and PANC1, colorectal cancer DLD-1 and HT29, and NSCLC H1650, H661, H460 and A549 cell lines (Fig. 3A and B), although the GI_{50} values were not associated with PIM2 expression (Fig. S2). Another pan-PIM inhibitor (AZD1208) was also tested, whose GI_{50} value for the acute myeloid leukemia cell line was 0.02 μM (26). It exhibited minimal efficacy in decreasing cell proliferation at only the highest dose of 30 μM in both the MDA-MB-231 and BT549 cells (Fig. 3C).

JP11646 treatment downregulates PIM2 protein expression. The present study then investigated the mechanisms underlying the effects of JP11646 on cancer cell proliferation. Previous research has demonstrated that JP11646 treatment induces the apoptosis of multiple myeloma cells, being associated with selective PIM2 downregulation (7), while the anti-apoptotic role of PIM2 is considered to be due to its phosphorylation of

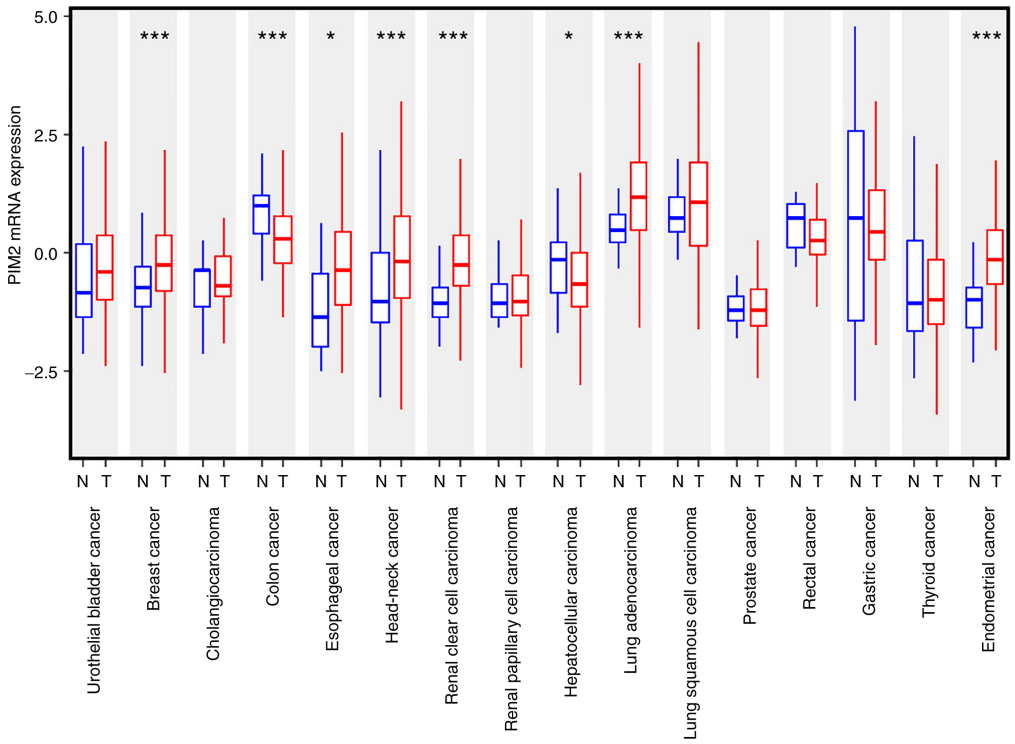

Figure 1. PIM2 expression comparison between cancerous and non-cancerous tissues of several types of solid cancers in The Cancer Genome Atlas datasets. T, tumor; N, normal tissue. PIM2, proviral integration of Moloney virus 2. *P<0.05, and ***P<0.001, vs. normal sample.

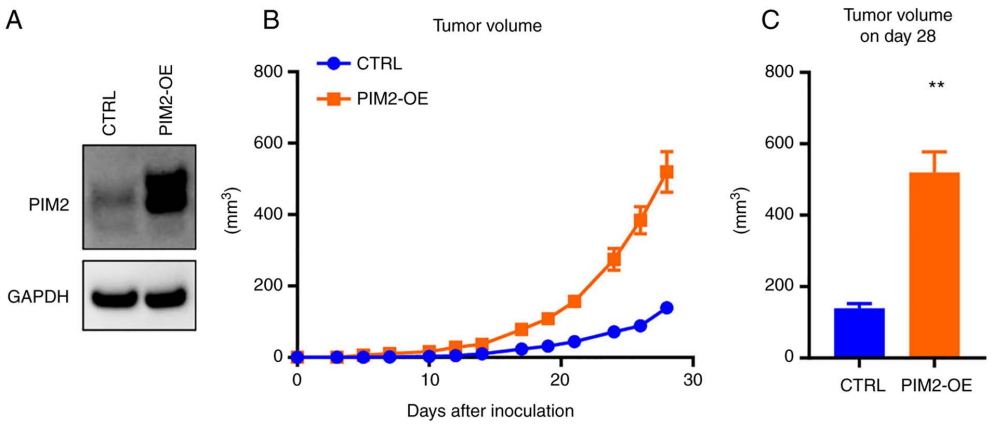

Figure 2. PIM2 overexpression promotes tumor growth *in vivo*. (A) PIM2 overexpression in MDA-MB-231 cells (PIM2: 34,38,40 kDa). (B) PIM2-overexpressing MDA-MB-231 cell-derived tumor growth in an *in vivo* orthotopic model (n=7 mice per group). (C) PIM2-overexpressing MDA-MB-231 cell-derived tumor volume on day 28. CTRL, empty vector transfected control; PIM2-OE, PIM2-overexpression; PIM2, proviral integration of Moloney virus 2. **P<0.01, vs. control samples.

downstream targets, including 4EBP1, TSC2 and BAD (9,27). The present study was able to recapitulate the observation that treatment with JP11646 resulted in the selective downregulation of PIM2, but not of PIM1 or PIM3 protein expression (Fig. 4A). The induction of cleaved PARP by JP11646 treatment was also confirmed in both MDA-MB-231 and BT549 cells (Fig. 4A), suggesting the induction of apoptosis. To examine the effects of PIM2 downregulation on apoptosis induction and the phosphorylation of downstream targets, BT549 cells were treated with either siRNA or the second pan-PIM inhibitor, AZD1208. PIM2 knockdown using siRNA also increased cleaved PARP expression together with the decreased phosphorylation of 4EBP1 and TSC2 (Fig. 4B). In addition, the results revealed the decreased phosphorylation of known PIM2 targets, 4EBP1 and TSC2, at 1 μM of AZD1208, indicating the inhibition of PIM2 kinase activity (Fig. 4B). However, AZD1208 did not decrease the PIM2 protein level and did not upregulate cleaved PARP, likely reflecting a lack of apoptosis induction (Fig. 4B).

Proteasome-dependent PIM2 protein degradation induces the apoptosis of breast cancer cells. The present study further examined the effects of targeting PIM2 on apoptosis induction; the MDA-MB-231 and BT549 cells were treated with either JP11646 or the second pan-PIM inhibitor, AZD1208. Consistent with the induction of cleaved PARP, JP11646 treatment significantly increased the apoptotic rate, and AZD1208

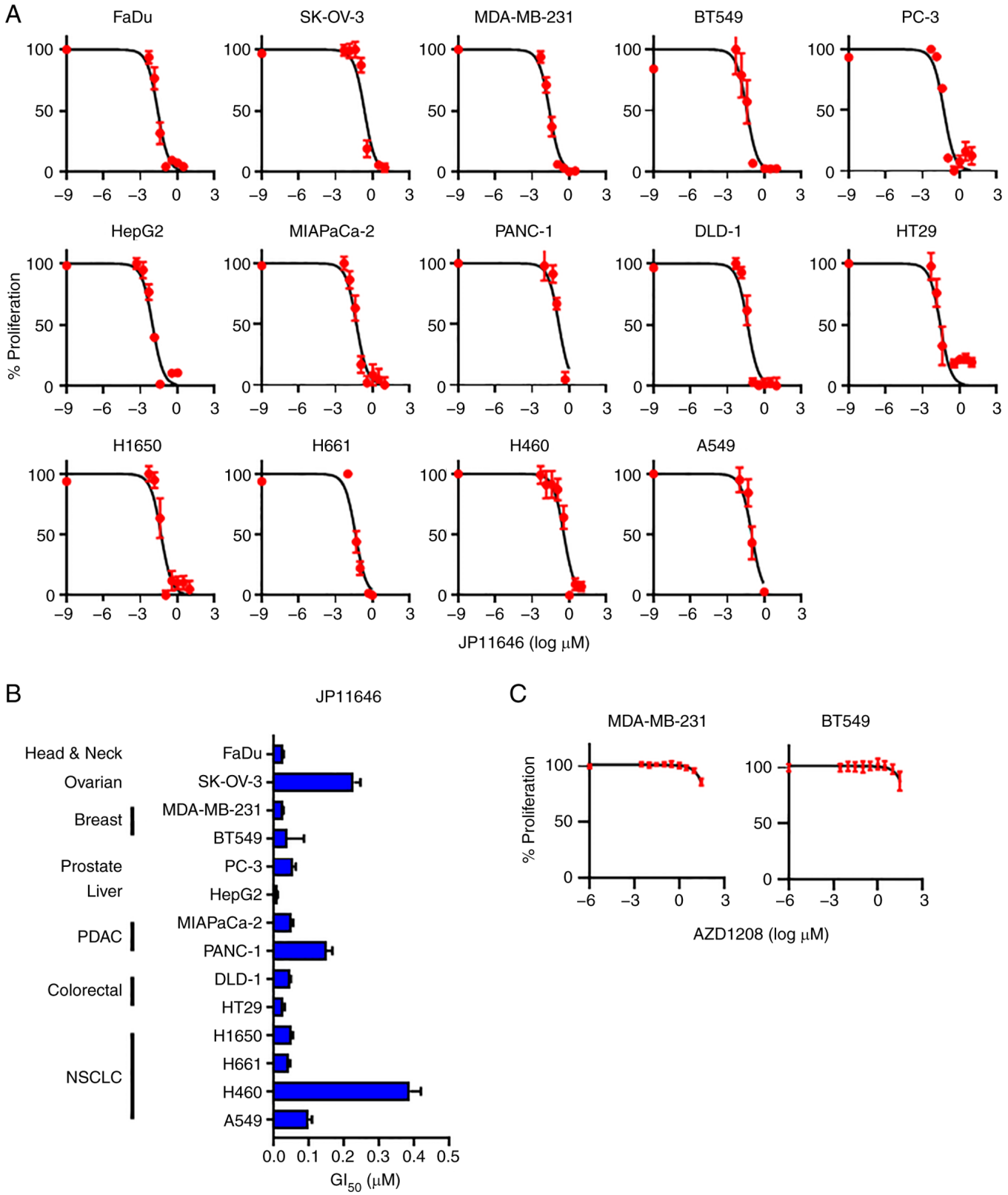

Figure 3. The pan-PIM inhibitor, JP11646 inhibits the proliferation of various cancer cell lines in a concentration-dependent manner. (A) Cell viability of JP11646 treatment for 72 h (n=12, each). (B) GI_{50} values of JP11646 in various solid cancer cell lines (n=12 repeats). (C) Viability of MDA-MB-231 and BT549 cells following AZD1208 (another pan-PIM inhibitor) treatment for 72 h in (n=6 repeats). PIM, proviral integration of Moloney virus.

treatment did not result in a change over the baseline levels in both MDA-MB-231 and BT549 (Fig. 5A-C). The present study then further examined the mechanisms of PIM2 downregulation by JP11646. The addition of the proteasome inhibitor, bortezomib, with JP11646 prevented PIM2 downregulation and decreased cleaved PARP levels, suggesting that proteasome activity leading to PIM2 degradation is required for JP11646 to induce cell death through the apoptosis of MDA-MB-231

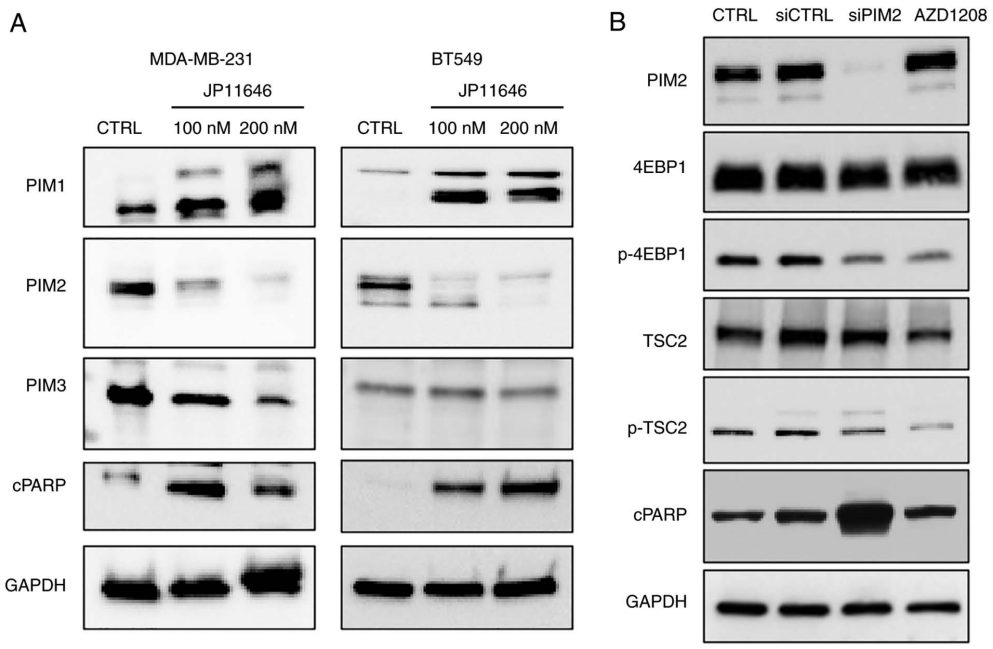

Figure 4. JP11646 treatment downregulates the PIM2 protein level. (A) Western blot analyses of PIMs (PIM1; 34, 44 kDa, PIM2; 34,38,40 kDa, PIM3; 35 kDa) and cleaved PARP (89 kDa) from JP11646-treated MDA-MB-231 and BT549 cells. (B) Western blot analyses of PIM2, 4EBP1 (20 kDa), p-4EBP (20 kDa), TSC2 (200 kDa), p-TSC2 (200 kDa) and cleaved PARP in BT549 cells transfected with siRNA or treated with AZD1208 (1 μM). PIM, proviral integration of Moloney virus; cPARP, cleaved PARP.

cells (Fig. 5D), although it did not rescue cell viability 72 h after treatment (Fig. S3).

PIM2 inhibition by JP11646 suppresses tumor growth in vivo. Finally, the present study examined the efficacy of JP11646 in multiple *in vivo* cancer models. JP11646 significantly suppressed tumor growth in five out of seven tested xenograft tumor models. These included breast cancer MDA-MB-231, liver cancer HepG2, PDAC MIAPaCa-2, and NSCLCs A549 and H1650 *in vivo* models (Fig. 6). However, JP11646 did not suppress tumor growth in PDAC PANC1 and colon cancer HT29 xenograft models (Fig. 6). Standard therapeutic agents were used for some models for the relative measure of JP11646 efficacy, sorafenib for liver cancer, gemcitabine for PDAC, paclitaxel for NSCLC and irinotecan for colorectal cancer. JP11646 resulted in comparable or improved antitumor efficacy when compared to most standard therapies. At the administered drug doses and schedules, no mouse exhibited a weight loss >20% or any other detectable severe side-effect.

Discussion

In the present study, it was demonstrated that PIM2 promoted *in vivo* tumor growth and may thus represent a potential therapeutic target. Targeting PIM2 using siRNA or the pan-PIM inhibitor, JP11646, resulted in the downregulation of PIM2 and the upregulation of cleaved PARP expression in breast cancer cells. JP11646-induced PIM2 degradation and the induction of apoptosis were dependent on proteasome activity and were associated with the inhibition of cancer cell proliferation *in vitro* and tumor growth *in vivo*.

Consistent with previous findings (7), the present study demonstrated that PIM2 downregulation induced apoptosis. The findings suggested that treatment with JP11646 resulted in a more profound inhibition of PIM2 signaling. It was found that the effect of JP11646 on PIM2 was proteosome-dependent in MDA-MB-231 cells. A previous study demonstrated the possibility of kinase-independent PIM2 activity (7), which may explain the enhanced anticancer efficacy of JP11646 as compared to other kinase inhibitors. Although bortezomib did not rescue cell viability at 72 h after treatment, the mechanisms through which JP11464 leads to cell death are not yet fully understood. This mechanism of JP11646 which leads to apoptosis by proteasome-dependent PIM2 protein degradation warrants further investigation in multiple cancer types in the future. It may lead to improvements in clinical efficacy, which have not yet been achieved by the current clinical testing of PIM inhibitors (16).

The present study also demonstrated the efficacy of JP11646 in preclinical mouse models of multiple solid cancers. As previously reported, it is necessary to demonstrate an efficacy in *in vivo* preclinical models before translating this into clinical practice (28-30). The present study broadly examined the efficacy of JP11646 in variety of cancers independent of PIM2 expression based on the hypothesis that targeting PIM2 for degradation highlights a potential novel mechanism. In total, five out of seven JP11646-treated cancers exhibited a significant tumor growth suppression compared to the controls with acceptable side-effect profiles. These results were compared to standard-of-care treatments with comparable results. This suggests that targeting PIM2 by JP11646 may be a potential novel therapeutic strategy for the treatment of solid cancers.

Even though the current experimental results demonstrated the anticancer efficacy of JP11646 in multiple cancers, JP11646 efficacy was not associated with PIM2 mRNA expression. This could be due to differences in mRNA translation,

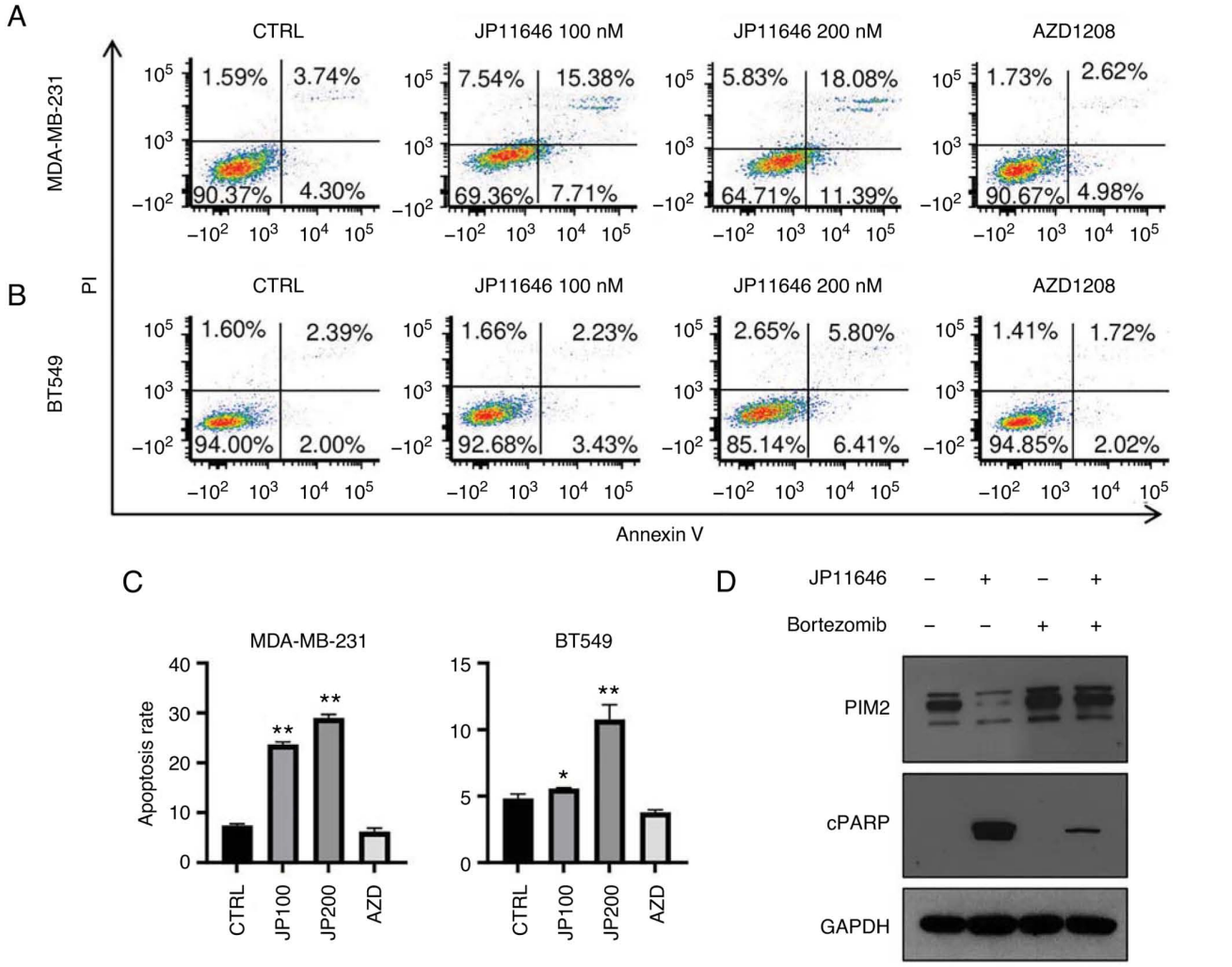

Figure 5. Proteasome-dependent PIM2 downregulation induces apoptosis. Apoptosis assay of (A) MDA-MB-231 and (B) BT549 cells treated with JP11646 (100 nM, 200 nM) or AZD1208 (1 μM). (C) Apoptotic cell rate comparison of MDA-MB-231 and BT549 cells. (D) Western blot analyses of PIM2 (34, 38 and 40 kDa) and cleaved PARP (89 kDa) in MDA-MB-231 cells treated with JP11646 (200 nM) and/or bortezomib (1 nM). PIM2, proviral integration of Moloney virus 2; cPARP, cleaved PARP. *P<0.05 and **P<0.01, vs. control samples.

the dependence of cells on PIM2 signaling or another yet undiscovered function of PIM2 protein. It may also be due to differences in PIM2 targeting for degradation or the existence of compensatory signaling pathways. The off-target effects of JP11646 have been extensively investigated (7). Although a previous study reported the specificity of PIM2 inhibition by JP11646 (7), there is a possibility that the response to JP11646 is more complex, as PIM2 signaling has been shown to be involved in numerous important pathways in cancer cell biology. Furthermore, JP11646 did not exhibit efficacy in some mouse models. The resistance mechanisms to JP11646 are under investigation. One possibility of resistance is the crosstalk between cancer cells and the tumor microenvironment. Although the present study used immune deficient mice, there are multiple components other than immune cells. The tumor microenvironment plays a role to support tumor progression (31); therefore, it may help cancer cells to acquire resistance. It may lead to the inability of JP11646 to achieve a necessary concentration intracellularly due to restricted diffusion or efflux pumps. Further studies are thus warranted to identify predictive markers of JP11646 sensitivity.

The present study demonstrated anticancer effects by targeting PIM2. However, the detailed mechanisms of PIM2 function and necessary inhibition have not yet been fully elucidated. The molecular mechanisms of PIM2 function and the effect of the JP11646 inhibitor in multiple cancer types, as well as the efficacy of other PIM2 inhibitors needs to be investigated in the future. Further studies are warranted to fill this gap in knowledge.

In conclusion, the present study found that PIM2 promoted cancer progression in solid tumors. PIM2 inhibition by JP11646 induced apoptosis via PIM2 protein degradation and suppressed cancer cell proliferation *in vitro* and *in vivo*. PIM2, if properly targeted, may serve as a novel therapeutic target for the treatment of solid cancers.

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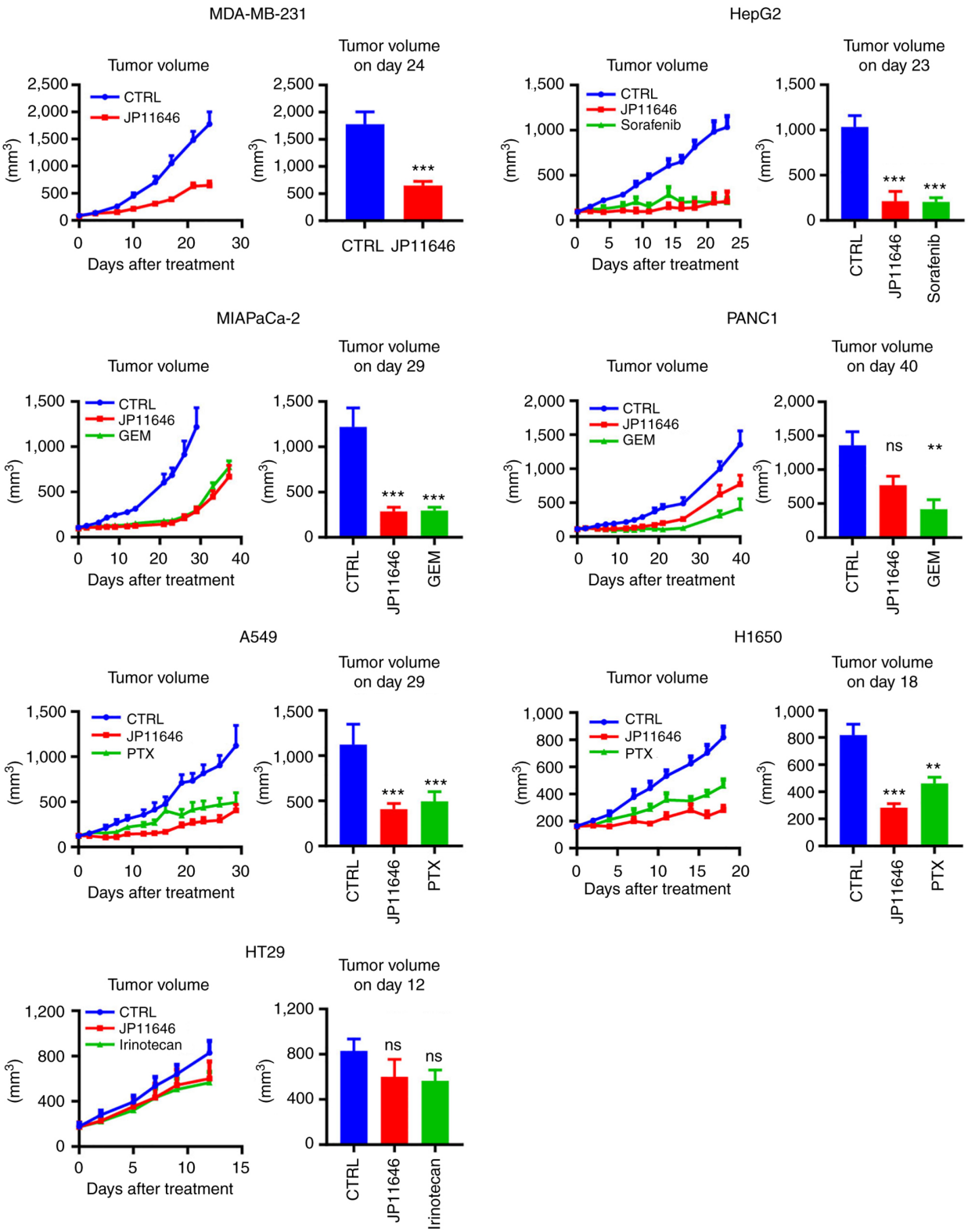

Figure 6. Tumor growth inhibitory effects of the pan-PIM inhibitor, JP11646, in multiple *in vivo* tumor models (n=5 per group). PIM, proviral integration of Moloney virus; CTRL, control; GEM, gemcitabine; PTX, paclitaxel. **P<0.01 and ***P<0.001, vs. control samples.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. TCGA data were downloaded through cBioportal website (https://www.cbioportal.org).

Authors' contributions

MGM, AAA and MO designed the study. EK, MGM, JM, MY and YD performed the experiments. EK, MGM, JM, AAA, JC, CMB, KPL, IHG, KT and MO. interpreted the results. JC and CMB provided the resources. EK, KPL, IHG, KT and MO prepared the article. MO provided supervision. EK and MO confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Since all the patients analyzed in the present study were from de-identified publicly available cohorts, institutional review board approval was waived. Informed consent was obtained by the researchers of the original publication (32).The present study was performed in accordance with the Declaration of Helsinki. All cellular experiments were approved by the Roswell Park Comprehensive Cancer Center Biosafety Committee. All animal experiments were conducted under approved Institutional Animal Care and Use Committee (IACUC) protocols and the Roswell Park Comprehensive Cancer Center Laboratory Animal Shared Resource is accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC).

Patient consent for publication

Not applicable.

Competing interests

M. Opyrchal has a Research support from Bayer and Pfizer and is an advisory board at AstraZeneca and Novartis. The other authors declare that they have no competing interests.

References

1. Cuypers HT, Selten G, Quint W, Zijlstra M, Maandag ER, Boelens W, van Wezenbeek P, Melief C and Berns A: Murine leukemia virus-induced T-cell lymphomagenesis: Integration of proviruses in a distinct chromosomal region. Cell 37: 141-150, 1984.
2. Eichmann A, Yuan L, Bréant C, Alitalo K and Koskinen PJ: Developmental expression of pim kinases suggests functions also outside of the hematopoietic system. Oncogene 19: 1215-1224, 2000.
3. Nawijn MC, Alendar A and Berns A: For better or for worse: The role of pim oncogenes in tumorigenesis. Nat Rev Cancer 11: 23-34, 2011.
4. Jinesh GG, Mokkapati S, Zhu K and Morales EE: Pim kinase isoforms: Devils defending cancer cells from therapeutic and immune attacks. Apoptosis 21: 1203-1213, 2016.
5. Yan B, Zemskova M, Holder S, Chin V, Kraft A, Koskinen PJ and Lilly M: The PIM-2 kinase phosphorylates BAD on serine 112 and reverses BAD-induced cell death. J Biol Chem 278: 45358-45367, 2003.
6. Ren K, Gou X, Xiao M, He W and Kang J: Pim-2 cooperates with downstream factor XIAP to inhibit apoptosis and intensify malignant grade in prostate cancer. Pathol Oncol Res 25: 341-348, 2019.
7. Nair JR, Caserta J, Belko K, Howell T, Fetterly G, Baldino C and Lee KP: Novel inhibition of PIM2 kinase has significant anti-tumor efficacy in multiple myeloma. Leukemia 31: 1715-1726, 2017.
8. Hideshima T, Nakamura N, Chauhan D and Anderson KC: Biologic sequelae of interleukin-6 induced PI3-K/Akt signaling in multiple myeloma. Oncogene 20: 5991-6000, 2001.
9. Fox CJ, Hammerman PS, Cinalli RM, Master SR, Chodosh LA and Thompson CB: The serine/threonine kinase Pim-2 is a transcriptionally regulated apoptotic inhibitor. Genes Dev 17: 1841-1854, 2003.
10. Cervantes-Gomez F, Stellrecht CM, Ayres ML, Keating MJ, Wierda WG and Gandhi V: PIM kinase inhibitor, AZD1208, inhibits protein translation and induces autophagy in primary chronic lymphocytic leukemia cells. Oncotarget 10: 2793-2809, 2019.
11. Chen LS, Yang JY, Liang H, Cortes JE and Gandhi V: Protein profiling identifies mTOR pathway modulation and cytostatic effects of pim kinase inhibitor, AZD1208, in acute myeloid leukemia. Leuk Lymphoma 57: 2863-2873, 2016.
12. Chen LS, Redkar S, Taverna P, Cortes JE and Gandhi V: Mechanisms of cytotoxicity to pim kinase inhibitor, SGI-1776, in acute myeloid leukemia. Blood 118: 693-702, 2011.
13. Yang Q, Chen LS, Neelapu SS, Miranda RN, Medeiros LJ and Gandhi V: Transcription and translation are primary targets of pim kinase inhibitor SGI-1776 in mantle cell lymphoma. Blood 120: 3491-3500, 2012.
14. Yang Q, Chen LS, Neelapu SS and Gandhi V: Combination of pim kinase inhibitor SGI-1776 and bendamustine in B-cell lymphoma. Clin Lymphoma Myeloma Leuk 13 (Suppl 2): S355-S362, 2013.
15. Cervantes-Gomez F, Chen LS, Orlowski RZ and Gandhi V: Biological effects of the pim kinase inhibitor, SGI-1776, in multiple myeloma. Clin Lymphoma Myeloma Leuk 13 (Suppl 2): S317-S329, 2013.
16. Cortes J, Tamura K, DeAngelo DJ, de Bono J, Lorente D, Minden M, Uy GL, Kantarjian H, Chen LS, Gandhi V, *et al*: Phase I studies of AZD1208, a proviral integration moloney virus kinase inhibitor in solid and haematological cancers. Br J Cancer 118: 1425-1433, 2018.
17. Uddin N, Kim RK, Yoo KC, Kim YH, Cui YH, Kim IG, Suh Y and Lee SJ: Persistent activation of STAT3 by PIM2-driven positive feedback loop for epithelial-mesenchymal transition in breast cancer. Cancer Sci 106: 718-725, 2015.
18. Han X, Ren C, Yang T, Qiao P, Wang L, Jiang A, Meng Y, Liu Z, Du Y and Yu Z: Negative regulation of AMPKalpha1 by PIM2 promotes aerobic glycolysis and tumorigenesis in endometrial cancer. Oncogene 38: 6537-6549, 2019.
19. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, Jacobsen A, Byrne CJ, Heuer ML, Larsson E, *et al*: The cBio cancer genomics portal: An open platform for exploring multidimensional cancer genomics data. Cancer Discov 2: 401-404, 2012.
20. Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, Sun Y, Jacobsen A, Sinha R, Larsson E, *et al*: Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Sci Signal 6: pl1, 2013.
21. Barretina J, Caponigro G, Stransky N, Venkatesan K, Margolin AA, Kim S, Wilson CJ, Lehár J, Kryukov GV, Sonkin D, *et al*: The cancer cell line encyclopedia enables predictive modelling of anticancer drug sensitivity. Nature 483: 603-607, 2012.
22. Ghandi M, Huang FW, Jané-Valbuena J, Kryukov GV, Lo CC, McDonald ER III, Barretina J, Gelfand ET, Bielski CM, Li H, *et al*: Next-generation characterization of the cancer cell line encyclopedia. Nature 569: 503-508, 2019.
23. Baldino CM, Caserta J, Lee CS, Nicewonger R, Flanders Y and Dumas S: Aminopyrimidine kinase inhibitors. Jasco Pharmaceuticals LLC, United States, 2013.
24. Flanders Y, Dumas S, Caserta J, Nicewonger R, Baldino M, Lee CS and Badlino CM: A versatile synthesis of novel pan-PIM kinase inhibitors with initial SAR study. Tetrahedron Lett 56: 3186-3190, 2015.
25. Zhang X, Song M, Kundu JK, Lee MH and Liu ZZ: PIM kinase as an executional target in cancer. J Cancer Prev 23: 109-116, 2018.
26. Dakin LA, Block MH, Chen H, Code E, Dowling JE, Feng X, Ferguson AD, Green I, Hird AW, Howard T, *et al*: Discovery of novel benzylidene-1,3-thiazolidine-2,4-diones as potent and selective inhibitors of the PIM-1, PIM-2, and PIM-3 protein kinases. Bioorg Med Chem Lett 22: 4599-4604, 2012.
27. Tamburini J, Green AS, Bardet V, Chapuis N, Park S, Willems L, Uzunov M, Ifrah N, Dreyfus F, Lacombe C, *et al*: Protein synthesis is resistant to rapamycin and constitutes a promising therapeutic target in acute myeloid leukemia. Blood 114: 1618-1627, 2009.

28. Katsuta E, DeMasi SC, Terracina KP, Spiegel S, Phan GQ, Bear HD and Takabe K: Modified breast cancer model for preclinical immunotherapy studies. J Surg Res 204: 467-474, 2016.
29. Katsuta E, Oshi M, Rashid OM and Takabe K: Generating a murine orthotopic metastatic breast cancer model and performing murine radical mastectomy. J Vis Exp: 2018.
30. Katsuta E, Rashid OM and Takabe K: Murine breast cancer mastectomy model that predicts patient outcomes for drug development. J Surg Res 219: 310-318, 2017.
31. Katsuta E, Rashid OM and Takabe K: Clinical relevance of tumor microenvironment: immune cells, vessels, and mouse models. Human Cell 33: 930-937, 2020.
32. Curtis C, Shah SP, Chin SF, Turashvili G, Rueda OM, Dunning MJ, Speed D, Lynch AG, Samarajiwa S, Yuan Y, *et al*: The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. Nature 486: 346-352, 2012.