Title: Unique anti-myeloma activity by thiazolidine-2,4-dione compounds with Pim inhibiting activity

Running head: Anti-myeloma Activity by Thiazolidine-2,4-dione compounds

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Summary

Pim-2 kinase is overexpressed in multiple myeloma (MM) cells, and regarded as an important therapeutic target. Here, we aimed to validate the therapeutic efficacy of different types of Pim inhibitors against MM cells for their possible clinical application. Intriguingly, the thiazolidine-2,4-dione-family compounds SMI-16a and SMI-4a reduced Pim-2 protein levels and impaired MM cell survival preferentially in acidic conditions, in contrast to other types of Pim inhibitors, including AZD1208, CX-6258 and PIM447. SMI-16a also suppressed the drug efflux function of breast cancer resistance protein, minimized the sizes of side populations, and reduced in vitro colony forming capacity and in vivo tumorigenic activity in MM cells, suggesting impairment of their clonogenic capacity. Pim-2 is known to be subject to ubiquitination-independent proteasomal degradation. Consistently, the proteasome inhibitors bortezomib and carfilzomib increased Pim-2 protein levels in MM cells without affecting its mRNA levels. However, SMI-16a mitigated the Pim-2 protein increase and cooperatively enhanced anti-MM effects in combination with carfilzomib. Collectively, the thiazolidine-2,4-dione-family compounds SMI-16a and SMI-4a uniquely reduce Pim-2 protein in MM cells, which may contribute to their profound efficacy in addition to their immediate kinase inhibition. Their combination with proteasome inhibitors is envisioned.

(190 words)

Key words: multiple myeloma, Pim-2, thiazolidine-2,4-dione compounds, BCRP, proteasome inhibitor
Introduction

Pim serine/threonine kinases, namely Pim-1, Pim-2 and Pim-3, have been demonstrated to be differentially overexpressed in a variety of malignant cells, and regarded as important therapeutic targets. Hematopoietic malignant cells preferentially overexpress Pim-1 and Pim-2, while Pim-3 is up-regulated in solid cancers such as pancreatic and hepatic cancers (Li, et al 2006, Mukaida, et al 2011, Wu, et al 2010). Of note, multiple myeloma (MM) has been reported to overexpress Pim-2 at the highest level among hematological malignancies (Johrer, et al 2012, Lu, et al 2013).

MM has a unique propensity to develop and expand almost exclusively in the bone marrow, and generates devastating bone destructive lesions in which bone marrow stromal cells (BMSCs) and osteoclasts (OCs) support MM cell growth and survival. We reported that Pim-2 is constitutively overexpressed and further up-regulated in MM cells by IL-6 and the TNF family cytokines TNFα, BAFF and APRIL elaborated from BMSCs as well as OCs, and that Pim-2 acts as an anti-apoptotic mediator in MM cells (Asano, et al 2011). Importantly, Pim-2 was found to be up-regulated in BMSCs as well in MM and plays a critical role in suppression of osteoblastogenesis as an intracellular mediator downstream of osteoblastogenesis inhibitors in MM, including IL-3, IL-7, TNF-α, TGF-β and activin A (Hiasa, et al 2015). Furthermore, Pim-2 is induced in osteoclastic cells during their osteoclastogenesis by RANK ligand, and acts as a critical mediator of osteoclastogenesis in MM (Teramachi, et al 2016). Therefore, Pim-2 plays a pivotal role in tumor progression and bone destruction and loss in MM, and Pim-2 inhibition may become an important therapeutic strategy targeting the MM cell-bone marrow interaction. Indeed, tumor reduction and bone restoration have been demonstrated in MM animal models with the Pim inhibitor (Z)-5-(4-propoxybenzylidene)thiazolidine-2,4-dione, named SMI-16a (Xia, et al 2009), suggesting the therapeutic impact of thiazolidine-2,4-dione-family compounds with Pim inhibiting activity on MM (Hiasa, et al 2015).

Acidic microenvironments are generally accepted to confer drug resistance and immune evasion capability in cancers (Kato, et al 2013). We recently reported that bendamustine reduces Pim-2 expression to impair MM cell viability preferentially in acidic conditions (Nakamura, et al 2016). These observations suggest that Pim-2 may play a critical role in MM cell survival in acidic conditions created in MM bone lesions. Although bone lesions confer drug resistance in MM cells, the role of Pim-2 in growth and survival in MM cells, as well as their drug resistance in acidic bone lesions in MM is largely unknown.

In the present study, we therefore aimed to clarify the therapeutic impact of the thiazolidine-2,4-dione-family compounds SMI-16a and SMI-4a (Xia, et al 2009) on MM cells and their progenitors in acidic conditions and on the ATP-binding cassette (ABC) transporter breast cancer resistance protein (BCRP)-expressing drug-resistant MM cells. We demonstrate here that these thiazolidine-2,4-dione-family compounds directly impair MM cell survival preferentially in acidic conditions and suppress BCRP function to restore drug sensitivity in MM cells. Interestingly, these compounds substantially reduced Pim-2 protein levels in MM cells preferentially in acidic conditions in contrast to other types of Pim inhibitors, including AZD1208, CX-6258 and PIM447. In addition, SMI-16a was suggested to have the ability to impair MM progenitors with self-renewal capacity. Although the proteasome inhibitors bortezomib and carfilzomib accumulated intact Pim-2 protein in MM cells through blocking its ubiquitination-independent proteasomal degradation, SMI-16a mitigated the Pim-2 protein increase and cooperatively enhanced anti-MM effects with the proteasome inhibitors. These data suggest that SMI-16a and SMI-4a may become an interesting anti-MM option and warrant further study on their combination with proteasome inhibitors.

Materials and Methods

Reagents

The following reagents were purchased from the indicated manufacturers: rh IL-6 from PeproTech EC (London, UK); rabbit anti-Pim-1 antibody from Abcam (Cambridge, UK); rabbit anti-Pim-2 antibody, horseradish-peroxidase-conjugated goat anti-rabbit
IgG, horseradish-peroxidase-conjugated goat anti-mouse IgG and bortezomib from Cell Signaling Technology (Beverly, MA, USA); mouse anti-β-actin antibody and puromycin from Sigma (St Louis, MO, USA); anti-puromycin antibody from Kerafast, Inc (Boston, MA); SMI-16a and SMI-4a from Calbiochem (Darmstadt, Germany); AZD1208 and pomalidomide from Tronto Research Chemicals (Tronto, Canada); CX6258 from Cayman Chemical Company (Ann Arbor, MI, USA); PIM447 from BOC Sciences (Shirley, NY, USA); Cycloheximide from Nacalai Tesque (Kyoto, Japan), carfilzomib from ChemieTek (Indianapolis, IN, USA); mouse anti-human BCRP from Millipore (Temecula, CA); mouse monoclonal anti-human multidrug-resistance-associated protein-1 (MRP-1) and rabbit polyclonal anti-Mcl-1 antibodies and lenalidomide from Santa Cruz Biotechnology (Santa Cruz, CA); FITC-labeled rabbit anti-mouse IgG from Zymed Laboratories (San Francisco, CA); and PE-labeled mouse anti-P-glycoprotein antibody, and PE-mouse IgG from BD Bioscience (San Jose, CA).

Cells and cultures

Human MM cell lines, RPMI8226, KMS-11 and U266 were obtained from American Type Culture Collection (ATCC) (Rockville, MD). The MM cell lines INA-6 and MM.1S were kindly provided by Dr. Renate Burger (University of Kiel, Kiel, Germany) and Dr. Steven Rosen (Northwestern University, Chicago, IL), respectively. TSPC-1 and OPC MM cell lines were established in our laboratory (Abe, et al 2002). MM cells were cultured in RPMI1640 supplemented with 5% fetal bovine serum, 2 mmol/L L-glutamine (Sigma), 100 U/mL penicillin G and 100 µg/mL streptomycin (Sigma). The pH levels in culture media were adjusted by adding lactic acid (Wako, Osaka, Japan). Bone marrow mononuclear cells were isolated from fresh bone marrow aspirates of patients with myeloma and primary CD138+ MM cells were further sorted using CD138 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) as described previously (Abe, et al 2002, Kitazoe, et al 2009). All procedures involving human specimens were performed with written informed consent according to the Declaration of Helsinki and using a protocol approved by the Institutional Review Board for human protection.

Cell viability assays
Cells were plated out in triplicate on 96-well culture plates and incubated with drugs. The number of viable cells was determined by the Cell Counting Kit-8 (WST-8) assay (DOJINDO, Kumamoto, Japan) according to the manufacturer’s instructions. The absorbance of each well was measured at 450 nm with a microplate reader (Model 450 micro plate reader; Bio-Rad Laboratories, Hercules, CA).

**Western blot analysis**

Whole cell lysate was lysed in RIPA buffer, and nuclear extract was lysed by NE-PER nuclear and cytoplasmic extraction reagent kit (Thermo Fisher Scientific, Rockford, IL, USA). These lysates were supplemented with 1 mmol/L phenylmethylsulfonyl fluoride and protease inhibitor cocktail solution (Sigma). Cell lysates and conditioned media were electrophoresed in a 10% SDS-PAGE gel and blotted onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). After blocking with 5% non-fat dry milk, the membranes were incubated with primary antibodies overnight at 4°C, followed by washing and addition of a horseradish-conjugated secondary antibody for 1 hour. The protein bands were visualized with the Enhanced Chemiluminescence Plus Western Blotting Detection System (Amersham Biosciences, Piscataway, NJ).

**RT-PCR**

Total RNA was extracted from cells using TRIZOL reagent (Gibco BRL, Rockville, MD). For reverse transcription-polymerase chain reaction (RT-PCR), 2 µg of total RNA was reverse-transcribed with Superscript II (Gibco) in a 20-µL reaction solution. One tenth of the RT-PCR products were used for subsequent PCR analysis with 24–30 cycles of 95°C for 30 seconds, 58°C for 30 seconds and 72°C for 30 seconds. The following primers were used: The primers used for RT-PCR were as follows: human Pim-2 sense 5’-GGTAAGGGATTGAGGATC-3’ and anti-sense 5’-TGGGGTATTGGAAGGAAAG-3’; human GAPDH sense 5’-TGTCTTCACCACCATGGAGAAGG-3’ and anti-sense 5’-GTGGATGCAGGGATGATGTTCTG-3’.

**Flow cytometry**

Cells were incubated in 100 µL of PBS with 2% human γ-globulin (Japan Blood Products Organization, Tokyo, Japan), and stained with PE-labeled mouse monoclonal
anti-P-glycoprotein antibody, or with mouse monoclonal anti-BCRP or anti-MRP1 antibody followed by FITC-labeled rabbit anti-mouse IgG antibody as described before. Samples were then washed and analyzed by flow cytometry using EPICS-Profile (Coulter Electronics, Hialeah, FL, USA). Apoptosis was evaluated by staining the cells with an annexinV-FITC and propidium iodide labeling kit (MEBCYTO Apoptosis Kit; MBL, Nagano, Japan) according to the manufacturer’s instructions.

Drug accumulation and efflux assay
Drug accumulation and efflux were analyzed as described previously (Abe, et al 2004). Cells were cultured for 30 minutes with 100 ng/mL mitoxantrone. Then, cells were washed, and intracellular fluorescence levels were analyzed by flow cytometry (accumulation phase, AP). For determining drug efflux levels, the cells were further incubated for 6 hours in the presence or absence of thiazolidine-2,4-dione compounds, and intracellular fluorescence levels were analyzed (efflux phase, EP).

Side population (SP) analysis
SP fractions were analyzed as described previously (Harada, et al 2013, Ikegame, et al 2012). Cells were incubated with 5 μg/mL Hoechst 33342 (Invitrogen) for 90 min at 37°C in the presence or absence of 100 μmol/L verapamil or 50 μmol/L SMI16a. After being washed, the cells were resuspended in ice-cold PBS supplemented with 1 μg/mL propidium iodide to detect dead cells. Hoechst 33342 was excited with a UV laser at 350 nm, and the SP fractions were analyzed by flow cytometry (EPICS ALTRA HyperSort, Beckman & Coulter) with 450 nm (Hoechst blue) and 675 nm (Hoechst red) filters.

Colony formation assays
Colony formation assays were performed as described previously (Harada, et al 2013, Ikegame, et al 2012). Cells were cultured in triplicate in 35-mm dishes containing H4034 methylcellulose medium (Stem Cell Technologies, Vancouver, BC, Canada) with 30% FBS, 100 U/mL penicillin G and 100 μg/mL streptomycin. After culturing for 2 weeks, colonies were visualized and counted under an Olympus BX50 microscope equipped with an UMPlanFI 40X/0.75 objective lens (Olympus, Tokyo, Japan). Images were recorded with an Olympus SC35 CCD camera and Viewfinder Lite Software.
Assessment of in vivo tumorigenic capacity

All animal studies were conducted in accordance with principles and procedures approved by the University of Tokushima Institutional Review Board (toku-dobutsu 14092). MM mouse models were well established and constantly reproduced by the intra-tibial inoculation of mouse 5TGM1 MM cells to ICR nu/nu mice (CLEA Japan, Tokyo, Japan) at 4–6 weeks old as previously described (D'Souza, et al 2011, Hiasa, et al 2015, Teramachi, et al 2016). Therefore, we used this model to determine in vivo tumorigenic capacity. We cultured mouse 5TGM1 MM cells for 24 hours in the presence or absence of 50 µmol/L SMI-16a, then the cells were washed and viable cell numbers were counted. The remaining living cells (2x10^5 cells) were directly inoculated to the tibia of ICR nu/nu mice. To estimate tumor growth, mouse IgG 2b protein levels in mouse sera were measured using Mouse IgG 2b ELISA Quantitation Set (Bethyl Laboratories, Inc., Montgomery, TX, USA). Tumor sizes were measured over time after detecting palpable tumors, and the tumor volume was calculated using the formula: \( \frac{ab^2\pi}{6} \), where \( a \) = the longest diameter and \( b \) = the shortest diameter (Ikegame, et al 2012).

Statistical analysis

Data are represented as means +/- standard deviations unless specified otherwise. Significance was determined by a one-way analysis of variance (ANOVA) with Scheffe’s post hoc tests. The minimal level of significance was \( P=0.05 \).

Results and Discussion

The thiazolidine-2,4-dione-family compounds SMI-16a and SMI-4a induce MM cell death preferentially in acidic conditions.

We first examined the expression of Pim-1 and Pim-2 at the protein level in MM cells. All MM cell lines except IL-6-dependent MM cell lines, INA-6, OPC and TSPC-1, clearly expressed Pim-2 at baseline, whereas Pim-1 expression was found only in RPMI8226 and OPC (Fig 1A). In cocultures with BMSCs, Pim-2 was substantially
up-regulated in all MM cell lines, including the above IL-6-dependent ones, suggesting Pim-2 overexpression in MM cells in the bone marrow. Therefore, Pim-2 appears to be a predominant Pim isoform overexpressed in MM cells, as previously reported (Lu, et al 2013).

As tumor acidity is known to confer drug resistance and because MM creates acidic bone lesions, we next looked at the effects of the thiazolidine-2,4-dione-family compounds SMI-16a and SMI-4a on MM cell viability at different pH values, including 7.4, 6.8 and 6.4. Both compounds dose-dependently induced cell death in RPMI8226, KMS11 and INA-6 MM cell lines (Fig 1B). Their cytotoxic activity was augmented as ambient pH values were lowered. The induction of apoptosis was confirmed with annexinV and propidium iodide dual staining in RPMI8226 and INA-6 cells (Supplementary Figure 1). We previously demonstrated that treatment with SMI-16a reduced the expression of the BCL family member Mcl-1 (Asano, et al 2011). However, phosphorylation of Bad and the levels of other apoptosis-related factors including Bcl-xL, Bcl-2 and Bim showed no appreciable change in MM cells upon treatment with SMI-16a. Treatment with SMI-4a as well as SMI-16a reduced the expression of Mcl-1 in MM cells, which was further enhanced in acidic conditions (Supplementary Figure 2). We also examined the cytotoxic assay with other commercially available Pim inhibitors. AZD1208 (Dakin, et al 2012, Harada, et al 2015, Keeton, et al 2014) is another member of thiazolidine-2,4-dione-family compounds; and CX-6258 (Haddach, et al 2012) and PIM447 (Peters, et al 2016) are structurally different types of Pim inhibitors. AZD1208, CX-6258 and PIM447 also induced dose-dependent MM cell death at pH 7.4 (Fig 1C). However, the cytotoxic activity of these compounds against MM cells was not increased in acidic conditions; of note, anti-MM effects of CX-6258 or PIM447 were rather blunted as pH values in culture media were lowered (Fig 1C). Therefore, augmentation of anti-MM effects in acidic conditions appears to be unique to these thiazolidine-2,4-dione-family compounds, which may allow them to target MM cells in acidic bone lesions.

SMI-16a and SMI-4a reduce Pim-2 protein levels in MM cells.

Pim kinases are autophosphorylated immediately after being synthesized in cells to become constitutively active; therefore, Pim-2 activity is reflected by its protein levels (Warfel and Kraft 2015). As Pim-2 protein has been demonstrated to be rapidly
degraded by a proteasome in a ubiquitination-independent manner (Adam, et al 2015),
Pim-2 activity appears to be largely determined by the balance of Pim-2 protein
production and degradation in cells. Therefore, we next investigated whether SMI-16a
and SMI-4a affect Pim-2 protein levels in MM cells. Although they are kinase inhibitors,
both SMI-16a and SMI-4a dose-dependently reduced Pim-2 protein levels in MM cells
at 24 hours (Fig 2A). However, Pim-2 expression was not affected in MM cells at
mRNA levels after exposure to SMI-16a or SMI-4a at pH 7.4, 6.8 and 6.4 (Fig 2B).
Interestingly, both compounds were able to further reduce Pim-2 protein levels in MM
cells in acidic conditions, which correlated well with their anti-MM activity. Although
Pim-2 protein levels were robustly increased in MM cells in cocultures with BMSCs,
SMI-16a or SMI-4a were able to mitigate the upregulation of Pim-2 protein levels in
MM cells by BMSCs (Fig 2C).

Although AZD1208, CX-6258 and PIM447 exerted the cytotoxic activities
against MM cells, these compounds did not reduced Pim-2 protein levels in RPMI8226
MM cells cultured alone or cocultured with BMSCs at the concentrations adequate to
induce MM cell death (Fig 2C). These results demonstrate that the
thiazolidine-2,4-dione-family compounds SMI-16a and SMI-4a uniquely reduce Pim-2
protein levels preferentially in acidic conditions, which may contribute to the
augmentation of their cytotoxic actions against MM cells.

SMI-16a and SMI-4a did not affect Pim-2 protein degradation.
To dissect the mechanisms of Pim-2 reduction, we first confirmed the effects of Pim
inhibitors other than the thiazolidine-2,4-dione-family compounds SMI-16a and SMI-4a
on PIM2 mRNA expression in MM cells. Similar to SMI-16a and SMI-4a, AZD1208,
CX-6258 and PIM447 did not affect PIM2 mRNA expression at the concentrations that
were able to impair MM cell viability (Fig 3A). These data suggest that Pim inhibition
itself does not affect PIM2 mRNA transcription, but that SMI-16a and SMI-4a uniquely
and intriguingly reduced Pim-2 levels in MM cells at protein levels. As Pim-2 is subject
to rapid degradation by proteasomes, we looked at the effects of SMI-16a and SMI-4a
on post-translational degradation of Pim-2 protein in MM cells upon treatment with
cycloheximide, an inhibitor of translation. As previously demonstrated in the other
types of cells (Adam, et al 2015), Pim-2 protein levels were rapidly degraded in MM
cells over time in the presence of cycloheximide; simultaneous treatment with SMI-16a
or SMI-4a did not noticeably affect the reduction of Pim-2 protein (Fig 3B), indicating Pim-2 protein reduction independent of its protein degradation. Therefore, it is plausible that SMI-16a and SMI-4a may reduce Pim-2 synthesis at translational levels. We previously reported that SMI-16a inhibited the phosphorylation of 4EBP1 to block the initiation of translation (Asano, et al 2011). To further clarify the mechanism of the Pim-2 protein reduction, we therefore investigated the effects of SMI-16a and SMI-4a on translation in MM cells using puromycin incorporation. Puromycin incorporation in neosynthesized proteins directly reflects the rate of mRNA translation (Schmidt, et al 2009). SMI-16a and SMI-4a dose-dependently reduced the puromycin incorporation in MM cells (Fig 3C), suggesting suppression of translation. Although Pim-2 protein degradation is quick in cells (Adam, et al 2015), transient global suppression of translation by SMI-16a and SMI-4a may decrease Pim-2 protein synthesis to cause the Pim-2 protein reduction in MM cells.

**Drug retention in MM cells by SMI-16a**

Drug-resistant cancer cells increase their expression of ABC transporters, including P-glycoprotein (ABCB1), BCRP (ABCG2) and MRP-1, which function as efflux transporters dependent on energy from the hydrolysis of ATP for a variety of chemotherapeutic drugs (Allen, et al 2000, Dean 2009, Dean, et al 2005, Doyle and Ross 2003, Fletcher, et al 2010) Pim kinases have been shown to be involved in the expression and activation of drug efflux transporters (Isaac, et al 2011). Pim-1 phosphorylates BCRP, and thereby promotes its multimerization with its stable membrane expression, leading to drug resistance (Xie, et al 2008). As drug-resistant MM cells are known to aberrantly express the ABC transporter BCRP (Zhou, et al 2002, Zhou, et al 2001), we hypothesized that Pim-2 overexpressed in MM cells may mediate BCRP function. To test this hypothesis, we next examined the effects of SMI-16a on intracellular retention of mitoxantrone in MM cells. Mitoxantrone is a substrate of BCRP and expelled by BCRP. Mitoxantrone emits auto-fluorescence, and its retention in MM cells can be estimated by flow cytometry. RPMI8226 cells aberrantly express BCRP on their surfaces; but U266 cells marginally express it (Fig 4A). After passive incorporation of mitoxantrone into these cells, intracellular drug accumulation was shown as an “accumulation phase (AP)” (Fig 4B). The cells were then washed and incubated in media without mitoxantrone. The intracellular drug levels decreased at 6
hours to the levels indicated as the “efflux phase (EP)”. Treatment with SMI-16a increased the drug retention levels in these cells. The drug retention by SMI-16a was more prominent in BCRP-expressing RPMI8226 cells than U266 cells. EP levels without SMI-16a were lower in these MM cells cocultured with bone marrow stromal cells compared with those cultured alone (Figure 4B). However, although Pim-2 was upregulated in MM cells in cocultures with bone marrow stromal cells, SMI-16a was able to similarly restore the drug retention in the MM cells in the coculture settings. The drug retention by SMI-16a tended to be increased in MM cells in acidic conditions. These results suggest that SMI-16a is able to restore drug retention in MM cells, preferentially in BCRP-expressing MM cells.

SMI-16a restores Dox’s anti-MM effects in acidic conditions

Doxorubicin (Dox) is clinically utilized as an anti-MM agent, and a substrate of BCRP. We next examined whether the blockade of BCRP function by SMI-16a is able to enhance Dox’s cytotoxic effects against BCRP-expressing RPMI8226 cells. Dox dose-dependently induced MM cell death at pH 7.4 (Fig 4C). In contrast to SMI-16a, which preferentially induced MM cell death in acidic conditions (Fig 1B), Dox’s cytotoxic activity against RPMI8226 cells was blunted at pH 6.8 (Fig 4C). However, simultaneous addition of SMI-16a instead restored and enhanced the cytotoxic effects of Dox in the acidic condition. The restoration of anti-MM activity of Dox in acidic conditions in combination with SMI-16a is suggested to be at least in part due to the blockade of BCRP function by SMI-16a.

SMI-16a reduces the sizes of SP fractions in MM cells

SP fractions are identified by their ability to efflux Hoechst 33342 dye, a substrate for BCRP, indicating that SP cells have high BCRP activity. SP has been demonstrated to be a drug resistant fraction in cancers, including MM, and is considered to contain cancer stem or initiating cells. We next examined the effects of SMI-16a on the size of SP fractions in MM cells. RPMI8226 and KMS11 cells clearly exhibited SP fractions as previously reported (Hanson, et al 2015, Ikegame, et al 2012, Jakubikova, et al 2011) (Fig 5A). The sizes of SP fractions were substantially decreased in both MM cells upon treatment with SMI-16a for 2 days at pH 7.4. Interestingly, exposure to SMI-16a at pH 6.8 still further reduced percent distributions of the SP fractions within remaining living
cells in these MM cell lines, suggesting a critical role of Pim-2 in maintaining SP fractions, especially in acidic conditions.

**SMI-16a abolishes clonogenic and in vivo tumorigenic capacity of MM cells.**

SP cells have also been reported as a drug resistant fraction in many cancers, and are considered to contain cancer stem cells (Bonnet and Dick 1997, Challen and Little 2006, Ho, *et al* 2007, Li, *et al* 2007, Ricci-Vitiani, *et al* 2007). Treatment with SMI-16a substantially minimized the sizes of SP fractions in RPMI8226 and KMS11 cells (*Fig 5A*). Given that SP fractions contain MM progenitors or MM initiating cells, there is a possibility that SMI-16a is able to target MM progenitors with stemness. To clarify the effects of SMI-16a on the clonogenic potential of MM cells, we examined colony formation of MM cells. RPMI8226 cells were pretreated for 24 hours with or without SMI-16a at pH 7.4 or pH 6.8, and then their capacity of colony formation was estimated, using methylcellulose assays. Spontaneous colony formation of RPMI8226 cells was substantially reduced when they were pretreated with SMI-16a at 7.4 as well as pH 6.8 (*Fig 5B*). We previously reported that SMI-16a efficaciously suppressed MM tumor growth and bone destruction in mouse models bearing murine 5TGM1 MM cells (Hiasa, *et al* 2015), which confirmed in vivo anti-MM actions of SMI-16a. From the above results (*Fig 5A and 5B*), it is plausible that SMI-16a can impair the self-renewal capacity of MM cells. To further investigate the effects of SMI-16a on the self-renewal capacity of MM cells, we next conducted experiments for in vivo tumorigenic capacity of MM cells. We cultured 5TGM1 cells for 24 hours in the presence or absence of SMI-16a. After the pre-treatment, the cells were washed, and remaining living cells were collected and counted. The same numbers of the viable MM cells with or without the pre-treatment with SMI-16a were inoculated into the tibiae of SCID mice. Without the SMI-16a pre-treatment, the MM cells rapidly formed tumors expanding outside of the tibiae (*Fig 5C*), and all the mice died within a month after confirming tumor growth at day 21. However, 5TGM1 cells with the pre-treatment with SMI-16a were not able to form tumors (*Fig 5C*); and 5TGM1 cell-derived IgG2b was not detected in the mouse sera (*Fig 5D*). No tumor was detected even at 3 months in the mice receiving the SMI-16a-pretreated MM cells, suggesting targeting clonogenic MM cells. These results suggest that SMI-16a effectively impairs MM cells, including their clonogenic fractions.
SMI-16a mitigates Pim-2 accumulation in MM cells by proteasome inhibitors.

Consisted with the previous observation with proteasomal degradation of Pim-2 protein (Adam, et al 2015, Warfel and Kraft 2015), treatment with the proteasome inhibitors bortezomib and carfizomib dose-dependently increased Pim-2 protein levels in KMS11 cells at 6 hours without increasing their Pim-2 mRNA expression (Fig 6A). However, the immunomodulatory drugs lenelidomide and pomalidomide did not increase Pim-2 protein levels. Addition of SMI-16a reduced the Pim-2 protein levels increased in RPMI8226 and KMS11 cells by bortezomib or carfizomib (Fig 6B). IL-6 markedly upregulated Pim-2 protein levels in these MM cells; bortezomib still further increased their Pim-2 protein levels in the presence of IL-6. SMI-16a was able to reduce their Pim-2 protein levels upon treatment with bortezomib even in the presence of IL-6.

Carfilzomib is the second generation proteasome inhibitor, which irreversibly inhibits proteasome actions and can induce the cytotoxic activity against MM cells resistant to bortezomib. Carfilzomib and SMI-16a cooperatively enhanced cytotoxicity against RPMI8226 cells (Fig 6C). KMS11 cells appeared to be resistant to carfilzomib; however, addition of SMI-16a was able to reduce their viability. These data suggest that SMI-16a enhances the cytotoxic activity of carfilzomib, which may be in part due to inhibit the action of Pim-2 accumulated through proteasome inhibition.

The present study demonstrates that the thiazolidine-2,4-dione-family compounds SMI-16a and SMI-4a impair MM cell survival preferentially in acidic conditions, while substantially reducing Pim-2 protein levels. Acidic enhancement of their anti-MM actions and Pim-2 protein reduction in MM cells appears to be unique to these compounds and is not observed in other types of Pim inhibitors, including AZD1208, CX-6258 and PIM447. The pKa of SMI-16a and SMI-4a and the zwitterionic character of AZD1208 can affect the difference in their activity against MM cells at the different pH values, although all of these are thiazolidine-2,4-dione-family compounds. At acidic pH values, an amino group of AZD1208 should be protonated rendering it hard to pass through a cell membrane via simple diffusion. On the other hand, SMI-4a and SMI-16a with no amino group have property of being insensitive to pH changes in solution. The basic character of CX-6258 and PIM447, structurally different Pim inhibitors, may cause the difference in the cytotoxic activity in acidic conditions and the effects on
Pim-2 levels in MM cells. The reduction by SMI-16a and SMI-4a of Pim-2 protein levels in MM cells may contribute to their marked efficacy along with their immediate kinase inhibition. Although these compounds did not affect transcription or degradation of Pim-2, precise mechanisms of Pim-2 protein reduction by these compounds still remain to be clarified in MM cells. In addition, compared to IC50 values for Pim kinase inhibition of these compounds (several to 150 nmol/L) according to the description in the previous report (Xia, et al. 2009) and the insertions of the compounds used in the present study, in vitro cytotoxic activity required relatively higher concentrations (several to more than 10 μmol/L); therefore, off-target effects may also affect their potencies of cytotoxic activity.

Chemotherapeutic drugs are generally accepted to have limited effects on clonogenic MM cells or SP cells because of their detoxification potential. SMI-16a was able to suppress BCRP drug efflux function to recover drug susceptibility in MM cells. Importantly, SMI-16a was able to target SP fractions with high BCRP function in MM cells, and impair their clonogenic capacity, indicating the merit of this compound. As BCRP is predominantly expressed in drug-resistant MM cells and their progenitors, the up-regulation of Pim-2 appears to involve drug efflux in MM cells to reduce the efficacy of drug treatment. Sensitization of MM cells to chemotherapeutic agents by Pim inhibition warrants further study on combinatory treatment of anti-MM agents, such as doxorubicin and carfilzomib, with Pim inhibitors.

Bortezomib and carfizomib were found to accumulate a large amount of Pim-2 protein in MM cells. As Pim-2 protein is subject to ubiquitination-independent proteasomal degradation, Pim-2 protein accumulated in MM cells by the proteasome inhibitors appears to be intact. Pim-2 is a critical survival factor in MM cells and the Pim-2 accumulation may therefore compromise the anti-MM effects of proteasome inhibitors. Therefore, the present results warrant further study on combinatory treatment of proteasome inhibitors with agents with Pim inhibitory and reducing activity such as SMI-16a and SMI-4a.

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**Author contributions**

S.F., S.N. and M.A. designed the research and conceived the project. S.F., S.N. A.O., H.M., H.T., J.T., M.H., Y.M., M.O., M.T., M.I., I.E., S.Y., K.A., K.K., T.H., K.K., performed the experiments. S.F., S.N., M.H., M.N., S.S. and M.A. analysed the data.

**Conflicts of interest disclosures**

The authors declare no competing financial interests related to this work.
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Figure legends

Figure 1. SMI-16a and SMI-4a induce MM cell death preferentially in acidic conditions. (A) Pim-1 and Pim-2 at protein levels in MM cells. The MM cell lines RPMI8226, INA-6, KMS11, OPM2 and U266 were cultured for 24 hours alone or cocultured with primary bone marrow stromal cells (BMSCs). Pim-1 and Pim-2 protein expression was analyzed in MM cells by Western blot analysis. β-actin was blotted as a loading control. (B, C) Viability of MM cells by Pim inhibitors. RPMI8226, KMS11 and INA-6 MM cell lines were cultured in triplicate at pH 7.4, pH 6.8 or pH 6.4. Lactic acid was added to adjust pH of media to the indicated values. SMI-16a or SMI-4a (B), or AZD1208, CX-6258 or PIM447 (C) were added at the indicated conditions. Cell viability was determined by a WST-8 assay after culturing for 2 days. Results are expressed as an optical density (means +/- SD).

Figure 2. SMI-16a and SMI-4a reduce Pim-2 protein levels in MM cells. RPMI8226, KMS11 and INA-6 MM cell lines were cultured at pH 7.4, pH 6.8 or pH 6.4. SMI-16a or SMI-4a were added at the indicated conditions. (A) Pim-2 protein levels were examined in the MM cells after culturing for 24 hours by Western blot analysis. β-actin was blotted as a loading control. (B) After culturing for 6 hours, PIM2 mRNA expression was analyzed by RT-PCR. GAPDH was used as an internal control. (C) RPMI8226 cells were cultured alone or cocultured with primary bone marrow stromal cells (BMSC). SMI-16a SMI-4a, AZD1208, CX-6258 and PIM447 were added at the indicated concentrations. After culturing for 24 hours, Pim-2 protein expression was analyzed in MM cells by Western blot analysis. β-actin was blotted as a loading control.

Figure 3. SMI-16a and SMI-4a did not affect Pim-2 protein degradation. (A) RPMI8226 and KMS11 cells were cultured in the presence or absence of SMI-16a SMI-4a, AZD1208, CX-6258 or PIM447 at the indicated concentrations. After culturing for 6 hours, PIM2 mRNA expression was analyzed by RT-PCR. GAPDH was used as an internal control. (B) RPMI8226 and KMS11 cells were cultured in the presence or absence of cycloheximide for the indicated periods of time. SMI-16a or SMI-4a at 50 µmol/L were added as indicated. The cells were harvested, and Pim-2 protein levels were examined in the MM cells by Western blot analysis. β-actin was blotted as a
loading control. (B) Puromycin incorporation. RPMI8226 and INA-6 cells were cultured alone or in the presence of 50 µmol/L SMI-16a or SMI-4a for 24 hours. Puromycin was added at 1 µmol/L for the last 15 minutes. The cells were harvested, and their puromycin incorporation was examined by Western blot analysis. β-actin was blotted as a loading control.

Figure 4. Drug retention in MM cells. (A) Surface expression of BCRP. Surface expression of the ABC transporters BCRP, P-glycoprotein and MRP1 was analyzed by flow cytometry in RPMI8226 and U266 cells (black line). Gray areas indicate background staining with isotype controls. (B) RPMI8226 and U266 cells were cultured for 24 hours alone or cocultured with primary bone marrow stromal cells (BMSC). Mitoxantrone was added at 100 ng/mL and incubated for 30 minutes, and then intracellular fluorescence levels were analyzed by flow cytometry (accumulation phase, AP), shown in gray. The MM cells with mitoxantrone incorporation were washed and further incubated in medium at the indicated pH values without mitoxantrone in the presence (red line) or absence (blue line) of 50 µmol/L SMI-16a. After incubating for 6 hours, intracellular fluorescence levels in the MM cells were analyzed (efflux phase, EP). (C) RPMI8226 cells were cultured in triplicate at pH 7.4 or pH 6.8. Doxorubicin was added at 1 µmol/L (gray) or 3 µmol/L (black) in the presence or absence of SMI-16a at 50 µmol/L as indicated. After culturing for 24 hours, cell viability was determined by WST-8 assay. Results are expressed as % change from the baseline with the mean +/- SD.

Figure 5. Targeting of SP and clonogenic MM cells. (A) RPMI8226 and KMS11 cells were cultured for 24 hours at pH 7.4 or pH 6.8 in the presence or absence of 50 µmol/L SMI-16a, and then the cells were stained with Hoechst 33342. Verapamil was added to determine SP cells, and distribution areas of SP cells were indicated. (B) RPMI8226 were cultured for 24 hours at pH 7.4 or pH 6.8 in the presence or absence of 50 µmol/L SMI-16a, then the cells were washed and cultured in semi-solid methylcellulose medium. Colony numbers were counted at 2 weeks. Data were expressed as mean ± SD. *p <0.05. (C, D) We cultured mouse 5TGM1 MM cells for 24 hours in the presence or absence of 50 µmol/L SMI-16a, then the cells were washed and viable cell numbers
were counted. The remaining living cells (2x10^5 cells) were directly inoculated to the tibia of ICR nu/nu mice (SMI-16a-treated and control cells to 4 and 3 mice, respectively). Tumor sizes were measured over time after detecting palpable tumors (C). * indicates the significant difference from the controls (p <0.05). To further estimate tumor growth, mouse sera were taken at 21 day after the inoculation, and 5TGM1 MM cell-derived mouse IgG2b protein levels in the mouse sera were measured (D). Data were expressed as mean ± SD. *p <0.05.

**Figure 6. SMI-16a mitigates Pim-2 accumulation in MM cells by proteasome inhibitors.** (A) KMS11 cells were cultured alone or with the indicated anti-MM agents. Bortezomib (Bor) and carfilzomib (Carfil) were added at the indicated concentrations. Lenalidomide (LEN) and pomalidomide (POM) were added at 1 μM. After culturing for 6 hours, Pim-2 protein and PIM2 mRNA levels were examined in the MM cells by Western blot analysis and by RT-PCR, respectively. β-actin was blotted as a loading control for the immunoblotting. GAPDH was used as an internal control in RT-PCR. (B) RPMI8226 and KMS11 cells were cultured for 24 hours in the presence or absence of 10 ng/mL IL-6. Bortezomib (Bor) and carfilzomib (Carfil) were added at the indicated concentrations. SMI-16a was added at 50 μmol/L as indicated. Pim-2 protein levels were examined in the MM cells by Western blot analysis. (C) RPMI8226 and KMS11 cells were cultured in triplicate for 48 hours and their viability was determined by WST-8 assay. Carfilzomib (Carfil) was added at 10 and 20 μmol/L for RPMI8226 and KMS11 cells, respectively. SMI-16a was added at 25 μmol/L. Cell viability was determined by a WST-8 assay after culturing for 2 days. Results are expressed as an optical density (means +/- SD).
Figure 1

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(B) 

- **SMI-16a**
  - RPMI8226
  - KMS11
  - INA6

- **SMI-4a**
  - RPMI8226
  - KMS11
  - INA6

(C) 

- **AZD1208**
  - RPMI8226
  - KMS11
  - INA6

- **CX6285**
  - RPMI8226
  - KMS11
  - INA6

- **PIM447**
  - RPMI8226
  - KMS11
  - INA6
Figure 2
### Figure 3

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β-actin
Figure 4

(A) Flow cytometry analysis of BCRP, P-glycoprotein, and MRP1 expression in RPMI8226 and U266 cells. RPMI8226 and U266 cells were stained with SMI-16a and analyzed by flow cytometry.

(B) pH-dependent expression of BCRP and P-glycoprotein in RPMI8226 and U266 cells with and without BMSC. Cells were exposed to pH values of 7.4, 6.8, 6.4, and 6.0.

(C) Viable cell counts of RPMI8226 and U266 cells after treatment with doxorubicin and SMI-16a at different pH levels (7.4 and 6.8). Bars represent the percentage of viable cells compared to baseline.
(A) RPMI8226

(-) SMI-16a

KMS11

(-) SMI-16a

pH 7.4

SP: 5.86 %

SP: 1.70 %

pH 6.8

SP: 4.78 %

SP: 0.52 %

SP: 2.47 %

SP: 0.86 %

SP: 3.64 %

SP: 0.57 %

(B) Colony numbers

RPMI8226

Colony numbers (per well)

SMI-16a

SMI-16a

pH 7.4

pH 6.8

(C) Control

SMI-16a

(D) Tumor volume (mm$^3$)

Serum IgG2b (ng/ml)

Control

SMI-16a

day0

day21

Figure 5
Figure 6

(A) Western blot analysis of Pim-2 and β-actin expression in RPMI8226 and KMS11 cells treated with different concentrations of Bor, Carfil, and POM, as well as LEN. PIM2 and GAPDH expressions are also shown.

(B) Immunoblot analysis of Pim-2 and β-actin expression in RPMI8226 and KMS11 cells treated with varying concentrations of Bor and SMI-16a, and further treated with IL-6.

(C) Graph showing the viability of RPMI8226 and KMS11 cells treated with Carfil, SMI-16a, and their combinations.