FULL PAPER *Pharmacology*

A Potential Therapeutic Application of SET/I2PP2A Inhibitor OP449 for Canine T-cell Lymphoma

Nobuyuki FUJIWARA[1)], Hideyoshi KAWASAKI[1)], Ryotaro YABE[1)], Dale J. CHRISTENSEN[2,3)], Michael P. VITEK[4)], Takuya MIZUNO[5)], Koichi SATO[1)] and Takashi OHAMA[1)]*

[1)]*Laboratory of Veterinary Pharmacology, Joint Faculty of Veterinary Medicine, Yamaguchi University, Japan*
[2)]*Department of Medicine (Hematology), Duke University, Durham, NC, U.S.A.*
[3)]*Oncotide Pharmaceuticals, Inc., Research Triangle Park, NC, U.S.A.*
[4)]*Departments of Medicine (Neurology) and Neurobiology, Duke University, Durham, NC, U.S.A.*
[5)]*Laboratory of Veterinary Internal Medicine, Joint Faculty of Veterinary Medicine, Yamaguchi University, Japan*

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ABSTRACT. Lymphoma is one of the most common malignant tumors in canine. Chemotherapy results in a high rate of remission; however, relapse and clinical drug resistance are usually seen within a year. Protein phosphatase 2A (PP2A) acts as a tumor suppressor and plays a critical role in mammalian cell transformation. Increased protein levels of SET, endogenous PP2A inhibitor, have been reported to correlate with poor prognosis in human leukemia. Here, we test the potential therapeutic role for a SET antagonist in canine lymphoma. We observed SET protein levels increased in multiple canine lymphoma cell lines compared with primary peripheral blood cells. A novel SET antagonist OP449 increased PP2A activity and effectively killed SET high-expressing canine lymphoma cells, but not SET low-expressing cells. Caspase-3 activation and enhanced Annexin V positive staining were observed after OP449 treatment, suggesting apoptotic cell death by OP449. Consistent with this, pan-caspase inhibitor Z-VAD-FMK blocked OP449 induced cell death. These data demonstrated the potential therapeutic application of SET antagonists for canine lymphoma.

KEY WORDS: apoptosis, canine lymphoma, OP449, protein phosphatase 2A, SET.

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The *set* gene was originally discovered as a component of the *set-can* fusion gene produced by a somatic translocation in a case of acute undifferentiated leukemia [26]. It has been reported that SET positively regulates multiple oncogenic pathways and SET protein levels are increased in various human tumors, including chronic myeloid leukemia, acute myeloid leukemia, B-cell non-Hodgkin lymphoma, choriocarcinoma and Wilms' tumor [4, 6, 7, 20, 27, 29]. Furthermore, elevated SET levels have been demonstrated to correlate with more aggressive disease in chronic lymphocytic leukemia and acute myeloid leukemia [7, 10].

SET, also known as I2PP2A, is a potent physiologic inhibitor of protein phosphatase 2A (PP2A). PP2A is a major protein serine/threonine phosphatase in cells and regulates wide range of biological processes including cell proliferation, apoptosis, development and motility [13]. Loss or inhibition of the PP2A has revealed a critical tumor suppressor function for PP2A [2]. PP2A exists as a heterotrimer with two common components, a catalytic subunit (PP2Ac) and a scaffolding subunit (PP2A A) forming the catalytic core dimer, with which one regulatory B subunit from four different families of genes. SET protein directly binds with PP2Ac through its both N-terminus and C-terminus regions, and inhibits PP2A phosphatase activity [1].

Peptides antagonist of SET has been reported, and COG133 was first developed as an anti-inflammatory and neuroprotective agent, which derived from amino acids 133–149 located in the receptor binding region of apolipoprotein E (apoE) based on the anti-inflammatory properties of apoE [16, 23]. COG112 is the fusion of COG133 with protein transduction domain derived from the *Drosophila* antennapedia which enhances the bioactivity of COG133 [17]. OP449 was recently created as a dimerised derivative of COG112 [7]. ApoE and COG peptides directly bind to SET and antagonize its function [7, 8, 24]. Recent reports showed that COG peptides enhance PP2A activity and induce apoptosis of human B-cell non-Hodgkin lymphoma and human B-cell chronic lymphocytic leukemia [7].

Lymphoma is one of the most common malignant tumors in canine. In spite of various therapeutic strategies, dogs with lymphoma have poor prognosis. Median duration of remission with CHOP-based chemotherapy is about 12 months [3, 12, 14]. Therefore, novel therapeutic targets must be identified. Here, we show the increased protein level of the SET in the various canine lymphoma cell lines and the specific anti-tumor effects of OP449 on canine lymphoma cells that are high SET expressing cells. Our data demonstrate the potential clinical application of SET antagonists for canine lymphoma.

MATERIALS AND METHODS

Cell culture: Canine lymphoma cell lines were kindly

*CORRESPONDENCE TO: OHAMA, T., Laboratory of Veterinary Pharmacology, Joint Faculty of Veterinary Medicine, Yamaguchi University, 1677–1 Yoshida, Yamaguchi 753–8515, Japan.
e-mail: t.ohama@yamaguchi-u.ac.jp

provided by Dr. Hajime Tsujimoto (CL-1 and UL-1), Dr. Munekazu Nakaichi (GL-1), Dr. Yasuhiko Okamura (Nody-1), Dr. Steven Suter (17–71), Dr. Barbara Ruetgen (CLBL-1) and Dr. Wellman Maxey (CLGL-90). Ema cells were previously established [25]. All cells were grown in RPMI1640 containing 10% FBS and 1 × anti-biotic/anti-mycotic (Life Technologies, Carlsbad, CA, U.S.A.).

Trypan blue exclusion assay: 5×10^3 of UL-1 cells and 1×10^4 of Ema cells were seeded into 96-well plates and treated with OP449 for 72 hr. Trypan blue solution was added to a 20 μl cell suspension in an equal volume, and survived cells were counted by microscopy.

PP2A activity assay: Ema cells were treated with OP449 (1 μM) for 2 hr and lysed in a buffer containing 50 mM MOPS (pH7.4), 0.1% NP-40, 0.1 mM EGTA and Roche's complete protease inhibitor cocktail (Roche, Indianapolis, IN, U.S.A.). Cell lysates were treated with or without type 2A phosphatase inhibitor okadaic acid (10 nM, LC Laboratories, Woburn, MA, U.S.A.) for 5 min, and phosphatase reaction was performed in a reaction buffer containing 50 mM MOPS (pH7.4), 24 mM $MgCl_2$, 2 mM $MnCl_2$, 0.03% 2-mercaptoethanol, 2.9% glycerol and 0.2 mM phospho-peptides (K-R-pT-I-R-R, Millipore, Billerica, MA, U.S.A.) for 20 min at room temperature. Reaction was stopped by adding 60% $HClO_4$ solution, and concentration of phosphate was analyzed by Malachite Green Assay as previously described elsewhere [31] with slight modification. PP2A phosphatase activity was calculated by subtracting 10 nM okadaic acid treated samples.

Immunoblotting: Immunoblotting was performed as previously described [21]. Briefly, cells were lysed in a buffer containing 50 mM Tris-HCl (pH 8.0), 5 mM EDTA (pH 8.0), 5 mM EGTA, 1% Triton X100, 1 mM Na_3VO_4, 20 mM Sodium Pyrophosphate and Roche's complete protease inhibitor cocktail. Proteins were separated by SDS-PAGE and transferred onto PVDF membrane (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Membranes were blocked with 0.5% skim milk and treated with primary antibodies: anti-SET (abcam, Cambridge, UK), anti-PP2A C subunit (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), anti-PP2A A subunit (Santa Cruz Biotechnology), anti-tublin (Thermo Scientific, Woburn, MA, U.S.A.) and anti-caspase3 (Cell Signaling Technology, Beverly, MA, U.S.A.). Bands were detected using ECL Western Blotting Detection System (GE Healthcare, Freiburg, Germany) and visualized using LAS-3000 (Fujifilm, Tokyo, Japan). Band densities were quantified using ImageJ densitometry analysis software (National Institutes of Health, Bethesda, MD, U.S.A.).

Flow cytometry: Ema cells were treated with OP449 (1 μM) for 8 hr, and apoptosis was examined by using the Annexin V-FITC Apoptosis Detection Kit (Bio Vision, Milpitas, CA, U.S.A.) according to manufacturer's protocol. The fluorescence intensity of 3,000 cells was measured using a CyFlow space flow cytometer (Partec, Munster, Germany)

Statistical analysis: The results are expressed as the means ± S.E. Comparisons between the groups were performed by one-way analysis of variance, followed by Student-Newman-Keuls test. For all of the analyses, a probability value of $P<0.05$ was considered to indicate statistical significance.

RESULTS

Although SET protein was reported to increase in human primary B-cell chronic lymphocytic leukemia and B-cell non-Hodgkin lymphoma (NHL) cell line cells [7, 20], it has not been analyzed in canine lymphoma. We first examined SET protein level in various canine lymphoma cell lines; 6 T-cell lymphoma cell lines (CL-1, CLGL-90, Ema, GL-1, Nody-1 and UL-1) and 2 B-cell lymphoma cell lines (17–71 and CLBL-1). SET band densities were normalized to canine peripheral blood mononuclear cells (PBMC) as 100%. The SET protein was overexpressed in 7 out of 8 cell lines at>1.5-fold compared with PBMC. Notably, the Ema cells expressed nearly 5 times the level of the PBMC (Fig. 1). On the other hand, there are few differences for protein levels of PP2Ac and A subunit, two components of PP2A holoenzyme. For further experiments, we used Ema and UL-1 cells as SET high- and low-expressing lymphoma cells, respectively.

Sequence for human SET protein (Accession number NM_001122821) and canine SET protein (Accession number XM_846114) were aligned using the Clustal X2. Alignment of the protein sequence of human and canine SET showed about 96% homology between 2 species with relatively low homology in N-terminus region, and 100% conservation in the C-terminal region (Fig. 2). Because COG/OP449 peptides (Oncotide Pharmaceuticals, Chapel Hill NC, U.S.A.) bind to C-terminal region of SET [8], we anticipated that OP449 would antagonize canine SET functions. We examined the anti-tumor effects of OP449 on canine lymphoma cell lines. Ema and UL-1 cells were treated with OP449 (0.1, 1, 10 μM) for 72 hr, and cell viability was assessed by trypan blue exclusion assay (Fig. 3A). Low dose of OP449 (0.1 and 1 μM) specifically killed Ema cells, suggesting beneficial effects of OP449 for SET high-expressing canine lymphoma. Meanwhile, high dose of OP449 (10 μM) killed both Ema and UL-1 cells, probably because of the non-specific cytotoxic effects. Additionally, we confirmed 1 μM of OP449 did not kill primary canine PBMCs (Fig. 3B).

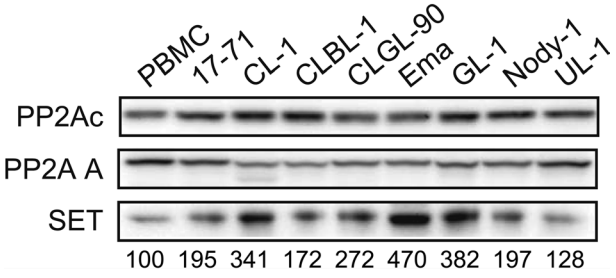

Fig. 1. Comparison of SET protein levels in various canine lymphoma cell lines.
Protein levels of SET, PP2Ac and PP2A A in various canine lymphoma cell lines were determined by immunoblotting. SET band densities were normalized to peripheral blood mononuclear cells (PBMC) as 100%.

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          *:*****.*.**.**** ***  *****:*  *** ******************************************
Human     MAPKRQSPLPPQKKKPRPPPALGPEETSASAGLPKKGEKEQQEAIEHIDEVQNEIDRLNEQASEEILKVEQKYNKLRQPF  80
Canine    MTPKRQSSLAPQTKKPRLPPAPKPEETSTSQHLPK-GEKEQQEAIEHIDEVQNEIDRLNEQASEEILKVEQKYNKLRQPF  79
          1.......10........20........30........40........50........60........70........80

          ********************************************************************************
Human     FQKRSELIAKIPNFWVTTFVNHPQVSALLGEEDEEALHYLTRVEVTEFEDIKSGYRIDFYFDENPYFENKVLSKEFHLNE  160
Canine    FQKRSELIAKIPNFWVTTFVNHPQVSALLGEEDEEALHYLTRVEVTEFEDIKSGYRIDFYFDENPYFENKVLSKEFHLNE  159
          ........90.......100.......110.......120.......130.......140.......150.......160

          ********************************************************************************
Human     SGDPSSKSTEIKWKSGKDLTKRSSQTQNKASRKRQHEEPESFFTWFTDHSDAGADELGEVIKDDIWPNPLQYYLVPDMDD  240
Canine    SGDPSSKSTEIKWKSGKDLTKRSSQTQNKASRKRQHEEPESFFTWFTDHSDAGADELGEVIKDDIWPNPLQYYLVPDMDD  239
          .......170.......180.......190.......200.......210.......220.......230.......240

          ************************************************
Human     EEGEGEEDDDDDEEEEGLEDIDEEGDEDEGEEDEDDDEGEEGEEDEGEDD  290
Canine    EEGEGEEDDDDDEEEEGLEDIDEEGDEDEGEEDEDDDEGEEGEEDEGEDD  289
          .......250.......260.......270.......280.......290
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Fig. 2. Protein sequences alignment of human and canine SET.
Sequences for human SET (Accession number NM_001122821) and canine SET (Accession number XM_846114) were aligned using the Clustal X2. "*", ":", and "." indicate "full", "strong" and "weak" conserved residues, respectively.

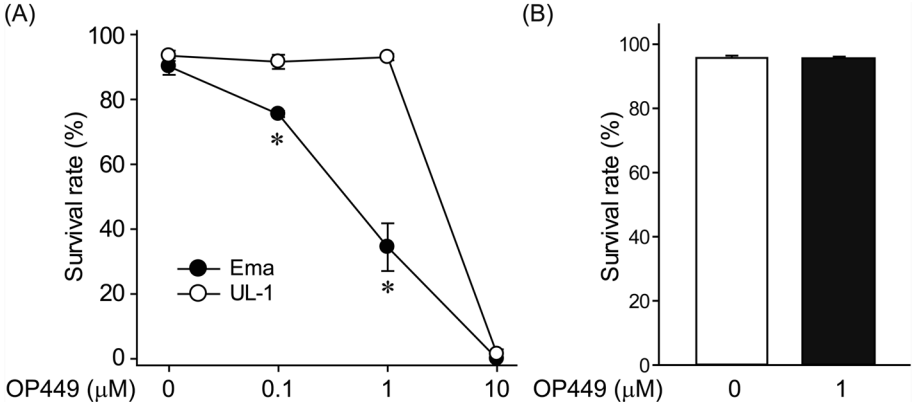

Fig. 3. OP449 induces cell death in SET high-expressing canine lymphoma cells.
Ema and UL-1 cells (A) and primary canine PBMCs (B) were treated with indicated concentrations of OP449 for 72 hr. Cell viability was assessed by trypan blue exclusion assay. Quantitative data from 3 independent experiments performed in duplicate are shown. *P<0.05 vs. UL-1.

We examined whether OP449 restored PP2A activity in Ema cells. Phosphatase activity in extracts of cells treated with or without 1 μM of OP449 for 2 hr was assayed with a relatively specific phosphopeptide (K-R-pT-I-R-R) as a substrate. As shown in Fig. 4, OP449 increased PP2A activity about 1.5 fold suggesting OP449 effectively antagonizes canine SET.

We further clarified the type of cell death induced by OP449 (1 μM) in Ema cells. The increase in the active caspase-3, a key event for apoptosis, was observed in OP449-treated cells by immunoblotting (Fig. 5A). Moreover, FACS analysis revealed that OP449 increased annexin V positive cells (Fig. 5B). We did not observe increased propidium iodide positive cells by OP449 treatment (data not shown). These data suggest that OP449 induces cell death through apoptotic signaling in SET high-expressing cells. Finally, we examined whether apoptosis inhibitor blocks the cell death induced by OP449. A pan-caspase inhibitor Z-VAD-FMK, significantly, but not completely, restored the cell survival rate (Fig. 6), indicating at least part of the cytotoxic effect of OP449 is exerted through apoptosis.

DISCUSSION

In this study, we tested the potential therapeutic role for a SET antagonist in canine lymphoma, the most common haematopoietic neoplasm in dogs. Standard therapeutic approaches are based on CHOP therapy, a combination of cyclophosphamide, doxorubicin, vincristine and prednisone. B-cell lymphoma occurs more often than T-cell lymphoma in canine, but T-cell lymphoma has a worse prognosis, mostly because of the resistance to conventional chemo-

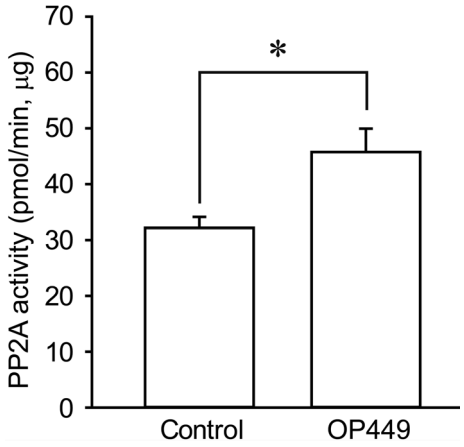

Fig. 4. OP449 increases PP2A activity in Ema cells.
Ema cells were treated with OP449 (1 μM) for 2 hr, and PP2A activity was assayed. Quantitative data from 3 independent experiments performed in duplicate are shown. *P<0.05.

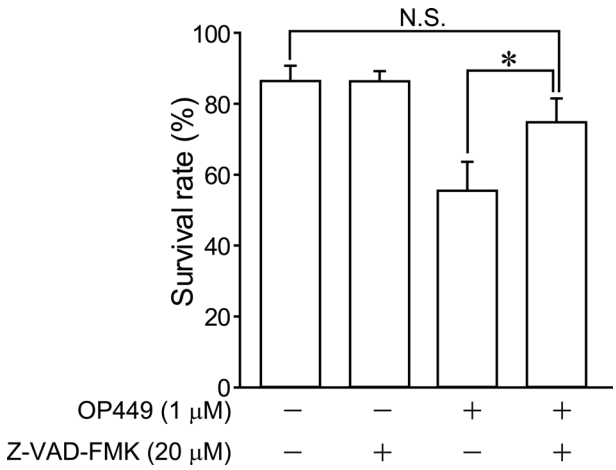

Fig. 6. Pan-caspase inhibitor suppresses OP449-induced cell death.
Ema cells were treated with a pan-caspase inhibitor Z-VAD-FMK (20 μM) for 1 hr before treating with OP449 (1 μM) for 72 hr. Cell viability was assessed by trypan blue exclusion assay. Quantitative data from 3 independent experiments performed in duplicate are shown. *P<0.05.

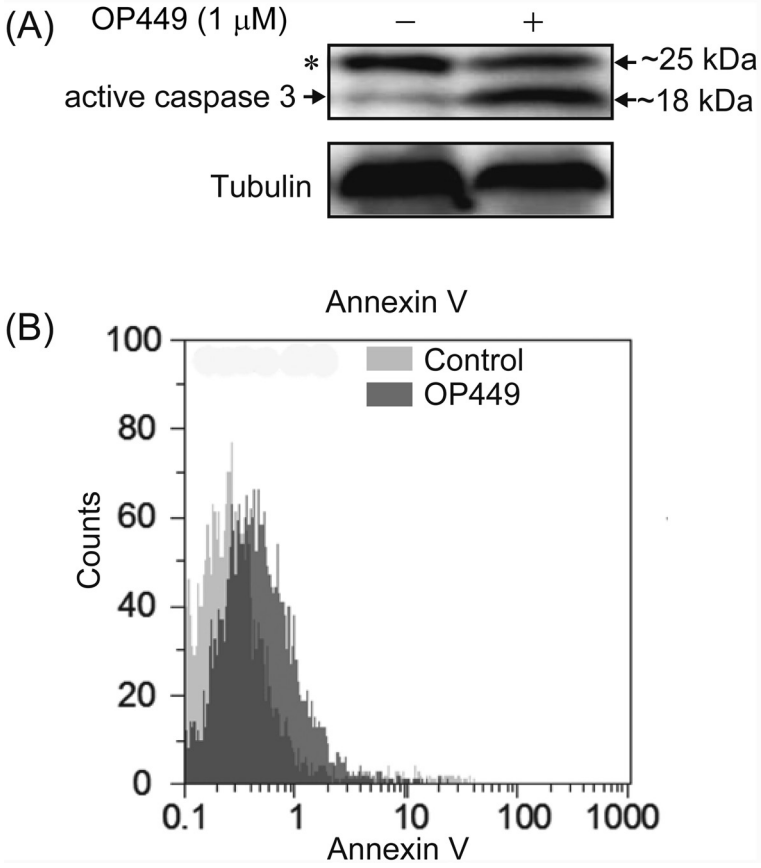

Fig. 5. OP449 treatment induces apoptosis in Ema cells.
Ema cells were treated with OP449 (1 μM) for 8 hr. (A) Levels of active caspase-3 were determined by immunoblotting. *:non-specific bands. (B) Annexin V positive cells are counted by flow cytometry. Representative images from two independent experiments are shown.

therapy [19]. For B-cell lymphoma, chemotherapy usually obtains 80–90% complete response rate [12, 14], however, relapse and clinical drug resistance are usually seen within a year. Therefore, a novel target of anticancer chemotherapy is required. In this study, we examined SET protein level in canine lymphoma cell lines and observed 7 out of 8 cell lines expressed>1.5 fold of SET proteins compared with PBMC. The SET specific antagonist OP449 selectively induced apoptosis for SET high-expressing canine lymphoma cells. We propose that SET antagonists represent an attractive approach to treatment of canine lymphoma and that OP449 may be an effective anticancer drug to treat these animals.

We demonstrated that SET antagonist activates apoptotic signaling in canine T-cell lymphoma cell line. However, it has not been completely clear how OP449 induces apoptosis. Because we observed the increased PP2A activity in OP449 treated cells, it is possible that restored PP2A activity plays an important role in OP449 induced apoptosis. PP2A is a critical tumor suppressor which regulates wide range of biological processes. PP2A exists as heterotrimeric complexes, each of which consists of a catalytic PP2Ac, a scaffolding PP2A A subunit, and one of the regulatory B subunit from 4 different families of genes, which controls PP2A specificity by targeting AC core dimer to substrates. Because releasing of SET from PP2Ac does not affect the subsequent PP2A complex formation [24], it is highly possible that OP449 increases overall active PP2A holoenzyme rather than complexes with specific regulatory B subunit. In this scenario, OP449 affects multiple pathways that are regulated by PP2A. One of the targets of PP2A is Akt, a serine/threonine kinase which has been shown to be a central node in a number of tumor-promoting pathways, and frequently deregulated in human cancers [5, 15, 18, 28]. PP2A directly dephosphorylates Akt at Thr308 and Ser473 which inhibits Akt activity and results in the inhibition of the pro-survival pathways and the overall growth retardation [15]. Therefore, it is possible that Akt pathway inhibition through PP2A activation is involved in the beneficial effects of OP449. This is supported by the demonstrated inhibition of Akt phosphorylation by a COG/OP449-like peptide [24].

Our data which showed a pan-caspase inhibitor significantly, but not completely block the cell death induced by OP449 raises the question of how OP449 kills cells. SET has also been described as an inhibitor for NM23-H1, a multifunctional protein which possesses 3 enzymatic activities, that is a 3′-5′ exonuclease, a nucleoside diphosphate kinase and a protein histidine kinase [30]. SET directly binds to NM23-H1 and inhibits its exonuclease activity [11, 31]. When cells are attacked by cytotoxic T lymphocytes, NM23-H1 is released from inhibition by granzyme A cleavage of SET, which leads to caspase-independent cell death that is characterized by single-stranded DNA nicks and other features of apoptosis [11]. Therefore, it is possible that part of the cell death induced by OP449 is through this caspase-independent pathway. Indeed, treatment of cancer cells with a COG/OP449 derivative demonstrated release of NM23-H1 from the SET complex, increased nuclear localization of NM23-H1 and enhanced exonuclease activity [24]. This suggests that multiple SET-associated pathways linked to cancer are antagonized by OP449 and could contribute to the anti-tumor activity.

Although SET protein levels in lymphoma cell lines are increased compared with PBMC, little is known about the regulatory mechanism of SET expression. In human chronic myeloid leukemia, Jak2, downstream target of Bcr-Abl, increases SET protein level [22]. Jak2 inhibition or knockdown reduces SET protein and increases PP2A activity. On the other hand, the potent immunosuppressive drug FTY720/fingolimod, a sphingosine analog, suppresses SET expression which leads to PP2A activation and caspase dependent apoptosis [9, 29]. Because FTY720 activates S1P1 signaling, downstream effectors of S1P1 such as PI3K/Akt, PLC/PKC or Raf/ERK signaling may suppress SET expression.

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