FULL PAPER *Pharmacology*

Anti-tumor effects of perphenazine on canine lymphoma

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ABSTRACT. Lymphoma is one of the most common malignant tumors in canine. Protein phosphatase 2A (PP2A), a well-conserved serine/threonine phosphatase, plays a critical role as a tumor suppressor. Perphenazine (PPZ) is one of the phenothiazines and widely used as an antipsychotic drug. Recently, it is reported that PPZ directly binds with scaffolding subunit of PP2A complex and exerts anti-tumor effects on human T cell acute lymphoblastic leukemia. However, the effect of PPZ on canine lymphoma has not been studied. Here, we investigated the potential therapeutic role of PPZ and its molecular mechanism in canine T-cell lymphoma. In canine T-cell lymphoma cell lines, UL-1 and Ema, PPZ decreased cell survival in a dose-dependent manner. Increased caspase 3 activity and Annexin V positive cells suggested that PPZ induced apoptosis. PPZ dephosphorylated Akt, MEK1/2 and ERK1/2. Akt inhibitor, but not MEK1/2 inhibitor and ERK1/2 inhibitor, induced cell death, indicating the importance of Akt dephosphorylation for the anti-tumor effect of PPZ. Finally, we observed enhanced PP2A activity by PPZ treatment. The present results for the first time revealed that PPZ induced canine lymphoma cells apoptosis through Akt dephosphorylation via PP2A activation. Our study suggests the possible therapeutic application of phenothiazines for canine T-cell lymphoma.

KEY WORDS: canine, lymphoma, perphenazine, PP2A

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Lymphoma accounts for about 20% of all canine cancers and about 85% of blood cancers [18]. Valli *et al*. divided canine lymphoma into 7 categories; (1) benighn hyperplasia; (2) low-grade B-cell; high-grade B- and T-cell; (3) high-grade B- and T-cell; (4) low-grade T-cell; (5) centroblastic large B-cell; (6) immunoblastic large B-cell and (7) high-grade peripheral T-cell. Amonge them, category 5 was the most populous, and categories 3 and 7 had poor prognosis [22]. In spite of various therapeutic strategies, dogs with lymphoma have poor prognosis. Average duration of remission with CHOP-based chemotherapy (combination of cyclophosphamide, doxorubicin, vincristine and prednisolone) is 26–51 months [9, 16]. Therefore, the identification of novel therapeutic targets is required to improve treatment outcomes.

Protein phosphatase 2A (PP2A) is a highly conserved serine/threonine phosphatase and an important tumor suppressor [19]. PP2A inhibits the tumorigenic potential, such as disordered cell proliferation and apoptosis, by suppressing tumor-promoting signals, including ERK1/2 and Akt/PKB signaling [17]. 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 [2]. In tumor cells, PP2A activity is suppressed by increased levels of PP2A inhibitory proteins, such as SET, CIP2A and PME-1 and/or Tyr307 phosphorylation of PP2Ac [19]. We previously reported that SET inhibitors activate PP2A and exert anti-tumor effects on canine T-cell lymphoma and melanoma [6, 7].

Perphenazine (PPZ) is a typical antipsychotic drug that is classified as a piperazinyl phenothiazine. In veterinary medicine, phenothiazines are widely used as tranquilizers [20]. Recently, it was reported that phenothiazines, including PPZ, have anti-tumor effects on human T cell acute lymphoblastic leukemia [10]. PPZ directly binds to PP2A A subunit and induces ERK1/2 and Akt dephosphorylation. Here, we show that PPZ exerts anti-tumor effects on canine lymphoma cell lines. We observed that PPZ treatment activates PP2A and dephosphorylates Akt, MEK1/2 and ERK1/2. Our data demonstrate the potential clinical application of phenothiazines for canine lymphoma.

MATERIALS AND METHODS

Cell culture: Canine T-cell lymphoma cell line (UL-1 and Ema) and canine peripheral blood mononuclear cells (PBMCs) were cultured as previously described [7]. Cells were grown in RPMI1640 containing 10% FBS and 1 × anti-biotic/anti-mycotic (Life Technologies, Carlsbad, CA, U.S.A.).

Antibodies and Drugs: Antibodies were obtained from the indicated supplier: anti-total PP2Ac (Merck Millipore,

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Billerica, MA, U.S.A.), anti-pTyr307 PP2Ac (R&D systems, Minneapolis, MN, U.S.A.), p97/VCP (GeneTex, Irvine, CA, U.S.A.), anti-cleaved caspase 3 Asp175, anti-pThr202/Tyr204 ERK1/2, anti-pThr308 Akt, anti-pSer473 Akt, anti-pSer241 PKD1, anti-pThr346 NDRG1, anti-pSer217/221 MEK1/2, anti-pSer445 B-Raf, anti-Ser338 C-Raf anti-ERK1/2, anti-Akt (Cell Signaling, Beverly, MA, U.S.A.), anti-SET (Abcam, Cambridge, MA, U.S.A.), anti-CIP2A (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) and anti-PME-1 (LifeSpan Bio, Seatle, WA, U.S.A.). Drugs were obtained from the indicated supplier: Perphenazine, FR180204 (Sigma-Aldrich, St. Louis, MO, U.S.A.), Akt inhibitor VIII (Santa Cruz) and U0126 (LC Laboratories, Woburn, MA, U.S.A.).

Cell Viability Assay: 4.0×10^4 cells were seeded on 96-well plates, and after 3 days of drug treatments, Cell Counting Kit-8 (CCK8, Dojindo, Kumamoto, Japan) was used to analyze cell proliferation according to the manufacturer's instruction.

Immunoblotting: Immunoblotting was performed as previously described [24]. Briefly, cells were lysed in a buffer containing 50 mM Tris-HCl (pH 8.0), 5 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.0), 5 mM ethylene glycol tetraacetic acid (EGTA), 1% Triton X100, 1 mM Na_3VO_4, 20 mM sodium pyrophosphate and Roche's complete protease inhibitor cocktail. The proteins were separated by SDS-PAGE and transferred onto ClearTrans Nitrocellulose Membrane (Wako, Osaka, Japan). Membranes were blocked with 0.5% or 3% skim milk, and treated with primary antibodies diluting 1:1,000 in TBS containing 0.05% Tween 20 (TBS-T). After treating with secondary antibodies diluting to 1:10,000 in TBS-T, immunoreactive bands were detected using ECL Pro Western Blotting Detection Reagent (PerkinElmer, Waltham, MA, U.S.A.) and visualized using a LAS-3000 luminescent image analyzer (Fujifilm, Tokyo, Japan). VCP was used as a loading control.

Flow Cytometry: UL-1 cells were treated with PPZ (20 μM) for 12 hr, and apoptosis was examined by using Annexin V-FITC Apoptosis Detection Kit (Bio Vision, Milpitas, CA, U.S.A.) according to manufacturer's protocol. The fluorescence intensity of 10,000 cells was measured using a BD Accuri flow cytometer (BD, Franklin Lakes, NJ, U.S.A.)

PP2A activity assay: Immunoprecipitation based PP2A specific activity was analyzed by using Active PP2A Duo-Set IC kit (R&D systems) according to the manufacturer's instruction.

Statistical Analysis: The results are expressed as means ± S.E. Student's *t*-test was used for comparison between two groups. Groups more than three were compared using one-way analysis of variance, after which Fisher LSD test was used. For all analyses, a probability value of $P<0.05$ was considered statistically significant.

RESULTS

We examined anti-tumor effect of perphenazine (PPZ) on canine lymphoma cell lines. UL-1 and Ema cells were treated with PPZ (2.5–12.5 μM) for 72 hr, and cell viability was assessed by CCK-8 (Fig. 1A). PPZ dose-dependently decreased cell survival in both UL-1 and Ema cells. To confirm the specific cytotoxic effect of PPZ on lymphoma cells, primary canine PBMCs were treated with 10 μM of PPZ (Fig. 1B); it had no effects on PBMCs.

We clarified the type of cell death induced by PPZ in UL-1 cells. The increase in the cleaved (active) caspase-3, a key event for apoptosis, was observed in PPZ-treated cells by immunoblotting (Fig. 2A). Moreover, FACS analysis revealed that even higher dose of PPZ (20 μM) increased Annexin V positive cells (Fig. 2B). These data suggest that PPZ induces cell death at least partially through apoptotic signaling in canine lymphoma cells. We also observed PPZ increased the level of cleaved caspase-3 in Ema cells (Supplementary Fig. 1).

Immunoblotting was performed to clarify the cell signaling affected by PPZ treatment. We observed rapid dephosphorylation of Akt Thr308 and Ser473 by PPZ treatment (Fig. 3A–C). Thr308 and Ser473 of Akt are phosphorylated by PDK1 and mammalian target of rapamycin complex 2 (mTORC2), respectively [1]. Therefore, we examined the effects of PPZ on these upstream kinases. NDRG1 phosphorylation is mediated by mTORC2 and used as an indicator for mTORC2 activity [8, 25]. PPZ had no effect on the phosphorylation levels of PDK1 and NDRG1 (Fig. 3D–F), indicating PPZ dephosphorylates Akt without affecting

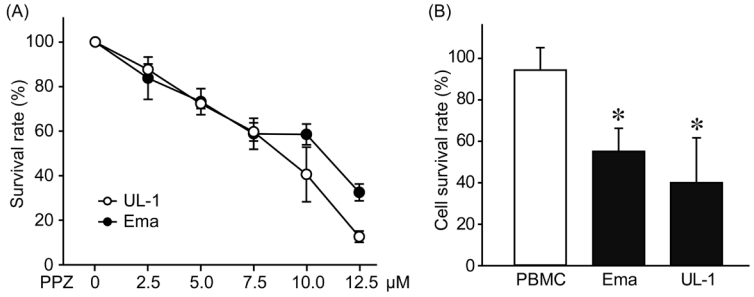

Fig. 1. Perphenazine induces cell death in canine lymphoma cells. (A) Ema and UL-1 cells were treated with indicated concentration of perphenazine (PPZ) for 72 hr. Cell viability was assessed by Cell Counting Kit-8. N=6. (B) Ema, UL-1 and primary canine PBMCs were treated with 10 μM of PPZ for 72 hr. Cell viability was assessed by Cell Counting Kit-8. N=4. *: $P<0.05$ vs. PBMC.

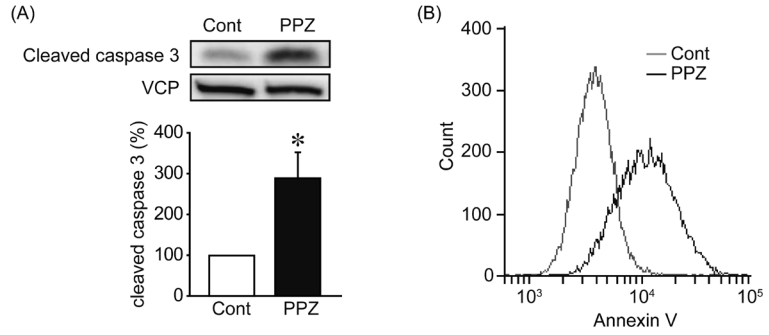

Fig. 2. Perphenazine induces apoptosis in canine lymphoma cells. UL-1 cells were treated with 20 μM of perphenazine (PPZ) for 12 hr. (A) Levels of active caspase 3 were determined by immunoblotting. Representative images and quantitative data from 3 independent experiments are shown. Cont: Control. *: P<0.05 vs. Cont. (B) Annexin V positive cells were counted by flow cytometry. Representative images from 2 independent experiments are shown.

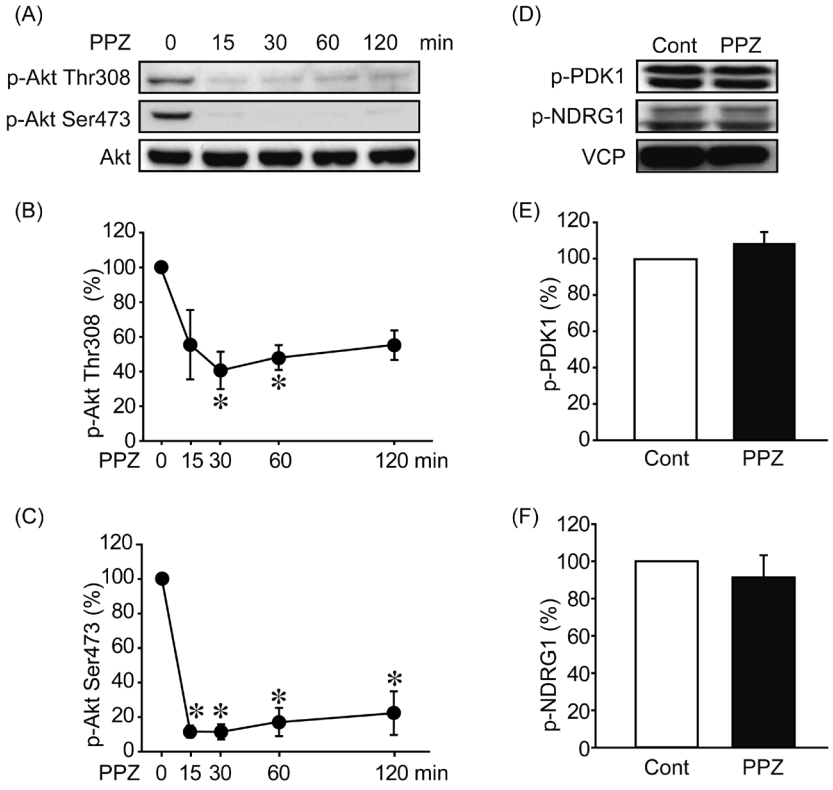

Fig. 3. Perphenazine induces dephosphorylation of Akt. (A-C) UL-1 cells were treated with 10 μM of perphenazine (PPZ) for indicated time period. Immunoblotting was performed using indicated antibodies to analyze the effect of PPZ on Akt phosphorylation. Representative images (A) and quantitative data for phospho-Thr308 (B) and phopho-Ser473 (C) of Akt from 3 independent experiments are shown. *: P<0.05 vs. 0 min. (D-F) UL-1 cells were treated with 10 μM of perphenazine (PPZ) for 15 min. Immunoblotting was performed using indicated antibodies to analyze the effect of PPZ on PDK1 and NDRG1 phosphorylation. Representative images (D) and quantitative data for phospho-PDK1 (E) and phopho-NDRG1 (F) from 3 independent experiments are shown. Cont: Control.

the upstream kinases. We also observed relatively gradual dephosphorylation of ERK1/2 (Fig. 4A and 4B). MEK1/2 is the responsible kinases for ERK1/2 phosphorylation, and B-Raf and C-Raf phosphorylate MEK1/2 [5]. PPZ decreased MEK1/2 phosphorylation level significantly but only slightly decreased B-Raf phosphorylation level, and did not affect C-Raf phosphorylation level (Fig. 4C–F). These data suggest that PPZ dephosphorylates MEK1/2

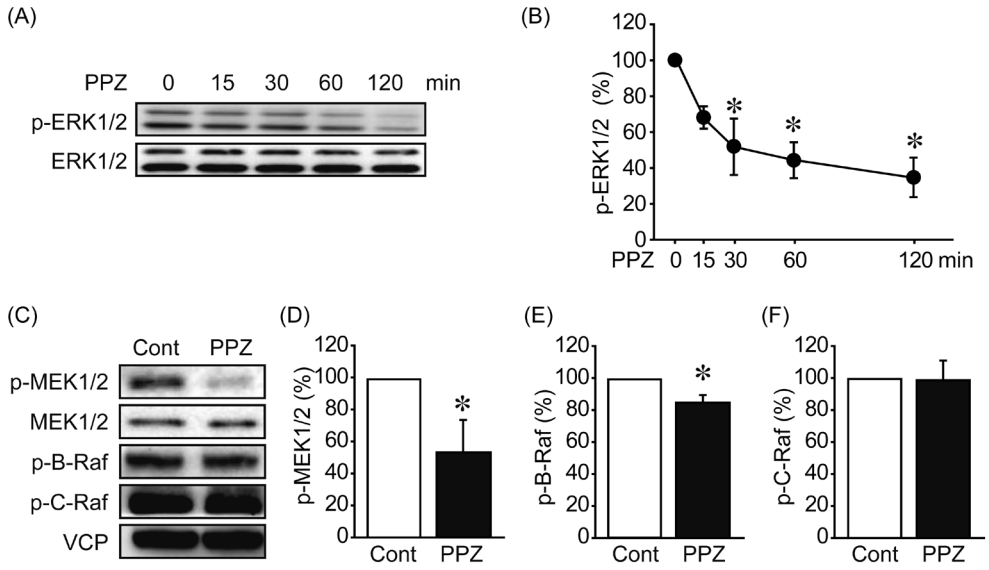

Fig. 4. Perphenazine induces dephosphorylation of MEK1/2 and ERK1/2. (A, B) UL-1 cells were treated with 10 μM of perphenazine (PPZ) for indicated time periods. Immunoblotting was performed using indicated antibodies to analyze the effect of PPZ on ERK1/2 phosphorylation. Representative images (A) and quantitative data from 3 independent experiments are shown. *: P<0.05 vs. 0 min. (C-F) UL-1 cells were treated with 10 μM of perphenazine (PPZ) for 15 min. Immunoblotting was performed using indicated antibodies to analyze the effect of PPZ on MEK1/2, B-Raf and C-Raf phosphorylation. Representative images (C) and quantitative data for phospho-MEK1/2 (D), phospho-B-Raf (E) and phospho-C-Raf (F) from 3 independent experiments are shown. Cont: Control. *: P<0.05 vs. Cont.

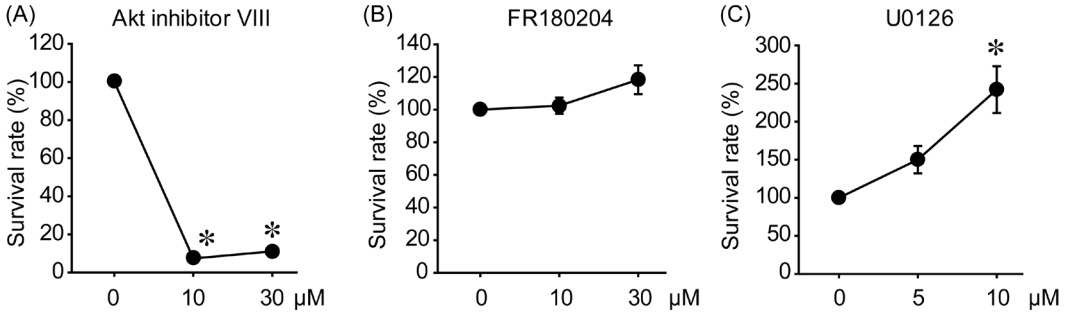

Fig. 5. Akt inhibitor induces cell death in canine lymphoma cells. UL-1 cells were treated with indicated concentration of Akt inhibitor VIII (A), FR180204 (B) and U0126 (C) for 72 hr. Cell viability was assessed by Cell Counting Kit-8. N=4. *: P<0.05 vs. 0 μM.

without significant effects on the upstream kinases, which leads to ERK1/2 dephosphorylation. We also observed PPZ decreased Akt and ERK1/2 phosphorylation levels in Ema cells (Supplementary Fig. 2).

PPZ inhibits both Akt and ERK1/2 signaling pathways, so we questioned which pathways are important for UL-1 survival. UL-1 cells were treated with Akt inhibitor (Akt inhibitor VIII, 10 and 30 μM), ERK1/2 inhibitor (FR180204, 10 and 30 μM) or MEK1/2 inhibitor (U0126, 5 and 10 μM) for 72 hr (Fig. 5A-C). Inhibition of Akt signaling drastically killed UL-1 cells. On the other hand, ERK1/2 inhibition did not affect cell survival, and MEK1/2 inhibition rather induced cell proliferation. These data indicate that PPZ exerts anti-tumor effects through inhibition of Akt signaling. We also observed that Akt inhibitor, but not FR180204 or U0126, drastically killed Ema cells (Supplementary Fig. 3).

We further examined whether PPZ restored PP2A activity. UL-1 cells were treated with PPZ, and PP2A was immunoprecipitated to analyze PP2A activity. We observed that PPZ slightly but significantly increased PP2A activity (Fig.6A). We also observed that PPZ increased PP2A activity in Ema cells (Supplementary Fig. 4). In tumor cells, PP2A activity is suppressed by increased levels of PP2A inhibitory proteins, such as SET, CIP2A and PME-1 and/or Tyr307 phosphorylation of PP2Ac. Therefore, we analyzed the effects of PPZ on protein levels of PP2A inhibitory proteins and phosphorylation level of PP2Ac (Fig.6B). PPZ did not affect protein levels of SET, CIP2A and PME-1, nor phosphorylation level

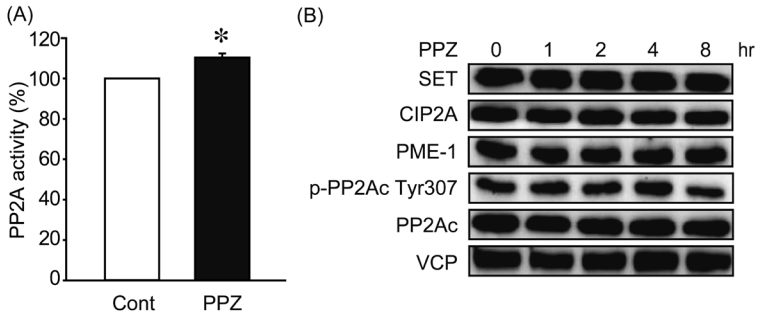

Fig. 6. Perphenazine increases PP2A activity. (A) UL-1 cells were treated with 25 μM of perphenazine (PPZ) for 15 min. PP2A activity was analyzed by using Active PP2A DuoSet IC kit. Cont: Control. N=3. *: $P<0.05$ vs. Cont. (B) UL-1 cells were treated with 10 μM of perphenazine (PPZ) for indicated time periods. Immunoblotting was performed using indicated antibodies. Representative images from 3 independent experiments are shown.

of PP2Ac Tyr307.

DISCUSSION

In this study, we found antipsychotic drug PPZ selectively killed canine lymphoma cell lines. PPZ activates PP2A and induces apoptosis. Although, PPZ suppresses both Akt and MEK1/2-ERK1/2 signaling pathways, a study with kinase inhibitors indicates that inhibition of Akt signaling is important for anti-tumor effects of PPZ.

A previous paper with human T cell acute lymphoblastic leukemia showed PP2A inhibitor, okadaic acid, blocked the anti-tumor effects of PPZ [10]. In UL-1 cells, treatment with okadaic acid itself induced cell death (data not shown). The mechanism of differential effects of okadaic acid is unclear, but this may be because okadaic acid inhibits all PP2A complexes. 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 one regulatory B subunit from four different families of genes. The substrate of PP2A complexes is determined by B subunit. Although, PP2A is highly conserved among mammals, we previously reported canine specific isoform of SET protein [24]. This indicates that the species differences may exist for the mechanisms controlling PP2A activity. Therefore, it is necessary to clarify which B subunit recruits PP2A core dimer to Akt. Moreover, it is also possible that anti-tumor effects of PPZ on lymphoma cells have differences among species.

PPZ has been reported to bind to scaffolding A subunit of PP2A complex and PP2A-dependently dephosphorylates Akt and ERK1/2 [10]; however, the precise mechanism how PPZ treatment leads to Akt and ERK1/2 dephosphorylation was unclear. In this study, we revealed PPZ increases PP2A activity. In cancer cells, PP2A activity is suppressed by association with inhibitory proteins, such as SET, CIP2A and PME-1, or by C-terminal Tyr307 phosphorylation [3, 4, 11, 13, 23]. We found protein levels of SET, CIP2A and PME-1 and phosphorylation levels of PP2A Tyr307 were not affected by PPZ. Therefore, it remains unclear how PPZ increases PP2A activity. It is possible that PPZ dissociates PP2A inhibitory proteins from PP2A. Previously, we reported that PP2A activation by SET inhibitor OP449 selectively killed SET-high expressing canine lymphoma cell line Ema, but not SET-low expressing UL-1 [7]. PPZ induced cell death in both Ema and UL-1 cells almost equally, suggesting SET-independent mechanism.

We found PPZ inhibits Akt and MEK1/2-ERK1/2 signaling. PPZ did not affect the activity of upstream kinases, suggesting direct dephosphorylation of these kinases by phosphatases. Association of PP2A and MEK1/2-ERK1/2 has been reported in cardiac myocytes [14]. Moreover, PP2A B55α regulatory subunit targets PP2A to Akt and regulates dephosphorylation of Akt Thr308 [12]. These observations suggest that PPZ dephosphorylates Akt and MEK1/2-ERK1/2 through PP2A activation.

Increased caspase 3 activity and Annexin V positive cells by PPZ treatment suggest the involvement of apoptosis in PPZ-induced cell death. Another type of PP2A activator, FTY720 and OP449, also induces apoptosis in canine lymphoma and human chronic lymphocytic leukemia, suggesting the important role of apoptosis in anti-tumor effects of PP2A activators [7, 26]. However, PP2A also plays a role in non-classical cell deaths, such as mitotic catastrophe and necroptosis. FTY720 was reported to dissociate PP2A inhibitory proteins from PP2A and induce necroptosis in human lung cancer cells [15]. Combined treatment with PP2A inhibitor and temozolomide induces mitotic catastrophe in human malignant glioma [21]. Therefore, it is possible that PPZ also induces these non-classical cell deaths.

Our data demonstrate the possible clinical application of phenothiazines for canine T-cell lymphoma. PPZ has antagonistic action for dopamine and serotonin receptors, and is treated as an antipsychotic drug. Therefore, using PPZ *in vivo* as an anti-cancer drug will develop neurogenic side effects. It is necessary to clarify the precise molecular mechanism for anti-tumor effects of phenothiazines and generate derivatives to diminish side effects.

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