Histone Deacetylase Inhibitor, Panobinostat Exerts Anti-proliferative Effect with Partial Normalization from Aberrant Epigenetic States on Granulosa Cell Tumor Cell Lines

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Abstract

The prognosis of the patients with inoperable or advanced granulosa cell tumors (GCTs) is still poor, and therefore it is important to establish a novel treatment strategy. Here we investigated the in vitro effects of a histone deacetylase inhibitor, panobinostat (PS) on two GCT cell lines (KGN and COV434).

GCT cell lines were found to be susceptible to PS treatment and it inhibited cell growth mainly by apoptosis. In cell cycle analysis, PS reduced only the ratio of S phase in GCT cell lines. Combined treatment of PS with a deubiquitinases inhibitor, VLX1570 exerted an additive anti-proliferative effect on KGN and COV434. The gene set enrichment analysis revealed that PS treatment suppressed DNA replication- or cell cycle-related gene expression which led to chemotherapeutic cell death and in addition, this treatment induced activation of the gene set of adherens junction towards a normalized direction as well as activation of neuron-related gene sets that might imply unexpected differentiation potential due to epigenetic modification by a HDAC inhibitor in KGN cells.

In the present study, we indicate a basis of a novel therapeutic availability of a HDAC inhibitor for the treatment of GCTs and further investigations will be warranted.

Introduction

Granulosa cell tumors (GCTs) account for approximately 70% of malignant sex cord–stromal tumors and represent 3 to 5% of all ovarian neoplasms. GCTs frequently occur in perimenopausal women and a half of those show atypical genital bleeding and menstruation disorder from the overproduction of estrogen[1,2]. A somatic missense mutation (C134W) of *FOXL2* which is involved in follicular development and granulosa cell differentiation is found in 95% of adult patients with GCTs[3]. Since more than 80-90% of the patients with GCTs is diagnosed at an early phase and can achieve remission surgically by removing the localized lesion and the five-year survival rate is more than 95% at stages I and II, while it worsens to 59% at stages III and VI. Although BEP therapy (bleomycin, etoposide and cisplatin) as a representative adjuvant chemotherapy is performed after surgery at stage II-VI, the response rate of this treatment is barely 37%[4]. Therefore, it has been expected to establish a novel chemotherapeutic strategy for the patients with advanced, recurrent or metastatic GCTs.

The aberrant gene expression by epigenetic change plays a critical role in the onset and progression of cancer[5]. Histone acetyltransferases (HATs) transfer acetyl groups to amino-terminal lysine residues in histone, which results in increased transcriptional activity, whereas histone deacetylases (HDACs) catalyze the removal of acetyl groups, leading to transcriptional repression[6,7]. HDACs has been recognized as targets to reverse aberrant epigenetic states that are involved in oncogenesis[8].

Panobinostat (PS) is a HDAC inhibitor classified into hydroxamate and strongly inhibits enzymatic activity of HDACs belonging to class I, II and IV[9]. It was previously reported to show an anti-proliferative effect on acute myeloid leukemia, Hodgkin's disease, multiple myeloma (MM) and various solid tumors[10-

[13]. Furthermore, it was reported that the combined treatment of PS with conventional therapeutic agents including gemcitabine or paclitaxel showed a synergistic effect on ovarian cancer cell lines[14-16].

In the present study, we investigated an anti-proliferative effect of PS on GCT cell lines and indicated that PS induced apoptosis and the combined treatment with a deubiquitinase inhibitor, VLX1570 showed an additive cytoreductive effect. The gene set enrichment analysis (GSEA) revealed that PS treatment suppressed DNA replication- or cell cycle-related gene expression which led to chemotherapeutic cell death and further induced activation of the gene set of adherens junction towards a normalized direction as well as activation of neuron-related gene sets that might imply unexpected differentiation potential due to epigenetic modification by a HDAC inhibitor in KGN cells.

Materials And Methods

Reagents

PS, Bortezomib and VLX1570 were purchased from Selleck Chemicals, (Houston, Texas, TX, USA). This was dissolved in dimethylsulfoxide and stored at -80°C with being protected from light. We used those at the concentrations up to 200 nM.

Cell lines and culture

A granulosa cell tumor cell line, KGN and COV434 were maintained in DMEM/Ham's F12 medium supplemented with 10% fetal bovine serum. An ovarian serous adenocarcinoma cell line, SK-OV-3 and a clear cell cancer cell line, RMG-I and OVISE were used in this study.

Cell growth assay and MTT assay

Cell growth was assessed by counting the number of living cells after trypan blue staining. Cell suspensions were plated into 96-well plates in the presence of the drug or solvent alone, incubated as above at 37°C for 1-4 days, and analyzed by the 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay[17].

Apoptosis assay

Apoptosis was examined using an AnnexinV Apoptosis Detection Kit (BD Pharmingen, San Diego, CA, USA) and all samples were analyzed with FACS Calibur flowcytometer and CellQuest software (Becton Dickinson, Franklin Lakes, NJ, USA)[18].

Cell cycle analysis

Cells were fixed with 70% methanol for 30 min and treated with 2 mg/ml ribonuclease A (Nacalai Tesque, Kyoto, Japan) for 30 min at 37°C, then with 50 mg/mL propidium iodide (PI; Sigma, St Louis, MO, USA) for further 20 min at room temperature[19].

Immunoblotting analysis

Cell lysates of all five cell lines were prepared in lysis buffer containing 50 mM Tris-HCL, 150 mM NaCl, 5 mM EDTA, 0.5% TritonX-100, 0.05% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 2 mM phenylmethylsulfonyl fluoride and 1 mM Na_3VO_4. The lysates were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting analysis was performed as previously described[20].

Primary antibodies were obtained from Santa Cruz Biotechnology (bcl2, bcl-XL/Xs; Santa Cruz, CA, USA), Cell Signaling Technology (cleaved-PARP (cPARP), p21, acetyl-H4 (Lys12); Danvers, MA, USA), Sigma-Aldrich (α-tubulin, acetyl-α-tubulin (Lys40); St. Louis, Mo, USA), Horse-radish peroxidase-conjugated mouse and rabbit antibodies were from GE Healthcare Life Sciences (Piscataway, NJ).

Gene expression profiling and gene set enrichment analysis (GSEA)

Gene expression profiling of KGN cells were analyzed in three independent experiments. Treated cells were harvested after 24 h treatment with 100 nM of PS. Total RNA was extracted with RNeasy Mini Kit (Qiagen,Germantown, MD, USA), converted to cDNA and amplified with GeneChip WT Terminal Labeling and Controls Kit (Affymetrix, Santa Clara, CA, USA). The fragmentation, the labeling and the hybridization of cDNA were treated with GeneChip Hybridization, Wash, and Stain Kit (Affymetrix). Chips were scanned with a GeneChip Scanner 3000 7G System (Affymetrix).

The gene set enrichment analysis (GSEA; Broad Institute Cambridge, MA, USA) was performed using the gene expression profiling data and by handling the GSEA software. The detail information of these experiments is described in references[21]. In this study, the whole expression change in the gene sets was defined as statistically significant if both the false discovery rate (FDR) q-values and the familywise error rate (FWER) p-values were less than 0.25.

Statistical analyses

All results are shown as the mean values with ranges. Comparisons between the groups were done using the Dunnett's and Scheffe's tests. Differences were considered statistically significant if p-values were less than 0.05. These analyses were carried out using SPSS for Windows version 14.0.

Results

PS inhibits the proliferation of ovarian cancer and GCT cell lines and mainly induce apoptosis.

To determine whether PS inhibits the growth of ovarian cancer and GCT cells, we treated three ovarian cancer cell lines including SK-OV-3, OVISE and RMG-I and two GCT cell lines including KGN and COV434 with indicated concentrations (0-100nM) of PS for 0-72 hr and examined the halfmaximal inhibitory concentration (IC50) of PS in all 5 cell lines (48 and 72hr) with MTT assay (Fig. 1a). The IC50 value of PS for SK-OV-3, OVISE, RMG-I, KGN and COV434 at 72 h was 34.4±0.11, 44.0± 0.46, 58.5±1.0, 34.7±0.94 and 53.5±8.4 nM, respectively (Table 1). We found that all 5 cell lines were susceptible to PS (Table 1). Cell counting by trypan blue staining indicated similar data to the result of MTT assay (data not shown). PS also induced the acetylation of Histone H4 and α-tubulin in SK-OV-3, KGN and COV434 cell lines. (Fig. 1b and S1)

Table 1
IC50 (nM) of PS in GCT and ovarian cancer cell lines

	48 hr	72 hr
SK-OV-3	43.1±1.3	34.4±0.11:
OVISE	*	58.5±1.0
RMG-I	75.2±1.8	44.0±0.46
KGN	89.5±1.0	34.7±0.94
COV434	59.4±1.4	53±8.4
PS: Panabinostat, GCT* Granulosa Cell Tumors. *Implies that exposure of OVISE to PS did not reach the IC_{50} in spite of being treates by the maximal concentration (100nM)		

Next, we examined the presence and the degree of apoptosis of PS treated cell lines by dual staining of annexinV and PI. Expectedly, PS induced apoptosis in 4 cell lines (Fig. 1c). The amount of cleaved PARP (cPARP), a marker of undergoing apoptosis, was increased with 100 or 200nM of PS treatment at 48 hr in SK-OV-3, KGN and COV434 cell lines (Fig. 1b). The expression of bcl-2 family members did not change in immunoblotting analysis (data not shown).

Because PS has been previously reported to affect the cell cycle[10-13], we performed the cell cycle analysis of PS-treated cell lines by flow cytometry and demonstrated that exposure of ovarian cancer cells including SK-OV3, OVISE and RMG-I to PS for 24 h led to the increase in the cell fraction at G0/G1 phase, whereas exposure of GCT cell lines including KGN and COV434 to PS did not significantly increase the G0/G1 cell fraction in spite of obvious decrease of cell fraction at S phase (Fig. 1d). In immunoblotting analysis, Exposure of SK-OV-3, KGN and COV434 to 0-200nM of PS for 48 hr increased the expression of cyclin dependent kinase inhibitor, p21 (Fig. 1e and S2).

Deubiqutinase inhibitor, VLX1570 collaboratively enhances the anti-proliferative effect of PS on GCT cell lines.

The ubiquitin proteasome system (UPS) is the procedure which degrades cellular unfolded or misfolded proteins distinct from autophagy lysosome system[22,23]. The inhibition of UPS by deubiqutinase inhibitor, b-AP15 or VLX1570 has been previously reported to induce apoptosis in various cancer cells[24-28]. We cultured KGN and COV434 cells with the combined treatment of PS with VLX1570 and demonstrated that VLX1570 enhanced the growth-suppressive effect of PS on MTT assay (Fig. 2a). This combined treatment additively increased the number of apoptotic cells in flow cytometry analysis using dual staining of annexinV and PI (Fig. 2b).

PS possibly restores the suppressed expression of the gene sets associated with adherens junction and neurotransmitter release cycle.

To further explore the action mechanisms of PS, we examined the gene expression profiling of KGN cells treated with or without 100 nM of PS for 24 hr. Genes whose expression changed by more than 1.5 fold or less than 0.66 following the treatment were defined as the affected genes. To know the gene targets of PS, we performed GSEA and found that some of the most positively affected GSEA sets were “adherens junction” (FDR q-val 0.000; FWER p-val 0.000), “cell adhesion molecules” (FDR q-val 0.005; FWER p-val 0.015) in KEGG pathway and “neurotransmitter release cycle” (FDR q-val 0.000; FWER p-val 0.000) in reactome pathway (Fig. 3). All genes included in each gene set were shown in supplementary Table S1 and Figure S3. Taken together, the gene expression profiling suggested that PS restored the genetic pathways involved in adherens junction, cell adhesion molecules and neurotransmitter release cycle which had been suppressed in KGN cells.

Discussion

In this study, we investigated the effects of PS on two GCT and three ovarian cancer cell lines and indicated that it suppressed cell proliferation mainly by inducing apoptosis. Some study groups previously analyzed the relationship between HDAC inhibition and the anti-proliferative effect by PS in various cancer cells[14-19]. From our data, PS also induced apoptosis of GCT cell lines as well as ovarian cancer cell lines (Fig.1a, c and table 1). Regarding the effects of PS on cell cycle, PS induced cell cycle arrest at G0/G1 with decrease in cell fraction at S phase in ovarian cancer cell lines, whereas it only reduced the ratio of S phase fraction without obvious G0/G1 arrest in GCT cell lines (Fig.1d), although it in unclear whether the difference of the effects of PS treatment on cell cycle is due to the different properties between GCT cell lines and ovarian cancer cell lines. Immunoblotting analysis for detection of acetyl-H4 and acetyl-α-tubulin in GCT and ovarian cancer cells revealed that PS actually worked as a HDAC inhibitor and it may be related with the suppression of cancer cell growth (Fig.1b).

Catley et al. investigated the effect of the combined treatment of PS with a proteasome inhibitor, bortezomib on multiple myeloma cells[29]. UPS works for quality control of the proteins by degrading unnecessary misfolded or unfolded proteins. If the amount of unnecessary proteins exceeds the capacity of UPS, they accumulate in aggresomes which are inclusion bodies formed by transport of aggregated protein on microtubules[30,31]. After HDAC6 binds both ubiquitinated misfolded proteins and motor protein dynein, it carries misfolded proteins to aggresomes along microtubules using dynein[32,33]. Since inhibition of UPS by bortezomib on myeloma cells leads to aggresome formation and PS inhibits the activity of HDAC6, the combined treatment shows the synergistic effect on myeloma cells. We evaluated the effect of combined treatment of bortezomib with PS on GCT cell lines in vitro but this combination barely showed an additive cytoreductive effect, probably because both cell lines were much susceptible to bortezomib, as it was reported that bortezomib alone strongly inhibited cell growth of these cells at low dose[34].

Next, we examined the effect of combined treatment of PS with a deubiqutinase inhibitor, VLX1570 on KGN and COV434 in vitro. VLX1570 is 19S proteasome inhibitor and inhibits the removal of ubiquitin chains from ubiqutinated proteins by UCHL5 and USP14, distinct from 20S proteasome inhibitor, bortezomib. Hillert EK, et al. examined the effect of combination of a similar deubiqutinase inhibitor, b-AP15 with PS on HCT116 and Hela cell lines and found that the combined treatment of PS with b-AP15 enhanced the proteotoxicity but it did not augment an anti-proliferative effect in those two cell lines[35]. In our study, the combined treatment of PS with VLX1570 additively suppressed the proliferation of KGN and COV434 and induced apoptosis (Fig.2a, 2b). Further study is necessary to clarify whether the additive anti-proliferative effect of PS and VLX1570 on GCT cells are associated with the inhibition of UPS and aggresome formation.

To further know the action mechanisms of PS and the gene targets of PS, we examined gene expression profiling of PS-treated KGN cells and performed GSEA. From GSEA using the data sets of KEGG pathways, the ontology groups “DNA replication”, “cell cycle”, “spliceosome”, “homologous recombination”, “mismatch repair” and “pyrimidine metabolism” were listed as markedly down-regulated gene sets with PS treatment, whereas the groups “adherens junction”, “endocytosis”, “cell adhesion molecules” and “axon guidance” were listed as significantly up-regulated gene sets with PS treatment. Most of down-regulated KEGG pathway gene sets with PS treatment could be explained as a result of the treatment with an anti-cancer agent. In contrast, among the groups listed as significantly up-regulated gene sets with PS treatment, we focused on “adherens junction”. Kranc et al. reported that genes responsible for proliferation, differentiation, and junction adhesion are up-regulated in human ovarian granulosa cells during in vitro culture and firstly raised the up-regulation of “adherens junction” in KEGG pathways[36]. Their finding is based on the changes of gene expression profiling of cultured normal granulosa cells in vitro, but our study might observe a part of restored gene expression of the tumor cell line as a result of PS treatment.

From GSEA using the data sets of reactome pathways, the ontology groups “mitotic spindle checkpoint”, “cell cycle checkpoints”, “resolution of sister chromatid cohesion”, “cell cycle” and “homology directed repair through homologous recombination” were listed as markedly down-regulated gene sets with PS treatment, whereas the groups “neurotransmitter release cycle”, “serotonin neurotransmitter release cycle”, “L1CAM interactions”, “dopamine neurotransmitter release cycle” and “acetylcholine neurotransmitter release cycle” were listed as markedly up-regulated gene sets with PS treatment.

As for down-regulated gene sets with PS treatment, the cytoreductive effects of PS were shown almost in line with the data from KEGG pathways. As for up-regulated gene sets with PS treatment, surprisingly, neurotransmitter- or neuron-related gene sets were accumulated. These data appeared intriguing but several previous reports suggested the possible relation of granulosa cells and neuronal subjects. Kossowska-Tomaszczuk et al. indicated that granulosa cells can acquire features of neuron under differentiating factors[37]. Up-regulation of NCS1 gene may take a role in the differentiation of granulosa cell towards neuronal cells[36]. Bence et al. reported that a HDAC inhibitor trichostatin A induced up-regulation of monoaminergic neurotransmission genes in neuroblastoma cells[38]. A similar gene-activation effect might occur ectopically on granulosa cell tumor cells, or perhaps it would imply some aspects of neuronal differentiation of granulosa cells. An axonal guidance factor or related gene sets were reported to be involved in granulosa cell function[39,40], and the axon guidance was actually one of up-regulated gene sets with PS treatment in KEGG pathways, as above described. Taken together, GSEA revealed that PS treatment suppressed DNA replication- or cell cycle-related gene expression which led to chemotherapeutic cell death and in addition, induced activation of adherens junction towards a normalized direction as well as activation of neuron-related gene sets that might imply unexpected differentiation potential due to epigenetic modification by a HDAC inhibitor.

In the present study, we investigated the effect of a HDAC inhibitor, PS by using GCT and ovarian cancer cell lines and demonstrated that PS exerted anti-proliferative effect mainly by apoptosis and also possess another potency such as activation of adherens junction toward a normalized direction. Further study regarding potential availability of PS will be needed to establish a promising therapeutic strategy for the patients with GCTs.

Declarations

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Author Contributions

TT was the principal investigator. YH and TT performed all the pilot experiments and most of analysis. The study was performed in KaT's group and KS group under the direction of TT. AK participated in the statistical analysis. YH, TT, AS and YM performed the benchwork. YH, TT, AS, YM and KaT discussed the steps of the study. YH, TT and KaT wrote the manuscript with contributions from the other authors.

Competing Interests Statement

The authors declared there is no competing interest.

Data availability

All data included in this study are fully described in the paper.

Role of funding sources

The funder has no role in the study design, data collection, data analysis, data interpretation or writing of the report.

References

1. Colombo, N., Parma, G., Zanagnolo, V. & Insinga, A. Management of ovarian stromal cell tumors. *J Clin Oncol* **25**, 2944-2951, doi:10.1200/jco.2007.11.1005 (2007).
2. Seagle, B. L., Ann, P., Butler, S. & Shahabi, S. Ovarian granulosa cell tumor: A National Cancer Database study. *Gynecol Oncol* **146**, 285-291, doi:10.1016/j.ygyno.2017.05.020 (2017).
3. Färkkilä, A. *et al.* Pathogenesis and treatment of adult-type granulosa cell tumor of the ovary. *Ann Med* **49**, 435-447, doi:10.1080/07853890.2017.1294760 (2017).
4. Homesley, H. D., Bundy, B. N., Hurteau, J. A. & Roth, L. M. Bleomycin, etoposide, and cisplatin combination therapy of ovarian granulosa cell tumors and other stromal malignancies: A Gynecologic Oncology Group study. *Gynecol Oncol* **72**, 131-137, doi:10.1006/gyno.1998.5304 (1999).
5. Baylin, S. B. & Ohm, J. E. Epigenetic gene silencing in cancer - a mechanism for early oncogenic pathway addiction? *Nat Rev Cancer* **6**, 107-116, doi:10.1038/nrc1799 (2006).
6. Roth, S. Y., Denu, J. M. & Allis, C. D. Histone acetyltransferases. *Annu Rev Biochem* **70**, 81-120, doi:10.1146/annurev.biochem.70.1.81 (2001).
7. Thiagalingam, S. *et al.* Histone deacetylases: unique players in shaping the epigenetic histone code. *Ann N Y Acad Sci* **983**, 84-100, doi:10.1111/j.1749-6632.2003.tb05964.x (2003).
8. Pandolfi, P. P. Histone deacetylases and transcriptional therapy with their inhibitors. *Cancer Chemother Pharmacol* **48 Suppl 1**, S17-19, doi:10.1007/s002800100322 (2001).

9. Bolden, J. E., Peart, M. J. & Johnstone, R. W. Anticancer activities of histone deacetylase inhibitors. *Nat Rev Drug Discov***5**, 769-784, doi:10.1038/nrd2133 (2006).
10. San José-Enériz, E., Gimenez-Camino, N., Agirre, X. & Prosper, F. HDAC Inhibitors in Acute Myeloid Leukemia. *Cancers (Basel)***11**, 1794, doi:10.3390/cancers11111794 (2019).
11. Maiso, P. *et al.* The histone deacetylase inhibitor LBH589 is a potent antimyeloma agent that overcomes drug resistance. *Cancer Res***66**, 5781-5789, doi:10.1158/0008-5472.Can-05-4186 (2006).
12. Lemoine, M. *et al.* The pan-deacetylase inhibitor panobinostat induces cell death and synergizes with everolimus in Hodgkin lymphoma cell lines. *Blood***119**, 4017-4025, doi:10.1182/blood-2011-01-331421 (2012).
13. Grassadonia, A. *et al.* Role of Hydroxamate-Based Histone Deacetylase Inhibitors (Hb-HDACIs) in the Treatment of Solid Malignancies. *Cancers (Basel)***5**, 919-942 (2013).
14. Garrett, L. A., Growdon, W. B., Rueda, B. R. & Foster, R. Influence of a novel histone deacetylase inhibitor panobinostat (LBH589) on the growth of ovarian cancer. *Journal of Ovarian Research***9**, 58, doi:10.1186/s13048-016-0267-2 (2016).
15. Budman, D. R., Tai, J., Calabro, A. & John, V. The histone deacetylase inhibitor panobinostat demonstrates marked synergy with conventional chemotherapeutic agents in human ovarian cancer cell lines. *Invest New Drugs***29**, 1224-1229, doi:10.1007/s10637-010-9467-6 (2011).
16. Chao, H. *et al.* Low dose histone deacetylase inhibitor, LBH589, potentiates anticancer effect of docetaxel in epithelial ovarian cancer via PI3K/Akt pathway in vitro. *Cancer Lett***329**, 17-26, doi:10.1016/j.canlet.2012.08.035 (2013).
17. Tsujioka, T. *et al.* Effects of DNA methyltransferase inhibitors (DNMTIs) on MDS-derived cell lines. *Exp Hematol***41**, 189-197, doi:10.1016/j.exphem.2012.10.006 (2013).
18. Ormerod, M. G., Collins, M. K., Rodriguez-Tarduchy, G. & Robertson, D. Apoptosis in interleukin-3-dependent haemopoietic cells. Quantification by two flow cytometric methods. *J Immunol Methods***153**, 57-65, doi:10.1016/0022-1759(92)90305-d (1992).
19. Matsuoka, A. *et al.* Lenalidomide induces cell death in an MDS-derived cell line with deletion of chromosome 5q by inhibition of cytokinesis. *Leukemia***24**, 748-755, doi:10.1038/leu.2009.296 (2010).
20. Kida, J. I. *et al.* An MDS-derived cell line and a series of its sublines serve as an in vitro model for the leukemic evolution of MDS. *Leukemia***32**, 1846-1850, doi:10.1038/s41375-018-0189-7 (2018).
21. Subramanian, A. *et al.* Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *Proceedings of the National Academy of Sciences***102**, 15545-15550, doi:10.1073/pnas.0506580102 (2005).
22. Rechsteiner, M., Hoffman, L. & Dubiel, W. The multicatalytic and 26 S proteases. *J Biol Chem***268**, 6065-6068 (1993).
23. Hershko, A. & Ciechanover, A. The ubiquitin system. *Annu Rev Biochem***67**, 425-479, doi:10.1146/annurev.biochem.67.1.425 (1998).

24. D'Arcy, P. *et al.* Inhibition of proteasome deubiquitinating activity as a new cancer therapy. *Nat Med***17**, 1636-1640, doi:10.1038/nm.2536 (2011).
25. Isaksson Vogel, R. *et al.* USP14 is a predictor of recurrence in endometrial cancer and a molecular target for endometrial cancer treatment. *Oncotarget***7** (2016).
26. Paulus, A. *et al.* Coinhibition of the deubiquitinating enzymes, USP14 and UCHL5, with VLX1570 is lethal to ibrutinib- or bortezomib-resistant Waldenstrom macroglobulinemia tumor cells. *Blood Cancer J***6**, e492, doi:10.1038/bcj.2016.93 (2016).
27. Wang, X. *et al.* The proteasome deubiquitinase inhibitor VLX1570 shows selectivity for ubiquitin-specific protease-14 and induces apoptosis of multiple myeloma cells. *Scientific Reports***6**, 26979, doi:10.1038/srep26979 (2016).
28. Mazurkiewicz, M. *et al.* Acute lymphoblastic leukemia cells are sensitive to disturbances in protein homeostasis induced by proteasome deubiquitinase inhibition. *Oncotarget***8**, 21115-21127, doi:10.18632/oncotarget.15501 (2017).
29. Catley, L. *et al.* Aggresome induction by proteasome inhibitor bortezomib and alpha-tubulin hyperacetylation by tubulin deacetylase (TDAC) inhibitor LBH589 are synergistic in myeloma cells. *Blood***108**, 3441-3449, doi:10.1182/blood-2006-04-016055 (2006).
30. Kopito, R. R. Aggresomes, inclusion bodies and protein aggregation. *Trends Cell Biol***10**, 524-530, doi:10.1016/s0962-8924(00)01852-3 (2000).
31. Johnston, J. A., Ward, C. L. & Kopito, R. R. Aggresomes: a cellular response to misfolded proteins. *J Cell Biol***143**, 1883-1898, doi:10.1083/jcb.143.7.1883 (1998).
32. Iwata, A., Riley, B. E., Johnston, J. A. & Kopito, R. R. HDAC6 and microtubules are required for autophagic degradation of aggregated huntingtin. *J Biol Chem***280**, 40282-40292, doi:10.1074/jbc.M508786200 (2005).
33. Kawaguchi, Y. *et al.* The deacetylase HDAC6 regulates aggresome formation and cell viability in response to misfolded protein stress. *Cell***115**, 727-738, doi:10.1016/s0092-8674(03)00939-5 (2003).
34. Chu, S., Alexiadis, M. & Fuller, P. J. Proteasome Inhibition by Bortezomib Decreases Proliferation and Increases Apoptosis in Ovarian Granulosa Cell Tumors. *Reproductive Sciences***16**, 397-407, doi:10.1177/1933719108327589 (2009).
35. Hillert, E. K. *et al.* Proteasome inhibitor b-AP15 induces enhanced proteotoxicity by inhibiting cytoprotective aggresome formation. *Cancer Lett***448**, 70-83, doi:10.1016/j.canlet.2019.02.003 (2019).
36. Kranc, W. *et al.* Genes responsible for proliferation, differentiation, and junction adhesion are significantly up-regulated in human ovarian granulosa cells during a long-term primary in vitro culture. *Histochemistry and Cell Biology***151**, 125-143, doi:10.1007/s00418-018-1750-1 (2019).
37. Kossowska-Tomaszczuk, K. *et al.* The multipotency of luteinizing granulosa cells collected from mature ovarian follicles. *Stem Cells***27**, 210-219, doi:10.1634/stemcells.2008-0233 (2009).
38. Bence, M., Koller, J., Sasvari-Szekely, M. & Keszler, G. Transcriptional modulation of monoaminergic neurotransmission genes by the histone deacetylase inhibitor trichostatin A in neuroblastoma cells. *J*

*Neural Transm (Vienna)***119**, 17-24, doi:10.1007/s00702-011-0688-4 (2012).

39. Basini, G. *et al.* The axonal guidance factor netrin-1 as a potential modulator of swine follicular function. *Mol Cell Endocrinol***331**, 41-48, doi:10.1016/j.mce.2010.08.001 (2011).
40. Wu, Y. *et al.* Transcriptome profile of one-month-old lambs' granulosa cells after superstimulation. *Asian-Australas J Anim Sci***30**, 20-33, doi:10.5713/ajas.15.0999 (2017).

Figures

Fig1

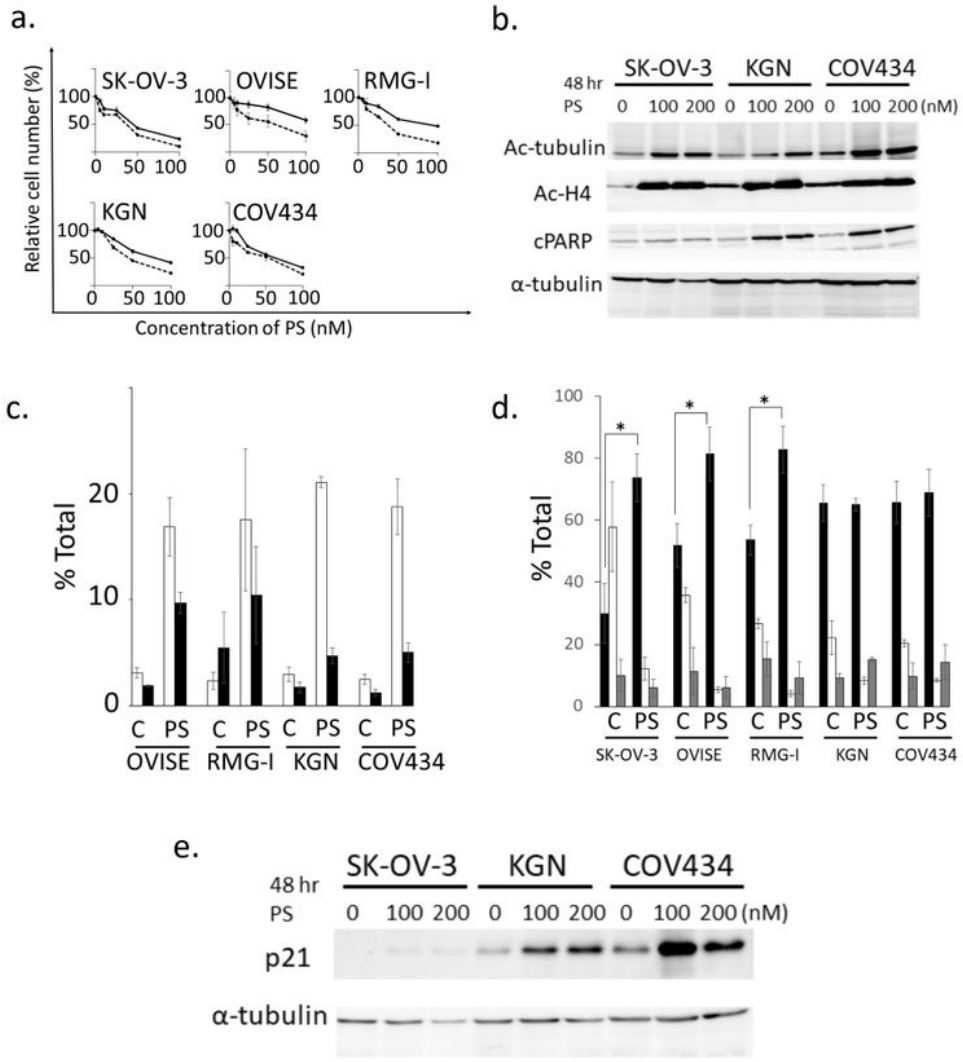


Figure 1

PS inhibits the proliferation of GCT and ovarian cancer cell lines and induces apoptosis. a) Three ovarian cancer cell lines (SK-OV-3, OVISE and RMG-I) and two GCT cell lines (KGN and COV434) were cultured with PS (0-100nM) for indicated times (48 and 72 hr). Cell viability was estimated by MTT assay. The value without PS was adjusted to 100%. The data represent the mean values with SD from five independent experiments. solid line:48hr, dash line:72hr. b) The cell lysates of each cell line were

analyzed by immunoblotting analysis for detection of acetyl-α-tubulin, acetyl-histone H4 and cleaved PARP. α-tubulin was used as a loading control. Ac-tubulin (acetyl-α-tubulin), Ac-H4 (acetyl-histone H4), cPARP (cleaved PARP). c) Four cell lines were cultured with or without 100nM of PS for 48 hr and apoptosis was assessed by flow cytometry using annexin V/PI dual staining. The single-positive fraction for annexin V (white bar) implies early apoptosis, and the double-positive fraction for annexin V/PI (black bar) implies late apoptosis. The data represent the mean values with SD from three independent experiments. C: DMSO control, PS: 100 nM of PS d) The cell cycle analysis by flow cytometory is shown. Five cell lines were treated with 100 nM of PS for 24 h, and cells were stained with PI and analyzed by flow cytometry. The data represent the mean values with SD from three independent experiments. The cell fractions at G0G1, S and G2/M phase are presented by black, white and gray bars, respectively. C: DMSO control, PS: 100 nM of PS e) The cell lysates of each cell line were analyzed by immunoblotting analysis for detection of p21. α-tubulin was used as a loading control. In d), statistical differences were evaluated and presented by asterisks in some cell lines, if p-values were less than 0.05.

Fig2

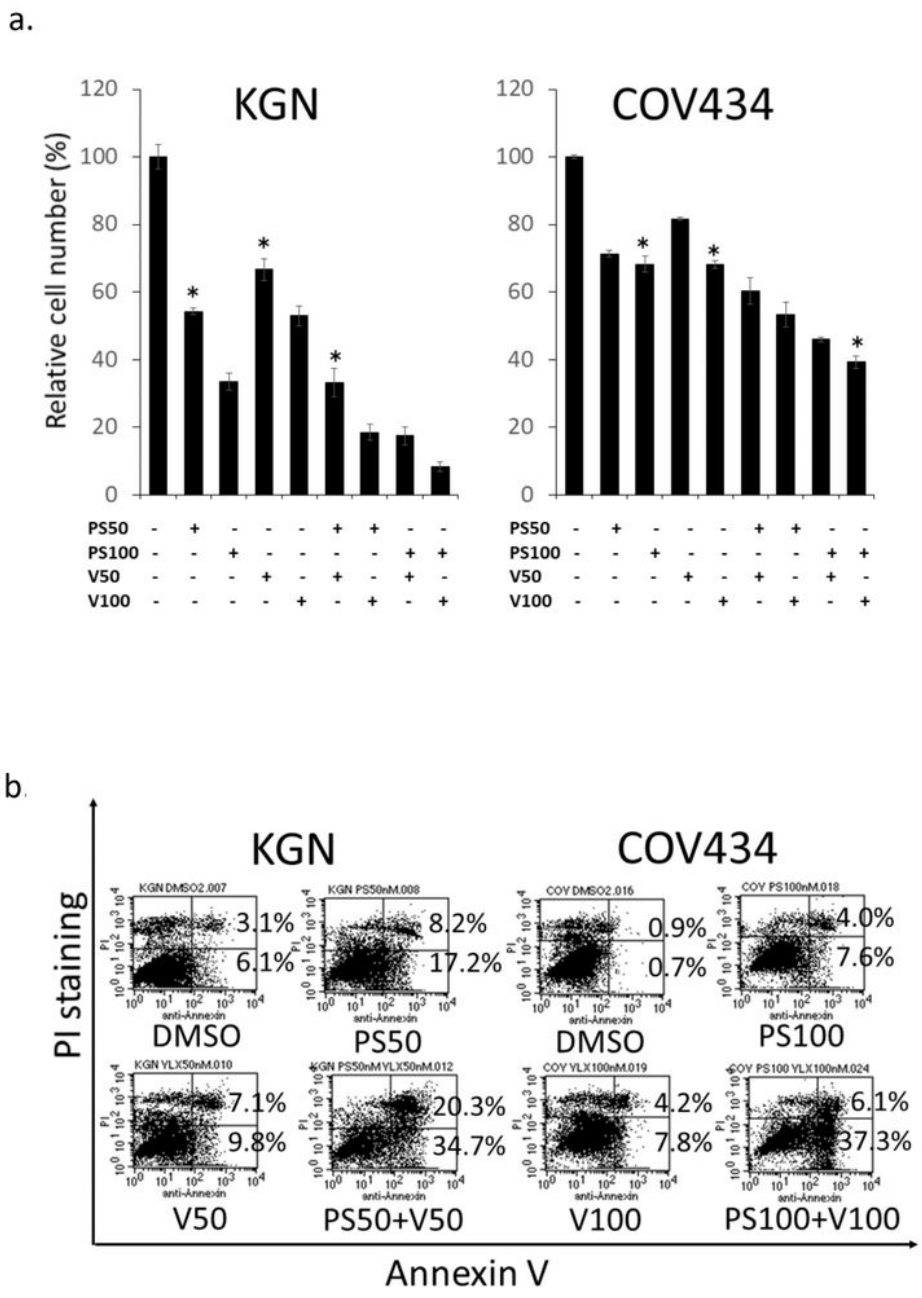
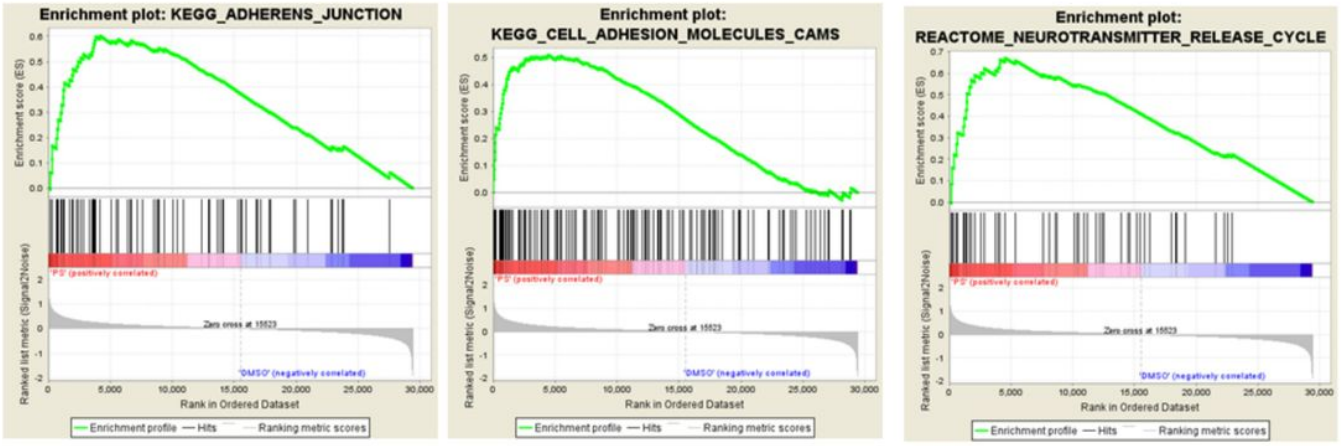


Figure 2

The combined treatment of PS with a deubiqutinase inhibitor, VLX1570 showed the additive effect on KGN and COV434 cell lines. a) KGN and COV434 were cultured with PS (0-100 nM), VLX1570 (0-100 nM) or both for 48 hr. Cytotoxicity was estimated by MTT assay. The data represent the mean values with SD from five independent experiments. Statistical differences were evaluated and presented by asterisks in two cell lines, if p-values were less than 0.05. PS: panobinostat, V: VLX1570 b) KGN and COV434 were

cultured with PS (0-100 nM), VLX1570 (0-100 nM) or both for 48 hr. The fraction of apoptosis cells was measured by flow cytometry analysis using annexin V/ PI dual staining. PS: panobinostat, V: VLX1570

Fig3



	adherens junction	cell adhesion molecules	neurotransmitter release cycle
ES	0.60166425	0.5079391	0.6715531
NES	2.112979	1.9569181	2.2229486
Nominal p-value	0	0	0
FDR q-value	0	0.005	0
FWER p-value	0	0.015	0

Figure 3

The gene enrichment analysis (GSEA) revealed the up-regulated gene sets: “adherens junction”, “cell adhesion molecules”, and “neurotransmitter release cycle” in PS-treated KGN cells. PS (100 nM)-treated or untreated KGN cells were harvested at 24 hr. Gene expression profiling of KGN cells was examined in triplicate experiments and obtained data were used for GSEA by handling the GSEA software and the Molecular Signatures Database according to the references. The gene set “adherens junction”, “cell adhesion molecules” and “neurotransmitter release cycle” was strongly up-regulated by PS treatment and several statistical values are also presented. ES: enrichment score, NES: normalized enrichment score

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- 210301FigS12300bpiPDF.pdf
- 210301SupplementarytableS1.xlsx