Histone Deacetylase Inhibitors and 15-Deoxy-Δ^{12,14}-Prostaglandin J_2 Synergistically Induce Apoptosis

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Abstract

Purpose: The clinically relevant histone deacetylase inhibitors (HDI) valproic acid (VPA) and suberoylanilide hydroxamic acid exert variable antitumor activities but increase therapeutic efficacy when combined with other agents. The natural endogenous ligand of peroxisome proliferator–activated receptor γ 15-deoxy-Δ^{12,14}-prostaglandin J_2 (15d-PGJ_2) is a potent antineoplastic agent. Therefore, we investigated whether these HDIs in combination with 15d-PGJ_2 could show synergistic antitumor activity in colon cancer DLD-1 cells.

Experimental Design: Cell viability was determined using a Cell Counting Kit-8 assay. Apoptosis and reactive oxygen species (ROS) generation were determined using flow cytometry analysis. Western blotting and real-time reverse transcription-PCR analysis were carried out to investigate the expression of apoptosis-related molecules. Mice bearing DLD-1 xenograft were divided into four groups (n = 5) and injected everyday (i.p.) with diluent, VPA (100 mg/kg), 15d-PGJ_2 (5 mg/kg), or a combination for 25 days.

Results: HDI/15d-PGJ_2 cotreatments synergistically induced cell death through caspase-dependent apoptosis in DLD-1 cells. Moreover, HDIs/15d-PGJ_2 caused histone deacetylase inhibition, leading to subsequent ROS generation and endoplasmic reticulum stress to decrease the expression of antiapoptotic molecules Bcl-X_L and XIAP and to increase that of proapoptotic molecules CAAT/enhancer binding protein homologous protein and death receptor 5. Additionally, VPA/15d-PGJ_2 cotreatment induced ROS-dependent apoptosis in other malignant tumor cells and was more effective than a VPA or 15d-PGJ_2 monotherapy in vivo.

Conclusions: Cotreatments with the clinically relevant HDIs and the endogenous peroxisome proliferator–activated receptor γ ligand 15d-PGJ_2 are promising for the treatment of a broad spectrum of malignant tumors. Clin Cancer Res; 16(8); 2320–32. ©2010 AACR.

Histone deacetylase (HDAC) inhibitors (HDI) are promising anticancer agents that are potent inducers of growth arrest, differentiation, and apoptotic cell death in various types of tumor cell lines (1, 2). HDIs achieve these effects through the alteration of gene expression by inhibition of HDAC and subsequent changes in chromatin structure (3). Valproic acid (VPA) and suberoylanilide hydroxamic acid (SAHA, vorinostat) are HDIs and exhibit antitumor effects in vitro and in vivo (4–7). VPA, an anticonvulsant clinically used for decades, is currently under investigation in clinical studies for myelodysplastic syndromes (8). SAHA was approved in the United States for the treatment of cutaneous T-cell lymphoma in 2006. However, a considerable proportion of patients with cutaneous T-cell lymphoma did not respond well to SAHA alone, although ~30% of patients overall achieved an objective response (9, 10). Recent clinical studies have shown that the combinations of VPA and all-trans retinoic acid, 5-aza-2'-deoxycytidine (decitabine), or the topoisomerase II inhibitor epirubicin are active in patients with hematopoietic malignancy or solid tumors (8, 11, 12). These combination therapies have led to exciting clinical responses in an otherwise refractory population. Therefore, additional mechanism-based therapeutic strategies for the use of HDIs in combination with other agents should be investigated to overcome resistance to HDIs or to increase the efficacy of HDIs.
Cyclopentenone prostaglandins (PG) are derived from arachidonic acid and are released from membrane phospholipids in response to various stress stimuli. It has been shown that the PGJ₂ series exert antitumor activity in vivo as well as in vitro (13, 14). PGD₂, which is the most abundant PG in normal tissue, is sequentially converted to PGJ₂ and then to Δ12,14-PGJ₂ or 15-deoxy-Δ12,14-PGJ₂ (15d-PGJ₂). Cotreatment with VPA and 15d-PGJ₂ was more effective than a VPA or 15d-PGJ₂ monotherapy in vitro. These results suggest that cotreatment with the clinically relevant HDIs and 15d-PGJ₂ may warrant clinical investigation as a rational combination therapy against malignant tumors.

Materials and Methods

Cell culture. Human colon cancer DLD-1 cells and human chronic myeloid leukemia K562 cells were maintained in RPMI 1640. Human immortalized keratinocyte HaCaT cells and human colon cancer HT29 cells were maintained in DMEM. Culture media were supplemented with 10% fetal bovine serum, 1-glutamine (2 mmol/L for RPMI 1640 and 4 mmol/L for DMEM), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂.

Reagents. 15d-PGJ₂, SAHA, and VPA were purchased from Cayman Chemical, Biomol, and Wako, respectively. N-acetylcysteine (NAC) and buthionine sulfoximine were purchased from Nacalai Tesque, Inc., and Wako, respectively. zVAD-fmk (pancaspase inhibitor), zLEHD-fmk (caspase-8 inhibitor), zAEVD-fmk (caspase-10 inhibitor), zLEHD-fmk (caspase-9 inhibitor), and zDEVD-fmk (caspase-3 inhibitor) were purchased from R&D Systems.

Cell viability assay. The number of viable cells was determined using a Cell Counting Kit-8 assay according to the manufacturer’s instructions (Dojindo). After the incubation of DLD-1 cells for 24 or 48 h with the indicated concentrations of various drugs, kit reagent WST-8 was added to the medium and incubated for a further 2 h. The absorbance of samples (450 nm) was determined using a scanning multiwell spectrophotometer that serves as an ELISA reader.

Detection of apoptosis. DNA fragmentation was quantified as the percentage of cells with hypodiploid DNA (sub-G₁). For flow cytometry analysis, cells were exposed to the agents for the indicated times. The cells were then treated with Triton X-100 and RNase A, and their nuclei were stained with propidium iodide before the DNA content was measured using a FACScalibur flow cytometer and CellQuest software (Becton Dickinson). For all assays, 10,000 cells were counted.

Western blot analysis. Cells were lysed in a radioimmunoprecipitation assay buffer containing PhosSTOP (Roche Applied Science). Western blot analysis was done as previously described (21), using mouse monoclonal anti–Bcl-2, anti–poly ADP ribose polymerase (Santa Cruz Biotechnology), anti-Bid, anti–caspase-8, anti–caspase-9, anti–caspase-10 (MBL), anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH; HyTest), anti–XIAP (R&D Systems), and anti–β-actin (Sigma). Rabbit monoclonal anti–caspase-3, anti–cleaved caspase-3 (Cell Signaling) and anti–GRP78/Bip (Santa Cruz Biotechnology), rabbit polyclonal anti–acetylated histone H4 (Millipore), anti–histone H4 (Abcam), anti–Bak, anti–Bax, anti–Bcl-X₁, anti–CAAT/enhancer binding protein homologous protein (CHOP/GADD153; Santa Cruz Biotechnology), anti–cIAP-1, anti–survivin (R&D Systems), and anti–death receptor 5 (DR5/TRAIL-R2; Prosci) were supplied as indicated. The signal was detected with an enhanced chemiluminescence Western blot analysis system (GE Healthcare).

Measurement of ROS production. For the measurement of ROS production, cells were treated with 20 μmol/L 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetylated ester dye (Molecular Probes) for...
15 min at 37°C, following a 12-h incubation with agents as indicated. Fluorescence was monitored in the FL-1 channel by a Becton Dickinson FACSCalibur using the CELL Quest software.

**RNA isolation and real-time reverse transcription-PCR analysis.** Total RNA was isolated from DLD-1 cells treated with agents for 24 h using Sepasol-RNA I (Nacalai Tesque, Inc.) according to the manufacturer's instructions. Total RNA (10 μg) was reversely transcribed to cDNA in a 20 μL reaction volume, with MMTV-reverse transcriptase (Promega), using oligo (dT) primers (Toyobo), according to the manufacturer's instructions. Quantitative real-time reverse transcription-PCR (RT-PCR) was carried out using a RT-PCR system GeneAmp5700 (Applied Biosystems) to quantify the expression level of Bcl-XL, survivin, cIAP-1, XIAP, CHOP, and DR5 mRNA normalized to 18S rRNA. Twenty hours after transfection, the medium was replaced and the cells were treated with or without VPA/15d-PGJ2 for 48 h and then harvested.

**In vivo studies.** Male BALB/c nu/nu mice were purchased from Shimizu Co., Ltd. On day 0, 7.5 × 10⁶ DLD-1 cells were resuspended in 1× phosphate-buffered NaCl solution, mixed with 1× Matrigel (BD Biosciences), and inoculated s.c. into the upper-right flank of each mouse. On day 3, when the tumors reached 6 to 8 mm in diameter, the mice were randomly divided into four groups (five mice per group) and treatment was initiated. Mice were injected everyday (i.p.) with diluent (10% DMSO in PBS, control group), VPA (100 mg/kg/d), 15d-PGJ2 (5 mg/kg/d) or a combination for 25 d. Tumors were measured with a sliding caliper twice weekly and the volumes (mm³) were calculated according to the formula: V = A × B² × 0.52, in which A is the largest superficial diameter and B is the smallest superficial diameter. On day 28, the tumors were excised from the euthanized mice. All experiments and procedures were done in accordance with the Institutional Care Use Committee guidelines.

**Statistical analysis.** Statistical evaluation of the data was done using the Student's t test for simple comparison between groups and treatments. \( P < 0.05 \) was considered statistically significant. Characterization of synergistic interactions was done using median dose effect analysis in conjunction with a commercially available software program (CalcuSyn, Biosoft).

## Results

**HDIs and 15d-PGJ2 synergistically induce caspase- and ROS-dependent apoptosis in DLD-1 cells.** To investigate the antiproliferative effects of 15d-PGJ2 or HDIs alone, we assessed the viability of DLD-1 cells treated with the indicated concentrations of agents for 24 or 48 hours. We observed that each agent showed minimally antiproliferative effects on DLD-1 cells in a dose- and time-dependent manner (Fig. 1A). Interestingly, cotreatments with 15d-PGJ2 and HDIs markedly inhibited the cell viability compared with that of cells treated with either 15d-PGJ2 or HDIs alone (Fig. 1B). Moreover, the combination index values for both 15d-PGJ2 and SAHA or VPA were <1.0, indicating synergistic cell death-inducing efficacy (Fig. 1B).

To clarify the mechanisms of synergistic inhibitory effects on cell viability by incubation with the combinations of 15d-PGJ2 and HDIs, we examined the effects of the combined treatments on apoptosis by measuring the sub-G₁ population. Cotreatments with 15d-PGJ2 and HDIs drastically induced apoptosis in a dose-dependent manner (Fig. 1C). As shown in Fig. 1D, the pan-caspase inhibitor zVAD-fmk, caspase-3, caspase-9, caspase-10, and caspase-8 inhibitors efficiently blocked apoptosis induced by
Fig. 1. HDIs and 15d-PGJ$_2$ synergistically induce caspase- and ROS-dependent apoptosis. A, DLD-1 cells were treated with DMSO alone (control) or the indicated concentrations of SAHA, VPA, or 15d-PGJ$_2$ alone for 24 h (□) or 48 h (■). Relative cell viabilities were examined using the Cell Counting Kit-8 assay. Columns, means (n = 3); bars, SD. B, DLD-1 cells were treated with 1.0 μmol/L SAHA or 0.5 mmol/L VPA with or without 6 μmol/L 15d-PGJ$_2$ for 24 h (□) or 48 h (■) and relative cell viabilities were examined as above (left). Columns, means (n = 3); bars, SD. DLD-1 cells were exposed to various concentrations of SAHA and 15d-PGJ$_2$ at a fixed ratio (1:2.33; middle) or VPA and 15d-PGJ$_2$ (500:1; right). Relative cell viabilities were examined as above. Combination index values of <1.0 correspond to a synergistic interaction. C, DLD-1 cells were treated with 15d-PGJ$_2$ and SAHA (left) or VPA (right) at the indicated concentrations for 48 h. Apoptosis (sub-G$_1$) was determined using flow cytometry analysis. Columns, means (n = 3); bars, SD. D, DLD-1 cells were pretreated with or without 20 μmol/L various caspase inhibitors (left), NAC, and/or buthionine sulfoximine (BSO; right) for 1 h followed by the addition of 1 μmol/L SAHA or 0.5 mmol/L VPA and 8 μmol/L 15d-PGJ$_2$ in combination for 48 h. Apoptosis was determined as above. P, pancaspase inhibitor; 3, caspase-3 inhibitor; 9, caspase-9 inhibitor; 10, caspase-10 inhibitor; 8, caspase-8 inhibitor. Columns, means (n = 3); bars, SD. *, P < 0.05 compared with cells treated with 15d-PGJ$_2$ and SAHA or VPA (left). ***, P < 0.05 (right).
Fig. 2. VPA/15d-PGJ₂ cotreatment alters the expression of apoptosis- or ER stress–related molecules. A, DLD-1 cells were treated with 0.5 mmol/L VPA for the indicated periods (top). DLD-1 cells were treated with DMSO alone or the indicated concentrations of VPA with or without 8 μmol/L 15d-PGJ₂ for 12 h (bottom). The expression of acetylated histone H4 and total histone H4 proteins was assessed by Western blotting. B and C, DLD-1 cells were treated with DMSO alone or 0.5 mmol/L VPA and/or 8 μmol/L 15d-PGJ₂ for 48 h. Activation of caspase-10, caspase-8, caspase-9, caspase-3, and Bid, and cleavage of PARP (B) and the expression of Bak, Bax, cFLIP-s, Bcl-2, Bcl-X₇, survivin, cIAP-1, XIAP, GRP78/ Bip, CHOP, and DR5 proteins (C) were assessed by Western blotting. GAPDH was chosen as a loading control in all blots. D, DLD-1 cells were treated with 0.5 mmol/L VPA and/or 8 μmol/L 15d-PGJ₂ for the indicated periods. The expression of Bcl-X₇, survivin, cIAP-1, XIAP, GRP78/Bip, CHOP, DR5, and GAPDH (a loading control) proteins was assessed by Western blotting.
Fig. 3. The alteration of apoptosis- or ER stress–related genes induced by VPA/15d-PGJ2 is mediated through ROS generation. A, DLD-1 cells were pretreated with or without 10 mmol/L NAC or 20 μmol/L zVAD-fmk for 1 h followed by the addition of 0.5 mmol/L VPA and/or 8 μmol/L 15d-PGJ2 for 12 h, after which ROS production was determined using flow cytometry analysis. Columns, means (n = 3); bars, SD. *, P < 0.05. B, DLD-1 cells were pretreated with or without 10 mmol/L NAC or 20 μmol/L zVAD-fmk for 1 h followed by the addition of 0.5 mmol/L VPA and/or 8 μmol/L 15d-PGJ2 for 12 h. The expression of acetylated histone H4 and total histone H4 proteins was assessed by Western blotting. C, DLD-1 cells were pretreated with or without 10 mmol/L NAC or 20 μmol/L zVAD-fmk for 1 h followed by the addition of 0.5 mmol/L VPA and/or 8 μmol/L 15d-PGJ2 for 12 h. The expression of cleaved PARP (left) and the expression of Bcl-XL, survivin, cIAP-1, XIAP, GRP78/Bip, CHOP, and DR5 proteins (right) were assessed by Western blotting. GAPDH was chosen as a loading control in all blots. D, DLD-1 cells were pretreated with or without 10 mmol/L NAC or 20 μmol/L zVAD-fmk for 1 h followed by the addition of 0.5 mmol/L VPA and 8 μmol/L 15d-PGJ2 for 24 h. The expression of Bcl-XL, survivin, cIAP-1, XIAP, CHOP, and DR5 mRNAs was examined using real-time RT-PCR. The levels of these mRNAs were standardized against that of 18S rRNA. Columns, means (n = 3); bars, SD. *, P < 0.05.
cotreatments with 15d-PGJ2 and HDIs, indicating that the induced apoptosis is mediated through the activation of both the intrinsic pathway (mitochondrial pathway) and the extrinsic pathway (death receptor pathway). It was reported that 15d-PGJ2– or HDI-induced lethality is associated with ROS production (18, 25). Pretreatment with the antioxidant NAC, which functions as a precursor of intracellular glutathione, significantly inhibited apoptosis induced by the combined treatments with 15d-PGJ2 and HDIs (Fig. 1D). On the other hand, a ROS inducer buthionine sulfoximine, which causes the depletion of intracellular glutathione, attenuated the apoptosis-inhibitory effect of NAC. These results suggest that cotreatments with 15d-PGJ2 and HDIs synergistically induce caspase- and ROS-dependent apoptosis.

VPA/15d-PGJ2 cotreatment alters the expression of apoptosis- or ER stress–related molecules. To elucidate the mechanism of apoptosis induced by VPA/15d-PGJ2 cotreatment, we carried out Western blot analysis. VPA at 0.5 mmol/L for 6 to 36 hours clearly induced acetylated histone H4 and the treatment with VPA for 12 hours increased acetylated histone H4 in a dose-dependent manner (Fig. 2A). Interestingly, VPA/15d-PGJ2 co-treatment for 12 hours significantly induced acetylated histone H4 compared with VPA alone. VPA and 15d-PGJ2 cooperated in the activation of caspases and the cleavage of Bid and PARP (Fig. 2B). On the other hand, VPA/15d-PGJ2 cotreatment significantly decreased the expression of antiapoptotic molecules such as a prosurvival Bcl-2 family member Bcl-XL and inhibitor of apoptosis proteins family members survivin, cIAP-1, and XIAP in dose- and time-dependent manners compared with 15d-PGJ2 alone (Fig. 2C and D).

ER stress was reported to be an event downstream of intense or persistent oxidative stress (26) and to induce ER stress markers such as a glucose-regulated protein GRP78/Bip and a proapoptotic molecule CHOP (27). We previously showed that an apoptosis-inducing membrane receptor, DR5, is a downstream target of CHOP (24). In the present study, VPA/15d-PGJ2 cotreatment significantly increased GRP78/Bip, CHOP, and DR5 expression in dose- and time-dependent manners compared with 15d-PGJ2 alone (Fig. 2C and D). Taken together, these results suggest that VPA/15d-PGJ2 cotreatment causes apoptosis through the downregulation of antiapoptotic molecules and the upregulation of CHOP and DR5, and that the
activation of both the intrinsic and extrinsic pathways are involved in the induced apoptosis.

VPA/15d-PGJ2 cotreatment alters the expression of apoptosis- or ER stress–related molecules through oxidative injury. Next, we further investigated whether ROS generation could relate to the induced apoptosis. VPA/15d-PGJ2 cotreatment markedly increased the ROS level, which was blocked by NAC pretreatment but not by zVAD-fmk (Fig. 3A). Moreover, neither NAC nor zVAD-fmk pretreatment decreased acetylated histone H4 induced by VPA/15d-PGJ2 cotreatment, suggesting that HDAC inhibition is an upstream event of oxidative injury– and subsequent caspase-dependent apoptosis caused by VPA/15d-PGJ2 (Fig. 3B). NAC and zVAD-fmk inhibited VPA/15d-PGJ2–induced activation of caspases and cleavage of PARP (Fig. 3C). VPA/15d-PGJ2 cotreatment down-regulated the expression of Bcl-XL and XIAP and upregulated that of CHOP and DR5 at both the protein and mRNA levels, and these alterations were restored by NAC pretreatment but not by zVAD-fmk (Fig. 3C and D). Unlike these genes, decreased cIAP-1 expression was recovered not only by NAC pretreatment but also by zVAD-fmk, raising the possibility that the down-regulation of cIAP-1 is a consequence of caspase activation and/or apoptosis induced by VPA/15d-PGJ2 cotreatment. Decreased expression of survivin was not significantly...
restored by NAC or zVAD-fmk (Fig. 3C and D). These results indicate that Bcl-X₅, XIAP, CHOP, and DR5 play important roles in the oxidative injury–mediated apoptosis induced by VPA/15d-PGJ₂ cotreatment.

SAHA at 1.5 μmol/L for 6 to 24 hours clearly induced acetylated histone H4 and the treatment with SAHA for 12 hours increased acetylated histone H4 in a dose-dependent manner (Supplementary Fig. S1A). Additionally, SAHA/
15d-PGJ2 cotreatment significantly induced ROS generation; decreased the expression of Bcl-XL and XIAP; and increased that of GRP78/Bip, CHOP, and DR5, which were inhibited by NAC pretreatment (Supplementary Fig. S1B and D). Neither NAC nor zVAD pretreatment decreased acetylated histone H4 induced by SAHA/15d-PGJ2 cotreatment (Supplementary Fig. S1C). These results suggest that SAHA has pretty similar effects to those by VPA when combined with 15d-PGJ2, although VPA has more clearly shown the synergistic effects with 15d-PGJ2 than SAHA. Taken together, these data indicate that significant HDAC inhibition is the first step in the oxidative injury– and caspase-dependent apoptosis caused by HDIs/15d-PGJ2.

**Bcl-XL, XIAP, CHOP, and DR5 are involved in VPA/15d-PGJ2–induced apoptosis.** To clarify whether the downregulation of the Bcl-XL and XIAP promoter was responsible for the inhibition of these mRNAs, we examined the promoter activities of Bcl-XL and XIAP. The promoter activities of these genes were not decreased by VPA/15d-PGJ2 cotreatment (data not shown). Therefore, this led us to investigate whether a posttranscriptional mechanism could be involved in the regulation of Bcl-XL and XIAP by analyzing the decay rate of these mRNAs. VPA/15d-PGJ2 cotreatment decreased the decay rate of XIAP mRNA but not that of Bcl-XL, indicating that these mRNAs are differentially regulated (Fig. 4A). Transient transfection of either Bcl-XL or XIAP showed that both Bcl-XL and XIAP partially protected against VPA/15d-PGJ2–mediated apoptosis (Fig. 4B). Moreover, the cells cotransfected with Bcl-XL and XIAP were found to be significantly more resistant to VPA/15d-PGJ2–induced apoptosis compared with those transfected with either Bcl-XL or XIAP. These results suggest that VPA/15d-PGJ2 cotreatment, at least partially, causes apoptosis through the downregulation of Bcl-XL and XIAP.

We next carried out a luciferase assay using reporter plasmids containing the CHOP or DR5 promoter. VPA/15d-PGJ2 cotreatment synergistically stimulated the promoter activity from pCHOP/-150 or pDR5/-347, which was attenuated by NAC pretreatment (Fig. 4C). Moreover, the mutation of ERSE abolished the activation of the CHOP promoter by VPA/15d-PGJ2. The promoter region from pDR5/-252 or pDR5/-347 with the CHOP mutation did not respond to VPA/15d-PGJ2. These results suggest that synergistic ROS generation induced by VPA/15d-PGJ2 cotreatment is an upstream event of ER stress, leading to the increase of the promoter levels of CHOP and DR5. Additionally, the siRNA of DR5 or CHOP almost completely decreased DR5 or CHOP protein and significantly attenuated apoptosis in DLD-1 cells treated with VPA/15d-PGJ2, respectively, compared with control LacZ siRNA (Fig. 4D). Importantly, concomitant with CHOP reduction by CHOP siRNA, VPA/15d-PGJ2–induced DR5 expression was at least partially suppressed in CHOP siRNA–transfected cells when compared with control LacZ siRNA–transfected cells. No significant difference in the attenuation of apoptosis among CHOP-, DR5- and CHOP/DR5 siRNA–transfected cells treated with 15d-PGJ2/VPA was detected. Moreover, no restoration of decreased Bcl-XL and XIAP by CHOP or DR5 siRNA was observed. Taken together, these results indicate that CHOP is involved in VPA/15d-PGJ2–induced apoptosis, mainly due to DR5 induction, and further support the notion that CHOP-mediated DR5 induction is an important step in the extrinsic pathway activated by oxidative injury and subsequent ER stress.

**VPA/15d-PGJ2 cotreatment causes apoptosis in other malignant tumor cells.** To determine whether the effects of VPA/15d-PGJ2 cotreatment on the induction of apoptosis or ROS generation could be observed more generally, other malignant tumor cell lines such as HaCaT,

**Fig. 6.** VPA/15d-PGJ2 cotreatment inhibits tumor growth in vivo. A, DLD-1 cells (7.5 × 10⁶) were s.c. inoculated into the upper-right flank of each mouse. Mice were randomly divided into four groups (five mice per group) and injected once daily through i.p. with diluent only (control group), VPA (100 mg/kg/d), 15d-PGJ2 (5 mg/kg/d), or their combination for 25 d. Points, mean of tumor volumes (n = 5); bars, SD. *, P < 0.05 compared with the control. B, representative of tumor tissue excised from each treatment group.
K562, and HT29 were similarly assayed. VPA/15d-PGJ_2 caused 74%, 44%, and 43% apoptosis in HaCaT, K562, and HT29 cells, respectively, which were effectively attenuated by NAC as seen in DLD-1 cells (Fig. 5A). VPA/15d-PGJ_2 cotreatment significantly downregulated the expression of Bcl-2, Bcl-X_L, cIAP-1 and XIAP in HaCaT cells, cIAP-1 and XIAP in K562 cells and survivin, cIAP-1 and XIAP in HT29 cells, and upregulated the expression of CHOP and DR5 in HaCaT cells, compared with VPA or 15d-PGJ_2 alone. These alterations were inhibited by NAC. Additionally, the combined treatment led to a significant increase in the level of ROS in these cells, which was inhibited by NAC (Fig. 5B). Moreover, we found that this combination did not lead to significant apoptosis in normal peripheral blood mononuclear cells (data not shown). These results suggest that VPA/15d-PGJ_2 cotreatment causes apoptosis in various malignant tumor cells with low toxicity to normal cells, correlating with the alteration of apoptosis-related molecules through ROS generation.

**VPA/15d-PGJ_2 cotreatment inhibits tumor growth in vivo.** Based on the in vitro antitumor efficacy of the combined treatment with VPA and 15d-PGJ_2, we evaluated the antitumor activity of the combined therapy in a nude mouse xenograft model inoculated with DLD-1 cells. Although either VPA or 15d-PGJ_2 treatment alone did not inhibit the growth of DLD-1 tumors, the combination therapy significantly suppressed the growth compared with the control on day 18 and later (Fig. 6A and B). These results suggest that a combination of VPA and 15d-PGJ_2 is more effective than a VPA or 15d-PGJ_2 monotherapy in vivo as observed in vitro.

**Discussion**

Recent preclinical studies have suggested that despite the promising anticancer effects of HDIs alone, they also enhance the antitumor activities of a large number of anticancer agents, including conventional chemotherapeutic and molecular-targeted drugs (28). It is likely that HDIs have a broader capacity to lower the threshold for tumor cells to undergo apoptotic cell death triggered by other agents. Therefore, to enhance the antitumor activity of HDIs and/or to overcome resistance to HDIs, mechanism-based therapeutic strategies using a combination of HDIs and other agents should be developed.

It has been shown that ROS-oxidation-reduction pathways are important mechanisms of HDI-induced transformed cell death as a monotherapy or combination therapy with other agents. Xu et al. (29) suggested that the downregulation of the endogenous ROS scavenger thioredoxin is associated with SAHA-induced ROS-dependent apoptosis. Moreover, ROS generation plays a crucial role in synergistic apoptosis caused by the combinations of HDIs and various compounds such as the proteasome inhibitor bortezomib, the purine analogue fludarabine, the MAP/ERK kinase 1/2 inhibitor PD184352, the alkyllysophospholipid perifosine, and the multiple receptor tyrosine kinase inhibitor AEE788 (30–34). The present study suggests that ROS generation through HDAC inhibition leads to caspase-dependent apoptosis caused by HDIs/15d-PGJ_2.

Fajas et al. (35) reported that VPA has a synergistic effect with the synthetic PPARγ ligands thiazolidinediones in the activation of PPARγ target genes. Therefore, VPA may render PPARγ permissive to activation by 15d-PGJ_2. However, pretreatment with the PPARγ irreversible antagonist GW9662 did not inhibit apoptosis induced by coadministration of 15d-PGJ_2 and HDIs, and unlike 15d-PGJ_2, the thiazolidinediones pioglitazone and ciglitazone failed to increase HDI-induced apoptosis even at a high concentration of 50 μmol/L, suggesting that the apoptosis induced by VPA/15d-PGJ_2 is independent of PPARγ activation (Supplementary Fig. S2A and B).

We showed that VPA/15d-PGJ_2 cotreatment drastically decreased Bcl-X_L and XIAP expression at the mRNA level through ROS generation. VPA/15d-PGJ_2 caused the destabilization of XIAP but not Bcl-X_L mRNA, indicating that these mRNAs are differentially regulated. Adenyate/uridylate-rich elements, often containing one to several copies of the consensus sequence AUUUA, are critical cis-acting regulatory motifs located in the 3′-untranslated region of many mRNAs and are targets for trans-acting proteins to modulate mRNA stability (21). We found that human XIAP mRNA contains 20 separate AUUUA repeats in the 3′-untranslated region, whereas Zhang et al. (36) reported that there are three AUUUA elements on the 3′-untranslated region of Bcl-X_L mRNA. Therefore, this raises the possibility that VPA/15d-PGJ_2 may affect XIAP mRNA stability through adenyate/uridylate-rich element–binding proteins that are different to those of Bcl-X_L.

We previously reported that treatment with 18 μmol/L 15d-PGJ_2 for 24 hours induces DR5 expression through mRNA stabilization in prostate cancer PC3 cells (37). Su et al. (38) showed that treatment with 30 μmol/L 15d-PGJ_2 for 24 hours triggers CHOP gene expression, resulting in the activation of the DR5 promoter in colon cancer HCT116 cells. These differences may depend on the concentrations of 15d-PGJ_2 used and/or particular properties of different cell lines. In the present study, VPA significantly enhanced DR5 promoter activity through the CHOP binding site and enhanced CHOP promoter activity through the ERSE in DLD-1 cells treated with 8 μmol/L 15d-PGJ_2, which were inhibited by NAC pretreatment. Taken together with the result that VPA/15d-PGJ_2 cotreatment for 12 hours cooperatively upregulated acetylated histone H4, these data suggest that ROS generation caused by HDAC inhibition as an upstream event of ER stress finally leads to an increase in the promoter levels of CHOP and DR5.

Based on the early preclinical data, VPA and SAHA were selected as candidates of HDIs for clinical trial. Kelly et al. (39) reported that in patients with advanced hematologic malignancies treated with oral-administered...
SAHA at 400 mg/d, the average maximum plasma concentrations of SAHA were 2.0 to 2.5 μmol/L. Münster et al. (12) suggested that the maximum tolerated dose and recommended phase II dose of VPA was 140 mg/kg/d in a phase I trial of VPA followed by the topoisomerase II inhibitor epirubicin in advanced solid tumors. Moreover, they showed that when patients with advanced solid tumors received a daily dose of 15 or 30 mg/kg, VPA reached serum concentrations between 50 and 130 μg/mL (0.3-0.8 mmol/L), which is the therapeutic range for antiepileptic treatment. Our in vitro data indicated that VPA at 0.5 mmol/L and SAHA at 1.5 μmol/L are clinically achievable concentrations, drastically induced apoptosis in DLD-1 cells when combined with 15d-PGJ2.

Regarding antitumor activities of 15d-PGJ2 in vivo, it has been reported that 15d-PGJ2 given at 2.5 mg/kg i.p. synergistically improves the angiogenic and antitumor effects of the thrombospondin peptide ABT510 in a bladder cancer xenograft (40) and that 15d-PGJ2 given at 1 mg/kg i.p. enhances docetaxel antitumor activity against a non–small cell lung cancer xenograft (41). Recently, Shin et al. (42) showed that 15d-PGJ2 given at a dose of 5 mg/kg through the tail vein every 3 days significantly decreased tumor growth in a mouse CT-26 s.c. colorectal cancer model or HL-60 human leukemia xenograft model. They suggested that mice treated with 15d-PGJ2 did not show signs of wasting and that body weights after 14 days were not significantly different from controls. Moreover, they assessed the toxicity of 15d-PGJ2 in mice by checking the level of glutamate-oxaloacetate transaminase and glutamate-pyruvate transaminase. They concluded that the dosage used in their experiment was safe based on the results for blood chemistry. In the present study, we showed that combination therapy with VPA at a dose of 100 mg/kg and 15d-PGJ2 at a dose of 5 mg/kg significantly suppressed the growth of a DLD-1 xenograft compared with the control, whereas either VPA or 15d-PGJ2 treatment alone did not inhibit the tumor growth. We observed no signs of toxicities such as skin rash, diarrhea, and wasting in mice treated with VPA at 100 mg/kg/d, 15d-PGJ2 at 5 mg/kg/d, or VPA and 15d-PGJ2 in combination, compared with control mice. Based on our in vivo study, clinical application of 15d-PGJ2 can be expected. However, a synthetic method to produce sufficient amount of 15d-PGJ2 for preclinical or clinical studies has not been established (43).

In conclusion, we have shown that the cotreatments with HDIs and 15d-PGJ2 synergistically induce apoptosis through HDAC inhibition and subsequent ROS generation and ER stress. The cotreatment with VPA and 15d-PGJ2 drastically induces apoptosis in various malignant tumor cells in vitro and inhibits tumor growth in vivo. These observations raise the possibility that the combined treatment with clinically relevant HDIs and the natural endogenous PPARγ ligand 15d-PGJ2 could prove to be promising for the treatment of a broad spectrum of malignant tumors in the clinic.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank R. Nakashishi and H. Watanabe for their experimental help.

Grant Support

Grant-in-Aid from the Japanese Ministry of Education, Culture, Sports, Science and Technology.

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Received 08/24/2009; revised 02/21/2010; accepted 02/24/2010; published OnlineFirst 04/06/2010.

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Histone Deacetylase Inhibitors and 15-Deoxy-\Delta^{12,14}\text{-Prostaglandin J}_2\text{ Synergistically Induce Apoptosis}


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