Novel SIRT1 inhibitor 15-deoxy-Δ^{12,14}-prostaglandin J\(_2\) and its derivatives exhibit anticancer activity through apoptotic or autophagic cell death pathways in SKOV3 cells

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Abstract. Clinically relevant sirtuin (SIRT) inhibitors may possess antitumor activities. A previous study indicated that 15-deoxy-Δ^{12,14}-prostaglandin J\(_2\) (15d-PGJ\(_2\)) exhibited potent anticancer activity by SIRT1 inhibition. Therefore, the aim of the present study was to investigate whether its derivatives (J11-C1 and J19) exhibited anticancer activity against ovarian cancer SKOV3 cells. Cell viability was determined using an MTT assay. Cell cycle arrest, apoptosis and autophagy were determined using flow cytometry or western blot analysis. J11-C1 and J19 were less cytotoxic to SKOV3 cells compared with 15d-PGJ\(_2\). Molecular docking studies supported the interactions of 15d-PGJ\(_2\), J11-C1 and J19 with various amino acids in SIRT1 proteins. Similar to 15d-PGJ\(_2\), J11-C1 and J19 inhibited SIRT1 enzymatic activity and decreased SIRT1 expression levels in a concentration-dependent manner. J11-C1 induced apoptotic cell death more effectively compared with J19, which was associated with markedly decreased expression of the anti-apoptotic molecule B-cell lymphoma 2 (Bcl-2). Furthermore, the levels of light chain 3-Ⅱ (LC3-II) and beclin-1 were clearly induced in SKOV3 cells treated with J11-C1. Thus, 15d-PGJ\(_2\) and its derivatives exhibited anticancer activity possibly by inducing apoptotic or autophagic cell death pathways. Collectively, the results of the present study suggest that 15d-PGJ\(_2\) and its derivatives exerted antitumor activity by selectively modulating the expression of genes associated with cell cycle arrest, apoptosis and autophagy. Notably, J11-C1 is a novel candidate SIRT1 inhibitor with anticancer activity.

Introduction

Sirtuins (SIRTs) are class III histone deacetylases (HDACs) that have been identified to serve important biological functions, including aging, energy mobilization and stress responses (1,2). Furthermore, SIRTs are involved in the regulation of cancer cell apoptosis and are potential targets for novel anticancer drugs that regulate the levels of deacetylated histone proteins, p53 and several transcriptional factors (3,4). Several SIRT1 inhibitors, such as Ex527, sirtinol and salermide, exhibit potent anticancer activity in various cancer cell lines (5,6). Previously, we demonstrated that a novel SIRT inhibitor, psammmaplin A, increased p53 acetylation and subsequently induced apoptotic death in MCF-7/Adr cells (7). In addition, Chu et al (8) demonstrated that patients with chemoresistant tumors overexpressed SIRT1; furthermore, the inhibition of SIRT1 expression decreased multidrug resistance 1 (MDR1) expression and increased drug sensitivity.

15-Deoxy-Δ^{12,14}-prostaglandin J\(_2\) (15d-PGJ\(_2\)) was revealed to exhibit pharmacological activities, including anti-inflammatory, anti-fibrotic and apoptotic effects, through peroxisome proliferator-activated receptor γ-independent signaling pathways such as the nuclear factor-κB (NF-κB), signal transducer and activator of transcription 1 (STAT1) and p53-dependent signaling pathways (9,10). Furthermore, 15d-PGJ\(_2\) was identified to induce apoptosis of various cancer cells through caspase-dependent signaling pathways (11). A previous study demonstrated that 15d-PGJ\(_2\) inhibited the migration of A2780/AD cells, possibly via NF-κB inhibition resulting from HDAC1 inhibition. The mechanisms of action underlying these novel effects of 15d-PGJ\(_2\) on SIRT1 and HDAC1 gene expression and enzyme activities were elucidated (12). In the present study, the effects of novel SIRT1 inhibitors (J11-C1 and J19), with a 15d-PGJ\(_2\) scaffold (11,12), on ovarian cancer cells were investigated.

Methyl jasmonate is a member of the jasmonate family of plant stress hormones, the most potent regulator of defense-associated mechanisms in plants (13). On the basis of its structural similarity to that of 15d-PGJ\(_2\), methyl jasmonate (J-11) was investigated for SIRT activity, and its functional mechanisms of regulation of cancer cell death pathways were...
investigated. A previous study indicated that an α-haloone analog, J7, exhibited enhanced in vitro anti-inflammatory potency (14,15).

Materials and methods

Reagents. 15d-PGJ$_2$ (87893-55-8) and 3-methyladenine (3-MA; 5142-23-4) were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). J11-Cl and J19 were synthesized in-house. The chemical structures of the drugs are presented in Fig. 1A. Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS) and cell culture supplements were obtained from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Primary antibodies against SIRT1 (cat. no. 8469; 1:1,000), SIRT2 (cat. no. 12672; 1:1,000), SIRT4 (cat. no. sc-135798; 1:500), SIRT5 (cat. no. 8779; 1:1,000), SIRT6 (cat. no. 8771; 1:1,000), B-cell lymphoma-2 (Bcl-2; cat. no. 15071; 1:500), Bcl-2-associated X protein (Bax; cat. no. 5023; 1:1,000), β-actin (cat. no. 3700; 1:1,000), light chain 3 (LC3; cat. no. 3868; 1:1,000), beclin-1 (cat. no. 4122; 1:1,000), autophagy-related 3 (Atg3; cat. no. 3415; 1:1,000), Atg5 (cat. no. 12994; 1:1,000), Atg7 (cat. no. 8558; 1:1,000), α-tubulin (cat. no. 3873; 1:1,000), cleaved caspase-3 (cat. no. 9661; 1:500), cleaved caspase-9 (cat. no. 7237; 1:1,000), poly(ADP-ribose) polymerase (PARP; cat. no. 9541; 1:1,000) and acetylated p53 (cat. no. 2570; 1:500) were purchased from Cell Signaling Technology (Beverly, MA, USA). Horseradish peroxidase-conjugated secondary antibodies [anti-mouse immunoglobulin G (IgG); cat. no. sc-2005; or anti-rabbit IgG; cat no. sc-2357] were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). All other chemicals were purchased from Sigma-Aldrich; Merck KGaA. All drugs were dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C until use. Chemical agents were diluted to appropriate concentrations with culture medium supplemented with 1% FBS. The final concentration of DMSO was <0.1% (v/v). DMSO was also present in the corresponding controls.

**SIRT1 enzyme activity.** SIRT1 enzymatic activity was assessed using commercial kits (cat. no. ab156065) from Abcam (Cambridge, UK), according to the manufacturer’s protocol. First, assay buffer [50 mM Tris/HCl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl$_2$, and 1 mg/ml bovine serum albumin (BSA)], SIRT1 enzyme, and either the solvent dimethylformamide (DMF) or different concentrations of the drugs (15d-PGJ$_2$, J11 or J11-Cl dissolved in DMF) were mixed with the substrate (p53) and co-substrate (NAD$^+$) for 45 min. Deacetylation reactions were conducted at 37°C for 60 min, and stopped by adding 50 µl stop solution containing the developer, followed by incubation at 37°C for 30 min. Fluorescence intensity was determined by reading fluorescence using a SpectraMax M2 microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA) with an excitation wavelength of 350 nm and an emission wavelength of 450 nm. Calculations of net fluorescence were made after subtracting values for a blank consisting of buffer without NAD.

**Docking simulations of sirtinol, 15d-PGJ$_2$, J11-Cl and J19 ligand and target structure.** The X-ray crystal structure of SIRT1 was selected from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (PDB; code 4I51) and prepared using the protein preparation wizard available in the Glide tool in Maestro (version 10.2; Schrödinger, LLC, New York, NY, USA). During the process, the missing side and back chains were included (16). The protein preparation wizard facility has two components: Preparation and refinement. Following ensuring the chemical accuracy, the preparation component adds hydrogen and neutralizes a side chain that is neither close to the binding cavity nor involved in the formation of salt bridges. The all-atom-optimized potentials for liquid simulations (OPLS-AA) force field was used for this purpose, and then the active site of protein was defined. Glide uses the full OPLS-AA force field at an intermediate docking stage and is considered to be more sensitive to geometrical detail compared with other docking algorithms. The water molecule occupying the protein structure was not suitable for the docking study, therefore it was removed. Finally, the optimization and minimization processes were performed until the average root mean square deviation of the non-hydrogen atoms reached 0.3 Å (17). This was followed by the generation of energy grids using the Glide protocol, as previously described (18-20). The docked ligand was used to define the energy grid boundaries with the default options. These grids were used to score the ligand ‘in place’ with the XP scoring function. It should be noted that considering the computational protocol followed for the generation of the covalent complexes, the XP Glide Score values can be used only in a qualitative sense to determine the binding of various ligands. The Glide XP scoring functions include improvements to the scoring of hydrogen bonds, the detection of buried polar groups, and the detection of π-cation and π-π stacking interactions. The result of the docking calculation is a top-scored predicted complex that is evaluated using the scoring function. The compounds sirtinol, 15d-PGJ$_2$, J11-Cl and J19 were prepared using the LigPrep software (version 3.4; Schrödinger, LLC), which can generate a number of structures from each input structure with various ionization states, tautomers, stereochemistries and ring conformatons. This process eliminates molecules using various criteria including the molecular mass, specified numbers and types of functional groups present with correct chiralities for each successfully processed input structure. The OPLS 2005 force field was used for the optimization, which produced the low-energy isomer of the ligand (21). Finally, all ligand molecules formed in the complex structure for input were docked.

**Cell culture.** Human ovarian cancer cell lines (SKOV3 and OVCAR3) and normal kidney epithelial cell lines (HK-2 and NRK-52E) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). SKOV3, OVCAR3, or NRK-52E cells were maintained in DMEM supplemented with 10% FBS, 4 mmol L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.). HK-2 cells were maintained in DMEM/Ham’s F12 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 5 µg/ml insulin (Gibco; Thermo Fisher Scientific, Inc.), 5 µg/ml transferrin (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin, 100 g/ml streptomycin, 0.1 µmol/l hydrocortisone, 2 nmol/l L-glutamine plus 10% FBS. The cell
cultures were incubated at 37°C in a humidified atmosphere containing 5% CO₂.

**Cytotoxicity assay.** Cell viability was determined using MTT (5 mg/ml; Sigma-Aldrich; Thermo Fisher Scientific, Inc.). The cells were seeded in 96-well plates at a density of 2x10⁴ cells/well. Following incubation at 24 h for 24 h, the cells were treated with 15d-PGJ₂ (0.05-20 µM), J11-Cl (1-160 µM) or J19 (1-160 µM) and cultured for a further 24, 48 or 72 h. Following incubation, 15 µl MTT reagent was added to each well and plates were incubated at 37°C for 3 h in the dark. The supernatant was aspirated and formazan crystals were dissolved in 150 µl DMSO at 37°C for 15 min with gentle agitation. The absorbance of each well was measured at 540 nm using a VersaMax microplate reader (Molecular Devices LLC, Sunnyvale, CA, USA). Three independent experiments were performed for each condition and normalized to the absorbance of wells containing medium only (0%) or untreated cells (100%). The half-maximal inhibitory concentration (IC₅₀) values were calculated from the sigmoidal concentration-response curves using SigmaPlot (version 12; Systat Software, Inc., San Jose, CA, USA).

**Cell cycle analysis.** Cells were treated with 15d-PGJ₂ (1, 5 or 10 µM), J11-Cl (1, 5 or 25 µM), or J19 (1, 5 or 25 µM) for 48 h or vincristine sulfate (BML-T117-0005; Enzo Life Sciences, Inc., Farmingdale, NY, USA) at 1x10⁵ cells/well and allowed to attach or vincristine sulfate for 25 µM or J19 (1, 5 or 25 µM) for 48 h when the cells reached 70% confluency. At the appropriate time points, the cells were treated with 15d-PGJ₂ (1, 5 or 10 µM), J11-Cl (1, 5 or 25 µM) or J19 (1, 5 or 25 µM), cultured for 48 h, harvested using trypsin digestion, and then washed twice with ice-cold PBS. To isolate total proteins, the cells were first suspended in PRO-PREP™ protein extraction solution (Intron Biotechnology, Inc., Seongnam, Korea). Protein concentrations were determined using a Bicinchoninic Acid protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA), according to the manufacturer's protocol. Protein samples (20 µg) of the cell extracts were separated by SDS-PAGE (6-15% gels) and transferred onto a polyvinylidene difluoride membrane (EMD Millipore), which was incubated for 1 h in TNA buffer (25 mM Tris/HCl pH 8.5, 192 mM glycine and 20% methanol) and blocked with 5% skimmed milk powder in PBS. Subsequently, the membrane was incubated with various primary antibodies against SIRTs, Bax, Bcl-2, β-actin, PARP, cleaved caspase-3, cleaved caspase-9, p53, acetylated p53, LC3, beclin-1, Atg3, Atg5 and Atg7 at 4°C overnight. Following washing for 1 h with TNA buffer, the membrane was incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (1:10,000) for 1 h at room temperature. The blots were developed using an Enhanced Chemiluminescence Plus kit (GE Healthcare Life Sciences, Little Chalfont, UK). The data were acquired and analyzed using an ImageSaver6 (ATTO Corp., Tokyo, Japan).

**Reverse transcription-polymerase chain reaction (RT-PCR).** Total RNA was isolated using TRIzol® reagent (Gibco; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Samples of 2 µg RNA were reverse-transcribed for 50 min at 42°C in a 20 µl reaction mixture containing 1 µl oligonucleotide (dT)₅ primer (0.5 µg), 10 mM dNTP mixture, 25 mM MgCl₂ (4 µl), 0.1 M dithiothreitol (2 µl), RNaseOUT inhibitor (1 µl; Invitrogen; Thermo Fisher Scientific, Inc.), Superscript II (50 units) and X10 reverse transcription buffer (2 µl), followed by denaturation at 68°C for 15 min. The cDNAs obtained were further amplified using PCR with the specific primers. The primers sets and PCR conditions are presented in Table I. The number of PCR cycles was estimated in a preliminary study and optimized in the exponential phase of PCR. The PCR products were subjected to electrophoresis on 2% agarose gels and visualized by ethidium bromide staining and UV transillumination (WSE-5600 CyanoView; ATTO Corp.). The molecular sizes of the amplified products were determined by comparison with a molecular mass marker (100 bp DNA ladder; Intron Biotechnology, Inc.) that was run in parallel with the RT-PCR products. Each assay was performed three times.

**Acridine orange staining.** SKOV3 cells were seeded in T-25 flasks and treated with 15d-PGJ₂ (1, 5 or 10 µM), J11-Cl (1, 5 or 25 µM) or J19 (1, 5 or 25 µM) for 48 h when the cells reached 70% confluence. At the appropriate time points, the cells were treated with 1 µg/ml acridine orange (2.7 µM) in serum-free medium at 37°C for 15 min at room temperature. Following washing with PBS, the formation of acidic vesicular organelles (AVOs) was observed using fluorescence microscopy (FV10i; Olympus Corp., Tokyo, Japan). The cytoplasm and nuclei of the stained cells fluoresced bright green, whereas the acidic AVOs...
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fluoresced bright red. Additionally, green (510-530 nm) and red (650 nm) fluorescence emission from 1x10⁴ cells exposed to blue (488 nm) excitation light was determined using a flow cytometer.

Monodansylcadaverine (MDC) incorporation assay. Autophagic vacuoles were also detected by incubation with 50 µmol/l MDC in PBS at 37°C for 10 min. Following incubation, the cells were washed four times with ice-cold PBS and were fixed with 3.75% paraformaldehyde in PBS. The cells were immediately analyzed using a confocal microscope (FV10i) equipped with a filter system (excitation wavelength, 380 nm; emission filter, 525 nm).

Statistical analysis. Statistical analyses were carried out using GraphPad Prism software (version 5.03; GraphPad Software, Inc., La Jolla, CA, USA). All numerical data are presented as the mean ± standard error of the mean. Statistical analyses were performed using Student's t-test or Mann-Whitney U test. One-way analysis of variance with Tukey’s post hoc test to assess differences among specific groups was used for comparison among multiple values. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of 15d-PGJ₂, J19 and J11-C1 on the viability of ovarian cancer cells. Different cancer cell lines were used to screen the in vitro cytotoxic activity of 15d-PGJ₂ and its derivatives. Cell viability was determined using an MTT assay. Human ovarian cancer SKOV3 cells were treated with the indicated concentrations of agents for 48 h. 15d-PGJ₂ significantly decreased cell viability.
viability in a concentration-dependent manner with an IC\textsubscript{50} of 7.58 µM after 48 h of treatment. Similarly, J11-C1 and J19 markedly affected SKOV3 cell viability in a concentration-dependent manner, but the IC\textsubscript{50} values of J11-C1 (17.6 µM) and J19 (83 µM) were much higher compared with that of 15d-PGJ\textsubscript{2} (Fig. 1B). During incubation with 15d-PGJ\textsubscript{2}, the cell morphology was altered to an enlarged and elongated form. However, SKOV3 cells also appeared to be more stretched following J11-Cl treatment, with a different cell morphology phenotype from that of the control cells. Following treatment with J19, the cells were initially rounded and shrunken with larger cytoplasm and distinct cellular boundaries (Fig. 1C).

The cytotoxicity of 15d-PGJ\textsubscript{2} and its derivatives against the other ovarian cancer cell line used, OVCAR3 cells, were compared. The results indicated that the cytotoxic effect of 15d-PGJ\textsubscript{2}, (IC\textsubscript{50}, 80.7 µM), J11-C1 (49.2 µM) or J19 (20.3 µM) was lower compared with that in SKOV3 cells (Fig. 1D), therefore, SKOV3 cells were selected for our subsequent experiments. The cytotoxic effect of 15d-PGJ\textsubscript{2} and its derivatives against normal cells was examined using an MTT assay. Normal kidney proximal tubule epithelial HK-2 or NRK-52E cells were treated with 15d-PGJ\textsubscript{2}, J11-C1 or J19 at various concentrations for 48 h. Cell viability was determined using an MTT assay. Results are presented as the mean ± standard error of the mean of three independent experiments. *P<0.05, **P<0.01 vs. control. 15d-PGJ\textsubscript{2}, 15-deoxy-\Delta\textsubscript{12,14}-prostaglandin J\textsubscript{2}.

Table I. Primer sequences for the polymerase chain reaction.

| Gene name | Primer sequence | Product length (bp) | T\textsubscript{m} (°C) | Cycling conditions |
|-----------|-----------------|---------------------|--------------------------|-------------------|
| SIRT1     | F: 5'-GACTCCAAGGCCACGGATA-3'  
R: 5'-GTGGAGGTATTGTTCCGGC-3' | 110 | 59.89 | 95°C for 5 min; 34 cycles of 95°C for 15 sec, annealing at 57°C for 15 sec and 72°C for 15 sec; 72°C for 10 min |
| SIRT2     | F: 5'-GGCATTTCAAGCCAACCATC-3'  
R: 5'-CCACCAAGTCCTCCTGTCC-3' | 132 | 59.76 | 95°C for 5 min; 35 cycles of 95°C for 15 sec, annealing at 57°C for 15 sec and 72°C for 15 sec; 72°C for 10 min |
| SIRT4     | F: 5'-AGGTCCTGTGGTGATGGATTG-3'  
R: 5'-GGTTTCAGATGGCCTCCACA-3' | 172 | 59.96 | 95°C for 5 min; 32 cycles of 95°C for 15 sec, annealing at 57°C for 15 sec and 72°C for 15 sec; 72°C for 10 min |
| GAPDH     | F: 5'-GATGCAACGGGATTTGGTGCGT-3'  
R: 5'-TTGTTGTCATGAGTCTCTCCA-3' | 512 | 58.21 | 95°C for 5 min; 32 cycles of 95°C for 15 sec, annealing at 56°C for 15 sec and 72°C for 15 sec; 72°C for 10 min |

SIRT, Siruin; F, forward; R, reverse.
SIRT enzymatic activity and expression in SKOV3 cells. The effect of 15d-PGJ2 and its derivatives on SIRT protein expression was examined in SKOV3 cells using western blotting with specific antibodies against acetylated SIRT1, SIRT2, SIRT4, SIRT5 and SIRT6. As presented in Fig. 3A, 15d-PGJ2 markedly decreased the expression of SIRT1, at 1 µM. However, a high concentration (5 µM) of J11-Cl and J19 markedly decreased SIRT1 protein levels (Fig. 3A and B). To confirm the expression of SIRTs in SKOV3 cells, the mRNA levels of SIRT1, SIRT2 and SIRT4 in SKOV3 cells treated with 15d-PGJ2 (1, 5 or 10 µM), J11-Cl (1, 5 or 25 µM), or J19 (1, 5 or 25 µM). As presented in Fig. 3C and D, mRNA levels of SIRT1 and SIRT4 were significantly decreased following 15d-PGJ2 treatment (5 and 10 µM). In addition, the mRNA levels of SIRT2 were markedly decreased in SKOV3 cells by 25 µM J11-Cl and J19 treatment (Fig. 3C). The effects of 15d-PGJ2, J19 and J11-Cl on total SIRT1 enzymatic activity were investigated. Sirtinol was used as a reference compound for SIRT1 inhibitor. As presented in Fig. 3E, sirtinol significantly inhibited SIRT1 enzyme activity in a concentration-dependent manner. It was identified that 15d-PGJ2 and J11-Cl significantly decreased SIRT1 enzymatic activity at high concentrations. Similar to the protein levels, J19 only significantly inhibited SIRT1 enzyme activity at high concentrations (Fig. 3E).

Molecular docking analysis. The aims of the molecular docking study were to elucidate whether 15d-PGJ2, J11-Cl or J19 modulate anticancer targets and identify the actual binding pocket against the molecular target of SIRT1. The orientations and binding affinities of the drugs to SIRT1 (PDB code 4I5I) were identified. The docking results for sirtinol revealed a high docking score of -9.41 to -8.27 and the formation of a hydrogen bond of 1.94 Å (-C=O) to the backbone of Gln145 and π-π stacking bond lengths of 4.7 and 5.2 Å with Phe273 and Phe141. In the docking pose, the chemical natures of the binding site residues within a radius of 3 Å from the bound compound are presented in Fig. 4A. Similarly, the docking results of 15d-PGJ2 revealed a docking score of -9.43 to -6.82 and formed three hydrogen bonds of 2.37 (O=C-NH2) and 1.98 Å (-C=O) to PRo and 1.77 Å (O=C-NH-) to Asp147 (Fig. 4B). Likewise, the docking results of J11-Cl revealed a docking score of -7.34 to -3.98 and formed two hydrogen bonds with lengths of 1.98 Å (-C=O) to PRo221 and of 2.51 Å (O=C-NH-) to Ile145 (Fig. 4C). Lastly, the docking results of J19 revealed a low docking score of -4.33 to -2.85 and formed two hydrogen bonds with lengths of 2.18 and 2.24 Å (-NH=C-NH2) to Arg274 (Fig. 4D). In the docking pose, the binding site residues were His363, Ile411, Ala292, Phe415, Phe273, Phe414, Phe297, Phe297 and Asn340, thus, the bound drugs exhibited good binding affinity and marked hydrophobic interactions, which may lead to greater stability and activity. The overall interaction of the binding site pocket (within a radius of 3 Å) is summarized in Table II.

Effects of 15d-PGJ2, J19 and J11-Cl on cell cycle progression in SKOV3 cells. SIRT inhibitors exhibited moderate cytotoxicity towards various human cancer cells through the induction of cell cycle arrest at a specific phase. The effects of 15d-PGJ2 and its derivatives on cell cycle progression were determined using flow cytometry, and 15d-PGJ2 was identified to significantly increase the number of cells in G2/M phase after 48 h of incubation (Fig. 5). Similarly, J11-Cl and J19 significantly increased the population of SKOV3 cells in G2/M phase.
phase following treatment for 48 h. Furthermore, the proportion of cells in G\textsubscript{1} phase was decreased following treatment of SKOV3 cells with the drugs (Fig. 5).

**Effects of 15\textit{d}-PGJ\textsubscript{2}, J19 and J11-C1 on apoptotic death of SKOV3 cells.** To evaluate the apoptotic death of SKOV3 cells following treatment with 15\textit{d}-PGJ\textsubscript{2}, J19 or J11-C1,
Annexin V-FITC and PI staining, and western blot analyses were performed. Concentration-dependent increases in apoptotic cell death were observed following treatment with 15d-PGJ$_2$ and its derivatives (Fig. 6A and B). A significant increase in Bax expression and a parallel decrease in Bcl-2 expression were also observed following treatment with these drugs (Fig 6C). In addition, the expression levels of cleaved caspase-3 and -9, cleaved PARP and acetylated p53 were markedly increased in SKOV3 cells following treatment with high concentration of 15d-PGJ$_2$ and its derivatives (Fig. 6D and E).

Effects of 15d-PGJ$_2$, J19 and J11-C1 on autophagic cell death. To evaluate autophagic cell death induced by 15d-PGJ$_2$, J19 and J11-C1, western blot analysis and acridine orange
staining were performed. The conversion of the soluble form of LC3-I into the autophagic vesicle-associated form LC3-II is considered to indicate autophagosome formation (22). As presented in Fig. 7A and B, a high concentration of 15d-PGJ$_2$, J11-Cl and J19 significantly increased the level of LC3-II, whereas unconjugated LC3-I levels were decreased. Similar to LC3-II, beclin-1 levels increased following treatment with 15d-PGJ$_2$, J11-Cl or J19 in a concentration-dependent manner (Fig. 7A and B). Subsequently, the induction of autophagy was confirmed by acridine orange staining. Acridine orange is a lysotropic dye that accumulates in acidic organelles in a pH-dependent manner (23-26). At neutral pH, acridine orange emits a green fluorescence, but emits bright red fluorescence within acidic vesicles when protonated and becomes trapped within the organelle (2). As presented in Fig. 7C and D, the control cells exhibited primarily green fluorescence and minimal red fluorescence, which indicated a lack of acidic vesicular organelles. However, drug-treated cells exhibited a more marked red fluorescence 48 h post-treatment compared with the control cells. The increased red fluorescence intensity observed in cultured SKOV3 cells following drug treatment indicated enhanced acidification of vesicular organelles (Fig. 7C). Fig. 7D presents the mean fluorescence intensity of the control and drug-treated cells.

**Autophagy inhibition ameliorates cytotoxicity of SKOV3 cells.** To investigate the anticancer mechanism of 15d-PGJ$_2$, J19 or J11-Cl against autophagic cell death in SKOV3 cells, 3-MA (1 mM), an autophagy-specific inhibitor, was incubated with SKOV3 cells. This agent blocks autophagy by inhibiting phosphoinositide 3-kinase, an enzyme required for autophagy (25). As presented in Fig. 8A, the morphological changes indicated...
that 3-MA alone was not cytotoxic to SKOV3 cells. However, the combination of 3-MA with 15d-PGJ$_2$, J19 or J11-C1 decreased cell death compared with individual treatment with 15d-PGJ$_2$, J19 or J11-C1 (Fig. 8A). To confirm the molecular mechanism and whether autophagic cell death was affected by 3-MA treatment, the expression levels of LC3, beclin-1,
Atg5 and Atg7 were determined using western blotting. The expression levels of LC3-II and beclin-1 in 15d-PGJ2-treated SKOV3 cells were markedly decreased in SKOV3 cells (Fig. 8B). Next, the induction of autophagy was confirmed using MDC staining. The expression level of the internal control. (C) MDC staining indicated inhibition of autophagy in SKOV3 cells following co-treatment with 3-MA (1 mM) and drugs. Cells were examined using confocal microscopy. Scale bar, 10 µm. 3-MA, 3-methyladenine; 15d-PGJ2, 15-deoxy-Δ12,14-prostaglandin J2; LC3, light chain 3; Con, control; Atg, autophagy-related.

Discussion

SIRT1 is the most widely studied member of the SIRT family and is known to modulate cell proliferation, differentiation, apoptosis, migration and invasion (3,7,28). SIRT1 controls cellular senescence and is overexpressed in specific cancer cell types (29,30). In the present study, the effect of 15d-PGJ2 and its derivatives on SIRT1-mediated cell death pathways were investigated in SKOV3 cells. First, in the comparison of the cytotoxicity of the tested drugs, 15d-PGJ2 exhibited more potent cytotoxicity compared with that of J11-Cl and J19, and decreased the expression levels of SIRT1 protein. Considering the reported electrophilic carbon atom (C9) of 15d-PGJ2, we hypothesized that the inhibitory activity of 15d-PGJ2 may be a consequence of the reactive carbon. The observation that 15d-PGJ2 inhibited the gene expression of SIRT1 led to the consideration of whether 15d-PGJ2 was able to directly inhibit the activity of SIRT1. To address this question, an acetylated p53 protein was selected as a substrate to determine SIRT1 activity. As expected, SIRT1/2 activity was significantly decreased by 15d-PGJ2. To investigate the contribution of the C9 electrophilic carbon atom to the direct inhibitory activity, the molecular mechanics of 15d-PGJ2, J11-Cl and J19 on SIRT1 were elucidated using docking simulations. Although the docking conformation of 15d-PGJ2 with the SIRT1 protein did not reveal any nucleophile that reacted with C9 within 3 Å of the chemical, it revealed a higher binding affinity (best docking score, -9.43) compared with that of the other drugs, with the formation of three hydrogen bonds with Asp148 and Ile347. The prior arrangement of the three hydrogen bonds also appeared to produce greater van der Waals interactions and electrostatic attractions for this drug. J11-Cl and J19 exhibited moderate (up to -7.34) and low docking scores (up to -4.33), respectively, to form two hydrogen bonds with Ile347 and Pro317 and a hydrogen bond with Arg724, these configurations were less stable and potent compared with that of 15d-PGJ2. Therefore, the strongly overlapping conformations of the tested drugs may explain the difference in binding affinity.

Next, to determine the mechanisms underlying the action of 15d-PGJ2 and its derivatives, their effects on apoptotic and autophagic cell death pathways were investigated. First, cell cycle analysis was performed and it was identified that 15d-PGJ2 and J19 significantly induced G2/M phase arrest in SKOV3 cells. Previous studies indicated that sirtinol, a class III HDAC inhibitor, induced senescent-like growth arrest in breast cancer cells (31). SIRT1 has a more prominent function in controlling cell growth and survival as it exists in the same intracellular compartment as most cell cycle and cell death regulators (7,32). As expected, 15d-PGJ2 and J19 treatment significantly increased cell cycle arrest in SKOV3 cells. These results are similar to those of a previous study, which indicated that the inhibition of SIRT1 activates p53 and Bax gene expression, thereby inducing cell cycle arrest and apoptosis (33). To investigate the mechanism underlying the anticancer effects of 15d-PGJ2 and its derivatives, apoptotic cell death was assessed. Flow cytometric analysis revealed that 15d-PGJ2 and J11-Cl markedly induced apoptosis and subsequently increased the sub-G1 phase cell population. The downstream mechanism of apoptotic cell death was investigated. 15d-PGJ2 and its derivatives increased the expression of Bax and decreased that of Bcl-2 in SKOV3 cells.

Autophagy, another cell death pathway that is important in tumor biology, was also investigated in SKOV3 cells following treatment with 15d-PGJ2 and its derivatives. The inhibition of autophagy was suggested to allow the continued growth of pre-cancerous cells and autophagy may act as a tumor suppressor (34,35). Generally, cancer cells require...
autophagy to survive nutrient-limiting and low-oxygen conditions, particularly in the poorly vascularized internal region of the tumor. According to the results of the present study, the autophagic process led to SKOV3 cell death following treatment with 15d-PGJ₂, or its derivatives. Significant increases in LC3B and Atg7 levels were observed following 15d-PGJ₂, J11-Cl and J19 treatment relative to the control cells, and these changes were markedly associated with the cytotoxic effects of these drugs. These results were confirmed by the induction of AVOs in the cytoplasm that was stained with acridine orange. Most importantly, novel mechanisms of action of 15d-PGJ₂, and its derivatives were revealed as the inhibition of SIRT1 regulated multiple mechanisms of tumor cell biology in SKOV3 cells.

In conclusion, the anticancer effects of newly synthesized 15d-PGJ₂ derivatives were investigated and the underlying molecular mechanisms of these drugs were elucidated. On the basis of the structural similarity of 15d-PGJ₂, J11-Cl and J19 bind to and inhibit SIRT1 enzyme activity. This indicated that J11-Cl and J19 may exert anticancer activity by binding to SIRT1, to enhance apoptotic and autophagic cell death of ovarian cancer cells. However, limitations to the present study require attention. Although the results of the present study clearly indicated that SIRT1 inhibition exhibited potent anti-cancer activity in ovarian cancer cells, further investigation to confirm the corresponding effect using in vivo xenograft models is warranted.

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Availability of data and materials
The datasets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions
HSK and MHK conceived and designed the experiments. IHT, EYP, PD and JYS performed the experiments and statistical analysis. SS, MHK and SYL performed the docking study. JHJ helped to analyze and interpret the data, and critically revised the manuscript. SYL, MHK and HSK drafted the manuscript. All authors read and approved the final paper.

Ethics approval and consent to participate
Not applicable.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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