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Sensitization of Hepatocellular Carcinoma Cells to Apo2L/TRAIL by a Novel Akt/NF-κB Signalling Inhibitor

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Abstract: Hepatocellular carcinoma (HCC) cells are intrinsically resistant to tumour necrosis factor-related apoptosis ligand (Apo2L/TRAIL), in part, due to the compensatory activation of nuclear factor-kappaB (NF-κB). To broaden the clinical utilization of Apo2L/TRAIL in HCC, OSU-A9, a potent indole-3-carbinol-derived Akt/NF-κB signalling inhibitor was used to overcome the intrinsic resistance. The antitumour effects of OSU-A9, Apo2L/TRAIL and the therapeutic combination were assessed by MTT assay, caspase activation and PARP cleavage, and the synergistic interactions were determined by Calcusyn analysis. NF-κB reporter gene and RT-PCR were tested for the activation of NF-κB and the expression of death receptors (DR)4 and 5. OSU-A9 could sensitize HCC cells to Apo2L/TRAIL with high potency through down-regulation of Akt/NF-κB signalling. OSU-A9 dose-dependently reduced Akt phosphorylation and the expression and nuclear localization of RelA/p65, accompanied by parallel decreases in the expression of NF-κB target products, including Bcl-xL, Mcl-1, cIAP1, cIAP2 and survivin. Moreover, OSU-A9 increased DR5 expression through a reactive oxygen species (ROS)-dependent mechanism. Concertedly, these mechanisms underlie the synergistic interaction between OSU-A9 and Apo2L/TRAIL in mediating apoptotic death in HCC cells. The ability of OSU-A9 to accentuate Apo2L/TRAIL-induced apoptosis by inactivating Akt/NF-κB signalling might foster a promising therapeutic strategy for HCC.

Hepatocellular carcinoma (HCC), the most common type of malignant primary liver tumours, is the third most frequent cause of cancer death in many Asian and African countries, accounting for over 600,000 deaths per year [1]. Treatment of HCC is complicated by many underlying conditions, including cirrhosis, thrombocytopenia, ascites, intravascular hypovolaemia and neutropenia, which renders most patients ineligible for potentially curative therapies [2]. In addition, the prognosis of HCC is poor because of the frequent *de novo* and acquired resistance to current chemotherapeutic regimens through the de-regulation of signalling pathways governing cell proliferation and survival, among which those mediated by Akt [3] and nuclear factor (NF)- κB [4] are especially noteworthy. Based on this rationale, we have previously used the phytochemical, indole-3-carbinol, as a scaffold to develop a novel Akt/NF-κB signalling inhibitor, OSU-A9, which exhibits potent *in vitro* and *in vivo* efficacy in suppressing the growth of HCC and other types of cancer cells [5–8]. From a mechanistic perspective, the unique ability of OSU-A9 to target Akt and NF-κB provides an effective strategy to overcome drug resistance in HCC cells associated with the up-regulation of these two critical pathways. Targeting NF-κB promotes apoptosis in cancer cells and has been used effectively to overcome resistance to many chemotherapeutic agents [9].

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In this study, we report the sensitizing effect of OSU-A9 on Apo2 ligand or tumour necrosis factor-related apoptosis-inducing ligand (Apo2L/TRAIL)-induced cell death in HCC cells. Apo2L/TRAIL has emerged as a promising anticancer agent due to its selectivity in inducing apoptosis in many types of malignant cells while paring normal cells [10–12]. However, HCC cells are intrinsically resistant to Apo2L/TRAIL-induced apoptosis, which could be due to several distinct mechanisms, including compensatory activation of Akt [13,14] and NF-κB [15–19], increased expression of antiapoptotic Mcl-1 and cIAP2 [20–22], and decreased caspase-8 and caspase-3 activities [19]. Our data indicate that cotreatment of HCC cells with Apo2L/TRAIL and OSU-A9 led to a dose-dependent reduction in the expression and nuclear localization of NF-κB/p65 by suppressing Akt activation and RelA expression, accompanied by parallel decreases in the expression of the NF-κB target gene products, including Bcl-xL, Mcl-1, cIAP1, cIAP2 and survivin. In addition, OSU-A9 up-regulated the expression of the death receptor (DR)5 via a reactive oxygen species-dependent mechanism, leading to increased caspase-3 and caspase-8 activities in Apo2L/TRAIL-treated HCC cells. Moreover, stable expression of NF-κB/p65 abolished this synergistic effect, which supports the role of NF-κB inhibition in OSU-A9-mediated sensitization of Apo2L/TRAIL in HCC cells.

Materials and Methods

Reagents, antibodies and plasmids. OSU-A9 {[1-(4-chloro-3-nitrobenzenesulfonyl)-1*H*-indol-3-yl]-methanol]} was synthesized as

described previously [23], of which the purity and identity were verified by HPLC and mass spectrometry analysis, respectively. OSU-A9 was dissolved in DMSO, diluted in culture medium and added to cells at a final DMSO concentration of 0.1%. Antibodies against various biomarkers were obtained from the following sources: Akt, p-^{473}Ser Akt, p-^{308}Thr Akt, NF-κB (p65/RelA), cIAP1, cIAP2, p-^{536}Ser NF-κB, Mcl-1, IKKα/β, p-$^{176/180}$ Ser IKKα/β, nucleolin, α-tubulin, caspase-3 and caspase-8 from Cell Signalling Technologies (Danvers, MA, USA); DR4, DR5, Bcl-xL and IκBα from Santa Cruz Biotechnology (Santa Cruz, CA, USA); survivin from R&D Systems (Minneapolis, MN, USA); c-Flip from BioVision, (Milpitas, CA, USA); β-actin from Sigma-Aldrich (St. Louis, MO, USA). Mouse antipoly (ADP-ribose) polymerase (PARP) monoclonal antibody was purchased from BD Pharmingen (San Diego, CA, USA). The pCMVp65 plasmid was kindly provided by Dr. Hung-Wen Chen (Academia Sinica, Taiwan) and Dr. Cheng-Wen Lin (China Medical University, Taichung, Taiwan). The enhanced chemiluminescence system for the detection of immunoblotted proteins was from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Apo2L/TRAIL was purchased from Peprotech (Rocky Hill, NJ, USA). Other chemicals and reagents were obtained from Sigma-Aldrich unless otherwise mentioned.

Cell culture. The three HCC cell lines used in this study were purchased from the following sources: Huh7 (JCRB0403) was obtained from the Health Science Research Resources Bank (Osaka, Japan). Hep3B, PLC5 and PC-3 were obtained from the American Type Culture Collection (Manassas, VA, USA). 4′,6-Diamidino-2-phenylindole (DAPI) nuclear staining indicated that these cells were devoid of mycoplasma contaminations. HCC cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA); and PC-3 cells were culturFed in RPMI1640 (Invitrogen) supplemented with 10% foetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and cells were cultured at 37°C in a humidified incubator containing 5% CO_2. Cells in log-phase growth were harvested by trypsinization for use in various assays.

Cell viability analysis. Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in six replicates as described previously [24]. In brief, cells were seeded at 4×10^3 cells per well in 96-well flat-bottomed plates and then, 24 h later, cells were treated with OSU-A9 and/or Apo2L/TRAIL at the concentrations indicated in individual figures. At the end of the treatment, the medium was removed, replaced by 200 μL DMEM containing 0.5 mg/mL of MTT and cells were incubated in the CO_2 incubator at 37°C for 2 h. Supernatants were aspirated from the wells, and the reduced MTT dye was solubilized in 200 μL/well DMSO. Absorbance at 570 nm was determined using a plate reader. Results were calculated by subtracting blank readings, in which cells were absent, from sample readings. IC_{50} and combination index (CI) values were determined using CalcuSyn® software (Biosoft, Cambridge, UK).

Immunoblotting. Western blot analysis was performed as reported previously [25]. Briefly, treated cells were washed with PBS, resuspended in SDS sample buffer, sonicated for 5 sec. and then boiled for 5 min. After brief centrifugation, equivalent amounts of proteins from the soluble fractions of cell lysates were resolved in 10% SDS–PAGE on a Minigel apparatus and transferred to a nitrocellulose membrane using a semidry transfer cell. The transblotted membranes were washed thrice with TBS containing 0.05% Tween 20 (TBST). After blocking with TBST containing 5% non-fat milk for 120 min., the membranes were incubated with the appropriate primary antibodies at 1:500 dilution (with the exception of anti-β-actin antibody, 1:2,000) in TBST–5% low fat milk at 4°C overnight and then washed thrice with TBST. Membranes were probed with goat antirabbit or antimouse IgG-horseradish peroxidase conjugates (1:2,500) for 90 min. at room temperature and washed thrice with TBST. The immunoblots were visualized by enhanced chemiluminescence [23]. Protein bands were analysed with image processing and analysis software, ImageJ version 1.45 (National Institutes of Health, Bethesda, MD, USA). The relative intensity of protein bands of drug-treated samples to that of the respective vehicle-treated control was presented as percentage after being normalized to the respective internal reference (total respective protein or β-actin).

Annexin V-FITC/PI flow cytometry. For the detection of apoptotic cell death, Apoptosis Detection Kit I (BD Biosciences, San Diego, CA, USA) was used according to the manufacturer's instructions. Hep3B cells were treated with OSU-A9 (2 μM), TRAIL (100 ng/mL) or their combination for 48 h. Cells undergoing apoptosis were detected using double staining with Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) and flow cytometry. Data analysis was performed using BD FACSDiva 6.0 software (BD Pharmingen, San Diego, CA, USA).

Preparation of nuclear and cytosolic extracts. Hep3B Cells (1×10^6) were treated with different concentrations of OSU-A9. One hour after treatment, 100 ng/mL Apo2L/TRAIL or vehicle was added to the medium. Six hours later, cells were harvested and nuclear and cytosolic extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (PIERCE®, Rockford, IL, USA) according to the manufacturer's instructions.

NF-κB-dependent reporter gene expression assay. One million Hep3B cells was transiently cotransfected with 2 μg of pNF-κB-Luc reporter plasmid (Dr. Cheng-Wen Lin, China Medical University, Taiwan) and 0.5 μg of the *Renilla reniformis* luciferase control reporter vector (pRL-CMV; Promega, Madison, WI, USA) using the Amaxa Nucleofector system (Lonza, Gaithersburg, MD, USA). Transfected cells were seeded into 12-well plates, and 24 h later, cells were treated in triplicate with different concentrations of OSU-A9 or vehicle with or without 100 ng/mL Apo2L/TRAIL (Peprotech, Rocky Hill, NJ, USA) for 6 h. The luciferase activities in cell lysates were determined using the Dual-Luciferase Reporter Assay System (Promega) and normalized to the constitutive *Renilla reniformis* luciferase activity.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Total RNA was extracted using the TRIzol® reagent (Invitrogen, Carlsbad, CA, USA). RT-PCR was run using a one-step RT-PCR kit and β-actin was used as internal control. The used upstream primers for DR4, DR5, and β-actin were 5′-CTGAGCAACGCA GACTCGCTGTC CAC-3′, 5′-GCCTCATGGACAATGAGATAAAGGTGGCT-3′ and 5′-G TCAGAAGGATTCCTATGTG-3′, respectively. The downstream primers for DR4, DR5 and β-actin were 5′-TCCAAGGACACGGC AGAGCCTGTGCCAT-3′, 5′CCAAATCTCAAAGTACGCACAA ACGG-3′ and 5′-ATCTCCTTCTGCATCCTGTC-3′, respectively. PCR products were a 506-bp fragment for DR4, a 502-bp fragment for DR5 and an 800-bp fragment for β-actin. PCR cycles were as follows: a denaturation step at 94°C for 30 sec., an annealing temperature of 62°C for 30 sec. and an extension period at 72°C. The amplification cycles of DR4, DR5 and β-actin were repeated 25, 22 and 22 times, respectively. Agarose gel electrophoresis was used to separate the amplified PCR products that were stained with 1.2% ethidium bromide and viewed under ultraviolet light. The integrated absorbance values of the DR4, DR5 and β-actin bands were analysed using the Bio-Rad image system Semi-quantitative analysis (Bio-Rad Laboratories, Hercules, CA, USA).

ROS measurement. Reactive oxygen species (ROS) production was detected using the fluorescence probe carboxy-H2DCFDA. Hep3B

cells plated on coverslips were washed twice with medium and then incubated in 25 μM DCFDA/DMEM for 30 min. at 37°C in the dark. Cells were then washed twice and treated with OSU-A9 for 1 h. Coverslips were rinsed with DMEM before mounting on slides, and fluorescence (FITC filter) images of cells were taken immediately using a fluorescence microscope. Ten fields (40× objective) for each coverslip were sampled (>400 cells), and the mean pixel values (0–255) of individual cells were analysed using ImageJ version 1.45 software. All treatments were performed in duplicate, and the data expressed were average values of all cells counted in each field.

For flow cytometry, drug-treated cells were analysed for staining with carboxy-H2DCFDA. Hep3B cells were plated onto 10-cm dishes, incubated overnight and then treated for 1 h with OSU-A9. Cells were then collected and stained with carboxy-H2DCFDA according to the vendor's protocols. FACScort flow cytometer was used, and the data were analysed by ModFitLT version 3.0 software (Verity Software House, Topsham ME, USA).

Caspase assays. Caspase-3/7 and caspase-8 activities in Hep3B cells treated with OSU-A9 or Apo2L/TRAIL or their combination were measured using a Caspase-Glo assay kit (Promega, Madison, WI, USA) following the manufacturer's instructions.

Overexpression of p65/RelA. Hep3B cells were transfected with 2 μg of pCMVp65 plasmid or the empty vector using the Amaxa Nucleofector system. Forty-eight hours later, cells were treated with different concentrations of Apo2L/TRAIL and/or OSU-A9. Cell viability was assessed after 48 h using MTT assay in six replicates as mentioned before. Expression of RelA and its effector protein cIAP2 was confirmed by Western blot analysis.

Statistical analysis. Each Western blot was performed 2–3 times. Differences in relative NF-κB activation were analysed for statistical significance using one-way analysis of variance followed by the Neuman–Keuls test for multiple comparisons. Differences were considered significant at $p < 0.05$. Statistical analyses were performed using SPSS for Windows (SPSS, Chicago, IL, USA). Using the software package Calcusyn (Biosoft), the values of combination index (CI) were calculated. The values of CI at different levels of growth inhibition were calculated based on the formula for mutually non-exclusive mechanism: (D1/Dx1) + (D2/Dx2) + (D1D2/DxlDx2) where Dx1 and Dx2 are the doses of drug 1 and drug 2 alone required to produce x% effect, and D1 and D2 are the doses of drug 1 and drug 2 in combination required to produce the same effect. The CI values less than 1, equal to 1 and greater than 1 are indicative of synergism, additive effect and antagonism, respectively.

Results

HCC cells are resistant to the antiproliferative effect of Apo2L/TRAIL due to the activation of NF-κB signalling.

Hepatocellular carcinoma (HCC) cells are known to be resistant to Apo2L/TRAIL and other cytotoxic drugs as a result of the activation of Akt and NF-κB, overexpression of Bcl-xL, Mcl-1 and other antiapoptotic proteins, and reduced caspase-3 and caspase-8 activities [4,13–15,18–22,26,27]. The antiproliferative effect of Apo2L/TRAIL was assessed in three representative human HCC cell lines, PLC5, Huh7 and Hep3B, and the TRAIL-sensitive PC-3 cells as a positive control by MTT assay. As shown, all three HCC cell lines showed resistance to the suppressive effect of Apo2L/TRAIL on cell viability up to 200 ng/mL irrespective of genetic abnormalities, while PC-3 cells exhibited an IC_{50} of 60 ng/mL (fig. 1A). Western blot analysis revealed increased phosphorylation of IκB kinase (IKK)αβ and the RelA/p65 subunit, accompanied by the decreased expression of the NF-κB inhibitor IκBα, in response to Apo2L/TRAIL (100 ng/mL) treatment in these three cell lines (fig. 1B). This finding is reminiscent with the report that Apo2L/TRAIL-induced NF-κB activation was preceded by IKK activation and IκBα degradation [17]. Moreover, this Apo2L/TRAIL-induced NF-κB activation was confirmed by increases in nuclear accumulation of RelA/p65 and NF-κB luciferase reporter activity in Hep3B cells (fig. 3B). Together, these data suggest a causative relationship between compensatory activation of NF-κB and Apo2L/TRAIL-resistant phenotype in these HCC cells.

OSU-A9 sensitizes HCC Cells to Apo2L/TRAIL-mediated antiproliferative effect.

OSU-A9, as a single agent, suppressed the viability of Hep3B, Huh7 and PLC5 cells with IC_{50} values of 2.8, 3.2 and 3.2 μM, respectively (fig. 2A). The effect of OSU-A9 on Apo2L/TRAIL-induced cell death was assessed by exposing Hep3B and Huh7 cells to a combination of escalating doses of OSU-A9 (1, 2 and 4 μM) and Apo2L/TRAIL (10, 50, 100 and 200 ng/mL). As shown, these combinations led to increased cytotoxicity to both cell lines in a synergistic manner, as

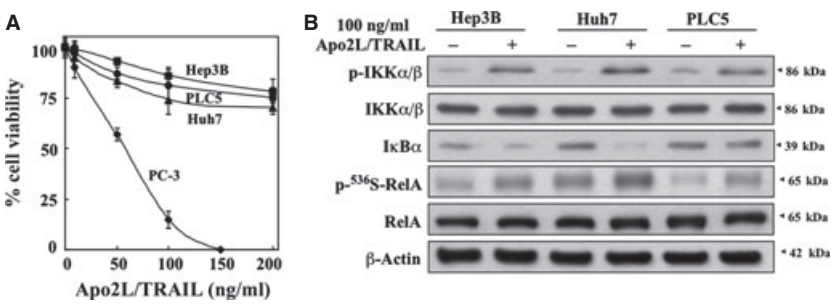

Fig. 1. NF-κB pathway activation is involved in hepatocellular carcinoma (HCC) cell lines resistance to the antiproliferative effect of Apo2L/TRAIL. (A) Cells were treated with Apo2L/TRAIL at the indicated concentrations in 5% FBS-supplemented Dulbecco's modified Eagle's medium (DMEM) in 96-well plates for 48 h, and cell viability was assessed by MTT assays. Points, mean; bars, S.D. (n = 6). (B) Western blot analysis showing the ability of Apo2L/TRAIL to activate NF-κB pathway, as manifested by increased phosphorylation of IKKα/β and RelA and decreased IκBα expression, in three HCC cell lines. Cells were treated with Apo2L/TRAIL (100 ng/mL) in 5% FBS-supplemented DMEM medium for 48 h.

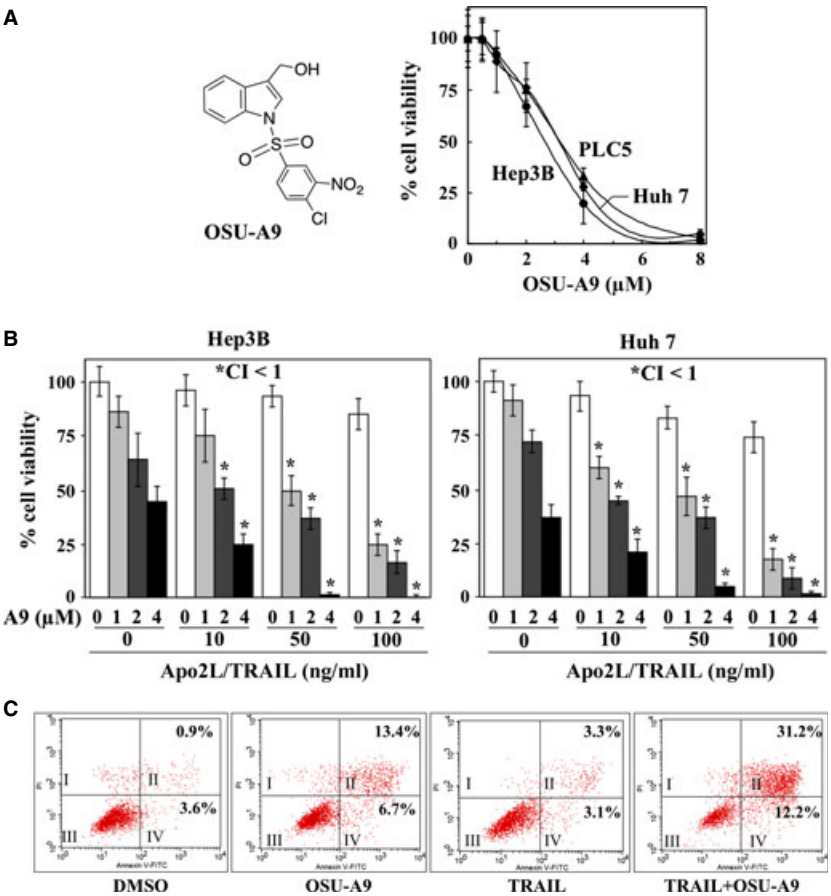

Fig. 2. OSU-A9 sensitizes hepatocellular carcinoma (HCC) cells to Apo2L/TRAIL-induced growth inhibition. (A) *Left panel*, structure of OSU-A9. *Right panel*, HCC cells treated with OSU-A9 in 5% FBS-supplemented Dulbecco's modified Eagle's medium (DMEM) in 96-well plates for 48 h, and cell viability was assessed by MTT assays. Points, mean; bars, S.D. (n = 6). (B) HCC cells treated with OSU-A9 in combination with Apo2L/TRAIL in 5% FBS-supplemented DMEM medium in 96-well plates for 48 h, and cell viability was assessed by MTT assays. Points, mean; bars, S.D. (n = 6). Combination indices were calculated by CalcuSyn software version 2.1. (C) Annexin V/PI staining assay of cell apoptosis. Hep3B cells were treated with OSU-A9 (2 μM), TRAIL (100 ng/ml) or their combination for 48 h, stained with Annexin V and propidium iodide, and analysed by flow cytometry. The total apoptotic cells were considered as the percentage of Q_{IV} (early apoptosis) + percentage of Q_{II} (late apoptosis).

indicated by the combination index (CI) values ranging from 0.68 to 0.74 (fig. 2B). Synergism of OSU-A9 with Apo2L/TRAIL was assessed using combination algebraic estimate (CI ± 1.96 S.D. *versus* fractional effect) calculated by CalcuSyn software. OSU-A9 at suboptimal doses could sensitize these cells to Apo2L/TRAIL-mediated antitumour effect. For example, in the presence of 1 μM OSU-A9 (IC_{10} for Hep3B and Huh7), the IC_{50} values of Apo2L/TRAIL in suppressing the viability of both cell lines cells were reduced to approximately 50 ng/mL (fig. 2B). These IC_{50} values were lowered to 10 ng/mL when cells were cotreated with 2 μM OSU-A9 (IC_{25} for Huh 7 and IC_{30} for Hep3B), which is comparable to that of the TRAIL-sensitive PC-3 cells. Furthermore, the synergistic induction of apoptosis by OSU-A9/Apo2L/TRAIL combination was confirmed by Annexin V-FITC/PI flow cytometry. The total apoptotic cells were considered as sum of the percentages of cells at early apoptosis and late apoptosis stages. As shown in fig. 2C, the combination showed a significant increase in the fraction of apoptotic cells (43.4%) compared to single treatment with either OSU-A9 (20.1%) or Apo2L/TRAIL (6.4%).

OSU-A9 antagonizes Apo2L/TRAIL-induced NF-κB activation. To investigate the role of NF-κB inhibition in OSU-A9-mediated sensitization of Apo2L/TRAIL-induced cell death, we examined the dose-dependent effects of OSU-A9 on various biomarkers associated with the activation status of Akt and NF-κB signalling in Apo2L/TRAIL-treated Hep3B cells by Western blot analysis. These biomarkers included the phosphorylation status of Akt and the expression levels of RelA/p65 and the antiapoptotic NF-κB target gene products Bcl-xL, Mcl-1, cIAP1, cIAP2 and survivin (fig. 3A). As shown, OSU-A9, alone or in combination, dose dependently reduced the phosphorylation of Akt at Thr-308 and Ser-473. Despite the ability of Apo2L/TRAIL to up-regulate the expression of these NF-κB-associated proteins, OSU-A9 was highly effective in suppressing their expression levels in a manner similar to that without Apo2L/TRAIL treatment. The ability of OSU-A9 to antagonize Apo2L/TRAIL-induced NF-κB activation was further confirmed by a dose-dependent reduction in RelA/p65 nuclear accumulation and NF-κB luciferase reporter activity in OSU-A9-treated Hep3B cells (fig. 3B).

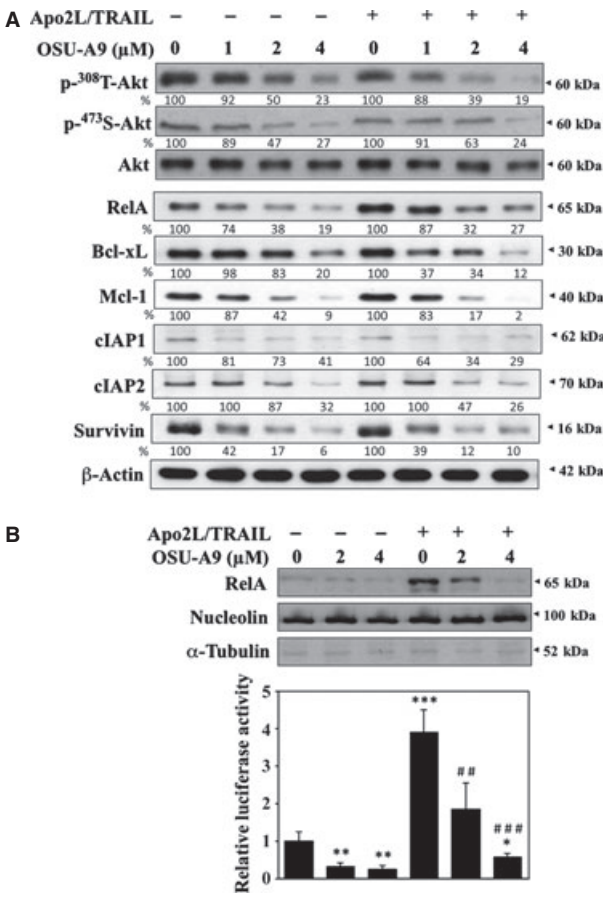

Fig. 3. Evidence that OSU-A9 antagonizes Apo2L/TRAIL-induced NF-κB activation. (A) Western blot analysis of the phosphorylation/expression levels of Akt, RelA (total), Bcl-xL, Mcl-1, cIAP1, cIAP2 and survivin in Hep3B cells treated with OSU-A9 in combination with 100 ng/mL TRAIL in 5% FBS-supplemented Dulbecco's modified Eagle's medium (DMEM) for 48 h. The values in percentage denote the relative intensity of protein bands of drug-treated samples to that of the respective vehicle-treated control after being normalized to the respective internal reference (total respective protein or β-actin). Each value represents the average of 2–3 independent experiments. (B) *Upper panel*, Western blot analysis of the dose-dependent effect of OSU-A9 on Apo2L/TRAIL-activated NF-κB nuclear translocation. Nuclear localization of total RelA was determined by Western blotting after treatments. Nucleolin and α-tubulin were used as controls to ensure purity of nuclear fraction. *Lower panel*: Dose-dependent effect of OSU-A9 on Apo2L/TRAIL-induced NF-κB transcriptional activity. Hep3B cells cotransfected with the pNF-κB-Luc reporter plasmid and Renilla Luciferase control reporter vectors (pRL-CMV) were treated with the indicated concentrations of OSU-A9 with or without 100 ng/mL Apo2L/TRAIL. Luciferase activity as an indicator of NF-κB-dependent transcription was determined as described in the experimental procedures. Columns, mean; bars, S.D. (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ as compared to vehicle (DMSO)-treated cells. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ as compared to Apo2L/TRAIL-treated cells.

OSU-A9 up-regulates DR5 expression via a reactive oxygen species (ROS)-dependent mechanism.

To further understand the mechanism by which OSU-A9 enhances the cell killing effect of Apo2L/TRAIL, we examined the two Apo2L/TRAIL death receptors, DR4 and DR5, as well as cellular FLICE-inhibitory protein (c-FLIP), a major inhibitor of death receptor-mediated caspase-8 activation, in Hep3B cells. As shown, OSU-A9, at 2 μM alone or in combination with Apo2L/TRAIL (100 ng/mL), increased the expression of DR5, at both mRNA and protein levels, in a dose-dependent manner, while that of DR4 and c-Flip were not affected (fig. 4A).

As ROS has been implicated in the effect of curcumin and sulforaphane on the up-regulation of DR5 in cancer cells [28,29], we examined the effect of OSU-A9 on ROS in Hep3B cells. Both immunocytochemical and flow cytometric analyses indicated the production of ROS in response to 2 μM OSU-A9, which could be abolished by *N*-acetylcysteine (NAC; fig. 4B,C). Equally important, NAC abrogated the ability of OSU-A9 to activate DR5 expression, confirming the mechanistic link between ROS- and OSU-induced DR5 expression (fig. 4D).

OSU-A9 increases caspase-3 and caspase-8 activities in Apo2L/TRAIL-treated Hep3B cells.

One of the plausible mechanisms that underlie Apo2L/TRAIL resistance in HCC cells is the lack of caspase-3 and caspase-8 activations in response to Apo2L/TRAIL treatment [19]. Thus, we examined the effects of Apo2L/TRAIL, alone or in combination with OSU-A9, on the activities of caspase-3/7 and caspase-8 in Hep3B cells. Our data show that Apo2L/TRAIL at 100 ng/mL had no significant effect on the activity of either caspase (fig. 5A). In contrast, OSU-A9 facilitated a dose-dependent increase in caspase-3/7 activity but had no effect on caspase-8. Nevertheless, the combination treatment gave rise to a robust increase in the activity of both caspases. These results were confirmed by Western blot data showing a higher degree of caspase-3 and caspase-8 activations, accompanied by a parallel increase in the extent of PARP cleavage in response to Apo2L/TRAIL/OSU-A9 combination treatment (fig. 5B).

Ectopic expression of RelA/p65 abrogates the sensitizing effect of OSU-A9 on Apo2L/TRAIL-induced cell death.

To validate the role of NF-κB inhibition in the synergistic interaction between OSU-A9 and Apo2L/TRAIL, we examined the effect of the ectopic expression of the RelA/p65 subunit on this synergy. As shown, this transient RelA/p65 overexpression rendered Hep3B cells more resistant to the dose-dependent suppressive effect of Apo2L/TRAIL, alone or in combination with 2 μM OSU-A9, on the viability of Hep3B cells (fig. 6A).

Discussion

Recently, the unique ability of Apo2L/TRAIL to selectively induce apoptosis in transformed cells but not in normal cells has received much attention and efforts aiming to its clinical development into a cancer therapeutic agent [10–12]. However, from a translational perspective, intrinsic resistance to Apo2L/TRAIL represents a major obstacle for HCC and many other types of cancers. Data from our and other laboratories

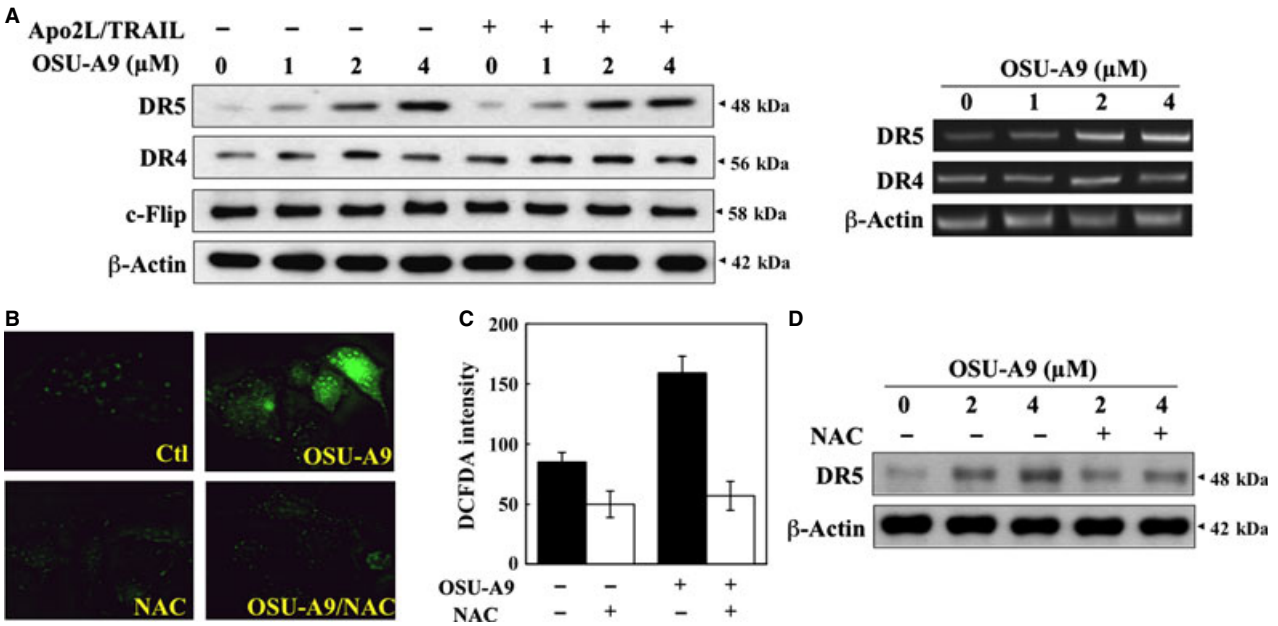

Fig. 4. Evidence that OSU-A9 up-regulates DR5 expression via a reactive oxygen species (ROS)-dependent mechanism. (A) The effect of OSU-A9, alone or in combination with Apo2L/TRAIL (100 ng/mL), on the expression of DR5, DR4 and c-Flip at both protein (*left panel*) and mRNA (*right panel*) levels in Hep3B cells treated in 5% FBS-supplemented Dulbecco's modified Eagle's medium (DMEM) for 48 h. (B) Immunohistochemistry of Hep3B stained by ROS fluorescence probe, carboxy-H2DCFDA after treatment with OSU-A9 in the presence or absence of *N*-acetylcysteine (NAC). (C) Par chart presentation of the flow cytometry analysis of Hep3B stained by carboxy-H2DCFDA after the indicated treatments. (D) Western blot analysis of the expression levels of DR5 in Hep3B cells after treatment with OSU-A9 for 48 h in the presence and absence of NAC.

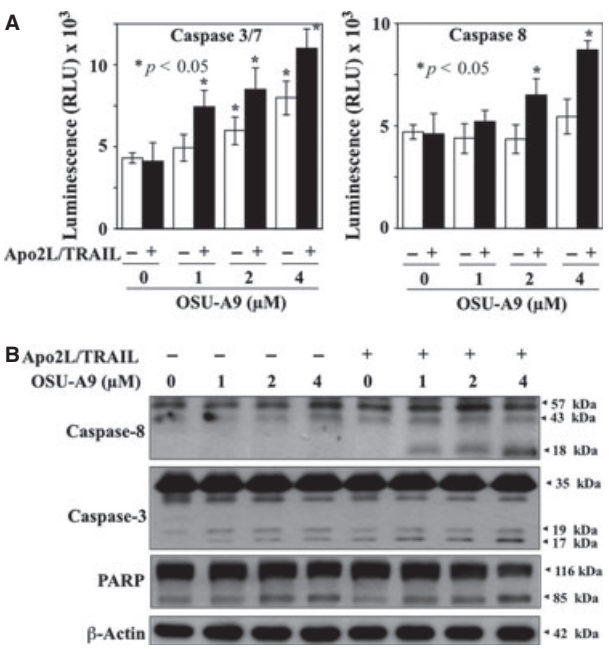

Fig. 5. OSU-A9 increases caspases activities in Apo2L/TRAIL-treated Hep3B cells. (A) Caspase 3/7 (*left panel*) and Caspase 8 (*right panel*) activities were measured using the corresponding Caspase-Glo Assay Kit and (B) Western blot analysis of the expression levels of caspase-8, caspase-3 and PARP in Hep3B cells after the indicated treatment for 48 h in 5% FBS-supplemented Dulbecco's modified Eagle's medium (DMEM). All data are depicted as mean ± S.D. *Significant difference from untreated control condition ($p < 0.05$).

have demonstrated that HCC cells acquire resistant phenotype to Apo2L/TRAIL through compensatory activation of NF-κB by facilitating IKK-mediated IκB degradation and subsequent nuclear translocation of RelA/p65, leading to up-regulated expression of many antiapoptotic proteins. A previous report indicates that this Apo2L/TRAIL-induced IKK activation is mediated through the death domain kinase RIP, which is recruited to Apo2L/TRAIL receptors upon ligand binding [30].

To overcome this drug resistance, various therapeutic agents have been reported to increase the sensitivity of HCC cells and other neoplasms to Apo2L/TRAIL by targeting relevant signalling effectors, which include Akt, bortezomib [14], NF-κB, actinomycin and nitrosylcobalamin [19,31]; DR5, curcumin [28], the synthetic cannabinoid WIN 55, 212-2 [32] and sulforaphane [29]; IKK, the bee venom component melittin [33]; caspase-8, 5-fluorouracil [34]; Mcl-1/cIAP2, sorafenib [21]; Stat3, sorafenib [35]; and survivin siRNA [36]. In the present study, our data showed that OSU-A9, a novel Akt/NF-κB signalling inhibitor [5–7,23], was highly effective in sensitizing HCC cells to Apo2L/TRAIL-induced apoptosis by concomitantly targeting many of the above signalling effectors (fig. 6B). The concerted effect of OSU-A9 on these targets culminated in the suppression of NF-κB transcriptional activities, leading to the down-regulation of its antiapoptotic gene expression. Equally important, OSU-A9 induced the expression of DR5 at both mRNA and protein levels, in a ROS-dependent manner, which is reminiscent with that reported in response to curcumin [28] and sulforaphane [29]. This

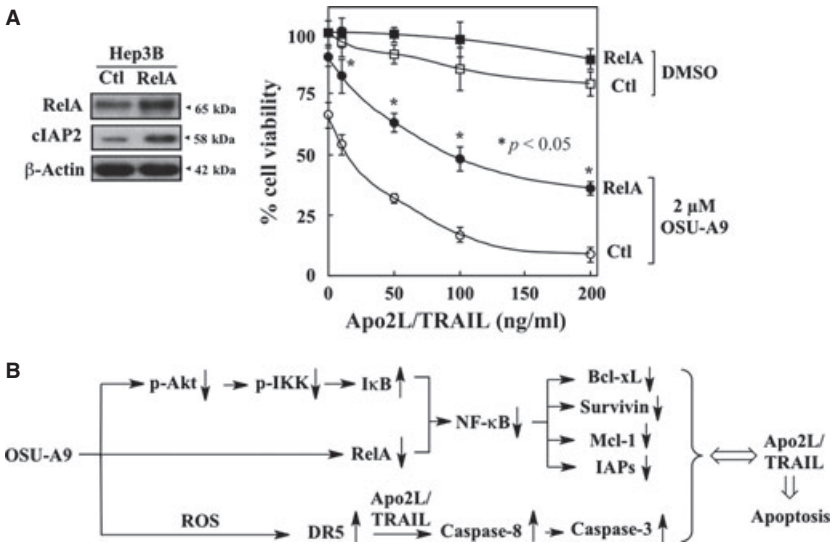

Fig. 6. Ectopic expression of RelA/p65 abrogates the sensitizing effect of OSU-A9 on Apo2L/TRAIL-induced cell death. (A) *Left panel*: Western blot analysis of the expression levels of RelA and cIAP2 in Hep3B cells transiently transfected with plasmids encoding RelA relative to untransfected Hep3B cells. *Right panel*: Effect of ectopic expression of RelA on the ability of OSU-A9 to sensitize Hep3B cells to the action of Apo2L/TRAIL on the cell viability of Hep3B relative to untransfected Hep3B cells. Points, mean; bars, S.D. (n = 6). (B) Proposed diagram depicting the mechanisms by which OSU-A9 sensitizes hepatocellular carcinoma (HCC) cells to Apo2L/TRAIL-mediated cell death.

induction, however, is highly specific because OSU-A9 did not affect the expression of DR4 or c-Flip, which contrasts the reported effect of indole-3-carbinol, the parent molecule of OSU-A9, on the expression of both DR4 and DR5 in LNCaP prostate cancer cells [37]. This discrepancy might underlie subtle differences in the mode of action between OSU-A9 and indole-3-carbinol.

In summary, the mode of action of OSU-A9 in targeting Akt-NF-κB signalling provides a mechanistic advantage to overcome Apo2L/TRAIL resistance in HCC cells, which is manifested by the ability of OSU-A9, even at 1 μM, to restore the sensitivity of HCC cells to Apo2L/TRAIL-induced cell death to a level comparable to that of a Apo2L/TRAIL-sensitive cell line. Together with our previous finding that oral OSU-A9 is well tolerated *in vivo*, these data underscore the translational value of this Akt/NF-κB signalling inhibitor in fostering new therapeutic strategies for HCC, which warrants continued investigations.

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