Regular Article

Alkaloids from *Stephania venosa* as Chemo-Sensitizers in SKOV3 Ovarian Cancer Cells via Akt/NF-κB Signaling

May Thuu Mon,¹,a Supachai Yodkeeree,²,a,c Wanisa Punfa,²,a,c Wilart Pompimon,² and Pornngarm Limtrakul*¹,a,c

¹Department of Biochemistry, Faculty of Medicine, Chiang Mai University; Chiang Mai 50200, Thailand; ²Department of Biochemistry, University of Medicine-2; Yangon 11031, Myanmar; ³Center for Research and Development of Natural Products for Health, Chiang Mai University; Chiang Mai 50200, Thailand; and ⁴Laboratory of Natural Products, Department of Chemistry, Faculty of Science, Lampang Rajabhat University; Lampang 52100, Thailand.

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Crebanine (CN), tetrahydropalmatine (THP), O-methylbulbocapnine (OMBC) and N-methyl tetrahydropalmatine (NMTHP) are isoquinoline derived natural alkaloids isolated from tubers of *Stephania venosa*. We investigated chemo-sensitizing effects of these alkaloids in ovarian cancer cells and evaluated underlying molecular mechanisms involved in chemo-sensitivity. Detection of cell apoptosis was evaluated by using flow cytometry. Cell viability was analyzed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Chou-Talalay median effect principle was used to evaluate potential drug interactions. Protein analyses were performed on ovarian carcinoma cells using Western blotting upon treatment with anticancer drug and alkaloids. Aporphine alkaloids, such as CN and OMBC, enhanced cisplatin sensitivity in intrinsic cisplatin resistant SKOV3 cells, but not in cisplatin sensitive A2780 cells. Protoberberine alkaloids, such as THP and NMTHP, had no synergistic effect on cisplatin sensitivity in either cell line. Chemo-sensitizing effects of CN and OMBC in SKOV3 cells were mediated via activating apoptosis-induced cell death through caspase-3, -8 and cleaved poly ADP-ribose polymerase (PARP) and via inhibiting anti-apoptotic and survival protein expression, such as Bcl-xL, Baculoviral IAP repeat-containing protein 3 (cIAP-2), survivin and interleukin (IL) -6. Cisplatin stimulated protein kinase B (Akt) and nuclear factor-kappaB (NF-κB) signaling pathways, but not mitogen-activated protein kinase (MAPK), activator protein 1 (AP-1) and signal transducer and activator of transcription 3 (STAT3) in SKOV3 cells. Akt/NF-κB signaling was blocked by CN and OMBC leading to increased sensitization to cisplatin. These findings demonstrate that CN and OMBC sensitizes SKOV3 cells to cisplatin via inhibition of Akt/NF-κB signaling and the down regulation of NF-κB mediated gene products. Our results suggest that alkaloids obtained from *S. venosa* could be used as chemo-sensitizers in ovarian cancer to sensitize and minimize the dose related toxicity of platinum-based chemotherapeutic drugs.

Key words ovarian cancer; aporphine; cisplatin sensitivity; apoptosis; chemo-sensitizer

Although platinum based chemotherapy is an effective treatment option for ovarian cancer patients, platinum drug resistance and the associated side effects are major limitations of the current therapeutic concepts. Cellular resistance to platinum can arise from various mechanisms related to decreased drug influx, increased DNA adduct repair, detoxification of platinum by intracellular thiols such as glutathione, alterations in intracellular signaling pathways and an increased efflux of drugs.¹,² Exposure to cisplatin induces several cellular responses with consequent changes in growth regulation. Recent data suggest that the mitogen activated protein (MAP) kinase pathway,³ the signal transducer and activator of transcription (STAT) pathway⁴ and the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathway⁵ get activated in response to cisplatin. Altered expression of intracellular signals by exposure to cytotoxic drugs may provide survival benefits to cells, leading to the development of drug resistance.

Nowadays, researchers have placed greater emphasis on the determination that natural products as chemo-sensitizers to enhance drug sensitivity and improve antineoplastic effects. Among several natural alkaloids, the herbal extracts obtained from the tubers of *Stephania venosa* have shown the presence of a wide variety of aporphine and protoberberine alkaloids.⁶ Crebanine (CN) and O-methylbulbocapnine (OMBC) are isoquinoline derived aporphine alkaloids isolated from *S. venosa* and they share a similar molecular structure with different side chains. Tetrahydropalmatine (THP) and N-methyl tetrahydropalmatine (NMTHP) are isoquinoline derived protoberberine alkaloids. In recent years, CN has gained much attention due to its biological activities and proven pharmacological safety.⁷,⁸ It was shown that CN more selectively decrease the survival and proliferation of cancerous cell lines than it does normal human fibroblasts.⁹ Moreover, CN has anti-invasive property in cancer cells by inhibiting constitutive nuclear factor kappa B (NF-κB) activation.¹⁰ On the other hand, THP has been stated to possess multi-drug resistant reversing properties in human breast cancer cells.¹¹ These established results have led us to hypothesize that these natural alkaloids may possibly improve the effectiveness of the traditional anticancer drugs.

Although the anti-cancer properties of CN have been studied in numerous cancer cell lines, it remains unidentified whether CN has chemo-sensitizing effect on drug-resistant cancer cells. Therefore, the purpose of the present study was to determine whether CN and its natural analogues show chemo-sensitizing effects in response to platinum drug in human...
ovarian cancer cells and to find out the mechanism of action.

**Experimental**

**Reagents and Chemicals** Roswell Park Memorial Institute (RPMI) 1640 medium, penicillin/streptomycin, and tryptophan–ethylenediaminetetraacetic acid (EDTA) were purchased from Gibco-BRL (Grand Island, NY, U.S.A.). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT, U.S.A.). P3K inhibitor (LY294002), antibodies specific to Bcl-2, Bax, and poly ADP-ribose polymerase (PARP) were obtained from Cell Signaling Technology (Boston, MA, U.S.A.). Antibodies specific to NF-κB, β-actin and monochromatic polyclonal antibodies (IgM) for mouse and poly ADP-ribose polymerase (PARP) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.).

**Ovarian Cancer Cell Lines** Human ovarian carcinoma cell line SKOV3 was attained from the American Type Culture Collection (Salisbury, U.K.). These cell lines were maintained in RPMI 1640 with 10% FBS, 50 mg/mL streptomycin and 50 mg/mL penicillin at 37°C in a humidified 5% CO2 atmosphere.

**Alkaloids Preparation or Extraction** The tuber part of *Stephania venosa* (BLUME) SPRENG (the Family Menispermaceae) was collected from Prachuap Khiri Khan province of Thailand and identified by Forest Herbarium. A voucher specimen (BKF No. 140583) has been deposited at the Forest Herbarium, Department of National Park, Wildlife and Plant Conservation, Ministry of Natural Resources and Environment, Bangkok, Thailand. The alkaloids CN, OMBC, THP and NMTHP were isolated and purified from the tube of *S. venosa* by Dr. Wilart Pompimon from Laboratory of Natural Products, Center for Innovation in Chemistry, Department of Chemistry, Faculty of Science, Lampang Rajabhat University. The extraction methodology, the yield of extracts, chemical profiles and structure elucidation of these alkaloids by extensive spectroscopic analysis had been reported in our previous studies.6,7 Chemical structures of four alkaloids are shown in Fig. 2A.

**Cell Viability Assay** Cells (3×103 cells/well) were harvested in 96-well plates and kept at 37°C in 5% CO2 overnight in RPMI with 10% FBS. The cells were then exposed to various concentrations of cisplatin or alkaloids or in combination for 48 h. Cell culture supernatants were then removed and incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye for 4 h, then MTT-formazan was dissolved in dimethyl sulfoxide (DMSO). The spectrometric absorbance at 540 nm was measured with a microplate reader with a reference wavelength of 630 nm.

**Combination Index Analysis** Combination index (CI) analysis was used in assessing the nature of drug interactions in combination chemotherapy. The Chou-Talalay median effect principle was used to evaluate potential drug interactions.22 CI is a numerical value calculated according to the following formula:

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CI = \frac{D1}{D1_i} + \frac{D2}{D2_i}
\]

D1 and D2 in the numerator represent the concentrations of compounds 1 and 2 in combination that are used to attain x% inhibition. D1i and D2i in the denominator stand for concentrations of compounds 1 and 2 that are used to show x% inhibition when present alone. CI<1 demonstrates synergy, CI=1 represents an additive effect, and CI>1 indicates antagonism.

**Annexin V Apoptosis Assay** SKOV3 cells were seeded in a six-well plate at a density of 2×105 cells per well and incubated overnight. Then, cells were treated with cisplatin (7.5 µM) with or without CN or OMBC (20, 40, 60 µM) for 36 h. After washing the cells with phosphate-buffered saline, they were mixed with binding buffer, and incubated for 20 min with annexin V-FITC (Merck Millipore Corporation, U.S.A.) at 25°C in the dark. Fluorescence was measured using a Guava easyCyte HT flow cytometer (Merck Millipore Corporation) with a minimum of 5000 events per sample.

**Enzyme-Linked Immunosorbent Assay (ELISA) Assay** The level of interleukin (IL)-6 in response to cisplatin with or without CN or OMBC in ovarian cancer cells was detected by sandwich ELISA assay. Ovarian cancer cells were seeded, incubated overnight and treated with CN or OMBC and cisplatin for 48 h. The supernatant was then collected, aliquoted and frozen at −20°C until being analyzed. The quantitation of IL-6 was assessed by using a human IL-6 ELISA kit (Biorad, San Diego, CA, U.S.A.).

**Protein Extractions and Western Blotting** The whole cell proteins and nuclear protein extractions were prepared as previously described.13,14 Determination of protein concentrations was done by the Bradford method. After electrophoresis, the separated proteins were relocated to a nitrocellulose membrane by electroblotting. The membranes were then probed with a specific antibody in 5% nonfat dry milk in Tris Buffer Saline (TBS) containing 0.1% Tween at 4°C overnight and then exposed to respective secondary antibodies. Finally, antibody binding was visualized by the use of an enhanced chemiluminescence detection system (Thermoscientific, Rockford, IL, U.S.A.).

**Statistical Analysis** All statistical analyses were performed using SPSS 17.0 software (SPSS, Chicago, IL, U.S.A.). Data were presented as mean±standard deviation (S.D.) of three separate experiments. Statistical analyses were calculated by one-way ANOVA and Student’s t-test. A difference between the test groups was considered statistically significant when the p value was <0.05.

**Results**

**Effects of Cisplatin and Alkaloids on Growth of Ovarian Cancer Cells** First, we observed sensitivity to cisplatin in two ovarian cancer cell lines, SKOV3 and A2780. We treated these cells with various concentrations of cisplatin for 72 h and cell viability was determined by MTT assay. The cytotoxicity of cisplatin and alkaloids in ovarian cancer cell lines are shown in Fig. 1. SKOV3 cells displayed 10-fold greater resistance to cisplatin than A2780 cells. Cisplatin sensitive A2780 cells showed more sensitivity to CN and OMBC than the cisplatin resistant SKOV3 cells. However, THP and NMTHP had no cytotoxic effect, even at high concentrations, in both the cisplatin sensitive and resistant cell lines. The non-cytotoxic concentrations (>80% cell survival) of the alkaloids were applied for subsequent experiments.

**Effects of Alkaloids on Cisplatin Sensitivity in Ovarian Cancer Cells** Aporphine alkaloids such as CN and OMBC enhanced cisplatin sensitivity in drug resistant SKOV3 (Figs. 2B, C), but not in drug sensitive A2780 cells (Figs. 2F, G).
Protoberberine alkaloids, such as THP and NMTHP, had no synergistic effect on cisplatin sensitivity in both cell lines (Figs. 2D, E, H, I). The combination index was calculated to define the synergistic, additive or antagonistic effects of the two compounds when given together. The CI values of the combination of cisplatin and CN in various doses (20, 40 and 60 µM) in SKOV3 cells were 0.89, 0.78 and 0.74, respectively, indicating their synergistic effects. CI values of the combination of cisplatin and OMBC in various doses (20, 40 and 60 µM) in SKOV3 cells were 0.91, 0.78 and 0.75, respectively and also indicated their synergistic interaction. However, CI values of CN and cisplatin in A2780 cells were 0.99, 0.97 and 1.18 indicating additive effects with 5 and 10 µM CN and antagonistic effect at 20 µM CN. Similarly, CI values of OMBC and cisplatin in A2780 cells were 0.95, 0.99 and 1.12 indicating additive effects with 5 and 10 µM OMBC and antagonistic effect at 20 µM OMBC.

**CN and OMBC Enhanced Cisplatin Sensitivity in SKOV3 Cells via Induction of Apoptosis-Induced Cell Death and via Suppression of Cisplatin-Induced Anti-apoptotic Proteins, Survival Proteins and Cytokine Expression** Since the effect of aporphine alkaloids on potentiating the cisplatin sensitivity was found in SKOV3 cells, we focused to demonstrate whether the combination of cisplatin and aporphine alkaloids could enhance SKOV3 cells apoptosis. To address this, SKOV3 cells were exposed to cisplatin alone or a combination of CN or OMBC and cisplatin for 36 h, then flow cytometry was done to define the apoptotic populations. As shown in Figs. 3A and B, CN or OMBC treated cells presented a low percentage of apoptosis, indicating that apor-
Apophine alkaloid alone, at up to 60 µM, did not cause apoptosis significantly. Cells exposed to a combination treatment showed an increase in apoptotic cells compared to the cisplatin-treated cells. The effect on apoptosis was confirmed by testing the expression status of apoptotic proteins such as caspase-3, -8 and cleaved PARP. The results showed that cisplatin treatment alone slightly increased the level of apoptotic proteins in SKOV3 cells while the co-treatment of the cells caused markedly increase expression of active caspase-3, -8 and formation of cleaved PARP (Figs. 3C, D). Next, we determined the expression of anti-apoptotic and survival proteins in response to cisplatin treatment. Our Western blotting results showed that Bcl-xL, cIAP-2, survivin expression were obviously increased after a single treatment of cisplatin while co-treatment could suppress cisplatin-induced anti-apoptotic and survival protein expression (Figs. 3E, F). Our results on ELISA assay showed that IL-6 levels were approximately three-fold increased after cisplatin treatment, while a combination treatment with CN or OMBC decreased the level of cisplatin-induced IL-6 production (Figs. 3G, H). Therefore, the enhancement of cisplatin sensitivity in SKOV3 cells by aporphine alkaloids may be mediated through the induction of apoptosis via a caspase dependent mechanism and by suppression of the cisplatin induced anti-apoptotic and survival protein expression.

Cisplatin Treatment Induced Phosphorylation of Akt and Enhanced NF-κB Activity  To investigate which signaling pathways were stimulated by cisplatin in SKOV3 cells, we treated the SKOV3 cells with cisplatin in a time dependent approach and the activities of STAT3, MAPKs, Akt, Activator protein 1 (AP-1) and NF-κB were investigated. At time 0, basal activity of p-STAT3, p-extracellular signal-regulated kinase (ERK), p-p38, p-Akt, AP-1 and NF-κB were observed. Western blotting results revealed that STAT3, AP-1, MAPKs were not responsive to cisplatin treatment, whereas phosphorylation of Akt and the translocation of NF-κB from cytosol to nucleus was upregulated for 30 min to 1 h after cisplatin exposure in SKOV3 cells (Figs. 4A–E). There was only the changes of NF-κB levels in the nucleus, whereas no changes of NF-κB in the cytoplasmic fractions was observed.

Akt/NF-κB Signaling Pathway Involved in Cisplatin Sensitivity in SKOV3 Cells In order to approve that Akt and NF-κB are involved in the cisplatin sensitivity in SKOV3 cells, we applied the selective inhibitor LY294002, which can prevent PI3K dependent Akt phosphorylation activity. First, we detected whether cisplatin induced NF-κB activity in SKOV3 cells via the Akt signaling pathway. With the co-treatment of cisplatin and inhibitor LY294002 (10, 20 µM) in SKOV3 cells, cisplatin-induced NF-κB activity was obviously decreased by Akt inhibition (Fig. 5A). Then, the significance of inhibitor on cisplatin sensitivity in SKOV3 cells was assessed by MTT assay. As shown in Fig. 5B, addition of PI3K/Akt inhibitor enhanced the growth inhibition effect of cisplatin in SKOV3 cells. These data propose that the activation of Akt/NF-κB signaling is certainly involved in platinum resistance in SKOV3 cells.

Effect of CN and OMBC on Akt/NF-κB Signaling In order to understand the molecular mechanism of the chemosensitizing effect of aporphine alkaloids, our attention was dedicated to the Akt/NF-κB signaling pathway. SKOV3 cells were pretreated with CN or OMBC and 2 h later, cisplatin was
exposed for 30 min and then the cell pellet was collected for Western blotting. The results showed that CN or OMBC could inhibit cisplatin-induced Akt phosphorylation and suppress cisplatin-induced NF-κB translocation from the cytosol to the nucleus (Figs. 5C–F).

Discussion

For the purpose of overcoming the dose-related toxicity of chemotherapeutic drugs in cancer, the development of natural products as chemo-sensitizers with higher potency and less toxicity have been intensively pursued. In this study, we examined the chemo-sensitizing properties of two alkaloid groups, aporphine (CN and OMBC) and protoberberine (THP and NMTHP), obtained from S. venosa in ovarian cancer cells. Our data demonstrated that non-toxic doses of aporphine (CN and OMBC), but not protoberberine (THP and NMTHP), revealed a synergistic effect on cisplatin in intrinsic cisplatin resistant SKOV3 cells.

The sensitivity of the cells to anticancer drug induced apoptosis was determined by analysis of the balance that existed between cellular apoptosis and survival. The ability of the cancer cells to escape apoptosis due to overexpression of genes that mediate anti-apoptosis (Bcl-xL, Bcl-2 and inhibitor of apoptosis proteins), reinforcement of survival signals (survivin, FLIP, NF-κB, etc.) and immune modulation (chemokines and interleukins) may contribute to the progression and development of tumor drug resistance.1,2) To understand the underlying mechanisms of the chemo-sensitizing action on cisplatin sensitivity by aporphine in SKOV3, we checked the anti-apoptotic and survival proteins expression by Western blotting. Our results exhibited that cisplatin enhanced the expression of Bcl-xL, cIAP-2, survivin and IL-6, whereas this
treatment together with aporphine could inhibit their expression.

Previous studies have proposed that one of the causes of cancer drug resistance is the overexpression of Akt and its upstream mediator PI3K.\textsuperscript{16,17} Peng \textit{et al.} revealed that Akt is frequently activated in ovarian carcinomas and is associated with activation of mammalian target of rapamycin (mTOR) leading to prevention of cisplatin-induced apoptosis, and subsequent cisplatin resistance. Moreover, STAT3 expression is also related to cisplatin drug resistance in squamous cell carcinoma of head and neck,\textsuperscript{18} while the suppression of activated STAT3 reverses chemo-resistance of gastric cancer cells.\textsuperscript{19} Some studies have established that exposure to cisplatin stimulates an AP-1 mediated ERCC-1 overexpression in ovarian cancer which would increase DNA repair activity and augment cancer cell survival.\textsuperscript{20} Furthermore, sustained stimulation of c-Jun N-terminal kinase (JNK)/p38 MAPK pathways in response to platinum drug leads to Fas ligand induction and ovarian cancer cell death.\textsuperscript{21} It was also stated that the loss of ability to induce p38 MAPK in response to platinum drug may be one of the mechanisms of chemo-resistance in SKOV3 cells.\textsuperscript{22} In this study, basal activity of p-STAT3, p-ERK, p-p38, p-Akt, AP-1 and NF-κB were observed. Basal activity of these proteins in our study was similar to those of many studies.\textsuperscript{3,7,15,23} However, cisplatin-induced activation was seen only in p-Akt and NF-κB. Increased phosphorylation of Akt was obviously noticed after cisplatin exposure in intrinsic cisplatin resistant SKOV3 cells. In addition, cisplatin treatment can cause NF-κB activation and translocation into the nucleus to counteract cisplatin induced apoptosis, favoring to cisplatin resistance in SKOV3 cells. However, STAT3, AP-1, p38, JNK and Erk were not responsive to cisplatin treatment in SKOV3 cells.

The PI3K/Akt pathway contributes to the tumorigenesis and chemo-resistance by inducing the anti-apoptotic activity of Akt. Akt hinders apoptosis through activation of the key transcriptional factor, NF-κB, which involved in cell survival, proliferation and drug resistance.\textsuperscript{24} In order to confirm that Akt and NF-κB are involved in cisplatin sensitivity in SKOV3 cells, we established by the use of an inhibitor compound.
that can suppress Akt phosphorylation activity. We revealed that pharmacological inhibition of Akt phosphorylation by its inhibitor leads to increased sensitivity to cisplatin in intrinsic cisplatin resistant SKOV3 cells. Therefore, the data suggest that Akt/NF-κB axis plays a significant role in platinum resistance in ovarian cancer cells. The present study demonstrated that cisplatin induced Akt activation in SKOV3 cells was inhibited by CN or OMBC leading to the suppression of the translocation of NF-κB from the cytosol to the nucleus, which in turn may lead to the downregulation of NF-κB mediated gene products.

Although aporphine alkaloids show the chemosensitizing effects in intrinsic cisplatin resistant SKOV3 cells via NF-κB signaling, they do not act as chemo-sensitizers in cisplatin sensitive A2780 cell type. One of the mechanisms might be probably due to the different characteristic between SKOV3 and A2780 in which SKOV3 cells could produce IL-6 which is one of the NF-κB regulated gene products, whereas there was no detectable IL-6 in A2780 (Supplementary Fig. 1).

Taken together, we have concluded that Akt/NF-κB signaling plays a significant role in cisplatin resistant SKOV3 cell survival and CN and/or OMBC is one of the natural alkaloids that can inhibit the pathway leading to increased platinum sensitivity. To our knowledge, this is the first report on the chemo-sensitizing effects of aporphine alkaloids obtained from S. venosa on platinum drug sensitivity in SKOV3 ovarian cancer cells. This may be a way to overcome the dose related toxicity and resistance to chemotherapeutic drugs in cancer cells. However, further in vivo experiments are required for clinical trials to pursue their potential as a chemosensitizing agent against cancer cells.

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Conflict of Interest The authors declare no conflict of interest.

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References
2) Niero E. L., Rocha-Sales B., Lauand C., Cortez B. A., de Souza M.


