# CHAPTER 3

# Results

## 3.1 Sensitivity to platinum drugs in ovarian cancer cells

First of all, the sensitivity to platinum drugs were compared in 3 human ovarian cancer cell lines: cisplatin sensitive A2780 ovarian cancer cells, acquired cisplatin resistance A2780/cis cells and intrinsic cisplatin resistance SKOV3 cells. The ovarian cancer cells were treated with various concentrations of cisplatin or carboplatin for 72 hours. Thereafter, the culture medium was discarded and growth inhibition was determined by using MTT assay. The data indicated in Table 3.1 shows that cisplatin inhibited the growth of all three ovarian cancer cell lines; A2780 cells were the most sensitive line whereas A2780/cis cells were the most resistant lines while SKOV3 cells fell in between with $IC_{50}$ at 1.10, 19.83 and 9.67 μM respectively. These data indicates that each ovarian cancer cell line has different sensitivity to platinum drugs.

Table 3.1 Sensitivity to platinum drugs in ovarian cancer cell lines

| Cell line | Cisplatin $IC_{50}$ (μM)[a] | Carboplatin $IC_{50}$ (μM)[a] |
|---|---|---|
| SKOV3 | 9.67 ± 2.99 | 99.63 ± 4.38 |
| A2780 | 1.10 ± 7.05 | 16.75 ± 6.01 |
| A2780/cis | 19.83 ± 3.05 | 155.40 ± 7.07 |

The values are presented as the mean ± SD of three independent experiments. [a] Cell survival was determined by the MTT assay. Ovarian cancer cells (SKOV3, A2780, A2780/cis), 50% inhibitory concentration ($IC_{50}$).

### 3.2 Cytotoxic effects of alkaloids from *S. venosa* in ovarian cancer cells

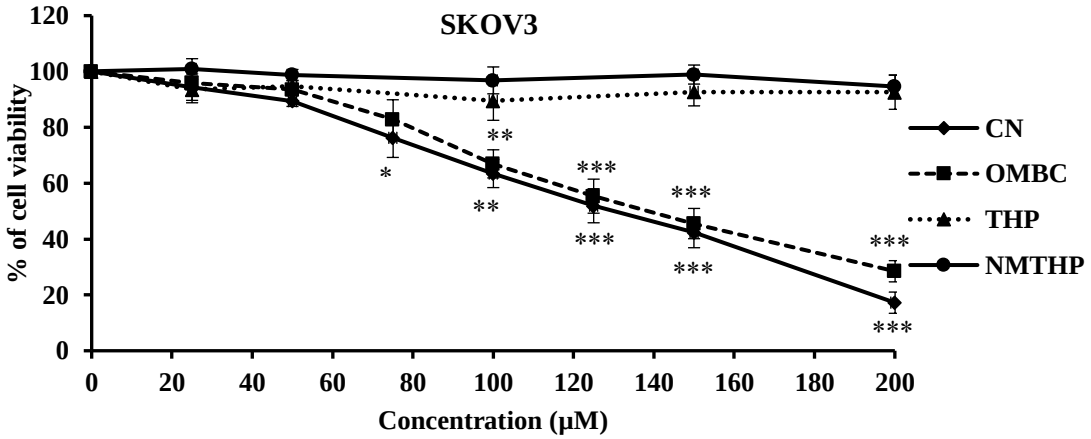
Before the effect of alkaloids on platinum sensitivity was determined, their cytotoxicity on the ovarian cancer cells were considered. Ovarian cancer cells were treated with CN, OMBC, THP or NMTHP in the range of concentration 0 to 200 μM for 48 hours. Thereafter, the culture medium was discarded and cell viability was determined by using MTT assay. Table 3.2 showed the $IC_{50}$ of the alkaloids in ovarian cancer cell lines. The data indicated that CN and OMBC showed potent cytotoxic effect on A2780 cells with $IC_{50}$ values 79.20 μM and 83.50 μM respectively while $IC_{50}$ values on A2780/cis cells were 89.33 μM and 94.12 μM respectively. $IC_{50}$ of CN and OMBC on SKOV3 cells were 139.67 μM and 141.70 μM respectively. However, THP and NMTHP had weak cytotoxic effect for doses up to 200 μM in all three cell lines (Figure 3.1). According to the results, the non-toxic concentrations (cell viability more than 80% relative to untreated control) of the alkaloids on each cell lines were further used for next experiments.

Table 3.2 $IC_{50}$ values of the alkaloids in ovarian cancer cell lines

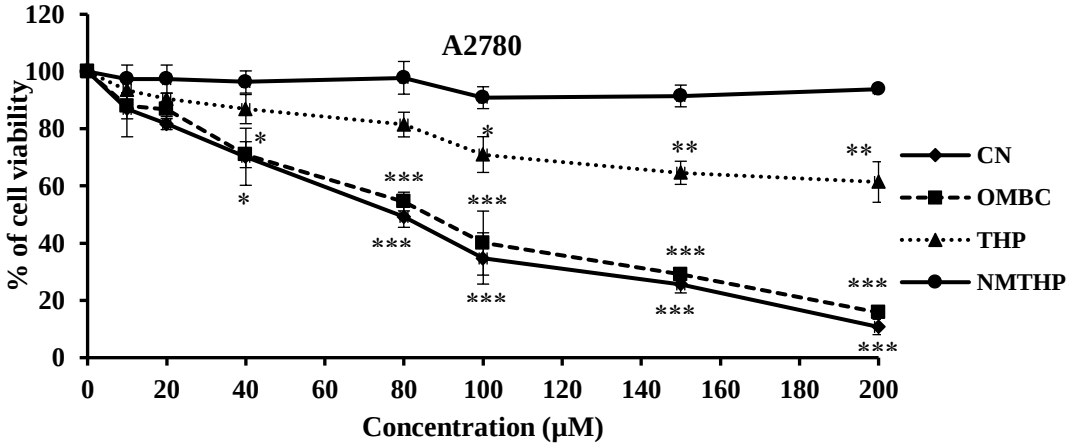
| Cell line | CN (μM) | OMBC (μM) | THP (μM) | NMTHP (μM) |
|---|---|---|---|---|
| SKOV3 | 139.67±2.06 | 141.70±6.51 | >200 | >200 |
| A2780 | 79.20±4.59 | 83.50±2.12 | >200 | >200 |
| A2780/cis | 89.33±7.18 | 94.12±5.78 | >200 | >200 |

The values are presented as the mean ± SD of three independent experiments.

(A)




(B)




(C)

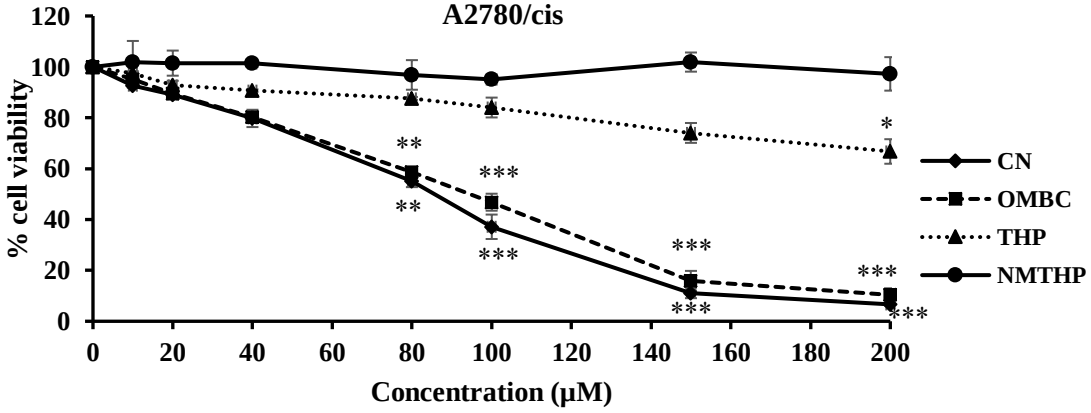



Figure 3.1 Cytotoxic effect of CN, OMBC, THP and NMTHP in ovarian cancer cell lines accessed by MTT assay. (A) SKOV3 cells (B) A2780 cells (C) A2780/cis cells were treated with indicated concentrations (0-200 µM) of CN, OMBC, THP and NMTHP for

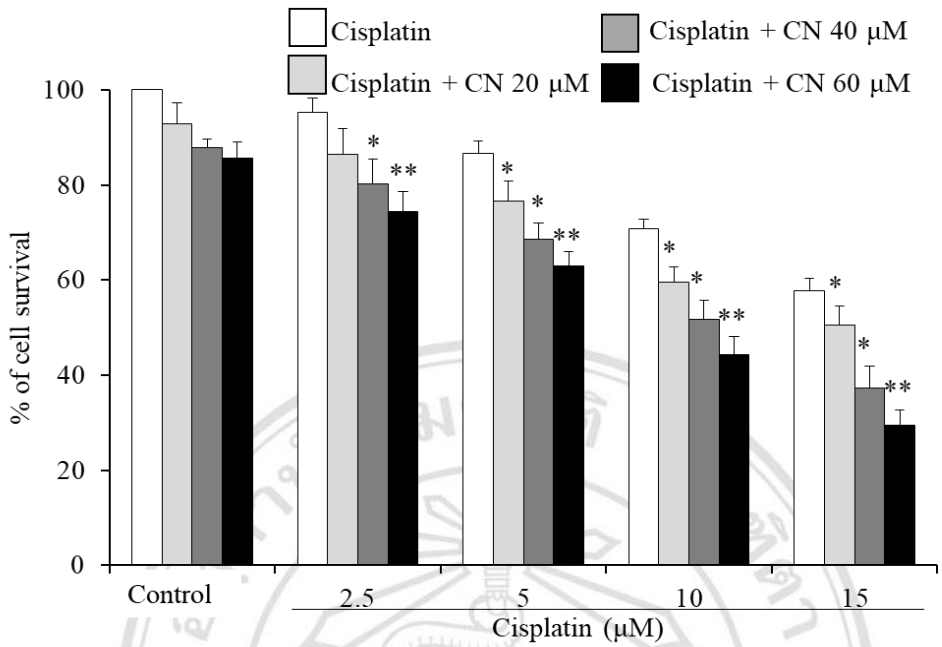
48 hours. The values are presented as the mean ± SD of three independent experiments. *$p$<0.05, **$p$<0.01, ***$p$<0.001 are compared with untreated control in each compound.

### 3.3 Effects of alkaloids on cisplatin sensitivity in ovarian cancer cells

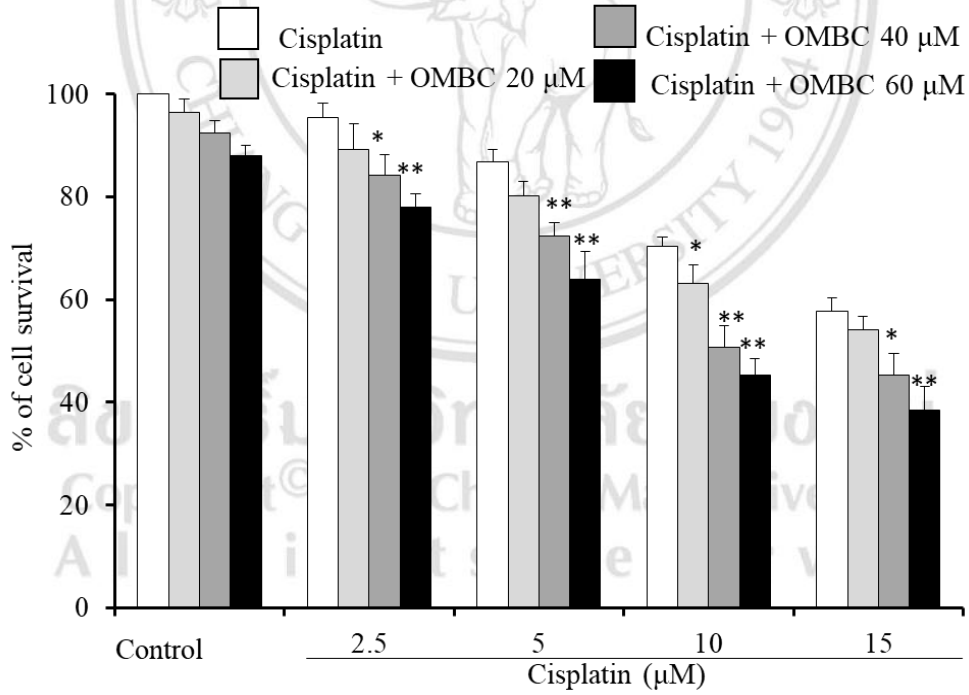
#### 3.3.1 Effects of alkaloids on cisplatin sensitivity in SKOV3 cells

To examine whether alkaloids from *S. venosa* could enhance cisplatin sensitivity on ovarian cancer cells, cell proliferation after combination treatment was measured using MTT assay. SKOV3 cells were pretreated with CN, OMBC, THP or NMTHP for 2 hours, followed by treatment with various concentrations of cisplatin for another 48 hours. CN and OMBC enhanced cisplatin sensitivity in intrinsic drug resistant SKOV3. The combination index was used 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 or OMBC are shown in Table 3.3. Combination of cisplatin with CN or OMBC at 40 μM and 60 μM exhibited moderate synergistic effects with CI values ranging from 7.4 to 7.8. However, THP and NMTHP had no effect on cisplatin sensitivity in SKOV3 cells (Figure 3.2).

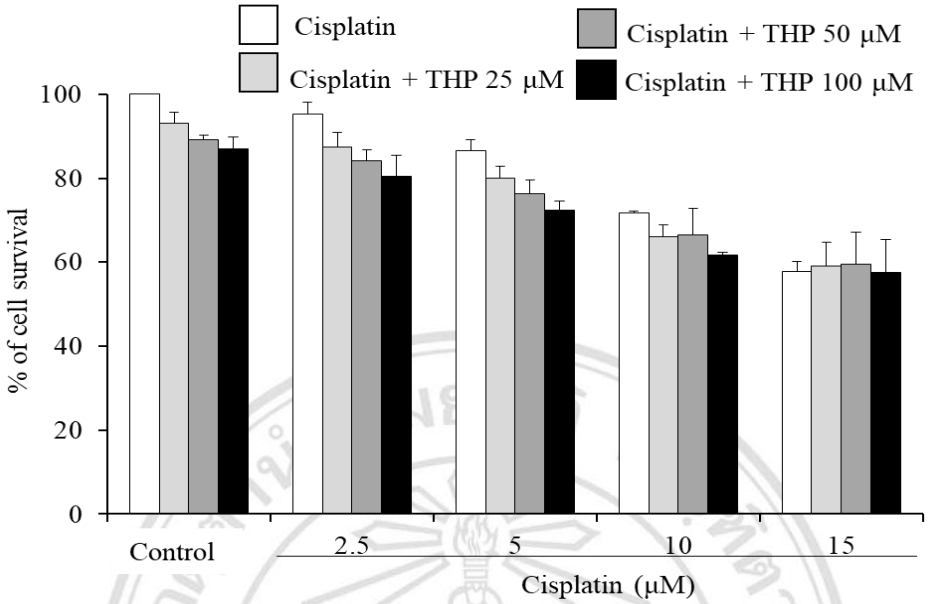
(A)


Cisplatin
Cisplatin + CN 40 µM
Cisplatin + CN 20 µM
Cisplatin + CN 60 µM
% of cell survival
100
80
60
40
20
0
Control
2.5
5
10
15
Cisplatin (µM)

(B)


Cisplatin
Cisplatin + OMBC 40 µM
Cisplatin + OMBC 20 µM
Cisplatin + OMBC 60 µM
% of cell survival
100
80
60
40
20
0
Control
2.5
5
10
15
Cisplatin (µM)

(C)

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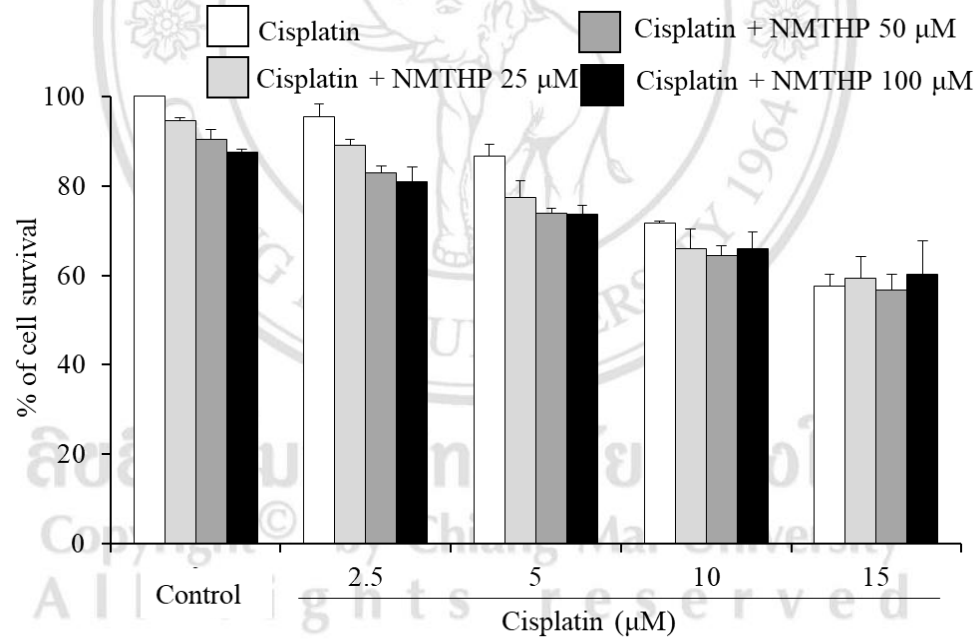



Figure 3.2 Effects of (A) CN, (B) OMBC, (C) THP and (D) NMTHP on cisplatin sensitivity in SKOV3 ovarian cancer cells. The SKOV3 cells were first treated with non-toxic concentrations of CN, OMBC, THP and NMTHP as indicated respectively. Two hours later, different concentrations of cisplatin were added and incubated for 48 hours, and then cell survival was assessed by MTT assay. The data was shown as mean ± SD of three independent experiments. *$p$<0.05, **$p$<0.01 are compared with cisplatin treatment in each concentration.

Table 3.3 Combination index (CI) values of cisplatin and CN or OMBC in SKOV3 cells

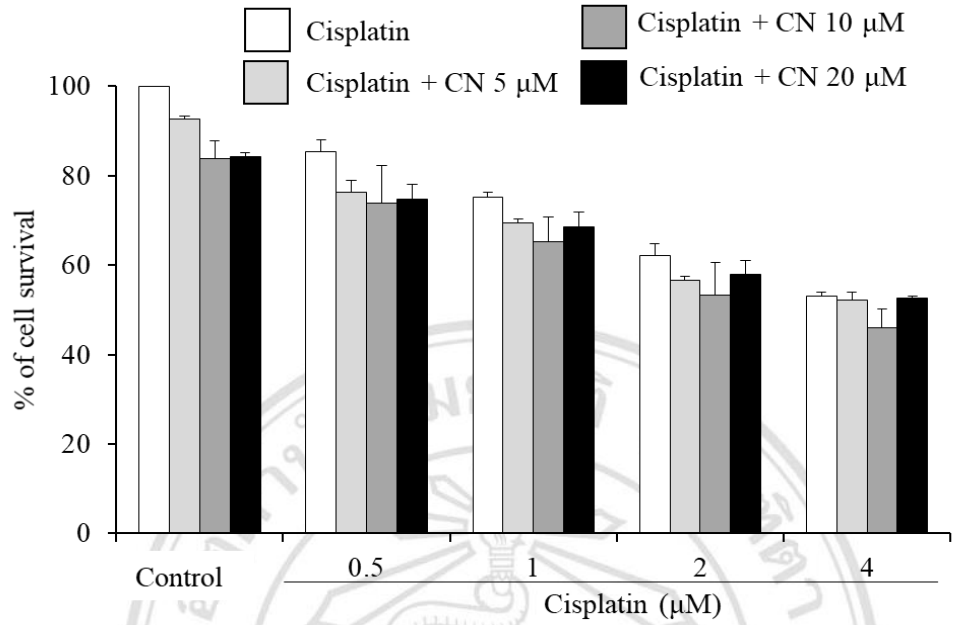
| | CI values | Description |
|---|---|---|
| Cisplatin + CN 20 μM | 0.89 | Slight synergism |
| Cisplatin + CN 40 μM | 0.78 | Moderate synergism |
| Cisplatin + CN 60 μM | 0.74 | Moderate synergism |
| Cisplatin + OMBC 20 μM | 0.91 | Nearly additive |
| Cisplatin + OMBC 40 μM | 0.78 | Moderate synergism |
| Cisplatin + OMBC 60 μM | 0.75 | Moderate synergism |

CI>1 indicates antagonism, CI=1 indicates additive effect and CI<1 indicates synergism.

### 3.3.2 Effects of alkaloids on cisplatin sensitivity in A2780 cells

To observe the effects of alkaloids from *S. venosa* on cisplatin sensitivity on A2780 ovarian cancer cells, cell proliferation after combination treatment with cisplatin and alkaloids was measured using MTT assay. The data indicates that neither aporphine nor protoberberine alkaloids showed any effect on cisplatin sensitivity in A2780 cell lines (Figure 3.3).

(A)

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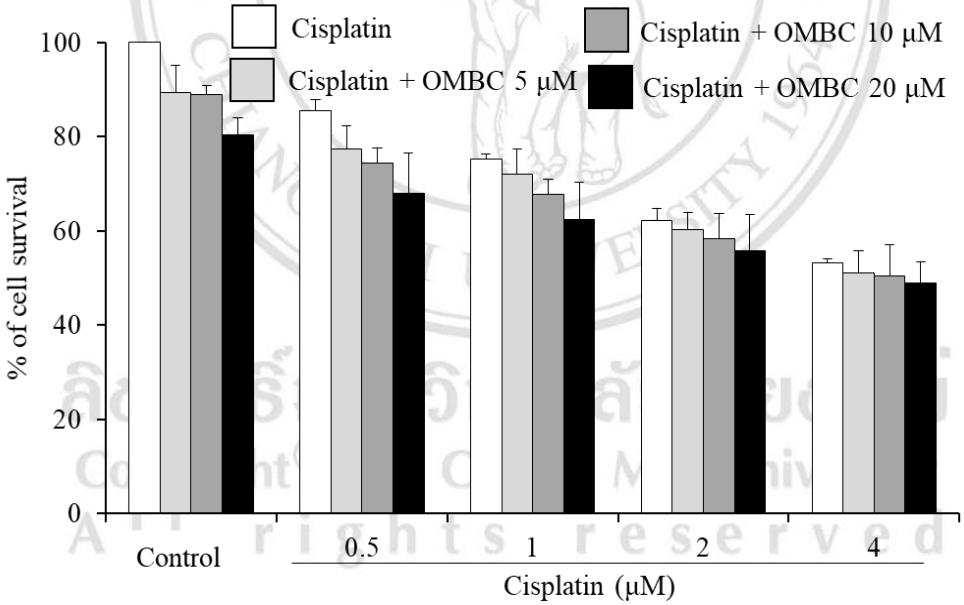

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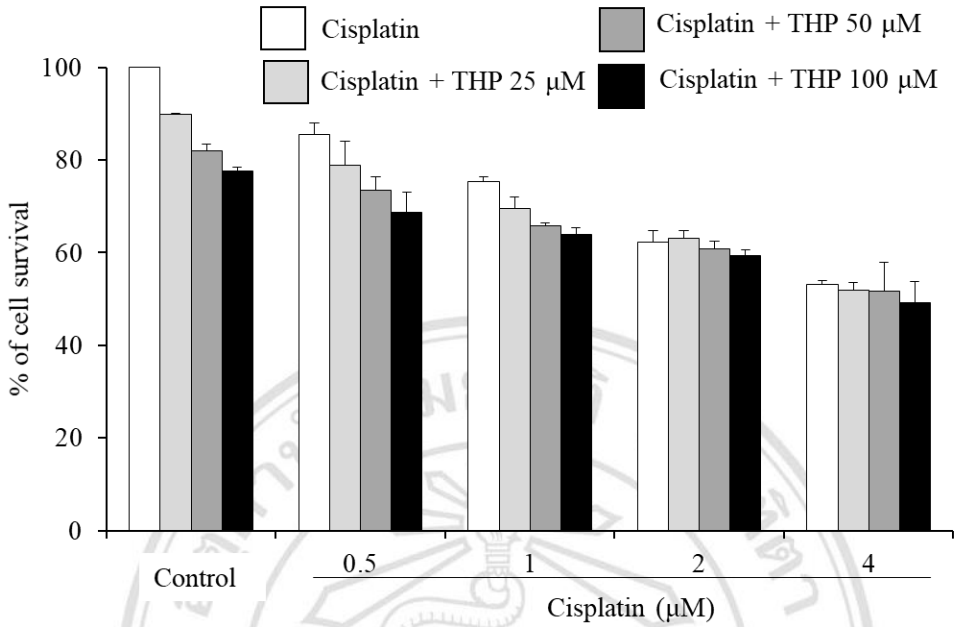


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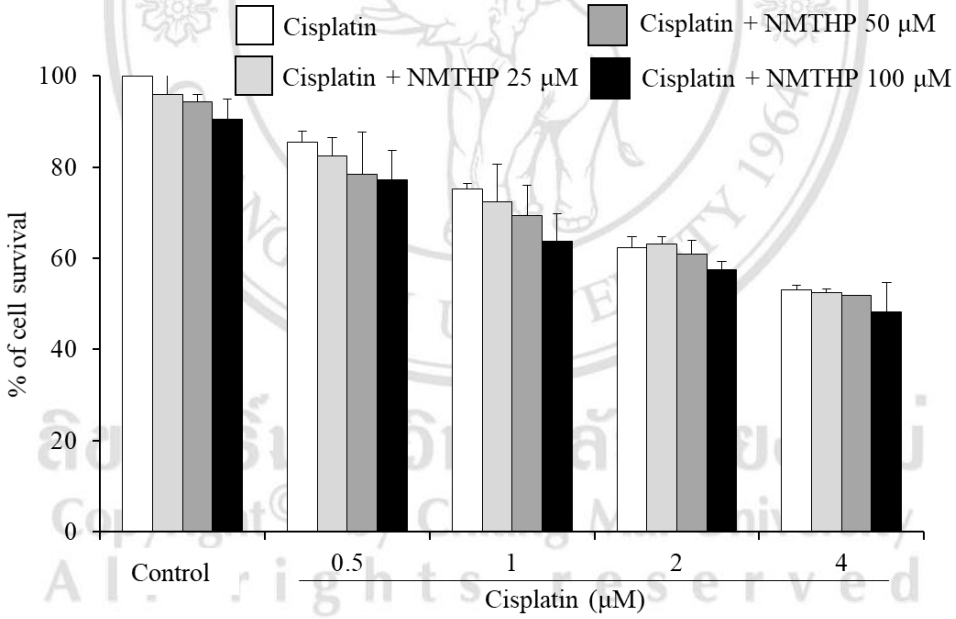
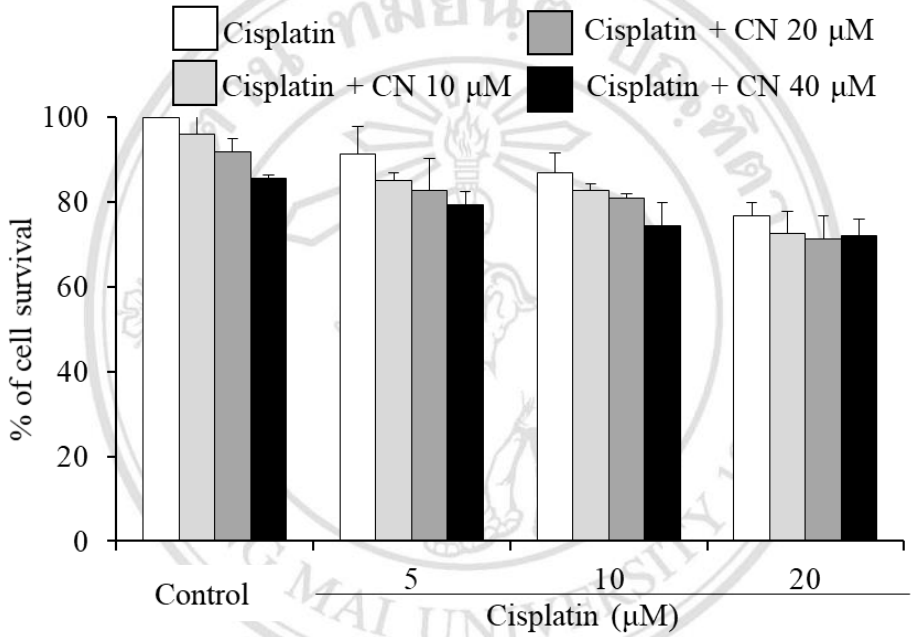



Figure 3.3 Effects of (A) CN, (B) OMBC, (C) THP and (D) NM-THP on cisplatin sensitivity in A2780 ovarian cancer cells. The A2780 cells were first treated with non-toxic concentrations of CN, OMBC, THP and NM-THP as indicated respectively. Two hour later, different concentrations of cisplatin were added and incubated for 48 hours, and then cell viability was assessed by MTT assay. The data are shown as mean ± SD of three independent experiments.

### 3.3.3 Effects of alkaloids on cisplatin sensitivity in A2780/cis cells

To survey the effects of alkaloids from *S. venosa* on cisplatin sensitivity in A2780/cis ovarian cancer cells, cell proliferation after combination treatment was assessed using MTT assay. Aporphine alkaloids, CN and OMBC, as well as protoberberine alkaloids, THP and NMTHP, had no effect on cisplatin sensitivity in A2780/cis cell line (Figure 3.4).

(A)

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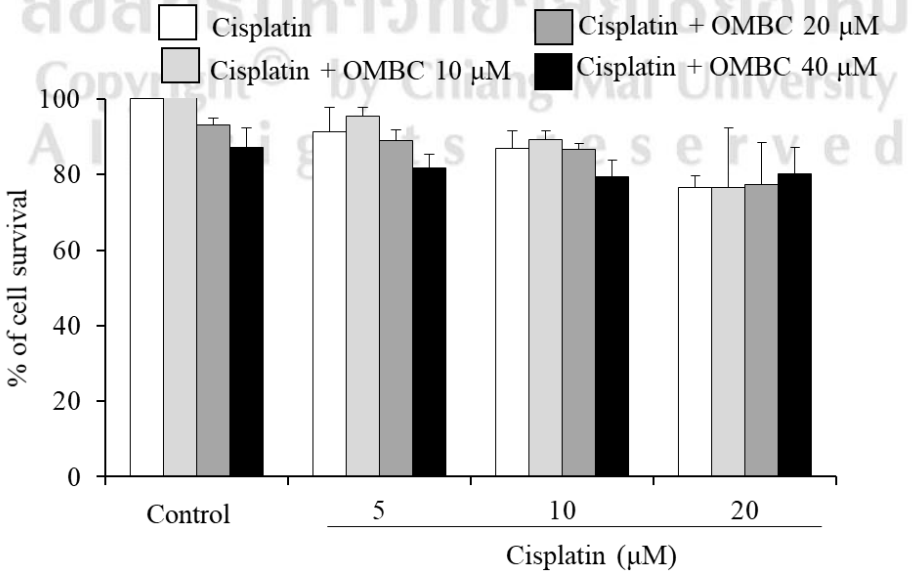

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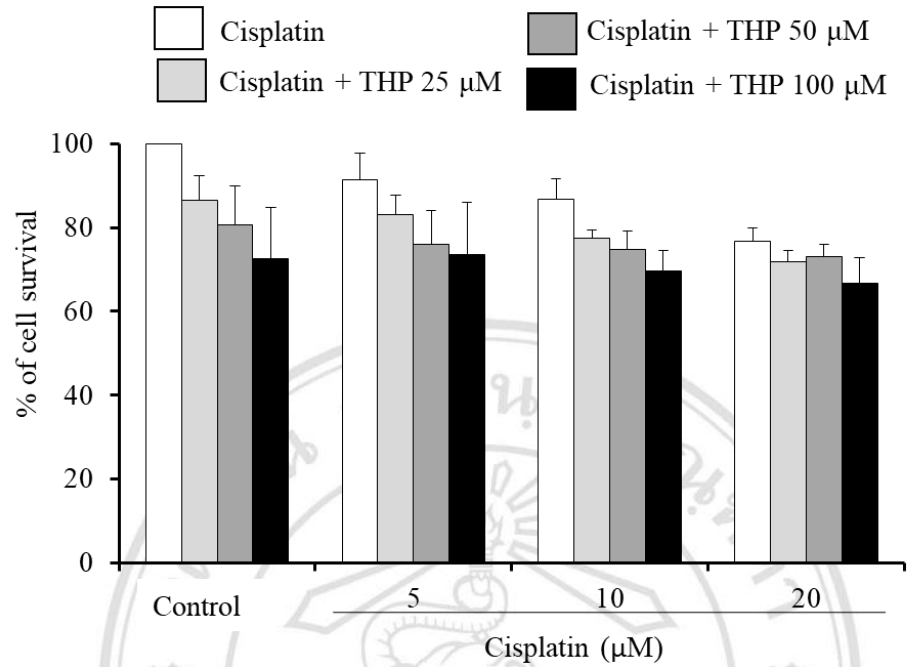


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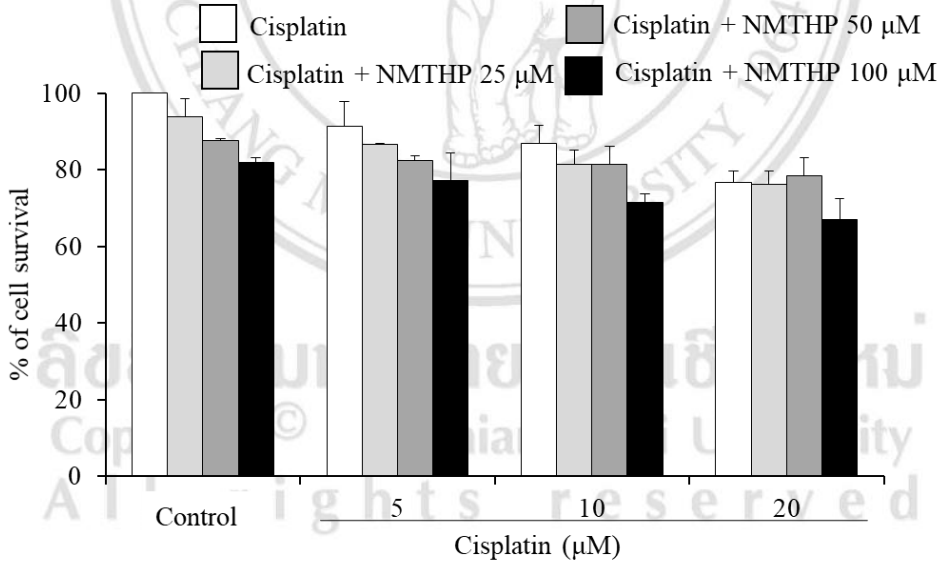


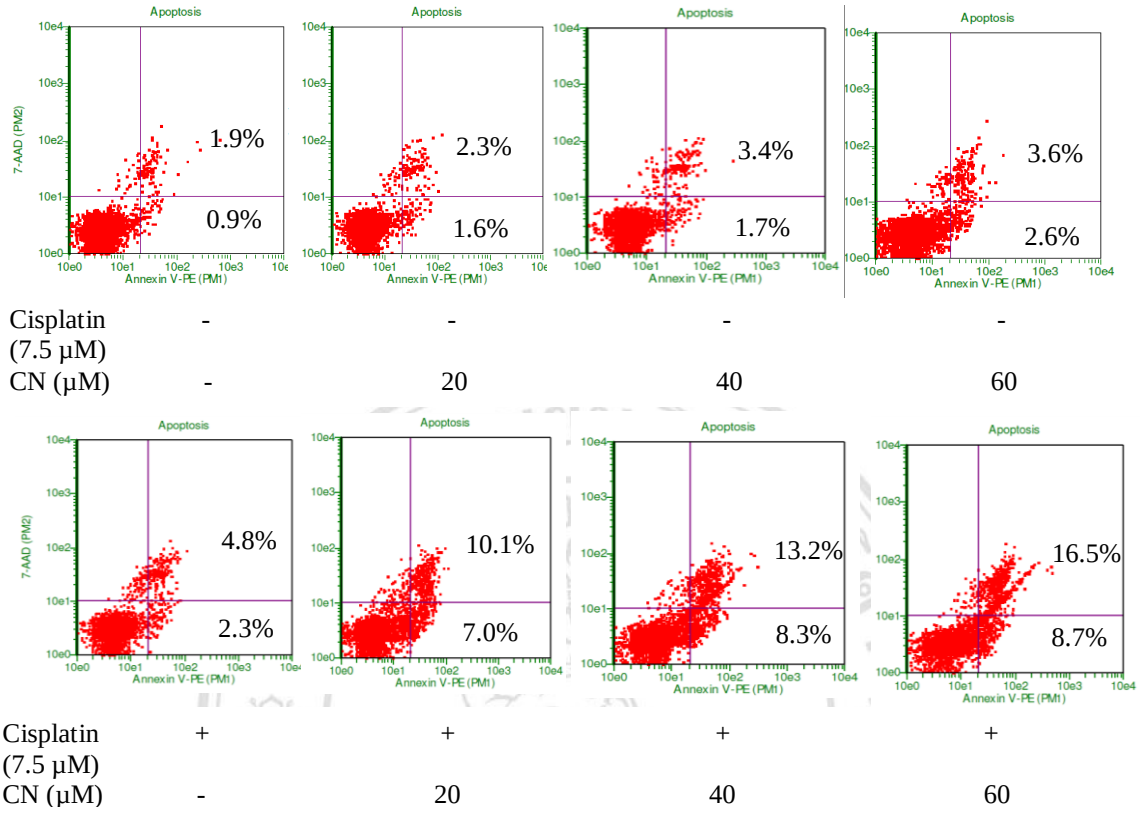

Figure 3.4 Effects of (A) CN, (B) OMBC, (C) THP and (D) NMTHP on cisplatin sensitivity in A2780/cis ovarian cancer cells. The A2780/cis cells were first treated with non-toxic concentrations of CN, OMBC, THP and NMTHP as indicated respectively. Two hour later, different concentrations of cisplatin were added and incubated for 48 hours, and then cell viability was assessed by MTT assay. The data are shown as mean ± SD of three independent experiments.

### 3.4 Effect of CN and OMBC on SKOV3 cells apoptosis

#### 3.4.1 Effect of CN and OMBC on SKOV3 cells apoptosis accessed by flow cytometry

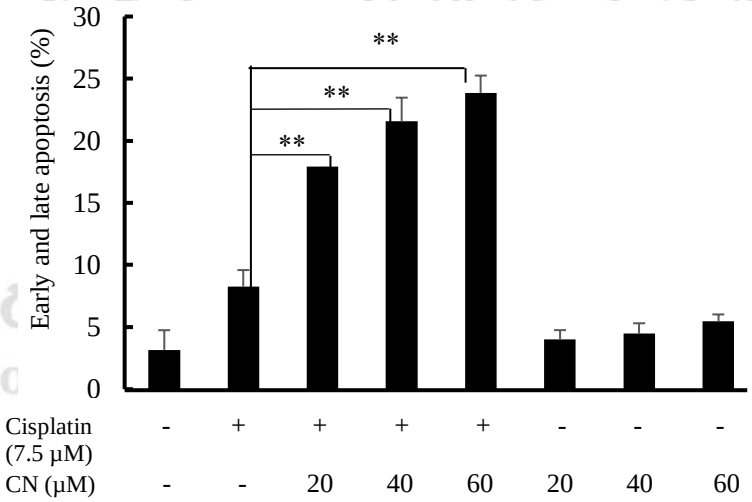
Since the effect of aporphine alkaloids on enhancing the cisplatin sensitivity was found in SKOV3 cells, assessment of SKOV3 cellular apoptosis was focused to know whether the combination of cisplatin and aporphine alkaloids could potentiate cellular apoptosis. To address this, SKOV3 cells were pretreated with CN or OMBC (20, 40, 60 μM) for 2 hours, then cisplatin (7.5 μM) was added and incubated for 36 hours. After that, cells were stained with annexin V-FITC to measure the total percentage of early and late apoptotic cells. As shown in Figure 3.5, CN treated cells exhibited a low percentage of apoptosis, indicating that CN alone, at up to 60 μM concentration, did not cause apoptosis significantly. Cisplatin-treated cells showed 7.1% of apoptotic cells, whereas, combination treatment of SKOV3 cells with cisplatin and CN at 20, 40 and 60 μM doses showed increased in the percentage of the apoptotic cells in dose dependent manner from 17.1%, 21.5% and 25.1% respectively. Similarly, cells treated with a combination treatment with OMBC displayed an increase in the total percentage of early and late apoptotic cells compared to the cisplatin-treated cells (Figure 3.6). The results of apoptosis evaluation suggested that the cytotoxic activity of CN and OMBC compounds in SKOV3 cells occurred via apoptosis.

(A)




| | | | | |
|---|---|---|---|---|
| Cisplatin (7.5 µM) | - | - | - | - |
| CN (µM) | - | 20 | 40 | 60 |
| Cisplatin (7.5 µM) | + | + | + | + |
| CN (µM) | - | 20 | 40 | 60 |

(B)

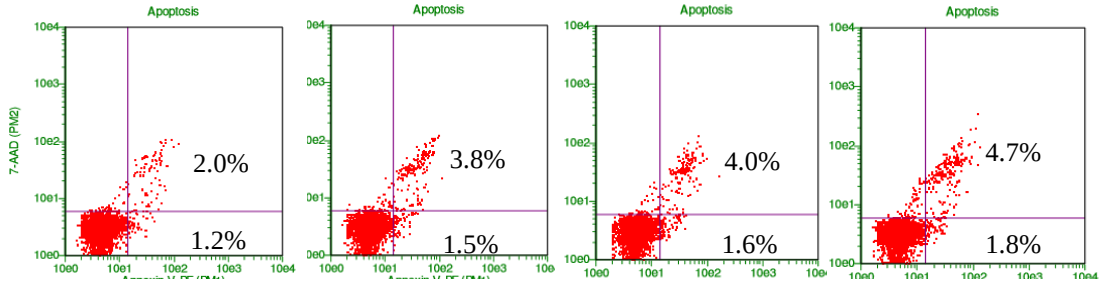



| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Cisplatin (7.5 µM) | - | + | + | + | + | - | - | - |
| CN (µM) | - | - | 20 | 40 | 60 | 20 | 40 | 60 |

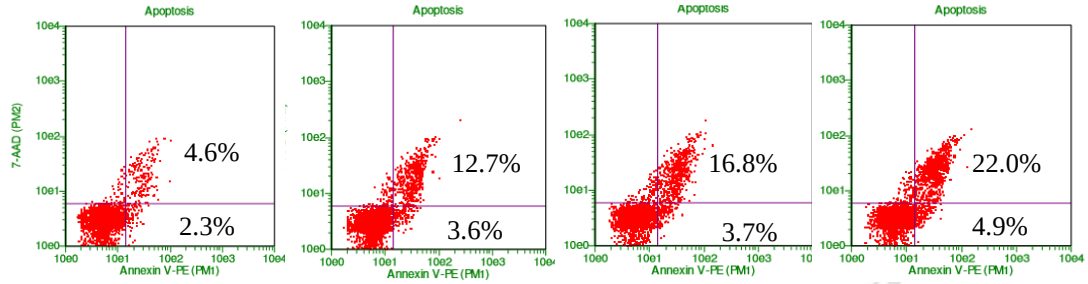
Figure 3.5 Effect of CN on SKOV3 cells apoptosis. SKOV3 cells were treated with cisplatin ± CN for 36 hours and the percentage of apoptotic cells was determined by Annexin V-FITC/PI staining. (A) Dot-plot graphs indicated the percentage of the cells at early stage of apoptosis in the right lower quadrent, at late stage of apotosis in the right upper quadrent. (B) Bar graph represents the mean ± SD of cell percentage in early and

late stages of apoptosis from three separate experiments. **$p$<0.01 are compared with the drug treatment.

(A)




| | | | | |
|---|---|---|---|---|
| Cisplatin (7.5 µM) | - | - | - | - |
| OMBC (µM) | - | 20 | 40 | 60 |




| | | | | |
|---|---|---|---|---|
| Cisplatin (7.5 µM) | + | + | + | + |
| OMBC (µM) | - | 20 | 40 | 60 |

(B)

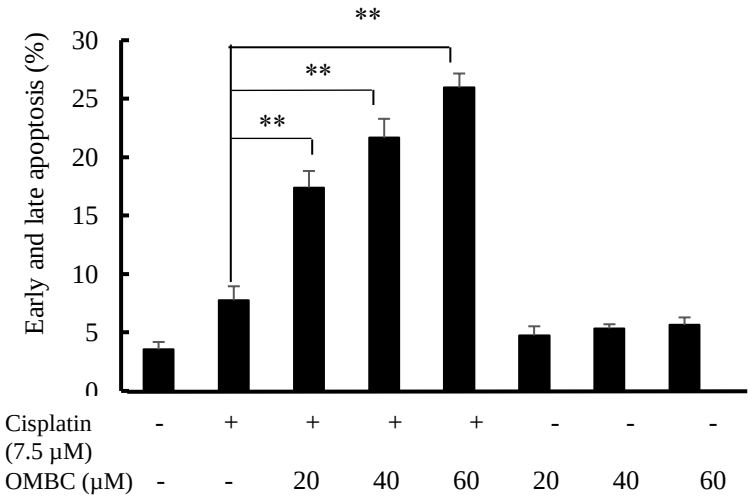



Figure 3.6 Effect of OMBC on SKOV3 cells apoptosis. SKOV3 cells were treated with cisplatin ± OMBC for 36 hours and the percentage of apoptotic cells was determined by Annexin V-FITC/PI staining. (A) Dot-plot graphs indicated the percentage of the cells at early stage of apoptosis in the right lower quadrent, at late stage of apotosis in the right upper quadrent. (B) ar graph represents the mean ± SD of cell percentage in early and late stages of apoptosis from three separate experiments. $^{**}p<0.01$ are compared with the drug treatment.

### 3.4.2 Effect of CN and OMBC on SKOV3 cells apoptotic protein expressions

Caspases are a family of cysteine proteases that are synthesized as inactive precursors and are only activated following an appropriate stimulus. The effect of CN and OMBC on apoptosis was confirmed by challenging the expression status of apoptotic proteins including caspase-3, -8 and cleaved PARP. SKOV3 cells were first treated with CN or OMBC (20, 40, 60 μM) as indicated respectively. Two hours later, 7.5 μM cisplatin was added and incubated for 48 hour, and then the cell pallet were collected for detection of apoptotic proteins expression by Western blotting. The results showed that single treatment with cisplatin slightly increased the level of apoptotic proteins in SKOV3 cells while the co-treatment of the cells obviously increased expression of active caspase-3, -8 and formation of cleaved PARP (Figure 3.7). Therefore, the potentiation effect of CN and OMBC on cisplatin sensitivity in SKOV3 cells might be mediated through the induction of apoptosis via a caspase dependent mechanism.

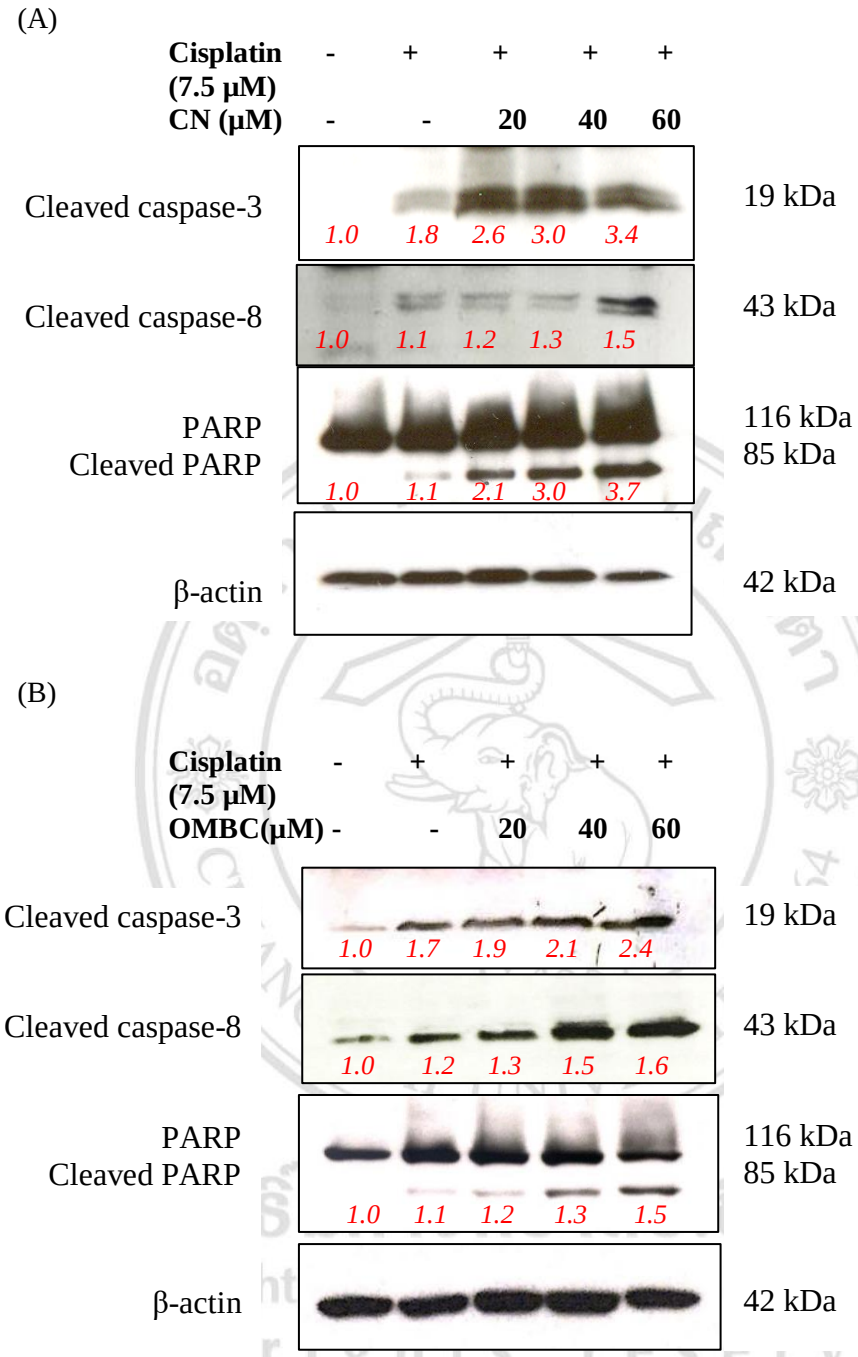


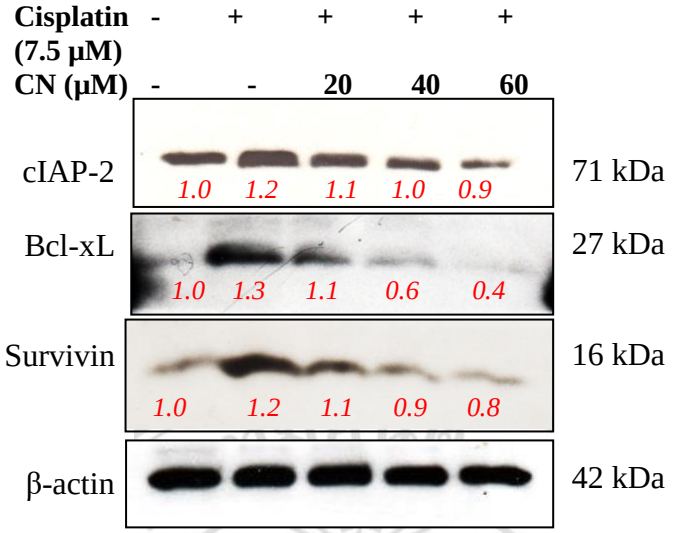

Figure 3.7 CN and OMBC enhanced cisplatin sensitivity in SKOV3 cells via induction of apoptosis-induced cell death. SKOV3 cells were pretreated with (A) CN or (B) OMBC for 2 hours, then cisplatin (7.5 µM) was added and incubated for 48 hours, and then the cell pellet was collected for detection of apoptotic proteins expression by Western blotting. Band densities are noted under each band in relative to untreated control.

### 3.5 Effect of CN and OMBC on SKOV3 cell survival protein expressions

#### 3.5.1 Effect of CN and OMBC on cisplatin-induced anti-apoptotic and survival proteins

Apoptotic pathways are frequently dysregulated in cancerous cells. As a result, malignant cells proliferate uncontrollably despite the presence of apoptotic signal. Many anticancer agents induce apoptosis of malignant cells by re-sensitizing them to apoptotic signals. Platinum resistance is often related to defects in the apoptotic pathway and it has been reported that resistance to chemotherapy is associated with the up-regulation of anti-apoptotic and survival proteins, leading to prevention of tumor cells from undergoing sufficient levels of programmed cell death or apoptosis, resulting in treatment failure [73]. In this study, the expressions of anti-apoptotic and survival proteins such as Bcl-xL, cIAP-2 and survivin were determined in response to cisplatin treatment and tested whether cotreatment with aporphine alkaloids could diminish their expression. The results showed that Bcl-xL, cIAP-2, survivin expression were noticeably increased after a single treatment of cisplatin while co-treatment could suppress cisplatin-induced anti-apoptotic and survival protein expression (Figure 3.8). Hence, the enhancement of CN and OMBC on cisplatin sensitivity in SKOV3 cells might be mediated through suppression of cisplatin-induced anti-apoptotic and survival protein expressions.

(A)

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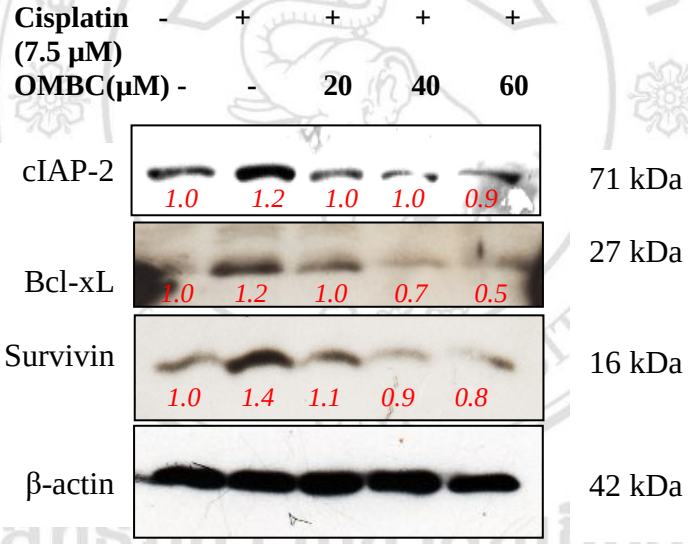





Figure 3.8 CN and OMBC enhanced cisplatin sensitivity in SKOV3 cells via suppression of cisplatin-induced anti-apoptotic and survival proteins expression. SKOV3 cells were pretreated with (A) CN or (B) OMBC for 2 hours, then cisplatin (7.5 µM) was added and incubated for 24 hours, and then the cell pellet was collected for detection of anti-apoptotic and survival protein expressions by Western blotting. Band densities are noted under each band in relative to untreated control.

### 3.5.2 Effect of CN and OMBC on cisplatin-induced cytokine expression

One of the reasons for chemotherapy resistance is the ability of tumor cells to induce IL-6 production upon chemotherapy treatment [53]. Since cytokines, especially IL-6, also play a critical role in the natural history of most cancers including ovarian cancer and drug resistance, the level of 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 hours. The supernatant was then collected and the quantitation of IL-6 was assessed. The data showed that IL-6 levels were approximately three-fold higher after cisplatin treatment, whereas the combination treatments lessened the production of IL-6 in dose dependent manner (Figure 3.9). Therefore, the improved cisplatin sensitivity in SKOV3 cells by CN and OMBC might be via inhibition of cisplatin-induced IL-6 production.

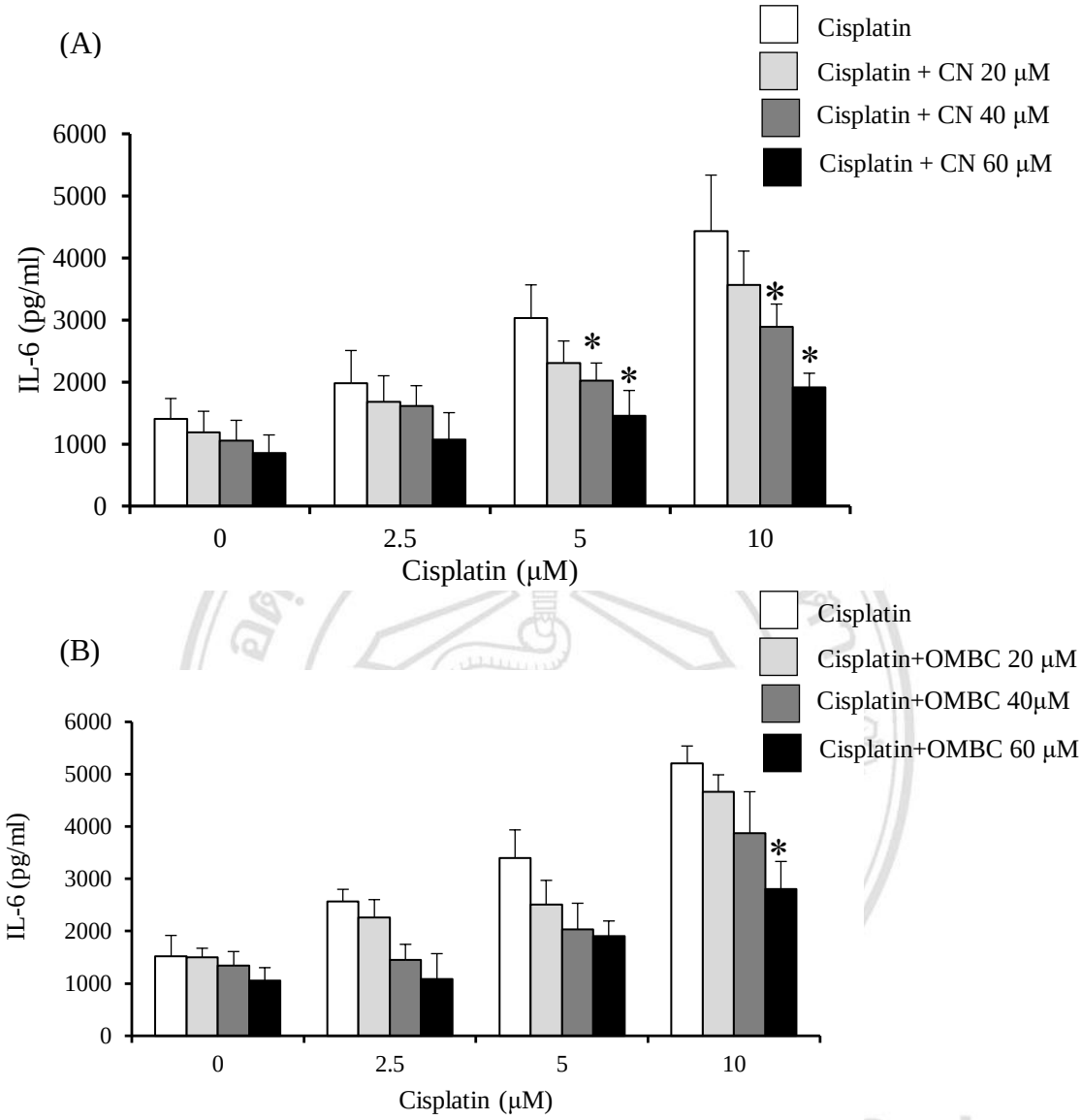


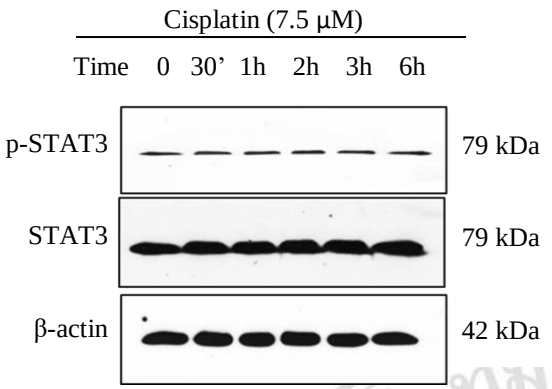



Figure 3.9 CN and OMBC enhanced cisplatin sensitivity in SKOV3 cells via inhibition of cisplatin-induced IL-6 production. SKOV3 cells were first treated with (A) CN or (B) OMBC for 2 hours and then cisplatin was added and incubated for 48 hours. The supernatant was collected to determine by ELISA analysis. *$p$<0.05 versus drug treatment.

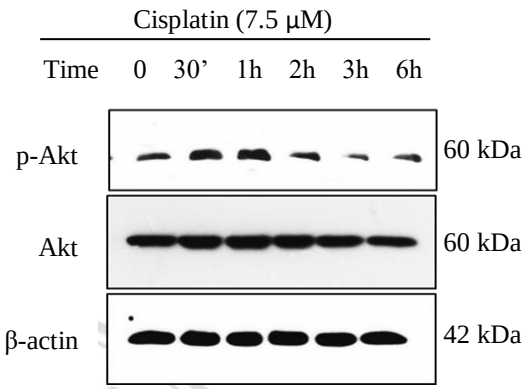
### 3.6 Signaling molecules and transcription factors induced by cisplatin treatment in SKOV3 cells

One of the key hallmarks of cancer is the ability of malignant cells to sustain proliferative signaling and execute an anti-apoptotic pro-survival program. Exposure to cisplatin provokes the induction of several cellular response signals with consequent changes in growth regulation. Altered expressions of genes concerned with the reinforcement of survival signals upon cytotoxic exposure may provide survival advantages to cancer cells. Therefore, dysregulation of signaling pathways might be critical in the development of drug resistance. To inspect which signaling pathways were enhanced by cisplatin in SKOV3 cells, we exposed the SKOV3 cells with cisplatin in a time dependent manner and the activities of Akt, STAT3, AP-1, NF-κB and MAPKs were measured. Western blotting results revealed that STAT3, AP-1, MAPKs were not responsive upon cisplatin treatment, whereas phosphorylation of Akt and the nuclear translocation of NF-κB were augmented after 30 minutes to 1 hour exposed to cisplatin in SKOV3 cells (Figure 3.10).

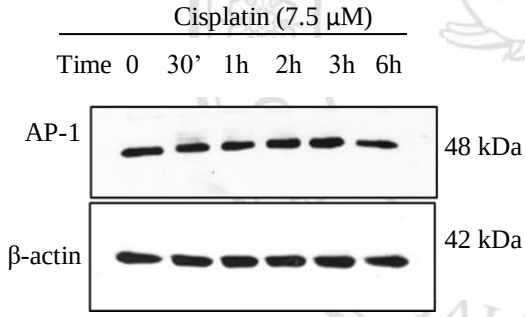
(A)




(B)




(C)

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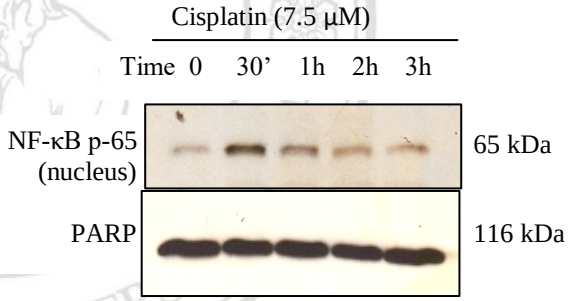


(E)

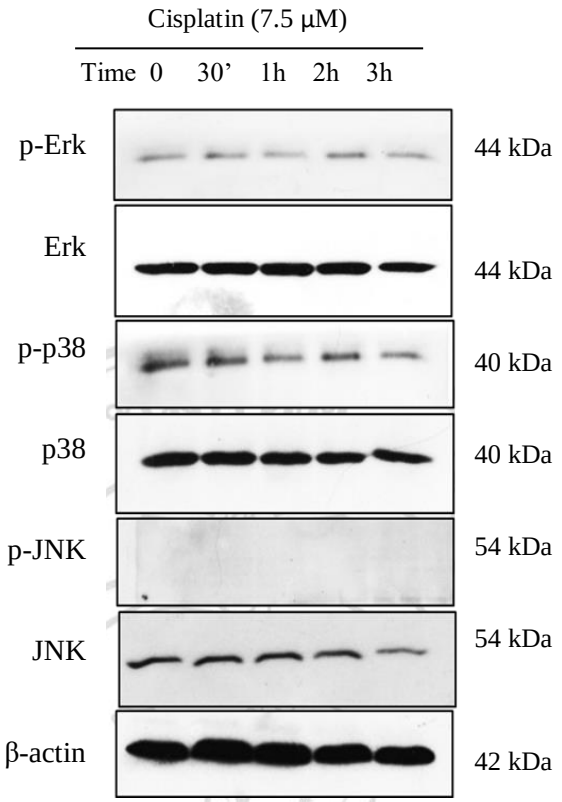



Figure 3.10 Cisplatin-induced augmentation of intracellular signaling molecules and transcription factors in SKOV3 cells. (A-E) SKOV3 cells were treated with 7.5 μM cisplatin for various times interval indicated as in figure. The nuclear lysates were extracted for NF-κB and whole cell lysates were prepared for p-Akt, Akt, p-STAT3, STAT3, AP-1, p-Erk, Erk, p-p38, p-38, p-JNK and JNK detection. The band is representative data of three independent experiments.

### 3.7 Akt/NF-κB signaling pathway is concerned with cisplatin resistance in SKOV3

#### 3.7.1 Effect of PI3K/Akt inhibitor on cisplatin-augmented NF-κB activity in SKOV3 cells

Based on the results of previous experiment, it was found that Akt and NF-κB were upregulated upon cisplatin exposure in SKOV3 cells. In order to observe whether Akt and NF-κB are involved in cisplatin resistance in SKOV3 cells, the selective PI3K/Akt inhibitor, LY294002, which can block PI3 kinase-dependent Akt phosphorylation activity was used [74]. Then, NF-κB activity after inhibition of Akt by selective inhibitor in SKOV3 cells upon cisplatin exposure was determined. The data indicated that cisplatin-augmented NF-κB activity was obviously decreased by Akt in SKOV3 cells (Figure 3.11).

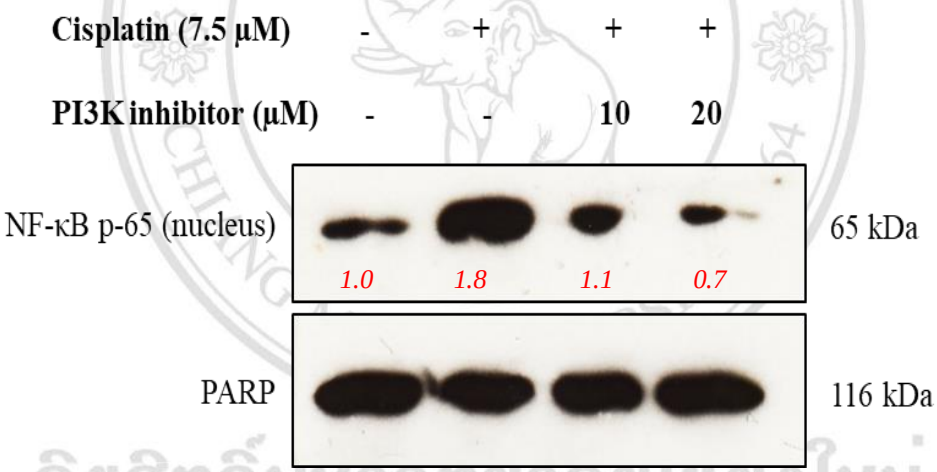



Figure 3.11 Effect of PI3K/Akt inhibitor on cisplatin-augmented NF-κB activity in SKOV3 cells. SKOV-3 cells were pretreated with 10, 20 μM PI3K/Akt inhibitor for 2 hours and then incubated with cisplatin (7.5 μM) for 30 minutes. The nuclear lysates were extracted for NF-κB detection by Western blotting. Band densities are noted under each band in relative to untreated control.

### 3.7.2 Effect of PI3K/Akt inhibitor on cisplatin sensitivity in SKOV3 cells

Next, the consequence of PI3K/Akt inhibitor on cisplatin sensitivity in SKOV3 cells was evaluated by MTT assay. As shown in Figure 3.12, cisplatin 7.5 μM treatment alone caused 20% growth inhibition whereas in combination with PI3K/Akt inhibitor 10 μM and 20 μM improved cisplatin-induced growth inhibition to 33% and 48% respectively in SKOV3 cells. These results suggest that the upregulation of Akt/NF-κB signaling is definitely involved in cisplatin resistance of SKOV3 cells.

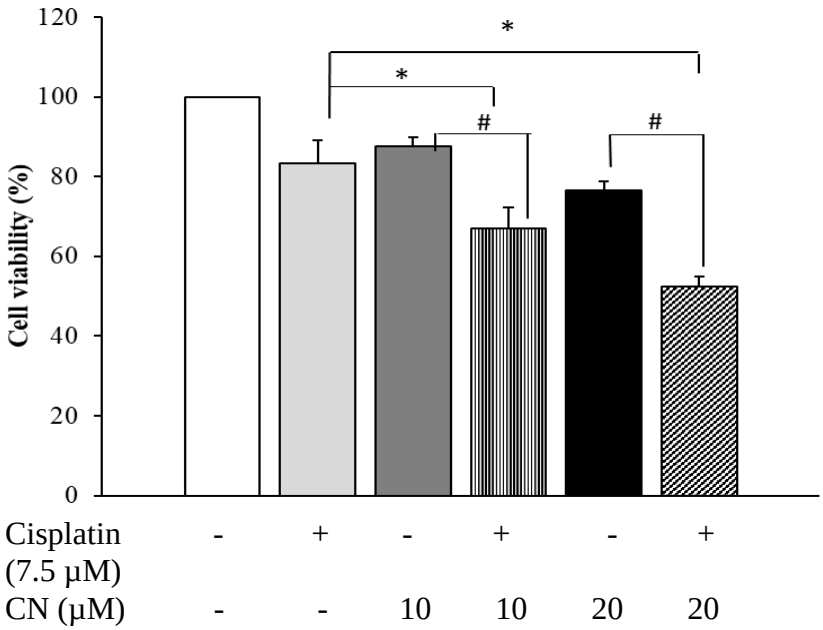


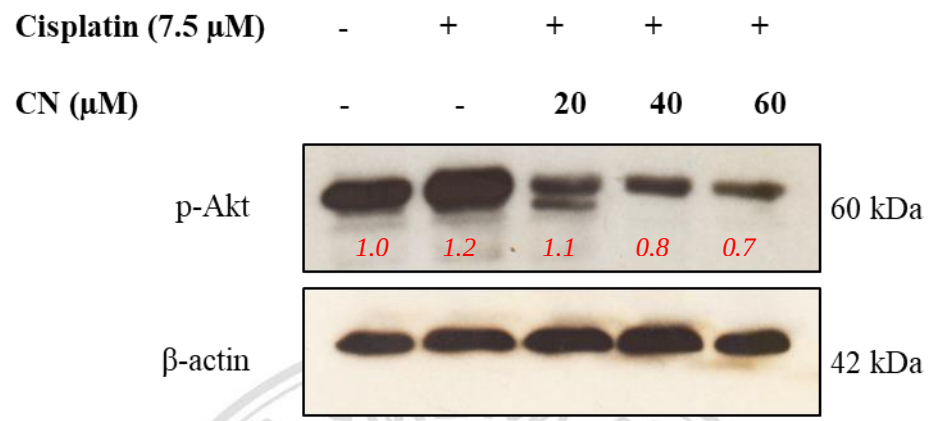

Figure 3.12 Effect of PI3K/Akt inhibitor on cisplatin sensitivity in SKOV3 cells. SKOV3 cells were treated with 7.5 μM cisplatin with or without PI3K/Akt inhibitor (10, 20 μM) for 48 hours and then cell proliferation was evaluated by MTT assay. *$p$<0.05 versus cisplatin treatment. $^{\#}p$<0.05 versus inhibitor treatment.

### 3.8 Effect of CN and OMBC on Akt/NF-κB signaling in SKOV3 cells

#### 3.8.1 Effect of CN and OMBC on cisplatin-augmented Akt activity in SKOV3 cells

Previous studies have demonstrated that Akt inhibits apoptosis through activation of the transcriptional factor, NF-κB, which is a key transcriptional factor involved in apoptosis, cell proliferation, cancer cell survival and drug resistance [75]. In order to evaluate the detail mechanism of the chemosensitizing effect of CN and OMBC on cisplatin sensitivity, the Akt/NF-κB signaling pathway was focused since cisplatin was found to induce the activity of Akt and the NF-κB signaling pathway in SKOV3 cells. SKOV3 cells were pretreated with CN or OMBC for 2 hours, followed by treatment with cisplatin for 30 minutes, and then the cell pellet was collected for Western blotting. The results showed that cisplatin treatment induced the phosphorylation of Akt in SKOV3 cells while CN or OMBC could suppress cisplatin-augmented Akt phosphorylation activity (Figure 3.13). The phosphorylation of Akt is routinely used as a type of marker for Akt activation. The inhibition of Akt phosphorylation by CN or OMBC is an important mechanism of action in cisplatin resistant ovarian cancer cells.

(A)




(B)

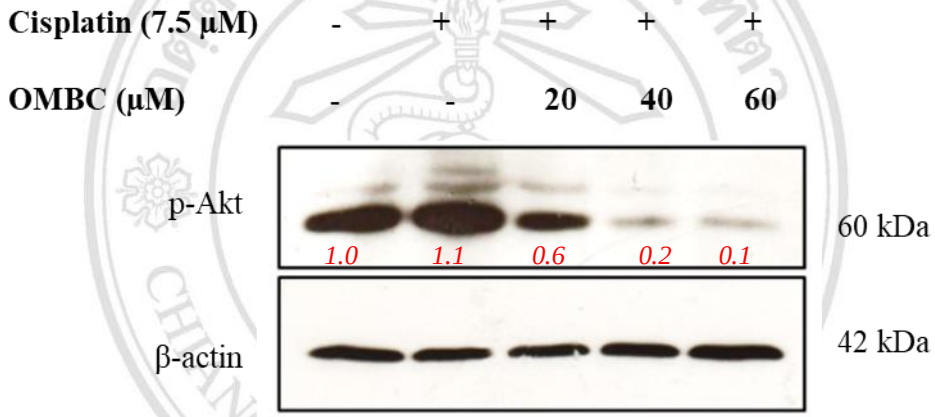
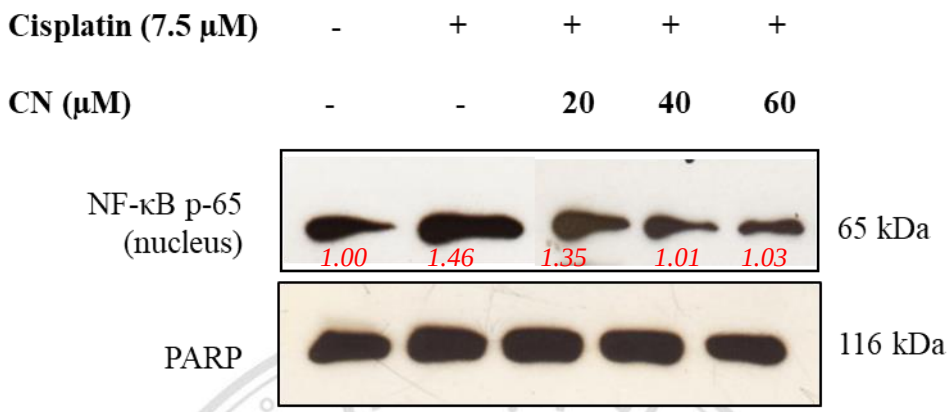



Figure 3.13 Suppressive effects of CN and OMBC on cisplatin-augmented Akt activity in SKOV3 cells. SKOV-3 cells were pretreated with (A) CN or (B) OMBC for 2 hours and then incubated with cisplatin (7.5 μM) for 30 minutes. The whole cell lysates were prepared for detection of p-Akt by Western blotting. Band densities are noted under each band in relative to untreated control. The band is representative data of three independent experiments.

### 3.8.2 Effects of CN and OMBC on cisplatin-augmented NF-κB activity in SKOV3 cells

Next, the effect of CN and OMBC on cisplatin-augmented NF-κB activity was investigated, since NF-κB is a key transcriptional factor involved in apoptosis, cell proliferation, cancer cell survival and drug resistance. SKOV3 cells were exposed to CN or OMBC for 2 hours, followed by treatment with cisplatin for 30 minutes. Then the cell pallet was collected and the nuclear lysate was prepared for Western blotting. The results showed that both CN and OMBC could suppress cisplatin-augmented NF-κB translocation from cytosol to nucleus (Figure 3.14). Taken the results into consideration in the current study, the observation of an inhibitory effect of CN or OMBC on Akt/NF-κB signaling might be involved in the chemosensitizing mechanism of aporphine alkaloids.

(A)




(B)

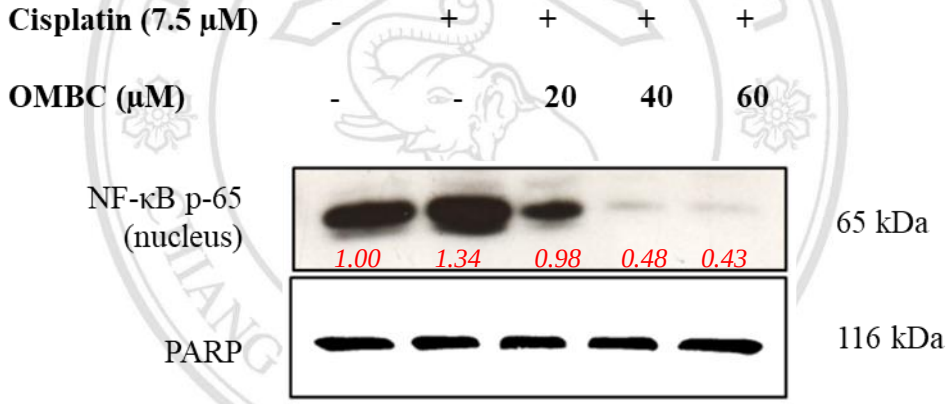



Figure 3.14 Effect of CN and OMBC on cisplatin-augmented NF-κB activity in SKOV3 cells. SKOV-3 cells were pretreated with (A) CN or (B) OMBC for 2 hours and then incubated with cisplatin (7.5 µM) for 30 minutes. The nuclear lysates were prepared for detection of NF-κB by Western blotting. Band densities are noted under each band in relative to untreated control. The band is representative data of three independent experiments.

### 3.9 Determination of IL-6 production from ovarian cancer cells

The levels of IL-6 in ovarian cancer cells after treatment with cisplatin were determined in the ovarian cancer cell lines. As shown in Figure 3.15, the results indicated that SKOV3 cells could produce IL-6 obviously (approximately 3-fold) in dose-dependent manner in response to cisplatin. However, A2780 and A2780/cis had no detectable IL-6 production before or after treatment with cisplatin. Therefore, IL-6 might implicate in intrinsic drug resistance mechanism.

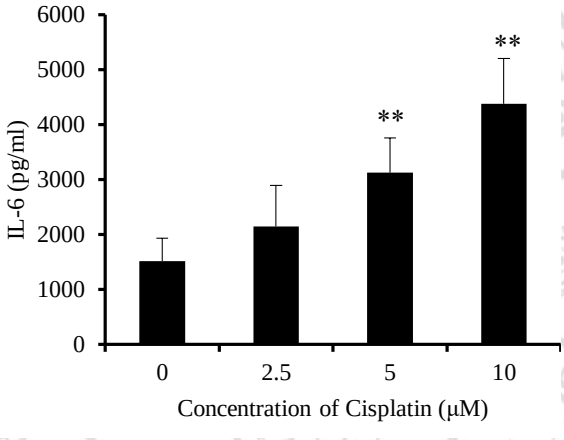



Figure 3.15 Platinum-induced cytokines secretion in SKOV3 ovarian cancer cells. SKOV3 cells were treated with different concentrations of cisplatin for 48 hours, and then supernatant was collected to determine cisplatin-induced IL-6 production by ELISA. Data are presented as the mean ± SD of three separate experiments. $^{**}p<0.01$ compared with untreated control.

### 3.10 Effects of neutralization and addition of exogenous IL-6 in SKOV3 cells

#### 3.10.1 Effect of neutralization of IL-6 on platinum sensitivity in SKOV3 cells

To confirm whether IL-6 signaling is involved in cisplatin resistance in ovarian cancer, platinum sensitivity in SKOV3 cells was detected after neutralization with anti-IL-6 antibodies. With 15 μg/mL anti-IL6 antibodies alone, there was no change in the proliferation or inhibition of SKOV3 cells over the 48 hours period. Anti-IL6 antibodies in combination with cisplatin 2.5 μM, 5 μM and 10 μM induced an increase in cytotoxicity of SKOV3 cells to 19%, 21% and 23% respectively (Figure 3.16). Similarly, treatment with carboplatin after neutralization of IL-6 increased in SKOV3 cells cytotoxicity. Therefore, we concluded that neutralization of IL-6 level could enhance the platinum drug sensitivity.

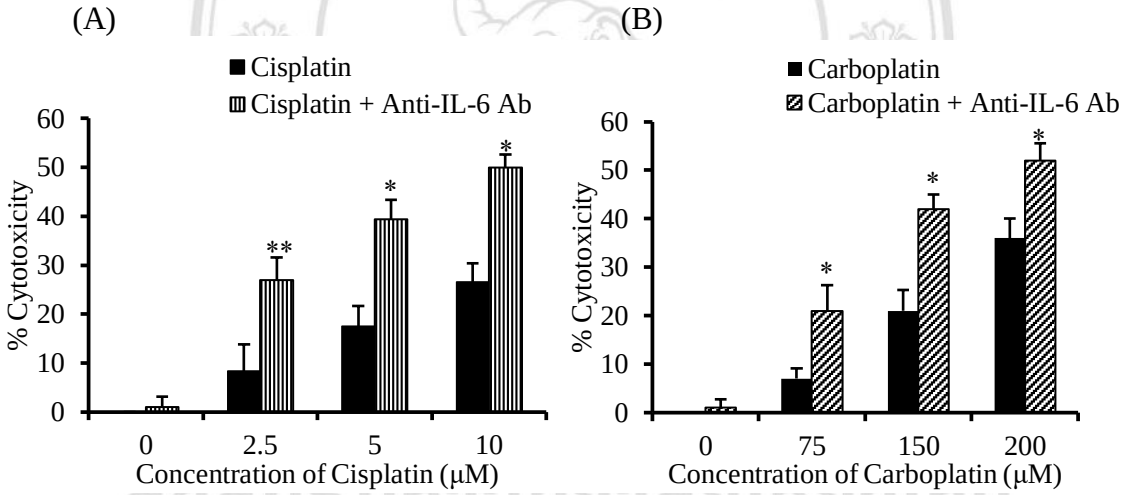



Figure 3.16 Effect of neutralization of IL-6 on platinum sensitivity in SKOV3 cells**.** SKOV3 cells were treated with different concentration of (A) cisplatin or (B) carboplatin in combination with anti-IL6 antibodies (15 μg/mL) for 48 hours, and then % cell cytotoxicity was assessed by MTT assay. The data was shown as mean ± SD of three independent experiments. *$p$<0.05, **$p$<0.01 was compared with cisplatin treatment in each concentration.

### 3.10.2 Effect of addition of exogenous IL-6 on platinum sensitivity in SKOV3 cells

To further demonstrate the role of IL-6 in platinum sensitivity, we aimed to add exogenous IL-6 to SKOV3 cells, at a level corresponding to the excess secretion after platinum treatment (based on ELISA assay). The results showed that addition of exogenous IL-6 (50 ng/ml) to SKOV3 cells led to approximately 20% increase in cell survival leading to more resistance to platinum drugs (Figure 3.17). The data indicated that IL-6 mediated platinum resistance and blocking IL-6 pathway was a way to sensitize SKOV3 ovarian carcinoma cells to platinum drugs.

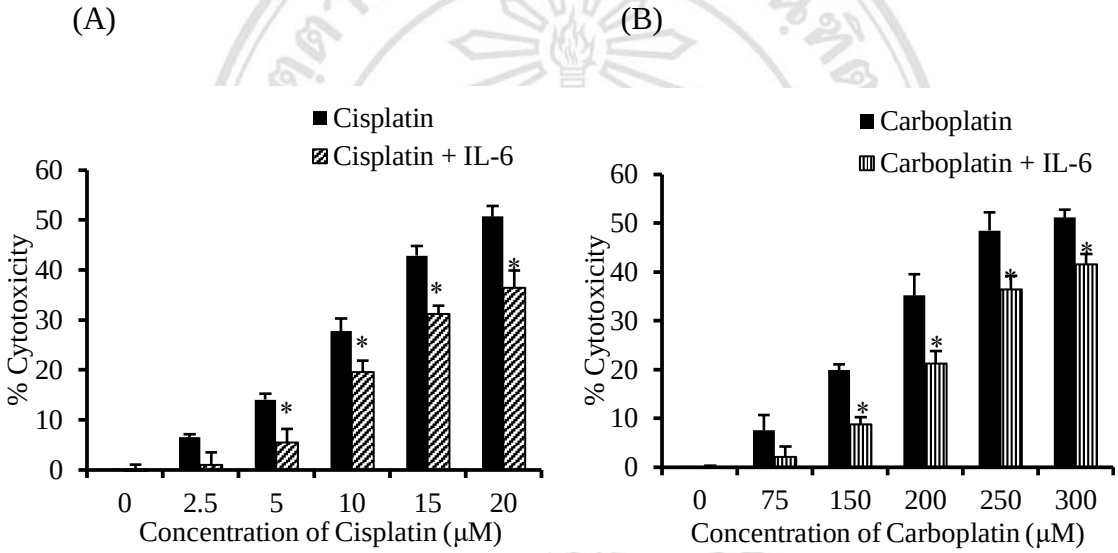



Figure 3.17 Effect of addition of exogenous IL-6 on platinum sensitivity in SKOV3 cells. SKOV3 cells were treated with different concentration of (A) cisplatin or (B) carboplatin in combination with exogenous IL-6 (50 ng/ml) for 48 hours, and then % cytotoxicity was assessed by MTT assay.*$p$<0.05 compared to drug treatment.

### 3.11 Effect of CN on IL-6 induced SKOV3 cells survival and proliferation

#### 3.11.1 Effect of CN on IL-6 induced anti-apoptotic proteins and survival protein expressions by Western blotting

Since CN has been reported to have anti-proliferative and apoptotic effects in human leukemic cells [7], the effect of CN on the IL-6 induced proliferative capacity of ovarian cancer cells was set out to evaluate. As shown in Figure 3.18, IL-6 treatment resulted in increased expression cIAP-2, Bcl-xL and survivin compared to untreated control. The levels of these proteins were found to be down-regulated when SKOV3 cells were treated in combination treatment with CN, which is suggestive of anti-IL-6 like ability of CN.

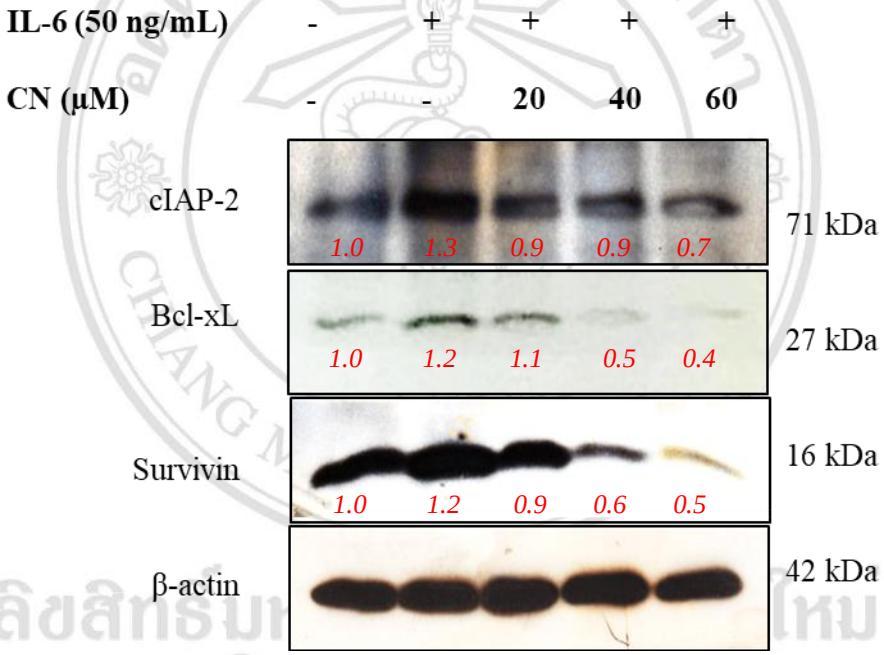


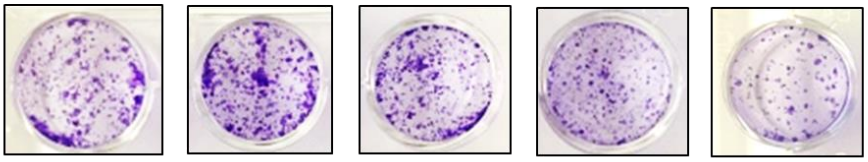



Figure 3.18 Effect of CN on IL-6 induced anti-apoptotic proteins and survival proteins expression. The whole cell lysates were prepared from SKOV3 cells that were pretreated with CN (20, 40, 60 μM) for 2 hour followed by treatment with or without IL-6 (50 ng/mL) for 24 hours and separated by SDS-PAGE followed by immunoblotting using specific antibodies. Band densities are noted under each band in relative to untreated control. The band is representative data of three independent experiments.

### 3.11.2 Effect of CN on IL-6 induced SKOV3 cells colony formation by clonogenic assay

Clonogenic assays are commonly used to investigate the ability of a cell to proliferate indefinitely, thereby retaining its reproductive ability to form a large colony or a clone. Here, the effect of CN on the IL-6 induced proliferative capacity of SKOV3 cells was determined by performing clonogenic assay. As shown in Figure 3.19, IL-6 treatment resulted in increased formation of colonies about 45% compared to untreated control. The colony formation was found to be 50% reduction when SKOV3 cells were treated in combination treatment with CN 60 μM. The clonogenic assay results were consistent with those of the Western blot analyses.

(A)



| | | | | | |
|---|---|---|---|---|---|
| **IL-6 (50 ng/mL)** | - | + | + | + | + |
| **CN (μM)** | - | - | **20** | **40** | **60** |

(B)

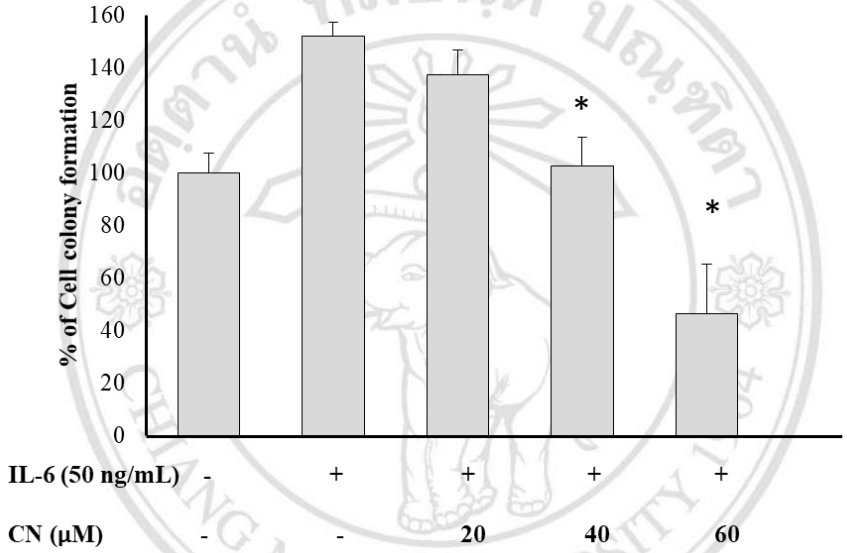


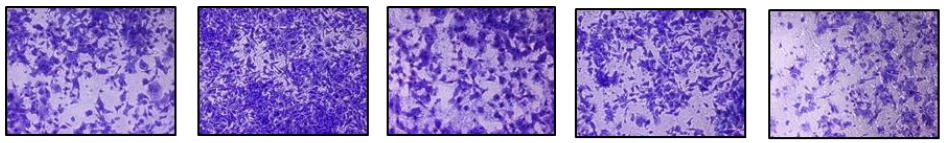

Figure 3.19 Effect of CN on IL-6 induced SKOV3 cells colony formation by clonogenic assay. (A) SKOV3 cells (300 cells/well in 12-well plate) were treated as indicated and allowed to form colonies for 10 days, then stained with crystal violet. The plate was then scanned by a photoimager. (B) The data analyzed by TotalLab Quant software. The data represent the mean ± SD of the three independent experiments. Combination with CN treated groups were significantly different from the IL-6 treated group (*$p$<0.05).

### 3.12 Effect of CN on IL-6 induced SKOV3 cells invasion and migration

#### 3.12.1 Effect of CN on IL-6 induced SKOV3 cells invasion detected by using transwell invasion chamber

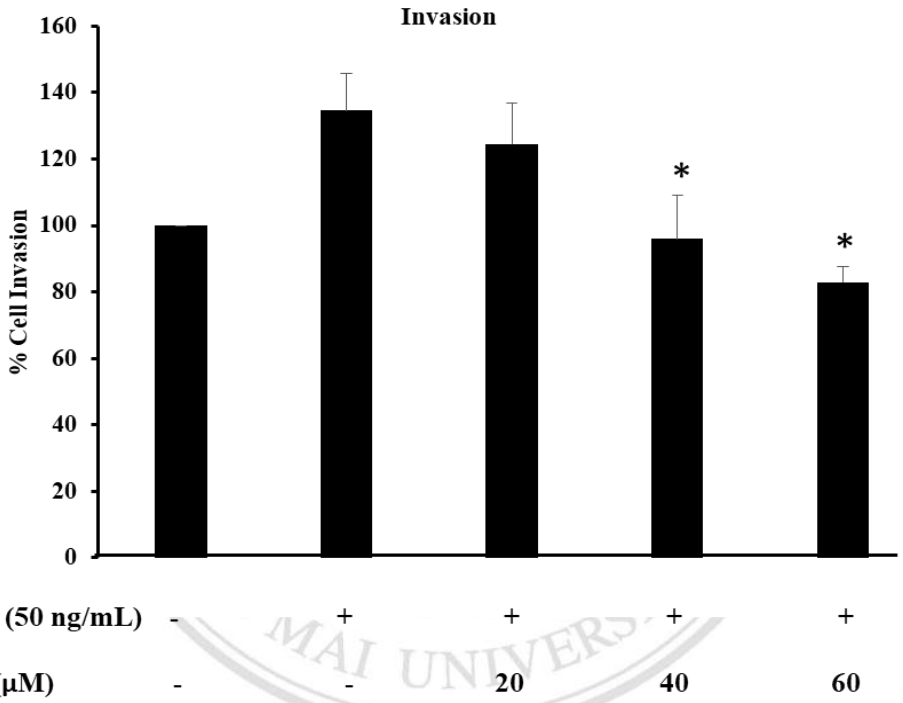
Tumor metastasis is a complex phenomenon that requires a number of specific steps such as decreased adhesion, increased motility and invasion, proteolysis, and resistance to apoptosis [76]. Recently, Lederle and colleagues showed that IL-6 promotes malignant growth of skin squamous cell carcinoma by regulating a complex cytokine and protease network [77]. Moreover, IL-6 is one of main chemokines present in serum samples of epithelial ovarian cancer patients and elevated IL-6 levels independently predict tumor recurrence, poor survival, and tumor metastasis [78, 79]. Therefore, we designed this study to further understand the role and mechanism(s) of CN on IL-6–mediated tumor cells invasion and aggressiveness. Here, we investigated the effect of CN on the invasive potential of SKOV3 cells using a transwell invasion chamber coated with Matrigel. We found that ovarian cancer cells were capable of invading through the Matrigel and this invasion was enhanced approximately 40% when the cells were treated with IL-6 (50 ng/mL). This increase in invasion was subsequently abrogated when CN was used in combination with IL-6 (Figure 3.20), suggesting that CN can block IL-6-induced invasive property of ovarian cancer cells and, thus, CN may be used to target a major step in ovarian cancer progression and metastasis.

(A)



| | | | | | |
|---|---|---|---|---|---|
| IL-6 (50 ng/mL) | - | + | + | + | + |
| CN (μM) | - | - | 20 | 40 | 60 |

(B)




| | | | | | |
|---|---|---|---|---|---|
| IL-6 (50 ng/mL) | - | + | + | + | + |
| CN (μM) | - | - | 20 | 40 | 60 |

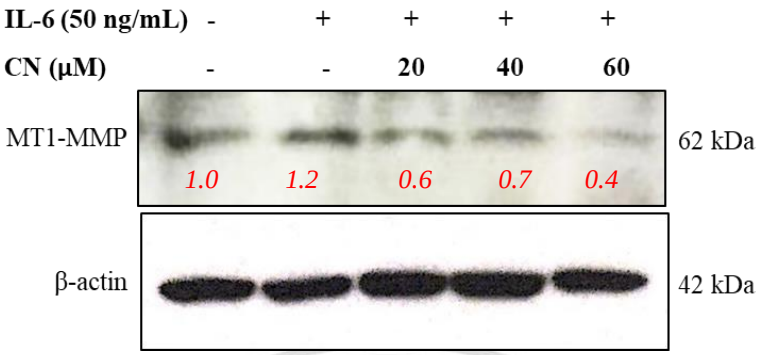


Figure 3.20 Effect of CN on IL-6 induced SKOV3 cells invasion. (A) The upper surfaces of the membrane filters were coated with Matrigel for invasion assay. SKOV3 ($2\times10^5$ cells) were seeded into the upper chamber with IL-6 (50 ng/ml) and/or CN (20, 40, 60 μM) and the lower chamber was filled with 10% FBS. After 24 hours of incubation, the invasive cells on the lower surface of the membrane were evaluated. (B) The data analyzed by Image J software. The data represent the mean ± SD of the three independent experiments. Combination with CN treated groups were significantly different from the IL-6 treated group (*$p$<0.05).

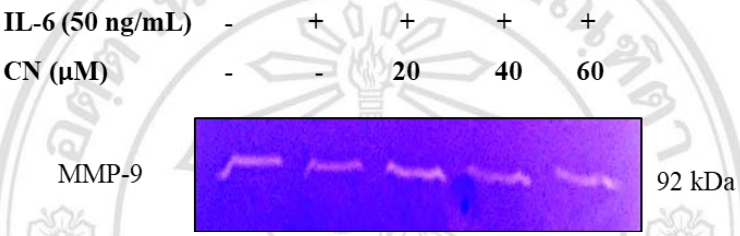
### 3.12.2 Effect of CN on IL-6 induced SKOV3 cells invasion and migration protein expressions

Tumor cells may acquire an enhanced ability to invade neighboring tissues. For this phenomenon, matrix metalloproteinases (MMPs) play an important role in tumorigenesis, invasion and metastasis. MMPs are a group of enzymes that are capable of degrading varieties of extracellular matrix proteins, but also can process a number of bioactive molecules. Therefore, they serve as reliable markers for tumor cell invasion and migration [80]. Here, MT1-MMP protein expression level was measured by performing Western blotting. IL-6 was able to induced MT1-MMP protein expression following 24 hours exposure to IL-6, whereas treatment with CN was able to suppress the IL-6-induced MT1-MMP expression in SKOV3 cells (Figure 3.21 A). MMP-9 protein secretion by SKOV3 cells into the culture supernatant was determined by gelatin zymography. Initially, when MMP-9 production was investigated in the presence of IL-6 in the culture medium, the stimulatory effect of IL-6 on MMP-9 secretion was not significant (Figure 3.21 B). We supposed that SKOV3 cells could produce IL-6 to a certain extent which was able to induce secretion of MMP-9 from the cell itself, making the medium supersaturated with IL-6. Moreover, the inhibitory effect of CN on MMP-9 secretion was not seen in the treatment with IL-6, which might be due to masking the effect of CN on MMP-9 secretion. Therefore, IL-6 was neutralized with anti-IL6-antibodies and the supernatant was collected to determine MMP-9 secretion. The results indicated that after neutralization of IL-6, MMP-9 secretion was reduced compared to the untreated control. CN was also able to suppress MMP- 9 protein expression in SKOV3 cells (Figure 3.21 C).

(A)




(B)




(C)

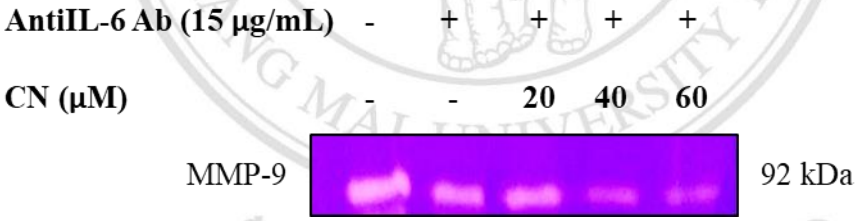


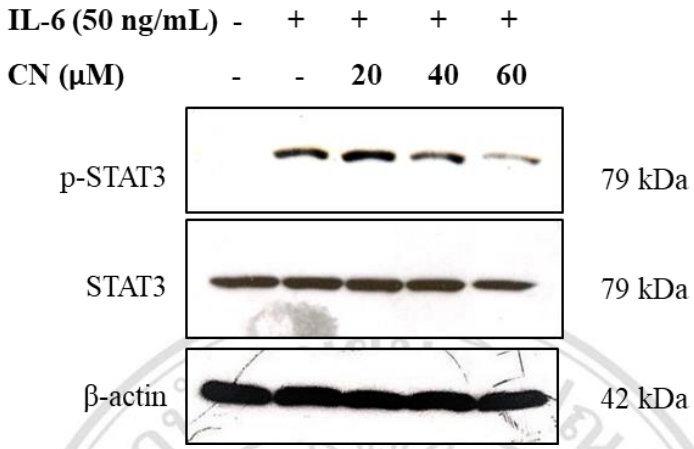



Figure 3.21 Effect of CN on IL-6 induced MT1-MMP protein expression by Western blotting and MMP-9 protein secretion by gelatin zymography (A) The whole cell lysates were prepared from SKOV3 cells that were pretreated with CN (20, 40, 60 μM) for 2 hours followed by treatment with IL-6 (50 ng/mL) for 24 hours and separated by SDS-PAGE followed by immunoblotting using specific antibodies. (B) SKOV3 cells were treated with IL-6 (50 ng/mL) ± CN or (C) anti-IL-6 Ab (15 μg/mL) ± CN for 24 hours. Then, supernatant was collected to detect for MMP-9 secretion by gelatin zymography.

### 3.13 Phosphorylation of STAT3 in SKOV3 cells was suppressed by CN

Since cell cycle progression, proliferation, survival and secretions of MMPs through IL-6 stimulation are known to be STAT3 dependent, and this stimulation can lead to tumor metastasis [81, 82]. Previous studies had demonstrated that activated nuclear STAT3 has been detected in various malignancies and enhanced activation of STAT3 has been suggested as a major contributor to platinum resistance [83, 84]. Conversely, conditional STAT3 disruption in gastric and other epithelial cell types was found to inhibit tumor development and progression [85, 86]. Therefore, activation and phosphorylation of STAT3 was detected to further elucidate the mechanism of action of IL-6 and CN on ovarian tumor aggressiveness. As shown in Figure 3.22, treatment with exogenous IL-6 induces activation and phosphorylation of STAT3 protein whereas treatment with CN 20, 40, 60 μM significantly suppress the phosphorylation of STAT3. Overall results indicate that CN is one of the natural alkaloids that targets IL-6/STAT3 axis leading to inhibition of ovarian cancer aggressiveness.

(A)




(B)

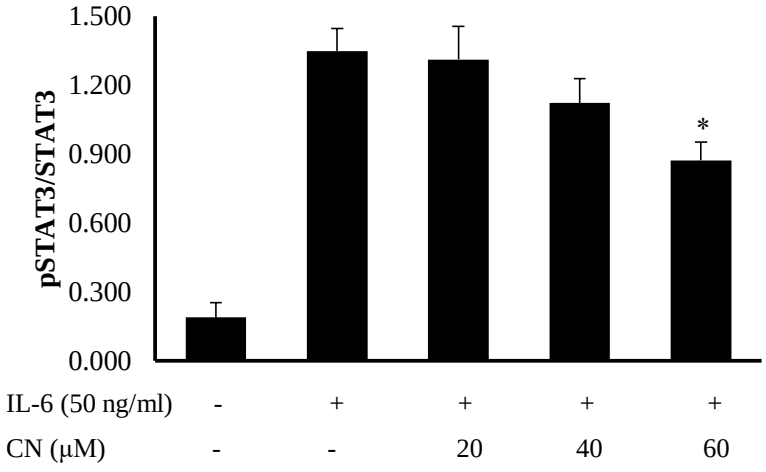





Figure 3.22 Effect of CN on IL-6 induced expression of STAT3 and STAT3 phosphorylation. (A) The whole cell lysates were prepared from SKOV3 cells that were pretreated with CN (20, 40, 60 μM) for 2 hours, then, treated with IL-6 (50 ng/mL) for 30 minutes. The whole cell lysates were separated by SDS-PAGE followed by immunoblotting using specific antibodies. (B) The band intensity was quantified by densitometry. The asterisks indicate a significant difference compared to IL-6 treated group (*$p$<0.05). The results were representative of three independent experiments.

### 3.14 Characteristics of the included patients and their clinical response to chemotherapy

In order to determine the relationship between IL-6 production and the responses to chemotherapy in clinical settings, the primary cancer cell culture method was established by using the tumor tissue of ovarian carcinoma patients who underwent surgery at the Department of Gynecologic Oncology, Division of Surgery, Maharaj Nakorn Chiang Mai Hospital. The records of the patients included in this study are summarized in Table 3.4. The median age of the enrolled patients was 52.8 (18-77) years. Among 19 patients, 12 patients (63.1%) were less than 60 years old, 13 patients (68.4%) were serous histological type adenocarcinoma and 11 patients (57.9%) were at advanced stage III and IV. After treatment with platinum-based chemotherapy in ten ovarian cancer patients, seven (70%) patients presented a complete or partial response to chemotherapy, and three (30%) patients showed progression and resistance to platinum-based chemotherapy. Two patients did not come for follow-up checkup, one patient died before chemotherapy could be started and six patients were under the chemotherapy treatment course. These patients were omitted from statistical analysis. Of the 19 patients who undertook platinum-based chemotherapy after primary cytoreductive surgery, ten cases met the criteria for statistical evaluation.

Table 3.4 Characteristics of the patients

| Clinical Parameters | Number (n=19) | Percentage (%) |
|---|---|---|
| **Age** | | |
| Medium age (years) | 52.8 | |
| Age range | 18 – 77 | |
| < 60 | 12 | 63.1 |
| ≥ 60 | 7 | 36.8 |
| **Histopathology** | | |
| i. Serous | 13 | 68.4 |
| ii. Mucinous | 2 | 10.5 |
| iii. Endometroid | 3 | 15.8 |
| iv. Granulosa cell carcinoma | 1 | 5.3 |
| **FIGO staging** | | |
| Early Stage I & II | 8 | 42.1 |
| Late Stage III & IV | 11 | 57.9 |
| **Clinical response to chemotherapy** | | |
| Complete or partial response | 7 | 36.8 |
| Recurrent, progression, drug resistance | 3 | 15.8 |
| No follow up | 2 | 31.6 |
| Under treatment | 6 | 10.5 |
| Death before chemotherapy | 1 | 5.3 |

### 3.15 Platinum-induced IL-6 production in *ex-vivo* studies

Since cytokines, especially IL-6, play a role in the drug resistance of various malignant diseases and platinum-induced IL-6 production was seen in intrinsic drug resistance SKOV3 cell lines, we determined whether the platinum-induced IL-6 production rates in the primary cell cultures of ovarian carcinoma patients were correlated with the clinical outcomes. Among platinum drugs, the treatment of the choice for ovarian cancer patients in clinical setting is carboplatin because it has lesser side effects on ototoxicity and nephrotoxicity compared to cisplatin. Therefore, we treated the primary culture cells with carboplatin for 72 hours and then determined IL-6 production by ELISA assay. Out of ten patients, we found that two patients showed inducible IL-6 production more than two fold from baseline level upon carboplatin treatment, whereas IL-6 level in the rest eight patients did not change upon drug treatment. Then, we evaluated the relationships between inducible IL-6 production and the clinical parameters by Chi square test. There was no significant association with age, staging and histological types with clinical response to chemotherapy. However, regarding with the IL-6 production, all responsive patients showed no or very little change in IL-6 level upon drug treatment. On the other hand, out of three patients who showed progression and resistance to platinum-based chemotherapy, two patients had inducible IL-6 production. Therefore, the data indicated that carboplatin-induced IL-6 production had a significant association with a poor clinical response to chemotherapy ($p$=0.016) (Table 3.5).

Table 3.5 The relationships of platinum-induced IL-6 production in *ex-vivo* studies and the clinical responses to chemotherapy among ovarian cancer patients

| Clinicopathological parameters | | IL-6 level after carboplatin treatment in primary cell culture | | |
|---|---|---|---|---|
| | | No or very little change (n=12) | Increased (>2 fold) (n=7) | *p* value |
| Age (y) (n=19) | < 60 (n=12) | 4 | 2 | 0.568 |
| | ≥ 60 (n=7) | 4 | 0 | |
| FIGO staging (n=19) | Early (n=8) | 3 | 0 | 0.061 |
| | Late (n=11) | 5 | 2 | |
| Histological types (n=19) | Serous (n=13) | 7 | 6 | 0.216 |
| | Mucinous (n=2) | 2 | 0 | |
| | Endometroid (n=3) | 3 | 0 | |
| | Granulosa (n=1) | 0 | 1 | |
| Clinical response (n=10) | Responsive (n=7) | 7 | 0 | 0.016* |
| | Nonresponsive (n=3) | 1 | 2 | |

### 3.16 Effect of CN on platinum sensitivity in patients' samples with high IL-6 production induced by carboplatin

We had already shown that CN enhanced platinum sensitivity in intrinsic resistant SKOV3 cell lines. Then, we would like to know whether CN could potentiate the platinum sensitivity in the primary cell cultures obtained from ovarian cancer patients. This study might provide important functional information for therapeutic outcomes to evaluate whether or not the chemosensitizing effect of CN identified in the *ex-vivo* studies could be inferred to clinical trials and beyond. Here, we determined the effect of CN on platinum sensitivity in patients' samples with high IL-6 production induced by carboplatin. The primary culture cells, which were capable of IL-6 secretion upon drug treatment, were treated with CN for 2 hours, followed by treatment with carboplatin for 48 hours and the cell proliferation was evaluated by MTT assay to determine the effect on platinum sensitivity. As shown in Figure 3.23, the results indicated that treatment with 200 μM carboplatin alone caused 65% and 56% cell survival in patient A and patient B respectively whereas in combination with CN 60 μM could enhance the percentage of cytotoxicity to 20 % in patient A and 18% in patient B respectively. Hence, CN has the ability to enhance platinum sensitivity in patients who showed poor clinical response and high IL-6 production induced by carboplatin, determined by *ex-vivo* studies.

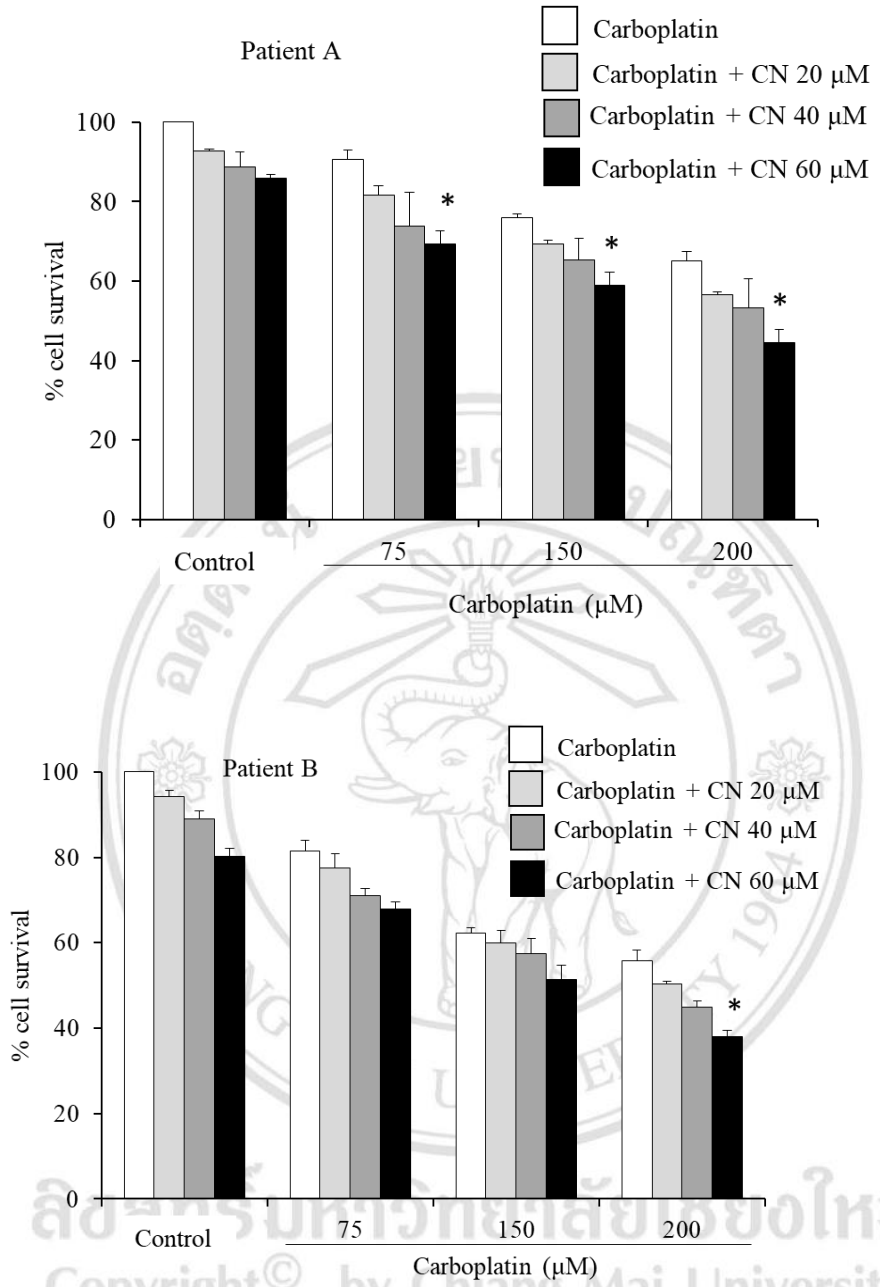





Figure 3.23 Effect of CN on platinum sensitivity in patients' samples with high IL-6 production induced by carboplatin. The primary culture cells, which were capable of IL-6 secretion upon drug treatment, were treated with CN for 2 hours, followed by treatment with carboplatin for 48 hours and the cell proliferation was examined by MTT assay. *$p$<0.05 compared to drug treatment.