# CHAPTER 4

## Discussion

Ovarian cancer is one of the principal causes of death from gynecologic malignancies worldwide. The standard therapy of late stage of ovarian cancer is cytoreductive surgery followed by combination of chemotherapy (a platinum-based drug and paclitaxel) [1]. According to global estimates, 230,000 new cases were discovered each year [10]. Despite significant advances in the understanding of the natural history of the disease, surgical and chemotherapeutic management, most patients relapse after primary treatment occurring more than 60% mortality rate within five years [10]. Thus, one of the major hindrances to an effective management in ovarian cancer is the failure of the initial chemotherapy to eliminate an appropriate number of cancer cells to avoid disease recurrence. The emergence of chemo-resistance is a time-dependent cellular process, in which several mechanisms and pathways are involved. The mechanisms of platinum resistance is believed to be multifactorial in nature and can be caused by a number of cellular adaptations, including increased DNA adduct repair due to overexpression of DNA repair proteins, alteration in intracellular signal transduction pathways, overexpression of anti-apoptotic proteins and interference in caspase activation [87]. For the purpose of overcoming the dose-related toxicity and resistance to chemotherapeutic drugs in cancer cells, researchers have emphasized on the discovery natural products as chemo-sensitizers that augment drug sensitivity and improve antineoplastic effects. Amongst the candidate natural alkaloids that play beneficial effects on cancer cells, the herbal extract from the tubers of *Stephania venosa* have been publicized the presence of aporphine alkaloids such as crebanine (CN) and *O*-methylbulbocapnine (OMBC) and protoberberine type natural alkaloids such as tetrahydropalmatine (THP) and *N*-methyl tetrahydropalmatine (NMTHP). In recent years, CN is interesting due to its biological activities such as anti-proliferative effect and anti-invasive effect in human cancer cell culture via regulated cell cycle proteins and via inhibition of constitutive NF-κB activation. Moreover, it had shown that although CN displayed growth inhibition of

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

cancerous cell lines, it had very little growth inhibition effect on normal human fibroblasts [7]. These established results have directed us to hypothesize that aporphine alkaloids may possibly improve the efficacy of the traditional anti-cancer drugs. Thereby, our study here examined the chemosensitizing effect of CN and its natural analogues found in *S. venosa* on the ovarian cancer cells when given together with platinum drugs.

In the study, wild type A2780, its acquired cisplatin resistant cell line A2780/cis and intrinsic cisplatin resistant SKOV3 cell line were considered as models for human ovarian cancer cells and had been used for the study to investigate the chemosensitizing properties of two alkaloid groups, aporphine (CN and OMBC) and protoberberine (THP and NMTHP). Our data revealed that non-toxic doses of aporphine (CN and OMBC), but not protoberberine (THP and NMTHP), revealed a synergistic outcome on cisplatin sensitivity in intrinsic cisplatin resistant SKOV3 cells. However, in A2780 cells and its acquired resistant type, there was no detected synergistic interaction between these alkaloids and platinum drug. One of the mechanisms might be probably due to the different characteristics between different cell lines in which SKOV3 cells could produce IL-6 which is one of the NF-κB regulated gene products, whereas A2780 and its resistant type had no detectable IL-6 production.

Functional representation of the apoptotic pathway has exposed how the apoptotic cascade is triggered upon stimulated by DNA damage, signaling discrepancy triggered by oncogene activation, survival factors insufficiency or hypoxia [88]. One of the key hallmarks of cancer cells is their ability to resist apoptosis. The conception that apoptotic resistance is the one of the major causes of tumor drug resistance due to overexpression of genes that mediate anti-apoptosis, reinforcement of survival signals and immune modulation has been well established [73, 89]. Altered expressions of these genes and dysregulation of intracellular signal transduction pathways by cytotoxic exposure may also provide survival advantages to cells which may contribute to the chemotherapy resistance and probably plays an essential role in cancer aggressiveness [90]. In this study, in order to identify the mechanisms underlying the chemosensitizing property of crebanine and OMBC upon cisplatin sensitivity in SKOV3 cells, the anti-apoptotic and survival proteins expression were checked by Western blot analysis. The data showed that cisplatin induced the expression of cIAP-2, Bcl-xL, survivin and IL-6, whereas

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

combination treatment with cisplatin and aporphine could inhibit these proteins expression.

There were several previous studies which described the occurrence of platinum resistance and altered intracellular signaling cascade. STAT3 overexpression is related to platinum resistance in squamous cell carcinoma of head and neck [91], while the inhibition of activated STAT3 converses drug resistance in some cancers such as gastric and head and neck carcinoma [49, 92]. Moreover, active PI3K/Akt pathway encourages cancer cell survival and chemo-resistance in ovarian cancer [93] whereas blockade of the PI3K/Akt pathway sensitize tumor cells to apoptotic cell death induced by platinum drugs [94]. In addition to PI3K/Akt pathway, some studies have revealed that persistent stimulation of MAPK pathways upon exposure to cisplatin leads to induction of Fas ligand and subsequent ovarian tumor cell apoptosis [95]. It was also reported that the inability to stimulate p38 MAPK after cisplatin exposure may be one of the chemoresistance mechanisms in SKOV3 cells [96]. Thereby, the study was interested in which signaling pathways were aberrant in SKOV3 cells. In the study, the basal activities of p-STAT3, p-Akt, p-p38, p-ERK, AP-1 and NF-κB were observed. Although STAT3, AP-1, p38, JNK and Erk were not responsive to cisplatin treatment in SKOV3 cells, cisplatin-induced increased phosphorylation of Akt and NF-κB activation and subsequent translocation into the nucleus were obviously noticed after cisplatin exposure in intrinsic cisplatin resistant SKOV3 cells. This might lead to counteract cisplatin induced apoptosis, favoring to cisplatin resistance in SKOV3 cells. These findings of basal proteins activities involved in various signaling cascades of SKOV3 cells were consistent to those of other studies [8, 40, 97, 98].

Copyright© by Chiang Mai University

The PI3K/Akt pathway contributes to the tumor survival and drug resistance by uplifting the action of Akt which involved the anti-apoptotic activity. Akt averts apoptosis by activation of NF-κB, which is an important transcriptional factor involved in anti-apoptosis, cell proliferation, cancer cell survival and drug resistance [75]. In the inactive cells, NF-κB is found in the cytosol via its tight association with inhibitory proteins called IκB. Akt activates IKK, which phosphorylates IκB and triggers the NF-κB signaling pathway. Triggering of NF-κB activity comprises of its dissociation from IκBα and the movement from cytosol to the nucleus, where it associates with the promoter region of

targeted genes, including survival and anti-apoptotic proteins including Bcl-2, survivin, Bcl-xL and cIAP-2 [4]. Hence, nuclear translocation of NF-κB and DNA binding activity are the hallmarks of its activation [99]. In order to prove that Akt and NF-κB are involved in platinum sensitivity in SKOV3 cells, we examined by means of using an inhibitor that can suppress PI3 kinase-dependent Akt phosphorylation and kinase activity. We found that inhibition of Akt activation by its pharmacological inhibitor LY294002 led to increased cisplatin sensitivity in ovarian cancer cells. Thus, the data suggested that activation of the Akt/NF-κB is a critical determinant of platinum resistance in ovarian tumor cells. The present study revealed that cisplatin treatment induced the phosphorylation of Akt in SKOV3 cells and that CN or OMBC repressed Akt phosphorylation. The Akt phosphorylation makes an attribution to Akt activation. The Akt phosphorylation activity suppressed by CN or OMBC denoted a vital role in cisplatin resistant ovarian cancer cells. Inhibition of Akt activation might lead to the suppression of the translocation of NF-κB from the cytosol to the nucleus, which in turn may lead to the down-regulation of NF-κB regulated gene products. The Akt-regulated NF-κB activity plays an essential role in cisplatin-induced chemo-resistance in ovarian cancer cells. In the present study, the finding of an inhibitory influence of CN or OMBC on Akt/NF-κB signaling might be associated with the chemosensitizing mechanism of aporphine alkaloids.

Tumor cells synthesize and secrete various chemokines and growth factors which can act in autocrine or paracrine manner in stimulation of tumor cell proliferation and drug resistance. Human IL-6 is made up of 184 amino acids and is produced by various host cells and tumor cells in the tumor microenvironment. IL-6 belongs to a cytokine family and its signaling is via a common receptor gp130. The association of IL-6 to its receptor leads to the activation of the linked Janus kinases (JAKs), followed by the recruitment and stimulation of STAT3 and STAT1 [100]. It has a broad range of biological activity concerning with inflammation, angiogenesis, immunity, differentiation, proliferation, and oncogenesis [101]. Constitutive activation of STAT3 has been displayed to involve in oncogenic change in cultured cells and induce tumor formation in mice [102]. Therefore, IL- 6/STAT3 has been suggested as a potential immunotherapeutic target for malignant diseases [103, 104]. Some studies proposed that the activation of STAT3 is enhanced in platinum therapy and is responsible for platinum resistance [105]. We therefore

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

hypothesize that IL-6/STAT3 pathway inhibition could improve platinum resistance in ovarian cancer cells.

Therefore, to determine the role of IL-6 in platinum resistance, ovarian cancer 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. We found that IL-6 production was induced by cisplatin in dose-dependent manner in intrinsic drug resistance SKOV3 cells. Nonetheless, A2780 and its acquired resistant type, there were no detectable IL-6 before and after drug treatment. Therefore, the induction of IL-6 might be correlated to the intrinsic drug resistance mechanism in ovarian cancer. The results showed that addition of exogenous IL-6 to SKOV3 cells led to increased cell survival and more resistance to platinum drugs and blocking IL-6 pathway is a way to sensitize SKOV3 ovarian carcinoma cells to platinum drugs. IL-6 mediates its downstream effects by triggering several signaling cascades including JAK/STAT pathway. In the study, we confirmed that treatment with CN significantly suppressed the phosphorylation of STAT3 whereas exogenous IL-6 induced activation and phosphorylation of STAT3 protein. As a transcription factor, STAT3 mediates the expression of various genes including Bcl-xL, surviving and cIAP-2. Matrix metalloproteinases (MMPs) are a family of calcium- and zinc-dependent proteolytic enzymes which are capable of degrading extracellular matrix components, releasing several factors, cytokines and chemokines that are present in ECM. They are reliable indicators for tumor cell invasion and migration [80]. Increased levels of MMPs and enhanced proteolytic activity were shown to be associated with advanced stage of ovarian tumor and a worse prognosis [106, 107]. Moreover, Jia *et al*., reported that p-STAT3 directly interacts with *MMP-9* gene promoter to induce gene expression of MMP-9 in ovarian cancer cells [81]. The inhibitory effect of CN on STAT3 pathway and MMP production led to oppose the invasion of cancer cells. This anti-IL-6-like activity of CN may be associated with the inhibition of ovarian cancer aggressiveness.

Although preclinical investigations involving immortal cancer cell lines have paved the way in the understanding of natural history of cancer biology and the development of an effective chemotherapeutic drug, the establishment of short-term primary culture of cancer cells from solid tumor tissues plays an essential setting in the management of personalized cancer therapy [108, 109]. Many studies stated that high IL-6 levels

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

measured in the ascites fluid of ovarian cancer patients were related with the rates of shorter progression-free survival [46, 110]. Some researchers have revealed that the increased serum level of IL-6 is correlated with advanced stage of cancer [111] and serum IL-6, IL-8 and C-reactive protein levels might be used as markers for prognosis in epithelial ovarian cancer patients [112]. In the present study, primary cell culture method was established to determine whether cytokines production rates in response to carboplatin in primary cancer cell culture derived from the samples of ovarian cancer patients were correlated with the clinical outcomes. Out of ten patients, two patients showed inducible IL-6 production upon exposure to carboplatin in *ex-vivo* studies, whereas IL-6 level in the rest eight patients did not change upon drug treatment. Additionally, there was significant association between inducible IL-6 production and poor clinical response to chemotherapy, such as recurrence, progression and resistance to platinum-based chemotherapeutic drugs ($p$=0.016). Therefore, IL-6 overproduction in response to carboplatin in the primary cancer cell cultures might be used as a predictive marker for the clinical and chemotherapeutic outcomes in ovarian cancer patients.

Though we found out that CN had chemosensitizing effect on platinum sensitivity in SKOV3 ovarian cancer cell line, we confirmed the effect of CN on platinum sensitivity in *ex-vivo* studies. The primary culture cells with inducible IL-6 production in response to carboplatin were treated with CN and assessed the platinum sensitivity by MTT assay. The result of combined treatment with CN could enhance the platinum sensitivity on two patients' samples with high IL-6 production induced by carboplatin. Hence, adjuvant therapy of CN would be possible way to increase the sensitivity to platinum-based chemotherapeutic drugs in ovarian cancer patients. However, additional *in vivo* experiments are necessary for clinical trials to assess the potential as a chemosensitizing agent against ovarian cancer cells.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

# CONCLUSION

1. Our findings are of the first report that identified the chemosensitizing effects of alkaloids isolated from the tubers of *S. venosa* on ovarian cancer cells. The aporphine alkaloids exhibited the chemosensitizing effect on SKOV3 ovarian cancer cells via inhibition of Akt/NF-κB signaling and the down regulation of NF-κB mediated gene products.
2. Our results on ex-vivo studies showed that platinum-induced IL-6 production predict poor response to chemotherapy.
3. CN could reduce IL-6-mediated augmentation of phosphorylation of STAT3 protein and its downstream signaling proteins involved in cancer cell survival and invasion.
4. Overall results draw attention to aporphine alkaloids from *S. venosa* as an interesting agent in ovarian cancer to sensitize and minimize the dose related toxicity of platinum-based chemotherapeutic drugs and to suppress IL-6 mediated ovarian tumor aggressiveness as summarized in Figure 4.1.
5. The current study's results will be of the great benefit in the development of further ovarian cancer preventive and/or therapeutic strategies concerning CN or OMBC as adjuvant therapy to conventional platinum-based chemotherapeutic agents. Importantly, since there are many mechanisms of platinum drug resistance, the effect of CN or OMBC versus different drug resistant mechanisms should be taken into consideration, as well as, which should be the focus of future investigations.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

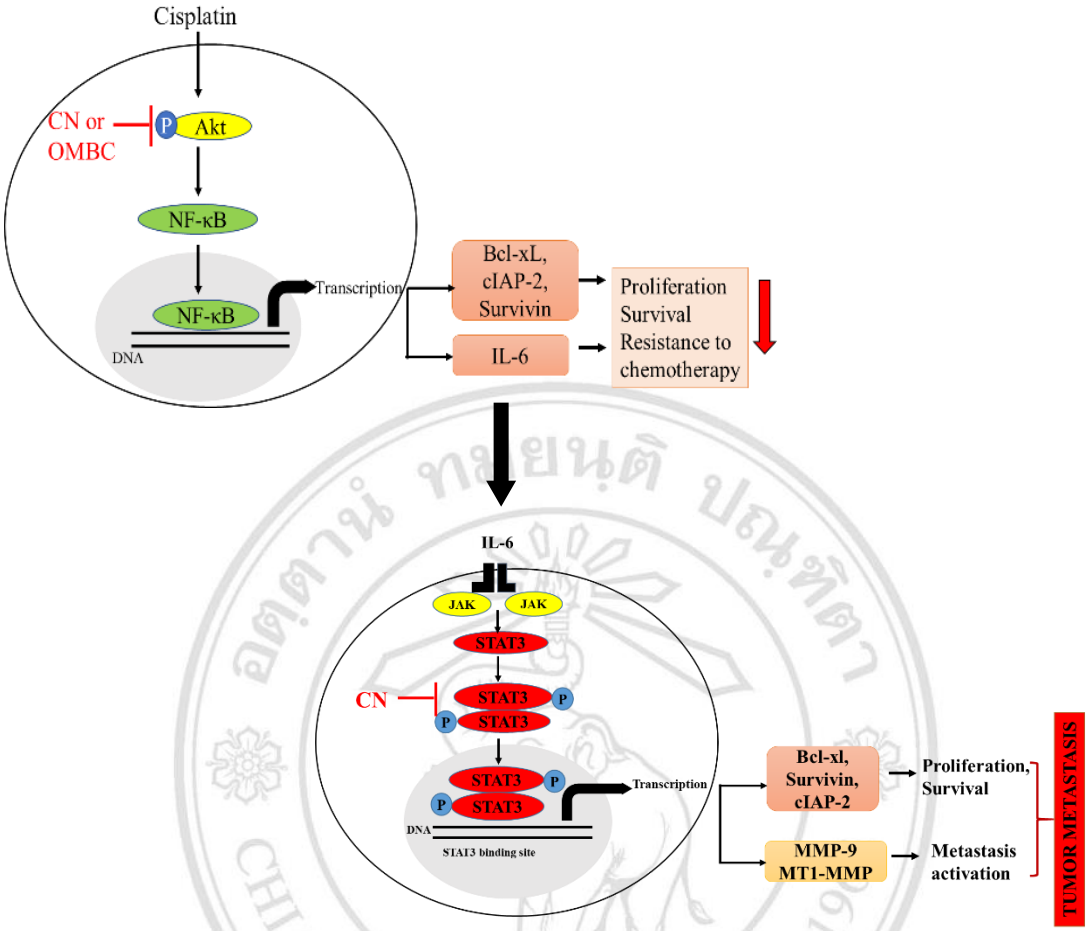


Figure 4.1 Scheme illustrates summary effects of aporphine alkaloids from *S. venosa* as chemosensitizer and inhibitor of ovarian cancer aggressiveness

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

# FURTHER STUDIES

1. Our study implied that CN and OMBC act as chemo-sensitizers in intrinsic drug resistance ovarian cancer cells. Hence, further investigation in experimental design that allows to investigate the drug resistance due to other mechanisms such as acquired drug resistance ovarian cancer cells are still needed in detail.
2. Our results on *ex-vivo* studies showed that platinum-induced IL-6 production predict poor response to chemotherapy and use of CN could enhance the platinum sensitivity. However, further *in vivo* experiments are required for clinical trials to pursuit their potential as a chemosensitizing agent against cancer cells.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved