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The Role and Regulation of Flice-Like Inhibitory protein (FLIP) in Cisplatin Resistance in Human Ovarian Cancer Cells *in vitro*

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The Role and Regulation of Flice-Like Inhibitory protein (FLIP) in Cisplatin Resistance in Human Ovarian Cancer Cells *in vitro*

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Abstract

Human ovarian cancer is the most lethal gynecological malignancy. Although cisplatin (CDDP) and paclitaxel are the first-line chemotherapeutic agents for ovarian cancer, chemoresistance is a major therapeutic problem with mechanisms involved still not well understood. Dysregulation of the apoptotic cascade may be a causative factor for chemoresistance. Indeed gene products involved in the regulation of apoptosis, including the PI3K/Akt and Flice-Like Inhibitory Protein (FLIP) are frequently over-expressed and/or dysregulated in chemoresistant cells.

FLIP, a caspase-8 analogue, modulates death receptor-mediated apoptosis by preventing caspase-8 activation. Since FLIP may have a critical involvement in ovarian cancer chemoresistance, it is importance to understand its role, the molecular and cellular mechanisms by which FLIP is regulated, and if and how dysregulated FLIP degradation is involved in the etiology of chemoresistance.

Human ovarian cancer cell lines were used to establish the possible involvement of FLIP in the regulation of CDDP sensitivity *in vitro* and to assess the mechanism by which CDDP regulates FLIP. We demonstrated that CDDP down-regulates FLIP content, induces caspase-8 and -3 activation and apoptosis in chemosensitive ovarian cancer cells, but not in their resistant counterparts. Moreover, we observed that FLIP over-expression in chemosensitive cells attenuates CDDP sensitivity, while FLIP down-regulation sensitizes chemoresistant cells to CDDP.

We further demonstrated that CDDP had no effects on FLIP mRNA abundance but down-regulated FLIP protein content in chemosensitive cells, a response attenuated by proteasome inhibitors. Itch is an E3 ligase protein involved in protein ubiquitination. CDDP also enhances FLIP-p53-Itch cell membrane co-localization, and FLIP ubiquitination in chemosensitive but not resistance cells. In addition, Itch silencing

attenuates CDDP-induced FLIP ubiquitination. p53 siRNA also attenuates FLIP-Itch and FLIP-p53 interactions and FLIP ubiquitination. While Akt activation inhibits CDDP-induced FLIP degradation and apoptosis in chemosensitive cells, these responses are facilitated by dominant-negative Akt expression in their resistant variants. Finally, p53 siRNA attenuates CDDP-induced and dominant negative (DN)-Akt-facilitated FLIP-p53 binding and FLIP ubiquitination in resistant cells.

These studies improve our understanding of FLIP involvement in chemoresistance, the mechanism by which CDDP regulates FLIP, and dysregulation of FLIP degradation in chemoresistance in ovarian cancer.

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LIST OF ABBREVIATIONS

ANP	atrial natriuretic peptide
ANF	atrial natriuretic factor
APAF-1	apoptotic protease activating factor-1
ATM	ataxia telangiectasia-mutated
ATP	adenosine triphosphate
ATR	ATM and Rad3-Related
ATRIP	ATR interacting protein
BAD	Bcl-2-antagonist of cell death
BAX	Bcl-2-associated X protein
BCL-2	B-cell lymphoma-2
BH	Bcl-2 homology
BID	BH3-interacting domain death agonist
BIR	baculovirus inhibitor of apoptosis repeat
CAD	caspase-activated deoxyribonuclease
CARD	caspase-associated recruitment domain
CDDP	Cis-diamminedichloroplatinum (II)
Chk1	checkpoint kinase 1
Chk2	checkpoint kinase 2
CHO	Chinese hamster ovary
DIABLO	direct IAP binding protein with low pI
DMEM	Dulbecco's modified Eagle Medium
DNA	deoxyribonucleic acid
DN-Akt	dominant negative Akt
DRs	Death Receptors

EDTA	ethylenediaminetetraacetic acid
EGTA	ethylenebis (oxyethylenenitrilo) tetraacetic acid
EGF	epidermal growth factor
eNOS	endothelial NOS
ER	endoplasmic reticulum
ER	estrogen receptor
ERCC1	Excision repair cross-complementing rodent repair deficiency, complementation group 1
FADD	Fas-associated death domain
FasL	Fas ligand
FIGO	International Federation of Gynecology and Obstetrics
FITC	fluorescein isothiocyanate
FLICE	FADD-Like ICE
FLIP	FLICE-like inhibitory protein
FSH	follicle stimulating hormone
GAPDH	glyceraldehyde phosphate dehydrogenase
HA	hemagglutinin
HIAP1	human inhibitor of apoptosis protein-1
HIAP2	human inhibitor of apoptosis protein-2
HRP	horseradish peroxidase
IAP	inhibitor of apoptosis protein
ICE	interleukin 1 β-converting enzyme
IgG	immunoglobulin G
ILK	integrin-linked kinase
iNOS	inducible NOS

IR	ionizing radiation
JNK	c-Jun N-terminal kinase
LPS	lipopolysaccharide
kD	kilo-dalton
LH	luteinizing hormone
MAPK	mitogen-activated protein kinase
MAPKAPK2	MAPK-activated protein kinase-2
MDM2	murine double-minute-2
MOI	multiplicity of infection
NAIP	neuronal apoptosis inhibitory protein
NF-KB	nuclear factor kappa B
OMIM	online mendelian inheritance in man
OSE	ovarian surface epithelium
P53AIP1	p53-regulated apoptosis inducing protein-1
PAGE	polyacrylamide gel electrophoresis
PARP	Poly (adenosine diphosphate) polymerase
PCR	polymerase chain reaction
PDK-1	phosphotidylinositol-dependent protein kinase-1
PFT	pifithrin-alpha hydrobromide
PI3K	phosphoinositol 3-OH kinase
PIP2	phosphatidylinositol-4,5-bisphosphate
PIP3	phosphatidylinositol-3,4,5-trisphosphate
PKB	protein kinase B
PH	pleckstrin homology
PMSF	phenylmethylsulfonyl fluoride

PTEN	phosphatase and tensin homologue
PUMA	p53-upregulated mediator of apoptosis
RB	retinoblastoma
RNA	ribonucleic acid
RPM	revolutions per minute
RPMI	Roswell Park Memorial Institute
ROS	reactive oxygen species
RT	reverse transcriptase
SDS	sodium dodecylsulfate
SHIP	src homology 2-containing inositol phosphatase
SMAC	second mitochondria-derived activator of caspases
tBID	truncated BID
TBS	tris-buffered saline
TBS-T	TBS-Tween
TE	Tris-EDTA
TNF-α	tumour necrosis factor-alpha
TP53	tumour suppressive protein 53
TRAIL	TNF-related apoptosis-inducing ligand
UTR	untranslated region
UV	ultraviolet
VEGF	vascular endothelial growth factor
XAF1	XIAP-associated factor-1
XIAP	X-linked inhibitor of apoptosis protein
XP	xeroderma pigmentosa

Chapter 1 - Introduction

1.1 Human Ovarian Cancer

Although human ovarian cancer accounts for only 5% of cancer deaths, it is the fifth most frequently occurring cancer among Canadian women. It is the leading cause of gynecological cancer deaths, resulting in an anticipated 1700 death and about 2500 new cases of ovarian cancer in 2008 (Canadian Cancer Society, 2008).

Patients with ovarian cancer often do not exhibit symptoms in the early stages of the disease. The symptoms are often vague and easily mistaken for more common pathologies. Therefore, ovarian cancer is usually diagnosed after the cancer cells have spread outside the ovary. If ovarian cancer is diagnosed and treated before it has spread beyond the ovary, the 5-year survival rate is 93% (American Cancer Society, 2008). However, less than 20% of all ovarian cancers are detected at this early stage (American Cancer Society, 2008). About 3 in 4 new patients with ovarian cancer survive 1 year after diagnosis (American Cancer Society, 2008).

The ovarian cancer incidence rate, defined as the number of new cases diagnosed each year per 100,000 women, has been slowly falling over the past 20 years. The average annual percent change in age-standardized incidence and mortality rates only showed 0.8% and 0.3 % decrease during 1995 -2004 for ovarian cancer, respectively (Canadian Cancer Society, 2008), suggesting that the therapeutic strategies for the treatment of ovarian cancer patients had little impact during the past decade.

1.1.1 Ovarian Cancer Stages

The International Federation of Gynecology and Obstetrics (FIGO) stage at the time of diagnosis is the most important prognostic factor for ovarian cancer. According to the following criteria, ovarian cancer is staged in four categories, as below (Kaku, Ogawa et al. 2003):

Stage I: Growth is limited to the ovary and is found in one or both ovaries;

Stage II: Growth involves one or both ovaries with pelvic extension;

Stage III: Tumor involves one or both ovaries with peritoneal implants outside the pelvis; or in the pelvis with malignant extension to the small bowel or omentum;

Stage IV: Growth involves one or both ovaries with metastasis to distant parts of body

Late diagnosis and drug resistance are two predominant factors responsible for the high mortality rate in patients with ovarian cancer. Ovarian cancer is usually diagnosed when it has reached stage III or IV, by which time it has spread beyond the ovaries. If ovarian cancer is diagnosed in stages I and II, the tumors are readily curable and the 5-year survival time approaches 90% for stage I and 65% for stage II (Nguyen, Averette et al. 1993). In contrast, patients with ovarian cancers in stage III or IV are typically more difficult to treat successfully, and the 5-year survival rates are 39% (stage III) and 15% (stage IV), respectively (Holschneider and Berek 2000). Since the early stage of ovarian cancer is asymptomatic, the disease is usually diagnosed in the late stages (Vanderhyden, Shaw et al., 2003). Knowing the stage and grade of the cancer is helpful in choosing the best treatment for the patient. If the tumor is confined to the ovary, cytoreductive surgery is the

initial therapeutic treatment for ovarian cancer. For the majority of patients with ovarian cancer, follow-up treatment with combination chemo-therapy is generally employed.

1.1.2 Ovarian Cancer Subtypes

The ovaries contain three cell types: germ cells which are located inside of the ovary and responsible for making ova, stromal and granulosa cells which are responsible for the female steroid hormone secretion, and epithelial cells that cover the surface of ovary. Ovarian cancer occurs upon transformation of ovarian cells and is classified based on the cells from which the tumor arises (e.g. surface epithelial cells, germ cells, and stroma cells). While a small fraction of ovarian tumors are derived from germ and stromal cells (~ 5% to 7%), ovarian tumors arising from surface epithelial cells accounts for almost 90% of ovarian cancers (Weiss, Homonchuk et al. 1977).

1.1.2.1 Germ cell tumors

Germ cell tumors account for almost 5% of ovarian cancers. Some germ cell tumors are malignant and could potentially be life threatening, although the majority of them are benign. Teratoma, dysgerminoma, endodermal sinus tumor are the most common germ cell tumors (Canadian Cancer Society, 2008).

1.1.2.2 Sex cord-Stromal cell tumors

Stromal cell tumors account for about 5% of ovarian cancers. The majority of them are granulosa cell tumors and are often found in women older than 50 years old. However, about 5% of stromal tumors occur in young girls. While thecomas and fibromas are benign

stromal tumors, low-grade cancers such as granulosa cell tumors and granulosa-theca tumors, are malignant stromal tumors (Canadian Cancer Society, 2008).

1.1.2.3 Epithelial ovarian tumors

Epithelial ovarian tumors originate from cells covering the surface of the ovary, and account for the majority (85-90%) of ovarian cancers. They are mostly diagnosed in post-menopausal women. Almost 70% of ovarian cancer patients are detected with stage III or IV of the disease. Once developed, the tumor is aggressive and becomes metastatic. Epithelial ovarian tumors are further divided into different histological groups and are classified into type I and type II (Shih Ie and Kurman 2004; Shih Ie and Kurman 2005). Type I tumors consist of low-grade micropapillary serous carcinomas (MPSCs), endometrioid, mucinous, and clear cell carcinomas. They are genetically stable and characterized by mutations of various genes, including Kirsten Ras *(KRAS), B-RAF (BRAF),* phosphatase and tensin homolog *(PTEN),* and *β-catenin* (Gershenson, Sun et al. 2006). In contrast, type II tumors are aggressive, genetically unstable, and consist of high-grade serous carcinoma, malignant mixed mesodermal tumors (MMMTs), and undifferentiated carcinomas (Bell and Scully 1994). Type II tumors are characterized by mutation in *TP53*, which is rarely found in type I tumors (Shih Ie and Kurman 2004; Singer, Stohr et al. 2005).

Serous ovarian tumors are the most frequently diagnosed ovarian cancer subtype. They are most frequently diagnosed in stage III or IV (Seidman, Horkayne-Szakaly et al. 2004). Serous tumors are usually large and frequently bilateral (two-thirds of the cases) (Kaku, Ogawa et al. 2003). They contain a combination of cystic, papillary, and solid growth patterns and frequently invade the ovarian capsule, and grow on the surface epithelium of ovary. Mutations of several

genes have been reported in serous ovarian carcinomas, including *KRAS* mutations at codons 12 and 13 in one-third of invasive low-grade MPSCs and one-third of serous borderline tumors (SBTs) (Singer, Kurman et al. 2002; Singer, Oldt et al. 2003), *BRAF* mutations at codon 600 in 30% of low-grade serous carcinomas and 28% of SBTs (Singer, Kurman et al. 2002), mutations of v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, *ERBB2* in less than 5% of these tumors, and mutation of *TP53* (> 80%) in advanced stage high-grade serous carcinomas (Kupryjanczyk, Thor et al. 1993; Wen, Reles et al. 1999; Chan, Cheung et al. 2000; Salani, Kurman et al. 2008) and in 37% of stage I and II high-grade serous carcinomas (Shelling, Cooke et al. 1995).

Mucinous tumors are the largest tumor types. They are predominantly solid (Kaku, Ogawa et al. 2003) and are usually found in only one ovary (Hart 2005) . Microscopically, mucinous tumors consist of glands and cysts which are lined by stratified mucinous epithelial cells. The majority of the mucinous ovarian cancers (71%) are diagnosed in stage I and are without stromal invasion (Riopel, Ronnett et al. 1999; Kaku, Ogawa et al. 2003). They can be uncharacteristically aggressive and lethal (Ludwick, Gilks et al. 2005). They occur mainly in women aged 30-50 and grow to an extremely large size. Frequent *KRAS* mutations at codons 12 and 13 have been reported in cystadenomas, mucinous borderline tumors (MBTs), and mucinous carcinomas (Enomoto, Weghorst et al. 1991; Mok, Bell et al. 1993; Ichikawa, Nishida et al. 1994; Caduff, Svoboda-Newman et al. 1999; Gemignani, Schlaerth et al. 2003).

Endometrioid tumors, adenocarcinoma of the endometrium, are characterized by the presence of elements of both epithelium and stroma cells (Kaku, Ogawa et al. 2003). They are the most common tumor generally present in stage I carcinomas [~ 50%; (Leitao, Boyd et al.

2004; Leitao, Soslow et al. 2004)] and are highly associated with cyst containing chocolate-coloured fluid (Kaku, Ogawa et al. 2003). They occur most often in women aged 50-70. Mutations of *KRAS* and *BRAF* in approximately 10% of endometrioid carcinomas (Cuatrecasas, Erill et al. 1998; Gemignani, Schlaerth et al. 2003; Hogdall, Hogdall et al. 2003; Okuda, Otsuka et al. 2003; Singer, Oldt et al. 2003; Mayr, Hirschmann et al. 2006), and *PTEN* mutation in 20% of endometrioid carcinomas have been reported (Obata, Morland et al. 1998). Importantly, it has been reported that the activating mutation of *KRAS* and inactivation mutation of *PTEN* (Dinulescu, Ince et al. 2005; Wu, Hendrix-Lucas et al. 2007) can produce an ovarian endometrioid carcinoma in mouse models (Dinulescu, Ince et al. 2005).

Clear cell ovarian cancer, most often presents in women aged 40-80, is usually found on only one ovary (unilateral) and is associated with endometriosis (50%). It is characterized by the presence of cells with cytoplasmic glycogen, and accounts for 5-10% of ovarian cancers in Western countries (Kaku, Ogawa et al. 2003). It constitutes a higher percentage (~30%) of ovarian cancer in Japanese women (Sugiyama, Kamura et al. 2000; Kaku, Ogawa et al. 2003). The cells from Clear cell carcinomas are typically polygonal and may form multiple papillae. They account for approximately 50% of stage I ovarian carcinomas (Leitao, Soslow et al. 2004; Mizuno, Kikkawa et al. 2006). Mutations in *KRAS, BRAF*, and *TP53* with low frequency are reported in some clear cell carcinomas (Mayr, Hirschmann et al. 2006).

Transitional cell tumors, the rarest type of ovarian cancer, are divided into Brenner tumors and Transitional-cell carcinoma. Almost 50% and 80% of these transitional cell

ovarian cancers are detected in stage I and II of the disease, respectively (Kaku, Ogawa et al. 2003).

Mixed epithelial ovarian cancers are made up of a mixture of the above epithelial subtypes as well as stromal components. They account for 10% of epithelial ovarian cancers.

1.1.3 Risk Factors of Ovarian Oncogenesis

The molecular mechanisms underlying the development of ovarian cancer are not clear. Due the lack of symptoms and reliable screening tests for early diagnosis, the majority of ovarian cancers (> 70%) are diagnosed at a late stage of the disease (Goff, Mandel et al. 2000), by which time the tumor cell biology might have changed in comparison to the initial stages of the disease. While a family history of ovarian cancer is an important risk factor for ovarian cancer, heredity accounts for approximately 5-10% of the disease (Holschneider and Berek 2000; Risch, McLaughlin et al. 2001). In addition to heredity, ovulation, hormones (e.g. gonadotropin and steroid), oncogenes and tumor suppressor genes, growth factors, cytokines and environmental agents have been shown to contribute in the etiology of ovarian cancer (reviewed in Salehi, Dunfield et al. 2008). However, the precise mechanisms of ovarian oncogenesis are yet to be fully elucidated. Moreover, understanding the molecular determinants and risk factors involved in the initiation of ovarian cancer and the mechanism implicated in this context has the potential to lead to successful treatment, including development of therapeutic strategies to target survival factors such as Akt and/or restore the activity of tumor suppressors such *PTEN* and *TP53*

1.1.3.1 Familial Ovarian Cancer

Family history is a major risk factor for ovarian cancer (Holschneider and Berek 2000). It has been reported that 5 to 10% of all cases of ovarian cancer are related to genetic factors (Ford and Easton 1995; Ford, Easton et al. 1995; Risch, McLaughlin et al. 2001; Prat, Ribe et al. 2005) and the estimated lifetime risk of developing ovarian cancer increases approximately 6-fold in the presence of a family history of the disease (Prat, Ribe et al. 2005). The identification of the *BRCA1,* breast and ovarian cancer susceptibility gene on chromosome 17q21 (OMIM# 113705) (Bowcock 1993; Miki, Swensen et al. 1995) and *BRCA2* on chromosome 13ql2-13 (Wooster, Bignell et al. 1995), provided a molecular association between ovarian cancer risk and the incidence of ovarian cancer in first- and second-degree relatives. Indeed, mutations in *BRCA1* (Hall, Lee et al. 1990; Miki, Swensen et al. 1995) and *BRCA2* (Wooster, Neuhausen et al. 1994; Wooster, Bignell et al. 1995) are frequently associated with ovarian cancer. While ninety percent of familial ovarian cancers are associated with mutations in both tumor suppressors *BRCA1* and *BRCA2* (Prat, Ribe et al. 2005), two-thirds of the cases are associated with *BRCA1,* and one-third with *BRCA2* gene mutations (Frank, Manley et al. 1998). Furthermore, women with *BRCA1/2* mutations have a 10 times increased risk in developing ovarian cancer compared with women without these mutations (Boyd, Sonoda et al. 2000). Taken together, these findings suggest that loss of *BRCA* gene product function may have a role in ovarian tumorigenesis. Both *BRCA1* and *BRCA2* influence a number of genes and proteins which are involved in diverse events such as DNA repair (Scully, Puget et al. 2000; Yoshida and Miki 2004), chromatin remodeling, DNA damage signaling via ATM/ATR, and apoptosis (Greenlee, Murray et al. 2000; Boulton 2006). A higher frequency of *TP53* mutation in ovarian tumors has been correlated with

germline *BRCA1* or *BRCA2* mutations (Ramus, Bobrow et al. 1999), suggesting that the function of the *BRCA* gene product is dependent upon the function of the p53 tumor suppressor and disruptions in p53 activity interferes with *BRCA*-mediated cell cycle arrest and/or apoptosis (Ramus, Bobrow et al. 1999).

1.1.3.2 Ovulation

Ovulation is associated with apoptotic cell death {Ackerman, 1993 #1} and repair of the ovarian surface epithelial (OSE) cells, resulting in the continuously remodeling of the OSE during the reproductive cycle. The 'incessant ovulation hypothesis' suggests that this frequent remodeling increases the risk of malignant transformation in OSE cells (Fathalla 1971). Several studies support the hypothesis, indicating that late menopause (Franceschi, La Vecchia et al. 1991) and nulliparity (Negri, Franceschi et al. 1991), both of which increase the total number of lifetime ovulations, slightly increase the risk of developing ovarian cancer. In contrast, suppression of ovulation during breastfeeding, pregnancy, and/or oral contraceptive intake are associated with decreased risk of ovarian cancer (Ford, Easton et al. 1994; Greenlee, Murray et al. 2000). In addition, it has been suggested that late menarche, which contributes to fewer ovulation events, decreases the incidence of ovarian cancer in Japan compared to western countries (reviewed in Salehi, Dunfield et al. 2008).

1.1.3.3 Gonadotropins

Ovarian cancers are mostly diagnosed in the postmenopausal period when serum FSH and LH levels reach their peak and when both normal ovarian surface epithelial cells and ovarian cancer cells express FSH and LH receptors (Zheng, Magid et al. 1996; Konishi,

Kuroda et al. 1999). These findings suggest that excessive gonadotropin production may play an important role in the etiology of ovarian cancer. Alternatively, it has been suggested that high level of gonadotropins, directly or indirectly, increases production of estrogen, thus promoting malignant transformation of the epithelial cells (Konishi, Kuroda et al. 1999). The fact that the surge of gonadotropic hormones is associated with ovulation and that gonadotropin levels are sustained at relatively high levels in menopausal women is consistant with the hypothesis that gonadotropins directly or indirectly may increase incidence of ovarian malignancies. Although LH hypersecretion and inhibin deficiency in mice develop granulosa and stromal cell tumors, respectively (Matzuk, Finegold et al. 1992; Keri, Lozada et al. 2000; Nilson, Abbud et al. 2000), the precise role of gonadotropins in ovarian epithelial tumors remains unclear.

1.1.3.4 Other Risk Factors of Ovarian Cancer

Besides those mentioned above, family history of breast, ovarian or hereditary colorectal cancer, increasing age and nulliparity are other risk factors for ovarian cancer. However, oral contraceptives, multiparity, breast feeding, and tubal ligation or hysterectomy could protect women against ovarian cancer (Risch, Weiss et al. 1983; Whittemore, Harris et al. 1992; Rosenberg, Palmer et al. 1994; Hankinson, Colditz et al. 1995; Purdie, Green et al. 1995; Mosgaard, Lidegaard et al. 1997; Mosgaard, Lidegaard et al. 1997).

1.1.4 Molecular Determinants of Sporadic Ovarian Tumorigenesis

Due to the late diagnosis of ovarian cancer, it has been difficult to elucidate the molecular mechanisms involved in the early stages of ovarian tumorigenesis. However, a number of candidate molecules have been implicated in this process.

1.1.4.1 p53

p53 (*TP53*; OMIM# 191170) is a transcription factor implicated in the regulation of genes involved in cell cycle progression, DNA repair, and apoptosis. The murine double minute-2 (MDM2; OMIM# 164785) facilitates the degradation of p53 via the 26S proteasome pathway by ubiquitinating p53 (Kubbutat, Jones et al. 1997), resulting in low basal levels of p53. p53 undergoes post-translational modification, including phosphorylation, following DNA damage-mediated cell stress. This inhibits MDM2-mediated p53 ubiquitination by attenuating MDM2 binding with the p53 N-terminus (Shieh, Ikeda et al. 1997), ultimately resulting in p53 up-regulation and facilitating its biological function.

Dysregulation of the *TP53* gene is a key determinant of tumorigenesis in human cancer. Although *TP53* alterations are uncommon in benign ovarian tumors (Skilling, Sood et al. 1996), *TP53* mutation is the most frequent genetic aberration in human tumors, including ovarian cancer, and *TP53* mutation occurs in approximately 50-60% of ovarian cancer (Aunoble, Sanches et al. 2000). Moreover, the incidence of *TP53* mutation is positively correlated with the disease stage [approximately 37% in stage I/II versus 58% of stage III/IV (Shelling, Cooke et al. 1995)]. While *TP53* knockout mice grow normally, they develop tumors in different sites by six months of age (Donehower, Harvey et al. 1992). Patients

with mutant *TP53* tumors demonstrated shorter survival time compared to those harboring wild-type *TP53* tumors (Hogdall, Kjaer et al. 2006). Taken together, these data suggest that alterations in *TP53* gene contribute to the disease progression.

Although *TP53* mutation or over-expression is more frequently found in serous ovarian cancers versus the other subtypes (Skilling, Sood et al. 1996; Morita, Ono et al. 2000), other studies found no association with the histological type of tumor (Wen, Reles et al. 1999; Fallows, Price et al. 2001; Reles, Wen et al. 2001; Rose, Robertson et al. 2003). This has been supported by Hogdel et al., studies that demonstrated no significant difference in the incidence of *TP53* mutation in 124 ovarian cancer patients with respect to histological subtype (Hogdall, Kjaer et al. 2006). It seems mutations are not associated to a single subtype of ovarian cancer. Moreover, serous ovarian cancer has been diagnosed much more frequently than any other histological subtypes, therefore sample bias may partially explain this discrepancy and it is unclear whether *TP53* mutations are more frequent in serous ovarian cancer than in other ovarian tumour types.

Recent studies suggest that p53 function may be an important component of BRCA-mediated tumorigenesis. While wild-type BRCA1 facilitates p53-mediated gene expression, mutant *BRCA1* reduces p53 function (Ouchi, Monteiro et al. 1998; Zhang, Somasundaram et al. 1998). Studies by Ramus et al. reported a higher frequency of *TP53* mutation in ovarian tumors with germline *BRCA1* or *BRCA2* mutations in comparison to sporadic cancers (Ramus, Bobrow et al. 1999).

It has been also reported that the retinoblastoma tumor suppressor gene *RBI* is aberrantly regulated in ovarian cancer (Gras, Pons et al. 2001). Inactivation of *TP53* and *RBI*

in the OSE of mice developed tumors with human ovarian tumor characteristics (Flesken-Nikitin, Choi et al. 2003).

1.1.4.2 KRAS

Mutation of KRAS (OMIM# 190070), a member of the small GTPase superfamily, constitutively activates the KRAS protein in human cancers (Yanez, Groffen et al. 1987). Once activated, KRAS binds to numerous downstream proteins, including those in the PI3K/Akt tumorigenic pathway (Kiss and Crilly 1995; Deora, Win et al. 1998; Espada, Perez-Moreno et al. 1999), the RAF pathway involved in the activation of the mitogen-activated protein (MAP) kinase/ERK (McCormick 1999), and up-regulates genes involved in metastasis (urokinase plasminogen activator; uPA) and angiogenesis (vascular endothelial growth factor; VEGF) (Rak, Mitsuhashi et al. 1995; Silberman, Janulis et al. 1997; Janulis, Silberman et al. 1999). It has been shown that constitutive activation of KRAS is associated with NIH3T3 cell and intestinal epithelial cell transformation (Oldham, Clark et al. 1996). Amplification of the KRAS oncogene in a patient with serous ovarian carcinoma (Filmus and Buick 1985) and frequent activating mutations of KRAS in patients with mucinous ovarian carcinoma (Enomoto, Inoue et al. 1990; Scambia, Masciullo et al. 1997; Dokianakis, Varras et al. 1999; Morita, Ono et al. 2000; Suzuki, Saito et al. 2000) have been reported. These findings suggest that dysregulation of KRAS function may be an important component of tumorigenesis in human cancer, including ovarian cancer.

1.1.4.3 The Phosphoinositol-3-kinase/AKT pathway and tumorigenesis

Phosphoinositol-3-OH-kinase (PI3K) is a lipid kinase involved in cytokine and growth factor signaling. It activates Akt, also known as protein kinase B (PKB), thereby promoting cell cycle progression and cell proliferation, and preventing apoptosis. While it has been reported that the *PIK3CA* gene (OMIM# 171834) is not altered in the human breast cancer and melanoma cell lines examined (0/5), or normal ovarian epithelial cells (0/1), its copy number is increased in cells from human ovarian tumor ascites fluid [100% (5/5)], human ovarian cancer cell lines [89% (8/9)] and primary human ovarian tumors [58% (7/12); (Shayesteh, Lu et al. 1999)]. The increases in copy number were associated with increased rates of *PIK3CA* transcription, protein content, and PI3K activity, suggesting that dysregulation of *PIK3CA* may be an important event in ovarian tumorigenesis. Moreover, while amplification of the *AKT2* gene (OMIM# 164731), a target of the PI3K, and elevated *AKT2* copy number have been reported in two of fifteen primary ovarian tumors, Akt2 mRNA is amplified 30-45 fold in human ovarian cancer cell lines compared to normal ovarian epithelial cells (Cheng, Godwin et al. 1992). Bellacosa et al. subsequently demonstrated that Akt2 is amplified in 12.1% (16/132) of ovarian cancers, but not in benign or borderline tumors (0/24) (Bellacosa, de Feo et al. 1995). In addition, Akt2 is activated in 47% (20/43) and over-expressed in 36% (33/91) of ovarian cancers, whereas the related kinase Aktl was activated in 10% (4/43) (Yuan, Sun et al. 2000). However, Akt2 amplification was observed in just 2.8% (3/106) of breast cancers, suggesting that this phenomenon may be unique to ovarian cancer. Another study demonstrated that Aktl is amplified in 72% (8/11) of ovarian cancers (Sun, Wang et al. 2001). Importantly, Akt2 over-expression facilitates NIH3T3 cell transformation and expression of Akt2 antisense reduces tumorigenicity and invasiveness in pancreatic carcinoma cells (Cheng, Ruggeri et al. 1996). Taken together, these studies

demonstrate that the PI3K/Akt pathway is frequently over-expressed and/or amplified in human ovarian cancer and suggest that up-regulation of this pathway is an important contributor to ovarian tumorigenesis.

1.1.4.4 Other Potential Mediators

Down-regulation of *OVCA1* and *OVCA2* tumor suppressor genes (OMIM# 603527 and 607896, respectively) (Phillips, Ziegler et al. 1996; Schultz, Vanderveer et al. 1996) has been observed in a large proportion of ovarian tumors and cell lines. Moreover, the inhibition of ovarian cancer cell growth by OVCA1 over-expression (Bruening, Prowse et al. 1999) and over-expression of OVCAl mRNA by BRCA1 (Atalay, Crook et al. 2002) suggest that dysregulation of OVCAl by loss of *BRCAl* function may be a critical factor in ovarian carcinogenesis. Endometrioid ovarian cancer is associated with mutation of the *PTEN* gene (OMIM# 601728), a negative regulator of the PI3K pathway (Sato, Kigawa et al. 1999; Gomes and Andrade 2006), suggesting that *PTEN* mutation may contribute to carcinogenesis of the endometrioid subtype.

Although several potential mediators of ovarian tumorigenesis have been described, the molecular mechanisms implicated are complicated. Ovarian tumorigenesis is likely due the alteration of several genes, including but not limited to those already described.

1.2 Chemotherapy and Chemoresistance in Human Ovarian Cancer

The main treatments for ovarian cancer consist of chemotherapy, tumor debulking by surgery, and radiation therapy. Chemotherapy for advanced ovarian cancer often includes a combination of a platinum agent, such as cisplatin or carboplatin, and a taxane, such as paclitaxol (McGuire 2003; McGuire and Markman 2003).

1.2.1 Cisplatin (CDDP)

Rosenberg et al. first described the anti-tumor activities of cisplatin [*cis*-Pt (II) $(NH3)_2Cl_2$; *cis*-diaminodichloroplatinum; (CDDP)] (Rosenberg, VanCamp et al. 1969). This group demonstrated that sarcoma and leukemia formation in mice is inhibited by several platinum components, such as CDDP, which were previously shown to inhibit cell division in Gram-negative bacteria. Since 1969, CDDP has been used as a routine anti-cancer agent in the treatment of numerous solid tumor types, including ovarian, testicular, and head and neck carcinoma.

CDDP consists of two amino groups (NH_2) and two chloride ions (Cl) bound to a central platinum ion in the *cis* configuration (Figure 1). Since the *trans*- isomer of the drug does not possess anti-tumor properties (Plooy, van Dijk et al. 1984), it seems that the *cis* configuration, as found in CDDP, is critical for the biological activities.

The CDDP molecule is usually non-ionized in its native state, so it easily crosses the cell membrane (simple diffusion). Since the intracellular chloride ion concentration is much lower than that in the extracellular environment, CDDP easily loses the chloride ions and produces a positively charged platinum core (Ise, Shimizu et al. 2005). The positively charged platinum

interacts with negatively charged cellular molecules such as DNA, thereby forming inter- and intra-strand DNA crosslinks (Perez 1998) often between adjacent guanines (Munchausen and Rahn 1975). These interactions destroy the DNA double helix, and initiate a cellular DNA damage response.

1.2.2 Direct Cellular Response to CDDP (ATM/ATR)

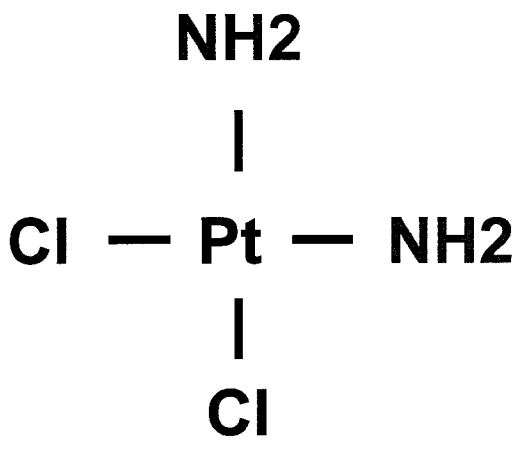
CDDP induces DNA damage and activates an evolutionarily conserved pathway involving members of the ataxia telangiectasia-mutated (ATM) family, including ATM and its related protein, ATR (ATM and Rad3 Related). Both ATM and ATR are members of the PI3K family (Savitsky, Bar-Shira et al. 1995; Savitsky, Sfez et al. 1995; Cimprich, Shin et al. 1996), which are critical mediators of the cellular response to DNA damage.

Ataxia Telangiectasia-Mutated (ATM)

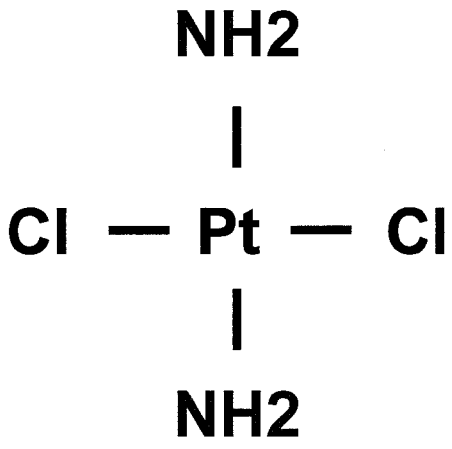
ATM is mostly responsible for sensing double strand breaks (DSBs) (Bakkenist and Kastan 2003). ATM has been shown to be rapidly activated by the histone deacetylase inhibitor (trichostatin A) via altering chromatin structure rather than direct binding to DSBs (Bakkenist and Kastan 2003). Moreover, mutations in the *ATM* gene (OMIM# 607585) gives rise to ataxia telangiectasia (AT), a rare childhood neurological disorder that causes degeneration in the part of the brain that controls motor movements and speech (Kapp, Painter et al. 1992; Savitsky, Bar-Shira et al. 1995; Savitsky, Sfez et al. 1995).

Figure 1: Representative structure of cisplatin and transplatin

A) Cisplatin contains a central platinum atom which is bound to two chloride ions and two amino groups in cis configuration. B) Transplatin has the same formula as cisplatin, but the chloride atoms and the amino groups are present in a trans configuration.



Cis-platin



Trans-platin

Figure 1

ATM-and-Rad3-Related (ATR)

ATR is a 301 kDa protein with a high degree of homology to the phosphoinositol kinases, including PI3K (Cimprich, Shin et al. 1996). Mutations in the *ATR* gene (OMIM# 601215) causes Seckel syndrome (O'Driscoll, Ruiz-Perez et al. 2003), which is characterized by hematological abnormalities such as Acute Myeloid Leukemia (AML), pancytopenia and chromosomal instability (Butler, Hall et al. 1987; Hayani, Suarez et al. 1994). ATR deficiency results in early embryonic death (Brown and Baltimore 2000), suggesting ATR is critical for normal cell function. ATR is responsible for sensing stalled replication forks (McGowan and Russell 2004). It is constitutively bound to ATR-Interacting Protein (ATRIP) and ATR-ATRIP interaction is necessary for its binding to the DNA binding protein Replication Protein A (RPA) (Zou and Elledge 2003; Zou, Liu et al. 2003).

Another protein complex, called the '9-1-1 complex' (Weiss, Matsuoka et al. 2002), is necessary for ATR signaling (Parrilla-Castellar, Arlander et al. 2004). The 9-1-1 complex is required for initial DNA damage sensing and, in combination with RPA binding to DNA, recruits and activates ATR. Different cell stresses, including CDDP (Damia, Filiberti et al. 2001), ultraviolet radiation (Abraham 2001), and ionizing radiation (IR) (Cortez, Guntuku et al. 2001) can induce ATR signaling such as BRCA1, Chk1 and p53 phosphorylation; however, genotoxic agents, including ultraviolet or ionizing radiation (Abraham 2001) are ineffective to activate ATR. These findings suggest that ATR biological activity may be limited by other factors such as its subcellular localization or interaction with specific co-factors.

Molecular Targets of ATM/ATR in Response to CDDP

Upon activation, ATR phosphorylates downstream targets such as the checkpoint kinases Chk1 (Liu, Guntuku et al. 2000) and Chk2 (Xu, Xin et al. 2001). Chk1 and Chk2 are implicated in initiating DNA repair, cell cycle arrest, and/or apoptosis (Takai, Tominaga et al. 2000; Hirao, Cheung et al. 2002; Peter, Varga et al. 2002; Gonzalez, Prives et al. 2003) (Figure 2).

It has also been shown that p53 is a substrate of ATM and ATR, which directly phosphorylate p53 on Serl5 (Canman, Lim et al. 1998; Tibbetts, Brumbaugh et al. 1999; Delia, Mizutani et al. 2000) (Figure 2). ATM/ATR also promotes Chk1 and Chk2 activation (Chehab, Malikzay et al. 2000; Hirao, Cheung et al. 2002), resulting in indirect p53 phosphorylation on Ser20 by Chk2 (Chehab, Malikzay et al. 2000) and p53 phosphorylation on Ser15 and 20 by Chk1 (Shieh, Ikeda et al. 1997; Shieh, Taya et al. 1999; Shieh, Ahn et al. 2000). p53 phosphorylation on Ser15 and Ser20 inhibits MDM2-mediated p53 degradation, activates p53-dependent gene transcription (Dumaz and Meek 1999) and/or p53-mediated apoptosis (Unger, Sionov et al. 1999). The interactions of BRCA1 with ATM, ATR, p53, Chk1 and Chk2 suggest that BRCA1 is involved in DNA repair and/or cell cycle checkpoint arrest (Scully, Chen et al. 1997; Zhong, Chen et al. 1999; Lee, Collins et al. 2000; Tibbetts, Cortez et al. 2000; Wang, Shyong et al. 2000; Garcia-Higuera, Taniguchi et al. 2001; Yarden, Pardo-Reoyo et al. 2002). BRCA1 is also phosphorylated by ATM and ATR following IR and UV irradiation, respectively (Tibbetts, Cortez et al. 2000; Gatei, Zhou et al. 2001). It has been suggested that BRCAl might be an ATM/ATR substrate, since BRCAl is required for ATM/ATR-dependent p53 phosphorylation (Foray, Marot et al. 2003), suggesting that p53 may be involved in BRCAl signaling. In this context, while phosphorylation of p53 is

impaired in mutant BRCA1 human ductal carcinoma cells (HCC1937 cells) following exposure to IR and occurred by UV radiation after 4h, overe-xpression of adenovirus BRCA1 facilitates these responses as early as 1h after exposure to IR and UV radiation. Although these studies suggest that phosphorylation of p53 is ATM- or ATR- dependent after exposure to IR or UV, respectively, the p53 phosphorylation events require a functional BRCA1 (Foray, Marot et al. 2003).

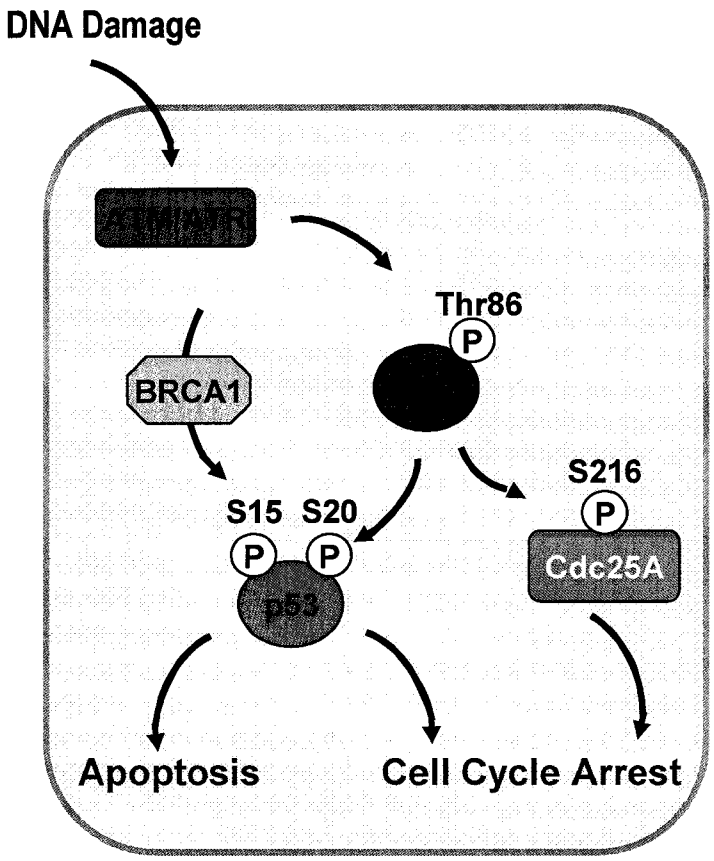
1.2.3 Mechanisms of Chemoresistance in Human Ovarian Cancer

The majority of ovarian cancer patients undergo cytoreductive surgery and combination chemotherapy with CDDP and taxol. CDDP and taxol combination is more efficient than the combination of CDDP with other agents, including cyclophosphamide (McGuire, Hoskins et al. 1996; McGuire, Hoskins et al. 1996). While about 80% of the patients demonstrate a good response to the therapeutic protocol (McGuire, Hoskins et al. 1996; McGuire, Hoskins et al. 1996), most of these patients have tumor recurrence which is mostly resistant to additional chemotherapy (Judson, Watson et al. 1999). This is due to chemoresistance, a clinical phenomenon which is now believed to be a major hurdle for successful treatment in human ovarian cancer.

Ovarian cancer is complicated and the molecular mechanisms involved in chemoresistance are multi-factorial. Chemoresistance and clinical chemotherapy outcome may depend upon disease stage, tumor type and several common determinants, such as drug transport and metabolism, DNA repair and the ability of the cells to undergo apoptotic cell death.

Figure 2: Activation of Chk1 and Chk2 by DNA Damage

DNA damaging agents, such as cisplatin (not shown), activate the related kinases ATM/ATR, which in turn catalyze the phosphorylation of Chk1 on Ser345 and Ser317, and Chk2 on Thr86 resulting in Chk1 and Chk2 activation. Activated Chk1 or Chk2 phosphorylates the phosphatase Cdc25A on Ser123 and Ser216, respectively, and facilitates its proteasomal degradation (not shown). The reduction in Cdc25A content promotes G/S2 cell cycle arrest. Furthermore, phosphorylation of p53 on Ser15 and Ser20 by Chk1, Ser20 by Chk2, or Ser15 by ATR/ATM, protects it from MDM2-mediated ubiquitination and proteasomal degradation, thereby inducing p53 activation and its up-regulation. p53 contributes to cell cycle arrest by up-regulating the cyclin-dependent kinase inhibitor p21 (not shown). However, if DNA damage is extensive or sustained, p53 contributes to the induction of apoptosis by up-regulating the expression of pro-apoptotic gene products such as Bax and PUMA (not shown).


DNA Damage
ATM/ATR
Thr86
P
BRCA1
S15 S20
P P
p53
S216
P
Cdc25A
Apoptosis
Cell Cycle Arrest

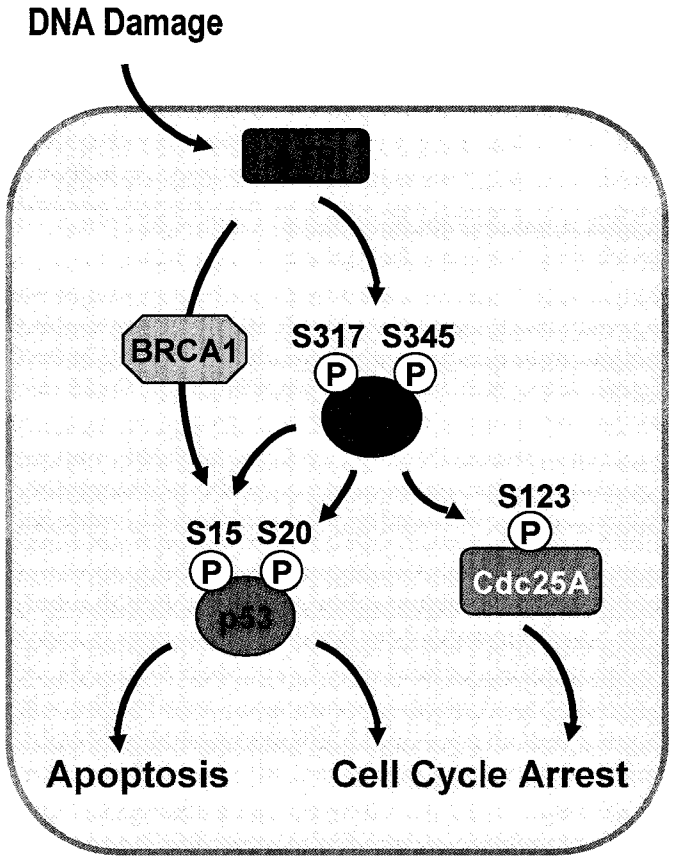

DNA Damage
BRCA1
S317 S345
P P
S15 S20
P P
p53
S123
P
Cdc25A
Apoptosis
Cell Cycle Arrest

Figure 2

1.2.3.1 Multidrug Resistance 'Pumps'

The 'drug efflux' pumps, such as p-glycoprotein, are involved in exporting chemotherapeutic agents from cells, limiting drug access to its target and thereby eliminating its biological effects. A recent study has suggested that increased efflux of CDDP from ovarian cancer cells may be important in the development of CDDP resistance. Indeed, this study demonstrates that ovarian cancer cells are rendered sensitive to CDDP by down-regulation of the gene product of *ABCB1* (OMIM# 171050; p-glycoprotein) (Zhang, Guan et al. 2005). It has also been suggested that platinum agents are not substrates for these efflux pumps (Lautier, Canitrot et al. 1996) and that the slight differences in CDDP uptake and DNA platination cannot account for the differences in CDDP sensitivity between sensitive and resistant ovarian cancer cells (Mansouri, Zhang et al. 2003). Over-expression of *ABCB1* in primary cultured ovarian cancer cells after recovery from CDDP treatment (Metzinger, Taylor et al. 2006) suggests that CDDP may influence the cellular response to other drugs that are substrates of *ABCB1* such as taxol, thereby inducing resistance to taxol *in vitro* (Judson, Watson et al. 1999). In this context, cross-resistance to CDDP and taxol is commonly reported in clinical chemotherapy (McGuire, Hoskins et al. 1996; McGuire, Hoskins et al. 1996). Taken together, these results suggest that aberrant *ABCB1* expression may be an important mechanism of taxol resistance in human ovarian cancer.

1.2.3.2 Drug Detoxification

CDDP can covalently binds with glutathione (γ-glutamylcysteinylglycine; GSH), initially forms GSH-CDDP complexes by catalytic activity of the enzyme glutathione-S-transferase (GST) or via a non-enzymatic reaction (Ishikawa and Ali-Osman 1993).

Following complex formation, CDDP can be removed from the cells by the activity of the ATP-dependent transporter GS-X (Zhang, Chew et al. 2001). In this context, elevated GSH level is associated with CDDP resistance in ovarian cancer, and cellular depletion of GSH and/or targeted GST down-regulation increases CDDP-induced cytotoxicity (Juvekar, Adwankar et al. 2000; Zhang, Chew et al. 2001; Zhang, Guan et al. 2005). Moreover, GSH levels are elevated in primary cultured ovarian cancer cells taken from the same patient after chemotherapy, and GSH levels are associated with multi-drug resistance in human ovarian cancer cells (Wolf, Hayward et al. 1987; Hamaguchi, Godwin et al. 1993). Taken together, these findings suggest that drug detoxification may be an important determinant of chemoresistance in this disease.

1.2.3.3 Enhanced DNA damage repair

Previous studies demonstrated that enhanced DNA repair is associated with chemoresistance (Perez, Hamilton et al. 1990). DNA repair involves a number of excision repair mechanisms that remove the damaged nucleotide and replace it with an undamaged nucleotide, including Base excision repair (BER; which repairs damage to a single nucleotide), Nucleotide excision repair (NER; which repairs damage affecting longer strands of 2–30 bases), and Mismatch repair (MMR; which corrects errors of DNA replication and recombination resulting in mispaired nucleotides following DNA replication) (Lindahl and Wood 1999; Wood, Mitchell et al. 2001).

The NER pathway is activated by CDDP-induced DNA damage (Gunz, Hess et al. 1996). It is a complex mechanism which implicates the activation of at least thirty proteins (Bernstein, Bernstein et al. 2002). Platinated DNA initially recruits members of the

xeroderma pigmentosa (XP) family such as XPC and XPE, subsequently recruits proteins XPB and XPD in order to form the transcription factor complex TFIID. XPB and XPD excise the damaged section of DNA, which is followed by a new synthesis of the excised portion facilitated by DNA polymerases through binding and recruiting xeroderma pigmentosa F/excision repair cross-complementing 1 (XPF/ERCC1) and xeroderma pigmentosa G (XPG) (van Steeg 2001).

Chemoresistance to DNA damaging agents may be enhanced by DNA repair, resulting in reversed DNA platination, and suppression of a downstream DNA damage cascade. In this context, while XPA expression is associated with improved clinical outcome in ovarian cancer (Stevens, Raffeld et al. 2005), Excision repair cross-complementing 1 (ERCC1) expression in non-small cell lung carcinoma (Rosell, Taron et al. 2003) and in CHO cells deficient in ERCC1 (Lee, Parker et al. 1993) are associated with increased CDDP resistance. Moreover, ERCC1 down-regulation in cultured ovarian cancer cells renders the cells sensitive to CDDP-induced cytotoxicity (Selvakumaran, Pisarcik et al. 2003). However, it is not clear if and how these factors are dysregulated in ovarian tumors and ovarian cancer cells.

1.2.3.4 Suppression of drug-induced apoptosis

Suppression of the apoptotic capacity is a key determinant of chemoresistance in human ovarian cancer, since the induction of apoptosis is a critical event in response to chemotherapeutic agents and is highly correlated with CDDP sensitivity in ovarian cancer cells (Gibb, Taylor et al. 1997; Sato, Kigawa et al. 1999). In this context, the ability of chemoresistant ovarian cancer cells to undergo apoptosis in response to chemotherapeutic agents is often attenuated. Moreover, poor responses to chemotherapy in human ovarian

cancer are frequently associated with the dysregulation of key determinants of apoptosis cascades such as Bcl-2, Bax, and p53 (Sato, Kigawa et al. 1999; Skirnisdottir, Seidal et al. 2002; Kupryjanczyk, Szymanska et al. 2003; Murata, Haisa et al. 2004). For instance, while X-linked inhibitor of apoptosis protein (XIAP) inhibits CDDP-induced apoptosis in cultured ovarian cancer cells (Sasaki, Sheng et al. 2000; Li, Feng et al. 2001), Survivin (another IAP family member) inhibits taxol-induced apoptosis. Moreover, survivin protein levels are correlated with a reduced remission rate after taxol-based chemotherapy in ovarian cancer patients (Zaffaroni, Pennati et al. 2002; Song, Song et al. 2003). Taken together, these findings support the hypothesis that apoptosis is critical for the sensitivity to chemotherapy and loss of apoptotic capacity may be an important factor in chemoresistance in human ovarian cancer.

1.3 Apoptosis in Human Ovarian Cancer

Programmed cell death (PCD) or apoptosis is a normal physiological process by which cells are eliminated from an organism in an ATP-dependent biochemical cascade (Kerr, Wyllie et al. 1972; Wyllie, Kerr et al. 1980). While Lockshin and Williams identified the existence of PCD in 1965 (Lockshin and Williams 1965), Kerr et al described 'apoptosis' for the first time as a natural occurring cell death during development and distinct from necrosis which happens during acute injury (Kerr, Wyllie et al. 1972). PCD serves as a regulatory mechanism for the maintenance of cell number and a defensive mechanism for removing unwanted and potentially dangerous cells, leading to normal tissue development and homeostasis (Jacobson, Weil et al. 1997). Cells undergoing apoptosis initially demonstrate some morphological changes such as nuclear/chromatin condensation and

membrane swelling followed by nuclear fragmentation. These changes are associated with subsequent pyknotic bodies (Kerr, Wyllie et al. 1972). The evidence for the existence of apoptosis arose initially from the research demonstrating that several genes are implicated in apoptosis in the nematode *Caenorhabditis elegans*. The first gene is the nuc-1 gene, which encodes a DNA endonuclease (Sulston 1976) and its loss results in defective DNA degradation and apoptosis. Two other genes (ced-1 and ced-2) are required for the phagocytosis of the remaining apoptotic cells by neighboring cells (Hedgecock, Sulston et al. 1983). Another gene, ced-3, is required for the initiation of apoptosis (Xue, Shaham et al. 1996).

1.3.1 Apoptosis in Mammalian Cells

Apoptosis in mammalian cells involves multiple activation and inhibition steps and usually occurs in two phases, including a commitment to cell death and an execution phase characterized by a dramatic stereotypical morphological change in cell structure (Takahashi and Earnshaw 1996). The mitochondrial pathway (intrinsic) and death receptor-mediated pathway (extrinsic) are two major mammalian apoptosis pathways, both of which ultimately result in a proteolytic cascade involving a family of cysteine-aspartic acid proteases called caspases (Takahashi and Earnshaw 1996; Salvesen and Dixit 1997; Song and Steller 1999). Upon receipt of a death signal, including exposure to DNA damaging agents, a signaling cascade is activated by pro-apoptotic molecules such as Tumor Necrosis Factor-alpha Receptor and Fas, resulting in activation of caspases which is ultimately responsible for the execution of apoptosis (Figure 3). However, recent evidence suggests that the endoplasmic reticulum (Szegezdi, Fitzgerald et al. 2003) and a caspase-independent apoptotic pathway involving

Apoptosis-Inducing Factor (AIF) (Susin, Zamzami et al. 1996; Yang, Fraser et al. 2008) may also play an important role in apoptosis induced by some cellular stressors (Figure 3).

1.3.1.1 Mitochondria-mediated Apoptosis – The Interinsic Pathway

In drug-induced mitochondria-mediated apoptosis, signals transmitted to the mitochondria trigger the release of pro-apoptotic proteins, including cytochrome c, Smac/DIABLO, HTR/Omi and AIF, from the mitochondrial matrix to the cytosol (Figure 3; (Susin, Zamzami et al. 1996; Du, Fang et al. 2000; Suzuki, Imai et al. 2001) and activate the caspase-9/caspase-3 cascade. Mitochondrial protein release is facilitated by the actions of pro-apoptotic proteins, such as Bax, Bad, Bak and Bid. Cell stress activates Bax-Bax dimerization at the mitochondrial membrane, thereby inducing membrane pores and releasing ions and small proteins from the mitochondria (Antonsson, Montessuit et al. 2001).

The action of Bax is prevented by anti-apoptotic Bcl-2 family members, such as Bcl-2 (Hengartner and Horvitz 1994) and Bcl-X_L (Antonsson, Conti et al. 1997; Antonsson, Montessuit et al. 2001) by the formation of heterodimer with Bax and attenuating Bax-Bax dimerization (Figure 3).

Upon release from the mitochondria, cytochrome c and APAF-1 (the mammalian homologue of the *C. elegans ced-4* gene product, CED-4) (Zou, Henzel et al. 1997), bind to procaspase-9 and form a 700 kDa complex called the apoptosome (Li, Nijhawan et al. 1997). The apoptosome activates pro-caspase-9 by cleaving it in an ATP-dependent manner (Li, Nijhawan et al. 1997). Caspase-9 then catalyzes the cleavage of procaspase-3 to form active caspase-3 (Fernandes-Alnemri, Armstrong et al. 1996; Li, Nijhawan et al. 1997).

Figure 3: Mammalian Apoptosis - The Intrinsic, Extrinsic and Endoplasmic Reticulum Pathways.

Cell stress, such as DNA damage, activates BH3-only Bcl-2 family members (PUMA and Bad), then attenuates the inhibitory interaction of Bcl-2 and Bax, thereby facilitating mitochondrial Bax-Bax dimerization. This leads to pore formation in the outer mitochondrial membrane, then ions and small molecules such as cytochrome C (Cyto-C), Smac, AIF, HtrA2/Omi, and APAF-1 are released from the mitochondrial matrix into the cytosol. Cytochrome C and APAF-1 bind with procaspase-9, induce its cleavage into active caspase-9. Similarly, active caspase-9 then activates pro-caspase-3. Caspase-3 activation leads to cleavage of various substrates such as Poly (ADP) Ribose Polymerase (PARP), and structural key proteins such as actin. While AIF translocates to the nucleus and serves as a caspase-independent endonuclease (not shown), Smac and Omi inhibit the Inhibitor of Apoptosis Proteins, such as XIAP, to facilitate apoptosis. These events ultimately results in DNA fragmentation, nuclear condensation, and loss of structural integrity.

Cell stress also activates cell death receptors through extracellular ligands such as FasL and TRAIL (not shown) leads to trimerization of Fas receptors, recruitment of Fas-Associated Death Domain (FADD) protein, and subsequently association of pro-caspase-8 to the activated receptor by interacting of death domains DD (FADD) and death effective domains (DED) from caspase-8, thereby leading to the formation of the death inducing signaling complex (DISC), pro-caspase-8 cleavage and activation. This in turn promotes the activation of caspase-3.

Caspase-8 also trunctates the Bcl-2 family member Bid into tBid, thereby facilitating crosstalk between the extrinsic and intrinsic pathways. FLIP inhibits apoptosis by

attenuating caspase-8 cleavage and activity, while XIAP inhibits apoptosis through blocking caspase-9 and caspase-3 activity.

Endoplasmic reticulum (ER)-induced apoptosis leads to caspase activation in a mitochondrial-dependent and -independent manner. ER stress activates caspase 12, which in turn activates procaspase-9 and pro-caspase-3 and induction of apoptosis. ER stress also facilitates the generation of mitochondrial ROS (not shown) by releasing Ca^{2+} and depolarization of the inner mitochondrial membrane (reviewed in (Malhotra and Kaufman 2007).

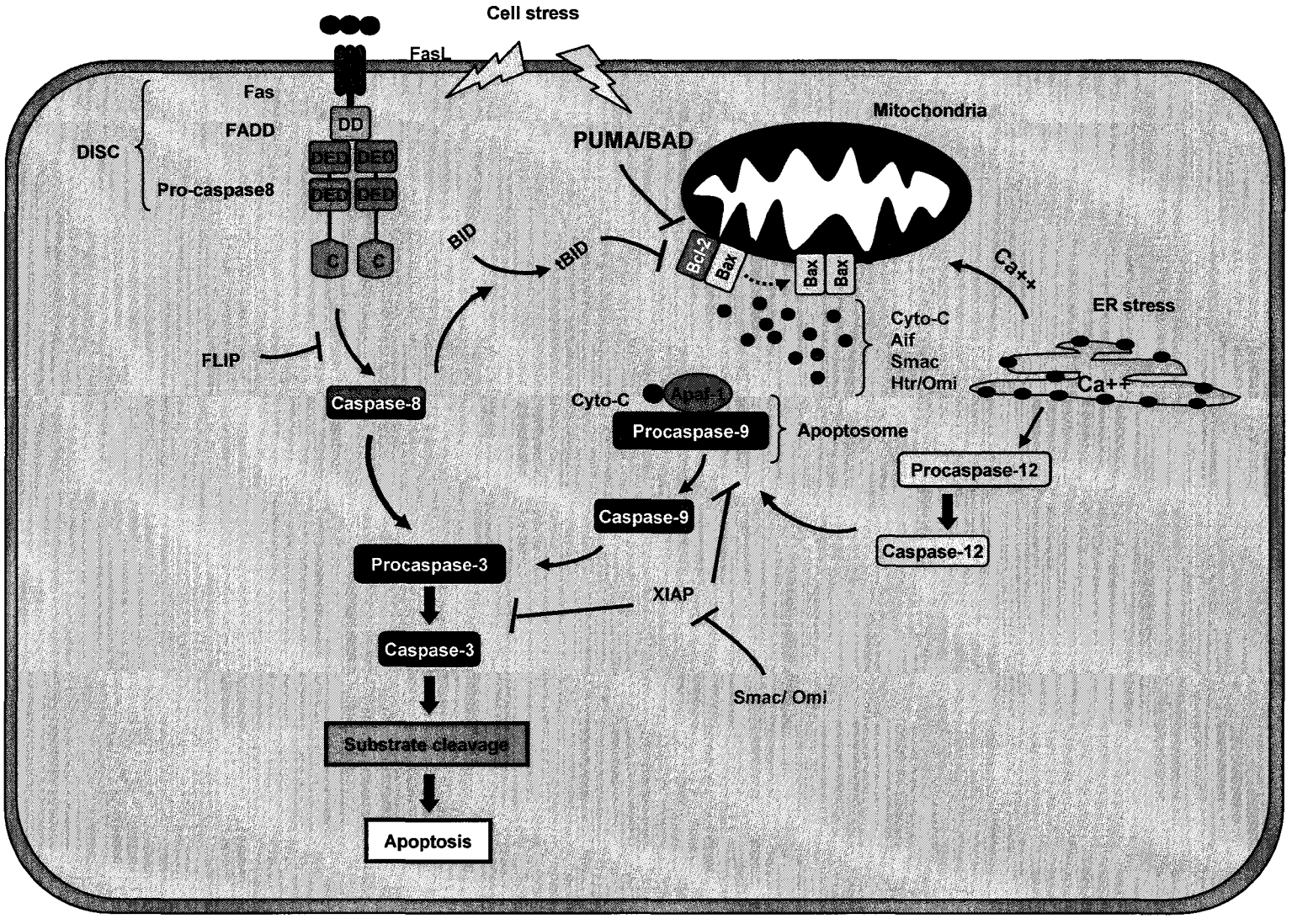

Cell stress
FasL
Fas
FADD
DISC
Pro-caspase8
DD
DED
C
BID
tBID
FLIP
Caspase-8
Mitochondria
PUMA/BAD
Bcl-2
Bax
Cyto-C
Aif
Smac
Htr/Omi
Ca++
ER stress
Cyto-C
Apaf-1
Procaspase-9
Apoptosome
Procaspase-12
Caspase-12
Caspase-9
Procaspase-3
XIAP
Caspase-3
Smac/ Omi
Substrate cleavage
Apoptosis

Figure 3

Caspase-3, along with the related caspase-7, is responsible for the ultimate execution of apoptosis (Figure 3) by facilitating the cleavage, at consensus DXXD motifs, of numerous proteins implicated in maintaining cell structure and the integrity of DNA. Poly (ADP) Ribose Polymerase (PARP) (de Murcia and Menissier de Murcia 1994; Tewari, Quan et al. 1995) and members of the Inhibitor of Apoptosis Protein (IAP) family including X-Linked Inhibitor of Apoptosis Protein (XIAP) (Johnson, Gastman et al. 2000) are substrates for caspase-3.

In addition to cytochrome c, proteins released from mitochondria include Second Mitochondria-Derived Activator of Caspases (Smac; also known as DIABLO) and HtrA2/Omi, both direct inhibitors of IAP proteins (Du, Fang et al. 2000; Suzuki, Imai et al. 2001), as well as Apoptosis Inducing Factor (AIF), a DNA endonuclease that facilitates DNA fragmentation and apoptosis independently of caspase activation (Susin, Zamzami et al. 1996).

1.3.1.2 Death Receptor-mediated Apoptosis – The Extrinsic Pathway

Activation of the extrinsic pathway of apoptosis results from the binding of an extracellular ligand to a death receptor. Two ligand/receptor systems, implicated in cell death receptor-mediated apoptosis, are the TNFα/TNFR-1 and Fas/Fas Ligand (FasL) systems. Several death receptors (DRs) have already been described including Fas (CD95), TNF-R1 (D120a), TNF-related apoptosis-inducing ligand receptor 1 (TRAILR1; DR4), TRAIL-R2 (DR5) (Wiley, Schooley et al. 1995; MacFarlane, Ahmad et al. 1997), TNF-R-related apoptosis-mediating protein (TRAMP; also known as DR3, Apo3 1), DR6, nerve growth factor receptor (NGF-R) and ectodermal dysplasia receptor (EDA-R) (Thome and Tschopp 2001). The Fas receptor is oligomerized upon binding to FasL (Kischkel, Hellbardt et al. 1995), resulting in the recruitment (and activation) of Fas-Associated Death Domain (FADD)

to Fas receptor through Death Domain-Death Domain (DD-DD) interaction as shown in Figure 3 (Chinnaiyan, O'Rourke et al. 1995; Chinnaiyan, Tepper et al. 1996). FADD activation in turn recruits pro-caspase-8/10 by Death Effective Domain-Death Effective Domain (DED-DED) interaction leading to formation of the death inducing signaling complex (DISC), cleavage of pro-caspase-8 and/or pro-caspase-10 into active caspase-8 and/or caspase-10, respectively results in initiation of apoptosis (Fernandes-Alnemri, Armstrong et al. 1996; Muzio, Chinnaiyan et al. 1996; Wang, Chun et al. 2001). Caspase-8 activation subsequently cleaves downstream effector caspases (caspase-3, -6 and -7) (Fernandes-Alnemri, Armstrong et al. 1996; Slee, Harte et al. 1999), induces the activation of a pro-apoptotic member of the Bcl-2 family, Bid (Luo, Budihardjo et al. 1998) and thereby linking the extrinsic and intrinsic pathways. The extrinsic pathway is activated by chemotherapeutic agents, including CDDP (Schneiderman, Kim et al. 1999) and is attenuated by the endogenous inhibitor Flice-Like Inhibitory Protein (FLIP) (Irmler, Thome et al. 1997; Thome, Schneider et al. 1997; Kinoshita, Yoshikawa et al. 2000; Xiao, Yan et al. 2003) through inhibition of caspase-8 activation.

1.3.1.3 Endoplasmic Reticulum-mediated Apoptosis –Intrinsic Pathway

The endoplasmic reticulum (ER) is implicated in several activities, including regulation of intracellular calcium levels, protein folding and trafficking and apoptosis. Apoptosis induced by ER stress can be initiated by different conditions, such as accumulation of unfolded proteins in the ER which occurs under a variety of situations, including mutation in secretary proteins or disruption of calcium homeostasis (Xu, Bailly-Maitre et al. 2005). Numerous apoptosis-regulatory proteins are involved in the ER-mediated apoptosis. These

include Bcl-2 (Krajewski, Tanaka et al. 1993), Bax and Bak (Zong, Li et al. 2003). BH3-only members such as Bid (Esposti, Erler et al. 2001) and Bim (Morishima, Nakanishi et al. 2004) may also be localized to the ER. Caspases including caspase- 12 (Nakagawa, Zhu et al. 2000), -7 and -9 (Chandler, Cohen et al. 1998; Zhivotovsky, Samali et al. 1999; Rao, Hermel et al. 2001) have also been implicated in the ER stress-induced apoptosis. While caspase-12 can be activated by ER stress, caspase-12-deficient mice are resistant to ER stress inducers (Nakagawa, Zhu et al. 2000; Martinon and Tschopp 2004), suggesting that caspase-12 is involved in ER stress-induced apoptosis. However, the mechanism by which ER activates caspase-12 is not clear.

In this context, CDDP induces cleavage of pro-caspase 12 resulting in caspases-3 and 9 in renal tubular epithelial cells (LLC-PK1 cells). The active form of Caspase-8 was not detected throughout the course of the study. Pre-incubation of the LLC-PK1 cells with the caspase-9 inhibitor did not attenuate Caspase-3 activation and provided no significant protection. Caspase-3 inhibitor provided only modest protection against CDDP-induced apoptosis. LLC-PK1 cells that were transfected with anti–caspase 12 antibody significantly attenuated capsulation-induced apoptosis. Taken together, these data indicate that Caspase-12 plays a pivotal role in CDDP-induced apoptosis. It is proposed that the oxidative stress that results from the interaction of CDDP with the ER cytochrome P450 leads to activation of procaspase-12, resulting in apoptosis (Liu and Baliga 2005).

1.3.2 Molecular Regulators of Apoptosis

Apoptosis in mammalian cells is modulated by numerous cellular mediators. Some of the important mediators of apoptosis are described below. Elaboration on the complete mechanisms implicated is beyond the scope of this dissertation.

1.3.2.1 The p53 Family

p53 was originally described in 1979 (Lane and Crawford 1979) as a protein expressed in human tumor cell lines (Crawford, Pim et al. 1981). It is implicated in multiple cellular events such as cell-cycle arrest and cell death (Lane 1992). It binds to DNA through its responsive element (p53RE) (el-Deiry, Kern et al. 1992; Bourdon, Deguin-Chambon et al. 1997) and regulates the transcription of genes, including p21 which is involved in cell cycle arrest (el-Deiry, Tokino et al. 1993) and PUMA (p53-upregulated mediator of apoptosis) (Nakano and Vousden 2001), thereby suppressing uncontrolled cell proliferation and cancer development. Two p53-related genes, p63 (Schmale and Bamberger 1997; Osada, Ohba et al. 1998; Trink, Okami et al. 1998) and p73 (Kaghad, Bonnet et al. 1997), were identified in 1997. They share > 60% amino acid identity with p53 in the DNA binding domain. This allows p63 and p73 to contribute to cell cycle arrest and apoptosis by transactivating p53-responsive genes. Together with p53, they make up a family of transcription factors (the p53 family). Both genes give rise to proteins that have both p53-related as well as entirely different functions.

p53 content and activity is maintained low by murine double-minute-2 (MDM2), which ubiquitinates p53, targeting it for proteasomal degradation (Honda, Tanaka et al.

1997). MDM2 binds p53 on its N-terminal between amino acids 17-27 (Chen, Marechal et al. 1993; Picksley and Lane 1994; Kussie, Gorina et al. 1996), and thereby facilitates p53 ubiquitination (Kubbutat, Jones et al. 1997). During cell stress, p53 is phosphorylated on Ser and Thr in or adjacent to the MDM2 binding pocket (Kussie, Gorina et al. 1996). This stabilizes and/or activates p53, facilitating its up-regulation by preventing MDM2-mediated p53 ubiquitination (Kussie, Gorina et al. 1996). CDDP induces phosphorylation at Ser15, 20, and 33 (Sanchez-Prieto, Rojas et al. 2000; Damia, Filiberti et al. 2001) via activation of ATM-and-Rad3-Related (ATR) and/or Chk1, Chk2, and p38, respectively. Moreover, p53-mediated trans-activation can be inhibited by MDM2, which seems to be distinct from the role of MDM2 in the regulation of p53 ubiquitination and degradation (Momand, Zambetti et al. 1992; Chen, Lin et al. 1995).

p53 up-regulates numerous genes involved in the intrinsic pathway, including PUMA which is a BH3-only member of the Bcl-2 family (Nakano and Vousden 2001). It also up-regulates Bax in a transcription-dependent manner (Miyashita, Krajewski et al. 1994; Selvakumaran, Lin et al. 1994), a response required for apoptosis induced by numerous stimuli (Knudson, Tung et al. 1995; Deckwerth, Elliott et al. 1996; Sakakura, Sweeney et al. 1996). NOXA, a related BH3-only protein, is also up-regulated by p53 (Oda, Ohki et al. 2000).

p53 gene mutations are a frequent event in human ovarian cancer (Kmet, Cook et al. 2003; Leitao, Boyd et al. 2004), and is often associated with chemoresistance (Buttitta, Marchetti et al. 1997; Kigawa, Sato et al. 2001; Fraser, Leung et al. 2003; Chan and Lung 2004; Koike, Fujita et al. 2004). Moreover, p53 can also induce apoptosis through directly targeting the mitochondria (Kmet, Cook et al. 2003).

Our laboratory has demonstrated that p53 is a determinant of CDDP sensitivity, and that CDDP induces p53 phosphorylation, p53 up-regulation, p53-mediated PUMA up-regulation and apoptosis in chemosensitive ovarian cancer cell lines but not or to a lesser extent in their resistant counterparts (Fraser, Leung et al. 2003; Fraser, Bai et al. 2008). p53 phosphorylation and p53 up-regulation by CDDP, induces mitochondrial cytochrome C, Smac and AIF release, and apoptosis in chemosensitive ovarian cancer cells, responses which are attenuated in their resistant variants and by Akt activation (Yang, Fraser et al. 2006; Yang, Fraser et al. 2008).

While sGC (soluble guanylyl cyclase) activity reduces p53 content and attenuates p53-dependent apoptosis in human ovarian cancer cells, inhibition of sGC activity by its specific inhibitor (ODQ) lowered cGMP content, induced phosphorylation of p53 on Ser15, increased p53 protein content and induced apoptosis in ovarian cancer cell lines. ODQ also up-regulates the p53-dependent gene products p21, MDM2, and Bax. Interestingly, these responses are attenuated by p53 inhibitor pifithrin or p53 siRNA, suggesting that sGC/cGMP activity regulates p53 protein stability, content, and function (Fraser, Chan et al. 2006).

We have also demonstrated that PTEN over-expression up-regulates p53 content and sensitizes chemoresistant cells to CDDP induced apoptosis, a response which is attenuated by p53 silencing in chemoresistant wild-type p53 ovarian cancer cells. Moreover, PTEN over-expression failed to sensitize the chemoresistant p53 mutant ovarian cancer cell line (A2780cp) to CDDP, unless wild-type p53 was reconstituted. These data suggest that PTEN over-expression may represent a novel therapeutic approach for chemoresistant human ovarian cancer and that this may involve a p53-mediated apoptotic cascade independent of the PI3K/Akt pathway (Yan, Fraser et al. 2006).

Taken together, these findings suggest that p53 is a key determinant of ovarian cancer sensitivity to CDDP, and demonstrate different actions of p53 in the regulation of apoptosis signaling.

1.3.2.2 The Bcl-2 Family

Bcl-2, the mammalian counterpart of the *C. elegans* CED-9 protein (Hengartner and Horvitz 1994), is a membrane protein constitutively located at the outer mitochondrial membrane (Janiak, Leber et al. 1994). It generally prevents mitochondrial activation by inhibiting the formation of Bax oligomers (Antonsson, Conti et al. 1997; Antonsson, Montessuit et al. 2001), an effect modulated by activated BH3 proteins through binding with Bcl-2 and suppressing its ability to prevent Bax oligomerization. DNA damaging agents activate BH3-only proteins (e.g. PUMA, NOXA, Bad, and Bid) through different processes, including transcriptional activation of PUMA and NOXA (Oda, Ohki et al. 2000; Nakano and Vousden 2001), dephosphorylation of Bad (Basu, Bayoumy et al. 1998), and/or cleavage of Bid (Luo, Budihardjo et al. 1998), and ultimately activates the mitochondrial cell death pathway. It has been demonstrated that CDDP down-regulates Bcl-2 mRNA and protein content and induces apoptosis in chemosensitive ovarian cancer cells (2000) but not in their resistant counterparts (2008 C-13), (Murata, Haisa et al. 2004). Moreover, while CDDP had no effect on CDDP-induced apoptosis in another chemoresistant ovarian cancer cells (SKOV-3), (Belanger, Cote et al. 2005). Taken together, these findings suggest that Bcl-2 may regulate CDDP sensitivity.

1.3.2.3 Inhibitor of Apoptosis Protein Family

Inhibitor of Apoptosis Proteins (IAPs) were characterized as mammalian homologues of baculovirus proteins, and consist of at least six members: Human Inhibitor of Apoptosis Protein-1 and -2 (HIAPl and HIAP2), X-Linked Inhibitor of Apoptosis Protein (XIAP), Neuronal Apoptosis Inhibitory Protein (NAIP), Livin, and Survivin (Roy, Mahadevan et al. 1995; Duckett, Nava et al. 1996; Liston, Roy et al. 1996; Uren, Pakusch et al. 1996; Ambrosini, Adida et al. 1997; Kasof and Gomes 2001). They are the mammalian homologues of baculovirus proteins implicated in suppression of apoptosis in infected host cells (Crook, Clem et al. 1993; Duckett, Nava et al. 1996; Liston, Roy et al. 1996; Uren, Pakusch et al. 1996). Each member of the IAP family proteins contains at least one Baculovirus Inhibitory Repeat (BIR) domain, which contributes to the anti-apoptotic properties of the molecule (Takahashi, Deveraux et al. 1998; Riedl, Renatus et al. 2001). XIAP, for example, contains three BIR domains, the most important of which are BIR2 and BIR3, which have been shown to be responsible for the direct inhibition of caspase-3 and caspase-9, respectively (Deveraux, Leo et al. 1999; Riedl, Renatus et al. 2001). However, other studies have suggested that the BIR domains are dispensable for XIAP-mediated caspase inhibition (Chai, Shiozaki et al. 2001). XIAP inhibits the conversion of pro-caspase-9 to caspase-9 (Takahashi, Deveraux et al. 1998), and blocks the activity of cleaved caspase-3 (Takahashi, Deveraux et al. 1998; Deveraux, Leo et al. 1999). Because of this property, XIAP attenuates both the intrinsic (Perkins, Kim et al. 1998; Finucane, Bossy-Wetzel et al. 1999) and extrinsic (Takahashi, Deveraux et al. 1998) apoptotic pathways, making XIAP an extraordinarily potent anti-apoptotic protein.

Our laboratory has shown that down-regulation of XIAP, but not HIAP2, induces apoptosis in chemosensitive and, to a lesser extent, in resistant ovarian cancer cells. XIAP

and HIAP-2 are present at highest levels in proliferative, but not apoptotic epithelial cells (Li, Feng et al. 2001). While CDDP decreases XIAP content and induced apoptosis in the chemosensitive cells but not in their resistant variants, over-expression of XIAP markedly attenuated CDDP-induced apoptosis (Sasaki, Sheng et al. 2000; Li, Feng et al. 2001; Fraser, Leung et al. 2003). Down-regulation of XIAP sensitizes chemoresistant cells to CDDP in the presence of wild-type p53. Furthermore, XIAP up-regulates PI3K/Akt pathway by increasing Akt phosphorylation (Fraser, Leung et al. 2003). XIAP down-regulation induces caspase-3 activation, caspase-mediated MDM2 processing, p53 accumulation, and apoptosis in the chemoresistant wild-type p53 cells, but not in the mutant (A2780cp) or null (SKOV3) cells. Restoration of wild type p53 in the p53-mutant or -null cells significantly enhanced the pro-apoptotic effect of XIAP antisense expression. Down-regulation of XIAP induces apoptosis in chemoresistant ovarian cancer cells, a process dependent on p53 status (Sasaki, Sheng et al. 2000; Fraser, Leung et al. 2003). Furthermore, CDDP induced focal adhesion kinase (FAK) cleavage in a caspase-3-dependent manner, the activation of which is modulated by XIAP (Sasaki, Sheng et al. 2000). These studies indicate that XIAP is an important element in the control of CDDP sensitivity in human ovarian cancer.

1.3.2.4 Akt Family

Phosphoinositide 3-kinase (PI3K) is a heterodimer containing a p85 regulatory subunit and a p110 catalytic subunit. PI3Ks are primarily involved in the phosphorylation of inositol-containing lipids such as phosphatidylinositol (Ptdins) at the 3'-position of the inositol ring (Fruman, Meyers et al. 1998). PI3K transduces the signals from growth factors and cytokines and is primarily implicated in the regulation of diverse cellular functions,

including cell death, growth, proliferation, differentiation, motility, survival and intracellular trafficking (Figure 4). Growth factors, such as epidermal growth factor (EGF), bind to their specific receptors, thereby phosphorylating specific receptor substrates, such as insulin receptor substrate-1 (IRS-1) (Hadari, Tzahar et al. 1992; Burgering and Coffer 1995). The phosphorylated receptor recruits the inactive p85-p110 complex to receptor tyrosine kinases (RTKs) through binding to the src-homology 2 (SH2) domain of p85 with consensus phosphotyrosine residues at the receptor (Cheng, Jiang et al. 2002). Following its recruitment to activated receptor tyrosine kinase at conserved phosphotyrosine sites in the plasma membrane, PI3K phosphorylates the membrane phospholipid phosphotidyl-inositol-4,5-bisphosphate (PIP2) at the 3' position of the inositol ring, thereby producing phosphatidylinositol-3,4,5-trisphosphate (PIP3) (Cheng, Jiang et al. 2002).

PIP3 is necessary for recruitment of proteins containing a plekstrin homology (PH) domain to the cell membrane (Haslam, Koide et al. 1993; Mayr, Hirschmann et al. 2006), including Akt/PKB (Konishi, Shinomura et al. 1994), phosphatidylinositol-dependent kinase-1 (PDK1) (Alessi, James et al. 1997), and integrin-linked kinase (ILK) (Hannigan, Leung-Hagesteijn et al. 1996) (Figure 4). PDK1 is constitutively recruited to the cell membrane due its strong affinity for PIP2 and PIP3 (Currie, Walker et al. 1999). It catalyses the phosphorylation of Akt on a threonine residue inside its kinase domain at amino acid 308 [(T308), (Thr309 in Akt2 and Thr305 in Akt3)] (Alessi, James et al. 1997; Stephens, Anderson et al. 1998; Chan, Rittenhouse et al. 1999). ILK is activated by binding to PIP3 via its PH domain and phosphorylates Akt on a serine residue at amino acid 473 [(S473), (Ser474 in Akt2 and Ser472 in Akt3)] (Alessi, James et al. 1997; Delcommenne, Tan et al. 1998; Stephens, Anderson et al. 1998; Chan, Rittenhouse et al. 1999). The integrity of the PH domain of Akt,

Figure 4: The PI3K Pathway and the Regulation of Cell Proliferation and Apoptosis

Upon activation, phosphotyrosine receptors (e.g. EGFR) recruit PI3K via its p85 subunit and activates its p110 catalytic subunit, thereby initiating its conformational change and enhancing its lipid kinase activity. PI3K then phosphorylates the membrane phospholipid phosphatidylinositol-4,5-bisphosphate (PIP2) to form phosphatidylinositol-3,4,5-trisphosphate (PIP3). PIP3 recruits proteins containing a pleckstrin homology (PH) domain, including Akt, PDK1 and PDK2 (ILK), and phosphorylates Akt on Thr308 by PDK1 and on Ser473 by ILK (not shown). This pathway is negatively regulated by lipid phosphatases, including PTEN and SHIP which convert PIP3 into PIP2 and phosphatidylinositol-3,4-bisphosphate, respectively. Activated AKT phosphorylates substrates plays, important roles in a variety of biological processes, including suppression of apoptosis. Akt facilitates cell cycle progression by phosphorylating of the inhibitor of cyclin-dependent kinase (CDK) $p21^{WAF1/CIP1}$, increases Estrogen Receptor-alpha (ERα) phosphorylation, and S-phase progression through cyclin Dl up-regulation. Akt phosphorylates IκB kinase (IKKα) resulting in the activation of NF-κB-mediated gene transcription. Akt attenuates p53-mediated apoptosis, and promotes cell cycle progression by phosphorylating MDM2 resulting in p53 degradation. Akt also suppresses apoptosis by facilitating XIAP phosphorylation and prevents XIAP autoubiquitation and its proteasomal degradation. Akt down-regulates FasL and cell death receptor-mediated apoptosis by phosphorylating of the Forkhead family of transcription factors (FKHRL1, FKHR and AFX). Akt phosphorylates caspase-9 and Bad, thereby suppressing mitochondrial pathway apoptosis.

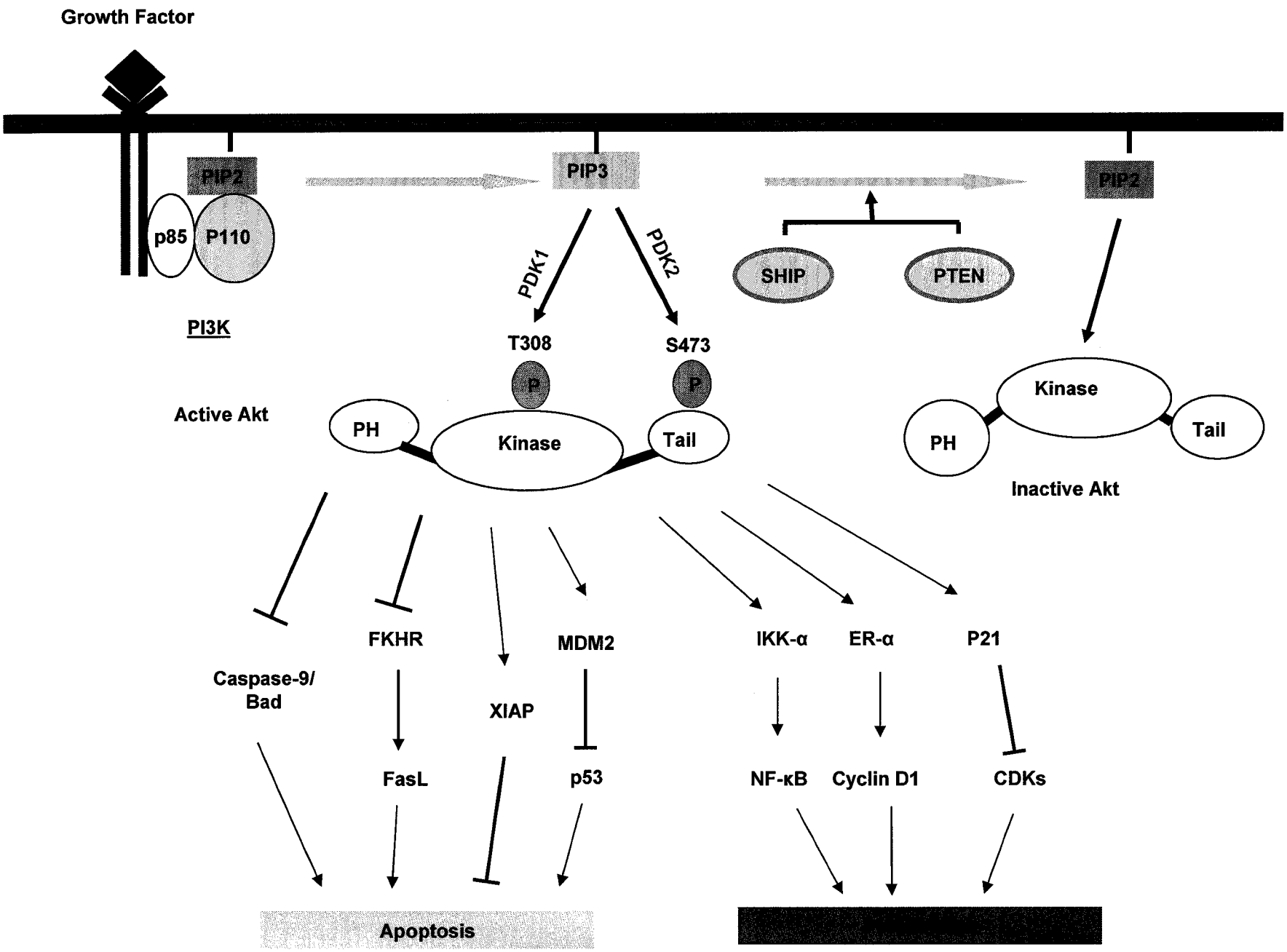

Growth Factor
PIP2
p85
P110
PI3K
PIP3
PDK1
PDK2
SHIP
PTEN
PIP2
T308
S473
P
P
Active Akt
PH
Kinase
Tail
Kinase
PH
Tail
Inactive Akt
FKHR
MDM2
IKK-α
ER-α
P21
Caspase-9/
Bad
XIAP
FasL
p53
NF-κB
Cyclin D1
CDKs
Apoptosis

Figure 4

which is required for its recruitment to the cell membrane, is key for Akt phosphorylation (Konishi, Shinomura et al. 1994). PI3K/Akt pathway activation can be inhibited by the actions of lipid phosphatases, such as Phosphatase and Tensin Homology (PTEN) (Stambolic, Suzuki et al. 1998), and SH2-Containing Inositol Phosphatase (SHIP)(Aman, Lamkin et al. 1998), which converts PIP3 to PIP2, thereby blocking Akt activation by preventing its PIP3-mediated membrane recruitment (Carver, Aman et al. 2000).

Akt, also known as protein kinase B (PKB), belong to a serine/threonine protein kinase B subfamily that are human homologues of the viral oncogene v-Akt (Bellacosa, Testa et al. 1991; Coffer and Woodgett 1991) known to cause leukemia in mice (Staal and Hartley 1988). There are three mammalian isoforms of Akt/PKB: Akt1/PKBα, Akt2/PKBβ and Akt3/PKBγ. They are transcribed from distinct genes and share >85% protein homology (Cheng, Jiang et al. 2002). They possess a very similar structure containing an N-terminal Pleckstrin homology (PH) domain, a central kinase domain, and hydrophobic C-terminus, a serine/threonine-rich domain (Figure 5).

Activated Akt, after phosphorylation at both T308 and S473 (Alessi, Andjelkovic et al. 1996), is translocated from the plasma membrane to the cytoplasm or nucleus (Andjelkovic, Maira et al. 1999) Akt phosphorylates Ser and Thr residues of its substrates within a consensus sequence (RXRXXS/T) (Cheng, Jiang et al. 2002). These include the c-myc proto-oncoprotein [for cell cycle progression (Ahmed, Grimes et al. 1997)] and IκB, the negative regulator of NF-κB signaling [increased expression of NF-κB-responsive gene products (Kane, Shapiro et al. 1999)]. It also activates NF-κB-mediated gene transcription independently of IκB (Wang, Chan et al. 2002). Akt phosphorylates the inhibitor of cyclin-dependent kinase (CDK) $p21^{WAF1/CIP1}$ on Thr145 (Rossig, Jadidi et al. 2001), thereby facilitating cell

Figure 5: Structure of Akt family members.

Three isoforms of AKT (Akt1, Akt2 and Akt3) share distinct functional domains: N-terminal pleckstrin homology (PH) domain of about 100 amino acids which is involved in protein-protein and protein-lipid interactions, a central kinase domain, and a serine/threonine-rich C-terminus catalytic domain.

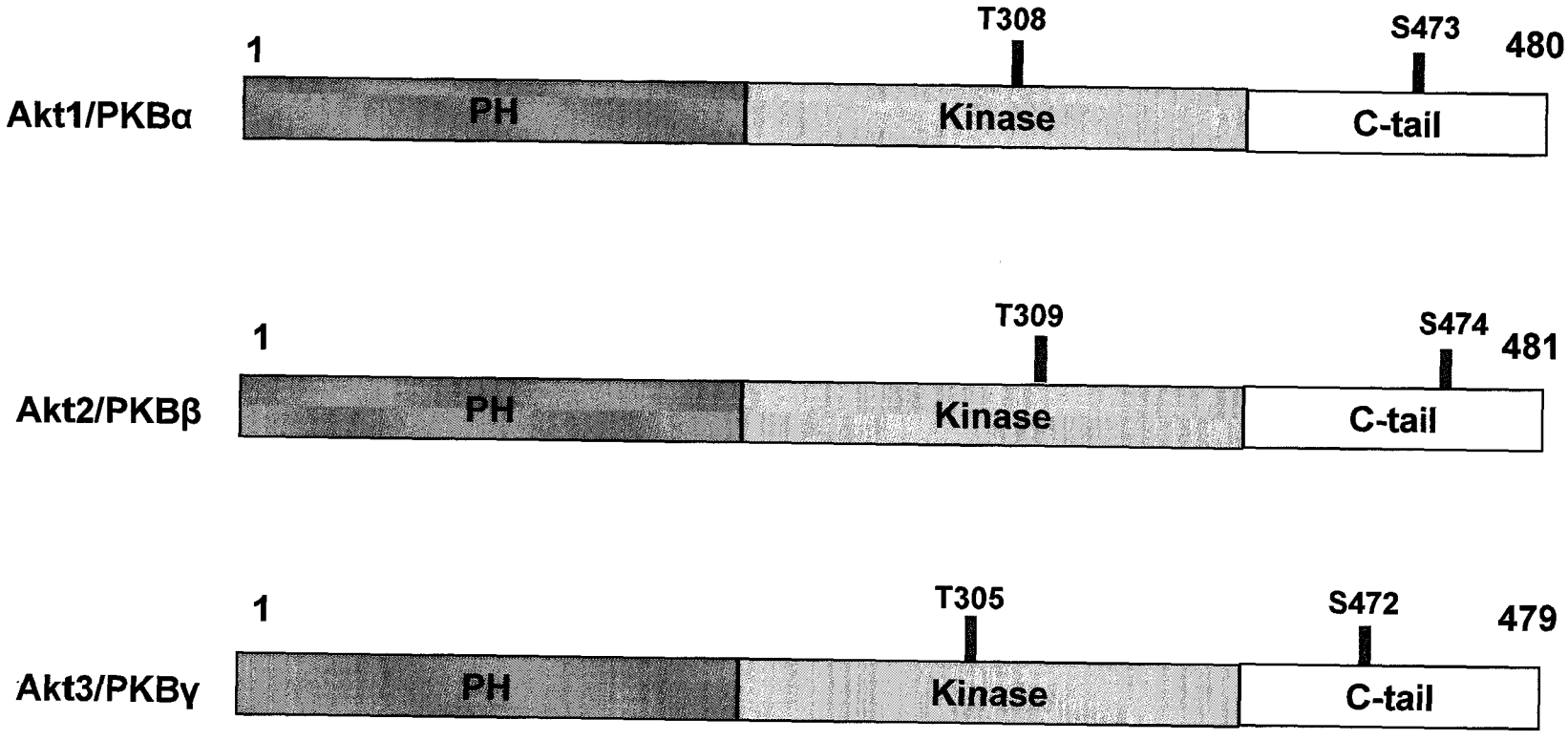

Akt1/PKBα
1
T308
S473
480
PH
Kinase
C-tail
Akt2/PKBβ
1
T309
S474
481
PH
Kinase
C-tail
Akt3/PKBγ
1
T305
S472
479
PH
Kinase
C-tail

Figure 5

cycle progression. Akt increases Estrogen Receptor-alpha (ERα) phosphorylation on Serl67 (Sun, Paciga et al. 2001), and S-phase progression through cyclin Dl up-regulation (Castoria, Migliaccio et al. 2001; Dupont and Le Roith 2001). Akt phosphorylates IκB kinase (IKKα) on Thr23 resulting in the activation of NF-κB-mediated gene transcription (Biggs, Meisenhelder et al. 1999).

Akt also inhibits apoptosis through phosphorylation of pro-apoptotic proteins. These include the forkhead-family transcription factor FKHRL1 which promotes transcriptional activation of the *FASLG* gene (OMIM# 134638; FasL) and the activation of the extrinsic apoptosis pathway (Brunet, Bonni et al. 1999). Akt phosphorylates transcriptional activators of FasL expression, such as members of the Forkhead family of transcription factors (FKHRL1, FKHR and AFX), (Biggs, Meisenhelder et al. 1999; Brunet, Bonni et al. 1999; Kops and Burgering 1999; Ciechomska, Pyrzynska et al. 2003), thereby down-regulating FasL and cell death receptor-mediated apoptosis. Akt phosphorylates FKHRL1 on Thr32 and Ser253 and prevents its nuclear localization, thereby inhibiting its activity as a transcriptional regulator (Brunet, Bonni et al. 1999). Akt mediates MDM2 phosphorylation (Mayo and Donner 2001; Zhou, Liao et al. 2001; Gottlieb, Leal et al. 2002; Ogawara, Kishishita et al. 2002), facilitates p53 degradation, thereby attenuates p53-mediated apoptosis, and promotes cell cycle progression.

Akt also attenuates the ability of Bad (a pro-apoptotic Bcl-2 family member) to bind and inhibit Bcl-2 (Scheid, Schubert et al. 1999) by phosphorylation on Ser 112 and Serl36 (Datta, Dudek et al. 1997; del Peso, Gonzalez-Garcia et al. 1997).

Akt can suppress the intrinsic apoptotic pathway by blocking the proteasomal degradation of XIAP through phosphorylation on Ser280 (Dan, Sun et al. 2004) and blocking of caspase-9 activation (Cardone, Roy et al. 1998).

Akt also phosphorylates caspase-9 (Cardone, Roy et al. 1998) and Bad, the pro-apoptotic Bcl-2 family member on Serl36 and Serll2 (Datta, Dudek et al. 1997; del Peso, Gonzalez-Garcia et al. 1997), thereby suppressing apoptosis.

Our laboratory has demonstrated that Akt is a physiological substrate of caspase-3 in ovarian cancer cells (Asselin, Mills et al. 2001; Jahani-Asl, Basak et al. 2007). We and others have also shown that Akt is a determinant of chemoresistance in ovarian cancer cells (Cheng, Altomare et al. 1997; Shayesteh, Lu et al. 1999; Sun, Wang et al. 2001; Fraser, Leung et al. 2003; Yang, Fraser et al. 2006; Fraser, Bai et al. 2008). Over-expression of active Akt attenuates p53 content and phosphorylation, modulates p53-mediated mitochondrial release of cytochrome C, Smac and AIF as well as of apoptosis induced by CDDP. Moreover, these responses are facilitated by down-regulation of Akt function (Yang, Fraser et al. 2006; Fraser, Bai et al. 2008; Yang, Fraser et al. 2008). Moreover, CDDP increases p53, decreases XIAP content, and induces apoptosis in chemosensitive ovarian cancer cells but not in their resistant counterpart. While expression of a constitutively active Akt2 prevents these responses in chemosensitive cells, dominant-negative Akt facilitates all of these responses in the resistant cells (Fraser, Leung et al. 2003).

Taken together, these results suggest that Akt may be an important regulator of both XIAP and p53 activation upon CDDP challenge. Inhibition of Akt expression or function may be a possible means of overcoming chemoresistance in ovarian cancer cells expressing either endogenous or reconstituted wild-type p53.

1.3.2.5 Nitric Oxide and Nitric Oxide Synthase Family

Nitric oxide (NO) was first described in 1987 as an endothelium-derived relaxation factor (EDRF) (Ignarro, Buga et al. 1987; Palmer, Ferrige et al. 1987). It is a key signaling molecule implicated in different physiological processes, including vasodilatation, neurotransmission, host-defense, platelet aggregation, and iron metabolism (Salvemini, de Nucci et al. 1989; Radomski, Palmer et al. 1990; Moncada, Palmer et al. 1991; Sanders and Ward 1992; Furchgott 1993; Moncada and Higgs 1995; Ignarro 1996; Pantopoulos, Weiss et al. 1996; Domachowske 1997). However, a growing body of evidence suggests that NO accumulation during chronic inflammation attributes to numerous pathological conditions, including cancer (Bredt and Snyder 1994; Tamir and Tannenbaum 1996; Ambs, Hussain et al. 1997).

NO is a short-lived gas with a half-life ~3–30 seconds with a lipophilic characteristics (Ignarro, Buga et al. 1987; Palmer, Ferrige et al. 1987) which facilitates its easy diffusion within cells. It is formed by a family of "NO synthases" (NOS) (Marletta 1994). Three isoforms exist for NOS: neuronal NOS (nNOS) and endothelial NOS (eNOS) which are generally constitutively expressed in neurons and endothelial cells, and inducible NOS (iNOS). Depending upon the cell type and concentration, NO is both pro-apoptotic and anti-apoptotic (Kolb 2000; Davis, Martin et al. 2001). NO-mediated apoptosis is attributed to its ability to induce oxidative stress and caspase activation (Klein and Ackerman 2003). However, endogenous NO or low amounts of NO suppresses apoptosis in *vivo* and in *vitro* (Liu, Wang et al. 1998; Chung, Pae et al. 2001).

NOS catalyzes NO production and influences NO-mediated lung neoplasia functions in tumor tissues (Puhakka, Kinnula et al. 2003). Elevation of NOS activity and increased

tumor-associated NO production have been observed in CDDP resistant lung cancer patients (Arias-Diaz, Vara et al. 1994; Fujimoto, Ando et al. 1997; Liu, Wang et al. 1998). Taken together, these findings suggest that NO may play a role in regulating CDDP sensitivity in cancer, including lung carcinoma. It has been shown that Akt activates eNOS, thereby promoting wild-type Ras nitrosylation and activation required for tumorigenesis. Moreover, suppression of eNOS phosphorylation inhibits tumor initiation and maintenance (Lim, Ancrile et al. 2008). NO has been shown to prevent Bcl-2 proteasomal degradation by facilitating S-nitrosylation of Bcl-2 in lung cancer cells (Chanvorachote, Nimmannit et al. 2006).

Recent studies from our laboratory have shown that CDDP up-regulates iNOS but decreases eNOS and nNOS in chemosensitive cells (Leung, Fraser et al. 2008). The NO donor SNAP (S-nitroso-N-acetylpenicillamine) enhances p53 protein levels and induces apoptosis in both cell types, and enhances CDDP-induced apoptosis in chemoresistant cells in a p53-dependent manner. CDDP-induced apoptosis in chemosensitive cells is partially inhibited by 1400W, a specific iNOS inhibitor. Blocking all NOSs, with NG-amino-L-arginine, renders chemoresistant cells markedly sensitive to CDDP-induced apoptosis (Leung, Fraser et al. 2008). These data demonstrate that CDDP differentially regulates the levels of all three nitric oxide synthases in human ovarian cancer cells, suggesting an important role of the three NOSs in regulating CDDP resistance in ovarian cancer cells.

1.3.2.6 Flice-Like Inhibitory Proteins

FLIP is an intracellular apoptosis suppressor protein. It suppresses FasL- and TRAIL-mediated cell apoptosis through the death receptor (Thome and Tschopp 2001; Yang, Xiao et

al. 2003), and also attenuates apoptosis induced by CDDP treatment (Kamarajan, Sun et al. 2003; Abedini, Qiu et al. 2004) (See in the section 1.5).

1.4 Regulation of Cellular Protein Content

Dysregulation of apoptotic machinery leads to changes in anti-apoptotic (e.g. Bcl-2, XIAP, FLIP and Akt) and/or pro-apoptotic (e.g. Bax, and p53) proteins in cancer, including ovarian cancer, and is frequently associated with poor responses to chemotherapy (Sato, Kigawa et al. 1999; Skirnisdottir, Seidal et al. 2002; Kupryjanczyk, Szymanska et al. 2003; Murata, Haisa et al. 2004). In this context, it has been shown that CDDP-induced apoptosis is associated with down-regulation of a number proteins either through alteration in their mRNA abundance or protein content due by post-translational modification. The latter includes caspase- and calpain-facilitated protein processing as well as proteasomal-mediated protein degradation. Therefore, in this section, the regulation of cellular protein will be reviewed in detail (See below).

1.4.1 Regulation of mRNA Abundance

The steady-state level of messenger RNAs (mRNA) depends upon its overall rate of synthesis and metabolism. The degradation of mRNA is an essential determinant in the regulation of gene expression, protein content and function. Down-regulation of gene expression is a complicated process implicating both translational suppression as well as accelerated mRNA turnover, and mostly facilitated by micro RNAs (miRNAs). Micro RNAs are endogenous RNA molecules 18–25 nucleotides (nt) long. They have been identified in viruses, plants and animals (Berezikov, Cuppen et al. 2006), and can negatively target up to

one-third of human mRNAs (Esquela-Kerscher and Slack 2006). The majority of miRNA genes are transcribed from primary miRNA transcripts (pri-miRNAs) by Polymerase II (Cai, Hagedorn et al. 2004; Lee, Kim et al. 2004); however, some of them are transcribed by polymerase III (Pol III) (Borchert, Lanier et al. 2006).

The pri-miRNA is cleaved in the nucleus by a multi-protein complex named Microprocessor, and then the pre-miRNA is exported into the cytoplasm by Exportin-5 via a GTP-dependent mechanism (Yi, Qin et al. 2003; Bohnsack, Czaplinski et al. 2004; Lund, Guttinger et al. 2004). The pre-miRNA is further cleaved into the mature ~22 nt miRNA (Hutvagner, McLachlan et al. 2001; Ketting, Fischer et al. 2001). Subsequently, it is assembled with Argonaut 2 to form an RNA-induced silencing complex (RISC) (Gregory and Shiekhattar 2005; Maniataki and Mourelatos 2005), thereby target the mRNA through base-pairing interaction. miRNAs down-regulate the expression of their target genes, induce the cleavage and degradation of the transcript initiated by deadenylation and decapping of the mRNA (Pillai, Bhattacharyya et al. 2007).

Previous studies suggest that miRNAs control cell proliferation, differentiation and apoptosis (Calin and Croce 2006), indicating that they may have an important role in cancer. It has also been reported that some miRNAs, including ''oncomirs'', can serve as both tumor suppressors and oncogenes (Esquela-Kerscher and Slack 2006). Therefore, modulation of miRNA expression and function may contribute to tumorigenesis.

1.4.2 Regulation of Protein Processing and Degradation

The cellular level of a protein is determined by relative rate of its synthesis, processing and degradation. While processing of a protein could be a consequence of caspase- and calpain-mediated cleavage, protein degradation generally happens through the lysosome and proteasome pathways.

1.4.2.1 Caspase-and calpain-mediated processing

Caspases are catalytically inactive zymogens. Caspase activation requires proteolytic cleavage of a caspase precursor between the large and small subunits in two Asp-X bonds to form a catalytically active fragment (Martin, Amarante-Mendes et al. 1996; Han, Hendrickson et al. 1997; Li, Bergeron et al. 1997; Srinivasula, Ahmad et al. 1998). Once activated, caspases recognize at least four contiguous amino acids in their substrates P4-P3-P2-P1, and hydrolyze peptide bonds on the carboxyl side of P1 (usually an aspartate). Proteins with a caspase-3 consensus site (DXXD) may be a physiologic caspase-3 substrate, and are recruited by caspase-3 upon activation. The proteins are then cleaved by caspase-3. Our laboratory demonstrated that Akt is a physiological substrate of caspase-3 in ovarian cancer cells (Asselin, Mills et al. 2001). Recent studies indicate that CDDP induces caspase3-mediated Akt cleavage and that the phosphorylation status of the kinase influences its ability to be processed by this protease (Jahani-Asl, Basak et al. 2007). In addition to the caspase-3 consensus site, there is also a number of non-consensus caspase-3 cleavage sites, the processing at which is largely influenced by the phosphorylation status of Akt. This finding suggest the existence of a possible feedback mechanism by which Akt activation prevents its processing by CDDP (Jahani-Asl, Basak et al. 2007).

Calpains, identified in rat brain in 1964, are calcium-dependent cysteine proteinases (Guroff 1964). Calpain μ and m (Micro- and milli-calpains) are two isoforms in this proteolytic system which have been extensively studied (Goll, Thompson et al. 2003). Both isoforms are heterodimers consisting of a 80 kDa subunit and a 28 kDa subunit. The large subunits are encoded by two different genes (capn1 and capn2) for μ- and m-calpain, respectively, while the 28 kDa subunit (Ohno, Minoshima et al. 1990) consists of two different small subunits (Css1 and Css2) which can dimerise equally with the large subunit to form either μ- and m-calpains (Schad, Farkas et al. 2002). Calpains, like many other proteases, are pro-enzymes and need to be activated for their physiologic function. Calpains are inactive in the absence of calcium. In the presence of calcium, they undergo autolysis at the N-terminal to form a 76 kDa and a 78 kDa large subunit for μ- and m-calpain, respectively as well as an autolysed 18 kDa regulatory subunit (Zimmerman and Schlaepfer 1991; Brown and Crawford 1993). This autolysis is associated with a reduction in the calcium concentration necessary for calpain activation. However, phosphorylation of milli- and micro-calpains on Tyr, Ser, and Thr residues (Goll, Thompson et al. 2003) by different kinases (e.g. ERK/MAPK), leads protease activation (Glading, Bodnar et al. 2004). On the other hand, phosphorylation of m-calpain on Ser 369/Thr 370 by PKA inhibits its activity (Shiraha, Glading et al. 2002).

Calpains are implicated in vitro proteolysis of cytoskeletal proteins (talin, vinculin, and MARCKS) (Dulong, Goudenege et al. 2004; Franco, Rodgers et al. 2004; Serrano and Devine 2004), signal transduction proteins (PKC and FAK) (Cooray, Yuan et al. 1996; Aragon, Poussard et al. 2002) and transcription factors (C/EBP, p53, p73 etc.) (Pariat, Carillo

et al. 1997; Wei, Yang et al. 2006), suggesting that calpain activity is important for a large number of physiological processes.

CDDP induces caspase-12 activation which is independent of DNA damage. It also activates caspase-3 (a response attenuated by the calpain inhibitor calpeptin and the calcium chelator BAPTA), increases expression of Grp78/BiP (a marker of ER stress) and induces apoptosis in the human melanoma cell line 224 and two variants of the colon cancer cell lines HCT116 (wt and p53-deficient), (Mandic, Hansson et al. 2003). Moreover, expression of *ARHI gene,* a putative tumor suppressor gene which is down-regulated in a majority of ovarian and breast cancers (Yu, Xu et al. 1999) induces apoptosis in ovarian and breast cancer cells, a response which is attenuated by calpain inhibitor but not caspase inhibitors (Bao, Le et al. 2002). These finding suggest that calpain may play important role in the regulation of apoptotic machinery in cancer cells.

1.4.2.2 Proteasomal Degradation

Proteasomes are ubiquitous intracellular multicatalytic enzymes and are responsible for the degradation of most cellular proteins (Rock, Gramm et al. 1994). Proteins destined for proteasomal degradation bind with ubiquitin units during poly-ubiquitination, and enter the proteasome for degradation (Ciechanover, Breitschopf et al. 1999).

Ubiquitin is a highly conserved 76-amino acid polypeptide involved in a variety of cellular functions including protein degradation. It serves as a tag for membrane protein internalization and as recognition proteins for the multi-subunit proteolytic complex known as the proteasome (Hicke 1997; Hershko and Ciechanover 1998; Hicke 2001). It covalently binds to substrate proteins via an isopeptide bond between a C-terminal glycine (Gly) in

ubiquitin and lysine residues (Lys) in the substrate molecules. Poly-ubiquitination of proteins involves three distinct enzymes (Figure 6). E1, the ubiquitin-activating enzyme, is responsible for ubiquitin activation by transferring a high-energy thio-ester bond (E1-S-ubiquitin) to ubiquitin in an ATP-dependent manner. Activated ubiquitin is then transferred to an ubiquitin-conjugating enzyme (E2). Ultimately, ubiquitin is transferred to the substrate by an ubiquitin ligase E3 (Rock, Gramm et al. 1994). This process can continue to add four or five ubiquitin molecules via binding of the glycine 76 in the C-terminus of ubiquitin and lysine 48 of another ubiquitin molecule, resulting in polyubiquitination of substrate molecules (Rock, Gramm et al. 1994; Hershko and Ciechanover 1998; Pickart 2001). Since ubiquitin contains several Lys residues, it can also be a substrate of ubiquitination and produce poly-ubiquitin chains of variable lengths (Pickart 2000; Pickart 2001a; Pickart 2001b).

Ubiquitinated proteins undergo an active proteolytic event and are cut into small peptides within the proteasome. The proteasome complex (Figure 6) consists of a pair of 19S-regulatory proteins, which recognizes polyubiquitinated protein at the pore opening, unfolds the ubiquitin-conjugated protein, and releases one ATP molecule. The proteasome also has a 20S core proteinase. The 20S proteasome, a cylindrical structure consisting of four rings stacked together with a pore (13 Å), possesses protease activity. When a polypeptide chain enters the pore, the protein is cleaved into smaller polypeptides through proteolytic events (Weissman 2001), and ubiquitin chains are released by deubiquitinating enzymes and recycled for subsequent protein ubiquitination (Hershko and Ciechanover 1998). In this context, recent studies have shown that CDDP decreases XIAP protein in CDDP sensitive

Figure 6: The ubiquitin-proteasome pathway

Ubiquitin (Ub) is covalently bound to a target protein via a multi-enzymatic system consists of ubiquitin-activating enzyme (E1) which activates an Ub monomer at its C-terminal cysteine residue to a high-energy thiol ester bond, ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3) enzymes. Activated Ub then can be transferred to a reactive cysteine residue of the E2 enzyme and ultimately is transferred to a reactive lysine residue of a substrate protein by the E3 enzyme. This process can be continued to attach 4-5 ubiquitin moieties by E3, results in poly-ubiquitin chain formation. Ubiquitin can be removed by DUBs from ubiquitinated substrate. Ubiquitinated proteins are directed to the 26S proteasome, via the 19S cap and degraded into oligopeptides in the catalytic 20S core. Then Ub molecules are released and recycled for a new reaction, (modified from (Passmore and Barford 2004).

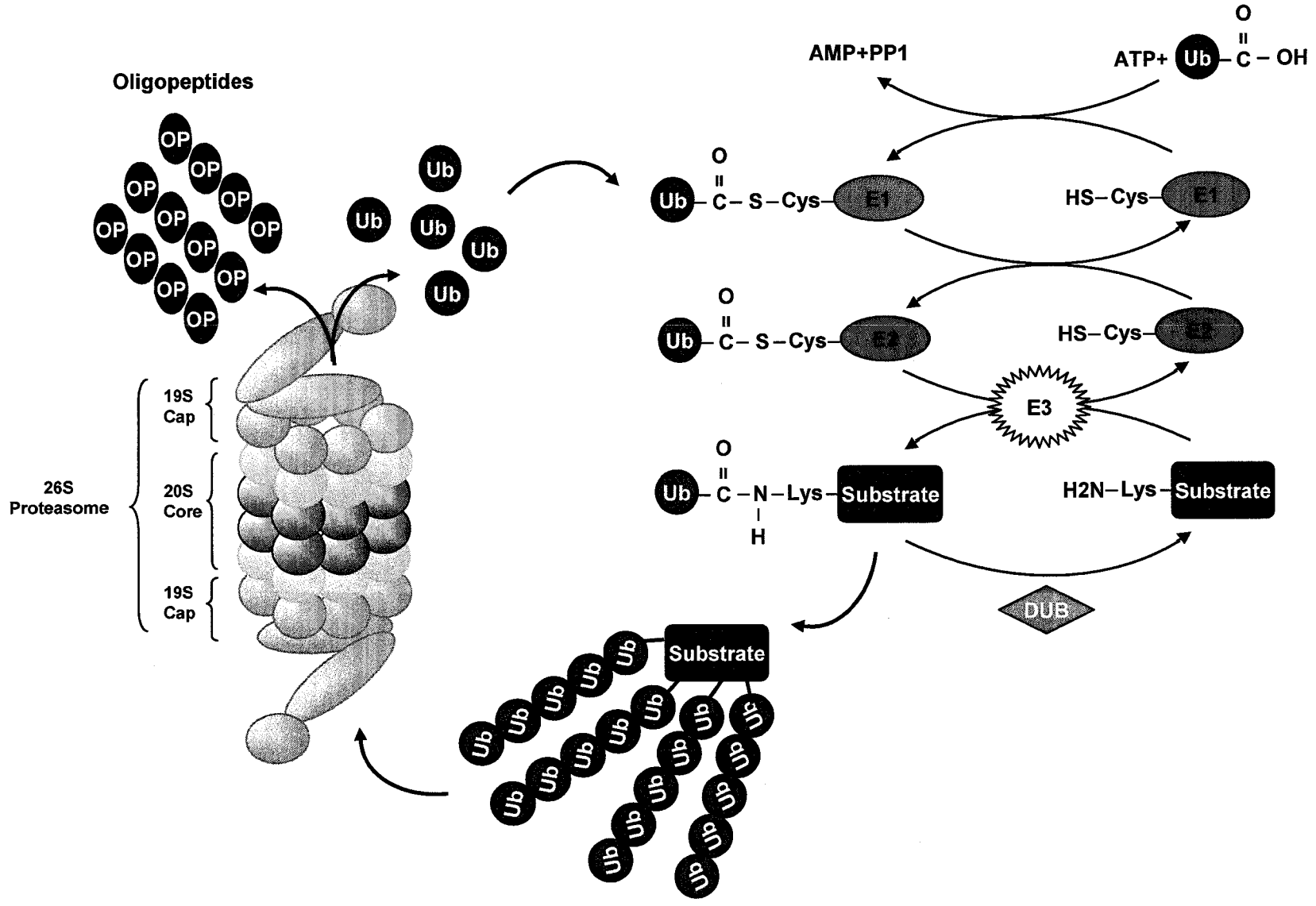

Oligopeptides
OP
Ub
26S
Proteasome
19S
Cap
20S
Core
19S
Cap
AMP+PP1
ATP+
Ub
C – OH
Ub – C – S – Cys – E1
HS – Cys – E1
Ub – C – S – Cys – E2
HS – Cys – E2
E3
Ub – C – N – Lys – Substrate
H
H2N – Lys – Substrate
DUB
Substrate

Figure 6

ovarian cancer cells through the proteasome pathway, a response which is inhibited by proteasome inhibition (Dan, Sun et al. 2004).

Itch

Itch, a HECT (Homologous to the E6-associated protein C Terminus) and WW-domain ligase, was originally identified as a mutant gene in the non-agouti-lethal 18H mice also called Itchy mice (Perry, Hustad et al. 1998). This mouse suffers from chronic lethal lymphoproliferative disease and constant itching. Itch serves as an E3 ligase for p73 and p63 ubiquitination and proteasomal degradation (Rossi, De Laurenzi et al. 2005; Rossi, Aqeilan et al. 2006; Rossi, De Simone et al. 2006). Itch also facilitates FLIP ubiquitination in response to TNF-α, thereby sensitizing cells to apoptosis (Chang, Kamata et al. 2006). Itch contains a N-terminal C2 domain which is responsible for its intracellular localization, a C-terminal HECT domain involved in ligase activity, and WW domains implicated in protein-protein interaction and recognition of substrates (Mouchantaf, Azakir et al. 2006). The negative regulator of Itch, N4BP1, inhibits its ubiquitination activity by binding to the WW domains of the Itch molecule (Oberst, Malatesta et al. 2007).

1.4.2.3 Lysosomal Degradation

The lysosomal proteolytic pathway is a major degradative pathway which is implicated in internalization and degradation of membrane proteins in the lysosomes (Hicke 1997; Jeffers, Taylor et al. 1997; Hicke 2001), including numerous mammalian receptors and the ENaC (epithelial sodium channel) which undergoes monoubiquitination and lysosomal degradation (Jeffers, Taylor et al. 1997). Unlike polyubiquitination, which consists of chains

with several ubiquitins bound together, monoubiquitination is restricted by one ubiquitin per lysine on multiple lysines on the target protein. Cytoplasmic protein ubiquitination leads to proteasomal degradation, while monoubiquitination of membrane proteins at one, two, or several different sites, serves as a signal for endocytosis and lysosomal degradation (Bonifacino and Weissman 1998; Rotin, Staub et al. 2000; Thrower, Hoffman et al. 2000; van Kerkhof, Sachse et al. 2001). It has been shown that some proteins can be degraded via both proteolytic pathways depending on their cellular localization. The cystic fibrosis transmembrane conductance (CFTR) is one such protein which is degraded through proteasome pathway (Hicke 1997) following polyubiquitination in the endoplasmic reticulum (ER); however, CFTR in the membrane is degraded in lysosomes (Peter, Varga et al. 2002). Monoubiquitination of membrane proteins can promote their internalization, suggesting that monoubiquitination is an important event for protein internalization (Strous, van Kerkhof et al. 1996).

1.5 Flice-Like Inhibitory Protein

Fas-Associated Death Domain-Like Interleukine-1ß-Converting Enzyme (FLICE)-Like Inhibitory Protein (FLIP) is an endogenous Fas associated death domain (FADD)-binding protein. It is an inhibitor of caspase-8 activation, thereby potently suppressing cell death receptor-mediated apoptosis (Irmler, Thome et al. 1997; Thome, Schneider et al. 1997). While the viral FLIP was identified and named Viral $FLIP_S$ (v-$FLIP_S$) (Thome, Schneider et al. 1997), the mammalian cellular homologue of v-FLIP is called c-FLIP (Irmler, Thome et al. 1997), caspase homologue (CASH) (Goltsev, Kovalenko et al. 1997), inhibitor of FLICE (I-FLICE) (Hu, Vincenz et al. 1997), caspase-like apoptosis-regulatory protein (CLARP) (Inohara, Koseki et al. 1997), FADD-like anti-apoptotic molecule (FLAME1) (Srinivasula,

Ahmad et al. 1997), MACH-related inducer of toxicity (MRIT) (Han, Chaudhary et al. 1997), Usurpin (Rasper, Vaillancourt et al. 1998) and caspase-eight-related protein (Casper) (Shu, Halpin et al. 1997). FLIP is the product of the CFLAR gene (OMIM# 603599), which is located in q33-34 on human chromosome 2 and clustered with caspase-8 and 10 genes (Han, Chaudhary et al. 1997; Inohara, Koseki et al. 1997; Srinivasula, Ahmad et al. 1997; Rasper, Vaillancourt et al. 1998). Although about 11 individual alternative splicing isoforms of FLIP gene have been reported (Djerbi, Darreh-Shori et al. 2001), two major isoforms detectable at the protein level are the full-length 55 kDa $FLIP_L$ and an alternatively spliced 28 kDa $FLIP_S$, both of which contain two death-effector domains (DED) (Irmler, Thome et al. 1997). While $FLIP_S$ contains a short C-terminus of about 20 amino acids, $FLIP_L$ possesses a catalytically inactive C-terminal caspase-like domain resulting from the substitution of tyrosine with the active cysteine residue in the conserved motif of caspase-8, which is necessary for enzymatic activity (Irmler, Thome et al. 1997) (Figure 7).

1.5.1 Involvement of FLIP in The Regulation of Apoptosis

FLIP is an intracellular apoptosis suppressor protein. It suppresses Fas ligand- and TRAIL-mediated apoptosis through the death receptor (extrinsic) pathway (Thome, Schneider et al. 1997; Kinoshita, Yoshikawa et al. 2000; Yang, Xiao et al. 2003), and also attenuates DNA damage-mediated apoptosis induced by CDDP (Kinoshita, Yoshikawa et al. 2000; Kamarajan, Sun et al. 2003; Abedini, Qiu et al. 2004). Previous studies on the anti-apoptotic action of FLIP have shown that it inhibits apoptotic signalling induced by Fas, TNF-R1, TRAIL-R and TRAMP activation via interaction with FADD and procaspase-8,

Figure 7: Molecular structure of FLIP Isoforms

Caspase-8 contains a catalytically active C-terminus and an N-terminus that consists of two death effector domains (DED). Flice-Like Inhibitory Protein (FLIP) are present in two spliced variants: a long splice variant of FLIP ($FLIP_L$) containing a catalytically inactive C-terminal caspase-like domain and two DED repeats, and a short form of FLIP ($FLIP_S$) consisting of two DEDs. Both $FLIP_L$ ad $FLIP_S$ are inhibitors of caspase-8 activation and suppress death receptor-mediated apoptosis by interacting with caspase-8 and preventing its dimerization (Irmler, Thome et al. 1997; Thome, Schneider et al. 1997).

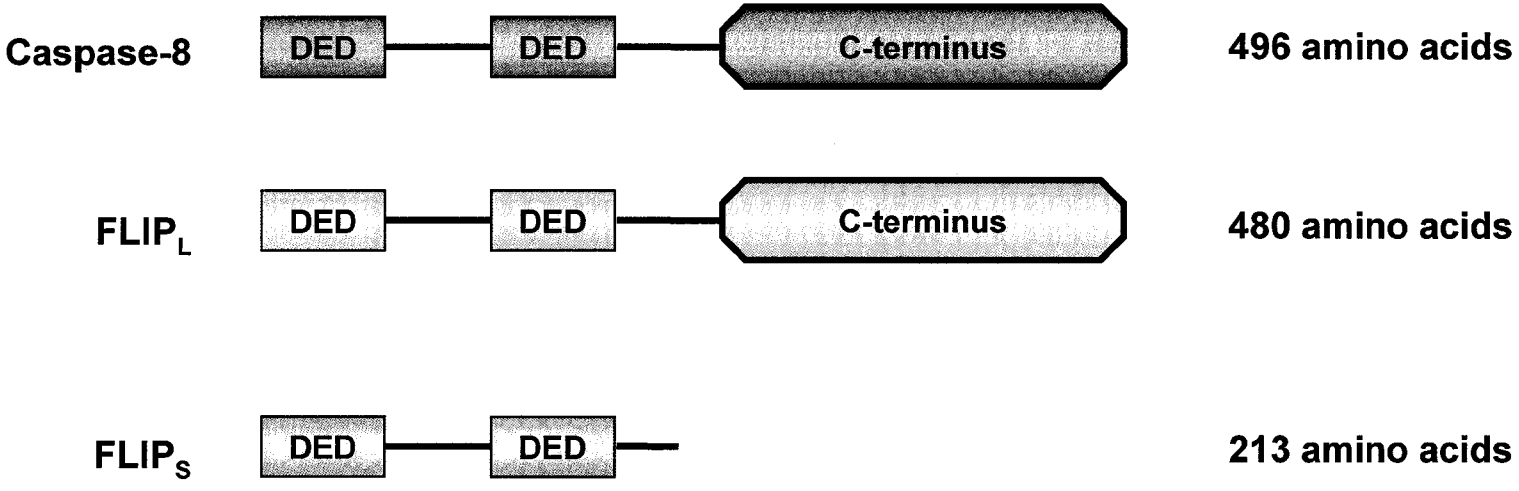

Caspase-8
DED
DED
C-terminus
496 amino acids
FLIP$_L$
DED
DED
C-terminus
480 amino acids
FLIP$_S$
DED
DED
213 amino acids

Figure 7

ultimately blocking caspase-8 activation (Goltsev, Kovalenko et al. 1997; Han, Chaudhary et al. 1997; Hu, Vincenz et al. 1997; Inohara, Koseki et al. 1997; Irmler, Thome et al. 1997; Shu, Halpin et al. 1997; Srinivasula, Ahmad et al. 1997; Rasper, Vaillancourt et al. 1998).

Controversies exist regarding the role of $FLIP_L$ in the regulation of apoptosis. While some have described it as a pro-apoptotic protein (Goltsev, Kovalenko et al. 1997; Han, Chaudhary et al. 1997; Inohara, Koseki et al. 1997; Shu, Halpin et al. 1997), others described it as being anti-apoptotic. Although the reason (s) for the differences is not apparent, it should be noted that the reports on the pro-apoptotic effects of $FLIP_L$ were based mainly on experiments using transient over-expression. It is possible that the excessive loads of DED-containing proteins form death effecter filaments, novel cytoplasmic structures that recruit Caspase-8 and induce apoptosis (Siegel, Martin et al. 1998). Moreover, data obtained from cells stably transfected $FLIP_L$ and from mice deficient in $FLIP_L$ clearly support its antiapoptotic effect (Irmler, Thome et al. 1997; Kataoka, Schroter et al. 1998; Rasper, Vaillancourt et al. 1998; Scaffidi, Schmitz et al. 1999; Yeh, Itie et al. 2000; Krueger, Baumann et al. 2001). Taken together, it seems $FLIP_L$ serves as an anti-apoptotic factor by attenuating CDDP-induced caspase-8 activation.

Upon FasL or TRAIL binding to their receptors, FADD/TRADD is recruited via its DD and interacts with procaspase-8 through its DED to form the Death-Inducing-Signaling Complex (DISC) (Kischkel, Hellbardt et al. 1995; Bodmer, Holler et al. 2000; Kischkel, Lawrence et al. 2000; Sprick, Weigand et al. 2000). Procaspase-8 is activated by autocatalytic cleavage following dimerization in the DISC, forming the large and small subunits. As a result, the DED-containing amino-terminal fragments of caspase-8 stays transiently at DISC, whereas the active caspase-8 protease dimer is released into the

cytoplasm to cleave executioner caspases such as procaspase-3 (Slee, Harte et al. 1999) and the pro-apoptotic member of the Bcl-2 family BID, and ultimately initiates the apoptotic cascade (Li, Zhu et al. 1998; Luo, Budihardjo et al. 1998; Fischer, Coelho et al. 2003; Fischer, Arunachalam et al. 2003).

Both FLIP isoforms inhibit apoptosis by blocking the cleavage and activation of caspase-8. However, the molecular mechanism of apoptosis inhibition by $FLIP_L$ is different from that of $FLIP_S$. While $FLIP_S$ binds to FADD and caspase-8, thereby blocking the initial cleavage of intact pro-caspase-8 into the 43 kDa intermediate form, $FLIP_L$ and caspase-8 are recruited and partially processed at the receptor level and block the cleavage of p43 pro-caspase-8 into the 20 kDa active caspase-8 (Wajant 2003). Therefore, $FLIP_S$ completely blocks cleavage and activation of caspase-8, while $FLIP_L$ allows only the first cleavage of procaspase-8 between large and small subunits. Since no cleavage between the caspase domain and DED occurs, the partially processed proteins stay bound to the receptor and active caspase-8 is not released into the cytosol (Krueger, Baumann et al. 2001; Thome and Tschopp 2001).

1.5.1.1 FLIP and Regulation of Apoptosis Signaling Pathways

The role of FLIP in the regulation of apoptosis by different stimuli has been studied and suggests that the anti-apoptotic properties of FLIP may involve its ability to regulate signaling through different pathways. Indeed, it has been demonstrated that FLIP down-regulation sensitizes cells to death receptor-mediated apoptosis by different stimuli.

The Fas/FasL system plays an important role in the regulation of apoptosis in ovarian cancer cells (Schneiderman, Kim et al. 1999). The involvement of FLIP in the regulation of

Fas-mediated apoptosis has been investigated and it has been demonstrated that over-expression of FLIP confers resistance to FasL-mediated cell death in various cell types, including ovarian cancer cells (Thome, Schneider et al. 1997; Kataoka, Schroter et al. 1998; Yeh, Hsu et al. 1998; Irisarri, Plumas et al. 2000; Kinoshita, Yoshikawa et al. 2000; Schlapbach, Spanaus et al. 2000; Wang, Lobito et al. 2000; Yoshikawa, Nakajima et al. 2000; Poulaki, Mitsiades et al. 2001; Suhara, Mano et al. 2001; Yang, Xiao et al. 2003). Moreover, down-regulation of FLIP by different biochemical and molecular strategies sensitizes a variety of cell types, including ovarian cancer cells, to Fas-mediated or CDDP-induced apoptosis (Fulda, Meyer et al. 2000; Kinoshita, Yoshikawa et al. 2000; Yoshikawa, Nakajima et al. 2000; Kamarajan, Sun et al. 2003; Wajant 2003; Xiao, Yan et al. 2003; Abedini, Qiu et al. 2004; Yang, Xiao et al. 2007). Taken together, these results suggest that FLIP contributes to resistance to Fas-mediated apoptosis.

Previous studies have shown that FLIPs is a major cellular inhibitor of TRAIL-induced apoptosis in HeLa cells (Bin, Li et al. 2002) and that CDDP decreases FLIPs protein expression, inhibits $FLIP_L$ phosphorylation and restores TRAIL-induced, caspase-8-mediated apoptosis in resistant melanoma cells (Song, Song et al. 2003). $FLIP_L$ up-regulation (Wang, Lobito et al. 2000), $FLIP_L$ phosphorylation (Song, Song et al. 2003) and $FLIP_L$ and $FLIP_S$ up-regulation (Moriyama and Yonehara 2007) inhibit TRAIL-mediated apoptosis. Moreover, down-regulation of FLIP protein by siRNA, NF-κB inhibition (Siegmund, Hadwiger et al. 2002; Chawla-Sarkar, Bae et al. 2004; Morales, Ruiz-Magana et al. 2007) and inhibition of $FLIP_L$ phosphorylation by CDDP (Song, Song et al. 2003) sensitize cells to TRAIL-mediated apoptosis through activation of caspase-8, suggesting that FLIP modulates TRAIL-induced cell death.

It has been shown that TNFα protects ovarian cancer cells (OV2008) from the cytotoxic (pro-apoptotic) effects of the cytokine by induction of NF-κB-mediated FLIPs mRNA and protein expression (Xiao, Yan et al. 2003). In the presence of cycloheximide (CHX), TNFα activates caspase-8 and -3 and induces apoptosis. It also induces IκB phosphorylation, nuclear factor κB activation, and the expression of $FLIP_S$ but not of $FLIP_L$. Over-expression of dominant negative IκB attenuates TNFα-induced $FLIP_S$ expression and enhances TNFα-induced apoptosis. While expression of $FLIP_S$ antisense facilitates TNFα- and CHX-induced apoptosis, transfection of these cells with $FLIP_S$ sense attenuates these responses. This study suggests that the induction of $FLIP_S$ expression by TNFα might contribute to the resistance of ovarian epithelial cancer cells (Xiao, Yan et al. 2003). In addition, c-FLIP modulates drug-induced apoptosis through inhibition of Fas- and TNFα-mediated cell death (Goltsev, Kovalenko et al. 1997) and activation of NF-κB pathway (Chaudhary, Eby et al. 2000; Kataoka, Budd et al. 2000; Kumar, Jasmin et al. 2001; Shivapurkar, Reddy et al. 2002; Mora, Corn et al. 2003), suggesting that FLIP plays an important role in the regulation of TNFα-mediated apoptosis.

Interestingly, FLIP is an NF-κB-responsive gene product (Micheau, Lens et al. 2001), and NF-κB protects cells from apoptosis induced by different stimuli including CDDP (Tsou, Tsai et al. 2003; Lu, Yeh et al. 2006). However, it has been reported that NF-κB facilitates cell death induced by CDDP in head and neck squamous carcinoma cells (Kim, Kim et al. 2006).

1.5.2 Regulation of FLIP Content

1.5.2.1 Caspase-3- mediated FLIP Processing

The presence of several caspase-3 consensus sequences (DXXD) in the FLIP protein structure raises the possibility that FLIP is a physiologic caspase-3 substrate, and that alteration in FLIP protein levels by different stimuli may be a consequence of caspase-mediated FLIP processing. In this context, it has been demonstrated that CDDP sensitizes HeLa cells to TRAIL-mediated apoptosis by inducing caspase-3-mediated $FLIP_S$ cleavage (Kim, Ajaz et al. 2003). Moreover, Fas-mediated $FLIP_L$ cleavage has also been demonstrated in B lymphocyte cells (Hennino, Berard et al. 2000). Upon the FasL stimulation on death receptors, $FLIP_S$ and $FLIP_L$ are also reported to be recruited along with caspase-8 to the Fas-FADD complex. While $FLIP_S$ inhibits caspase-8 cleavage (activation), $FLIP_L$ allows the first cleavage of caspase-8 to generate caspase-8 (p43) but inhibits caspase-8 activation by preventing the second cleavage (Krueger, Baumann et al. 2001). Recruitment of caspase-8 and $FLIP_L$ in the DISC generates cleavage of $FLIP_L$ (p12) at Asp376 and $FLIP_L$ (43 kDa) (Shu, Halpin et al. 1997; Srinivasula, Ahmad et al. 1997; Krueger, Baumann et al. 2001). Moreover, $FLIP_L$ (43 kDa), similarly to full length of $FLIP_L$, can inhibit Fas-mediated cell death and renders the cell resistant by inhibiting the second cleavage of caspase-8, thereby suppresses caspase-8 activation (Krueger, Baumann et al. 2001)

1.5.2.2 FLIP and Proteasome Pathway

$FLIP_L$ content in human prostate and ovarian cancer cells is decreased through the proteasome pathway following FLIP ubiquitination (Fukazawa, Fujiwara et al. 2001; Kim, Suh et al. 2002). It has also been reported that $FLIP_S$ is down-regulated through the ubiquitin

proteasome pathway in the cervical carcinoma HeLa cells (Perez and White 2003). These findings indicate that both $FLIP_L$ and $FLIP_S$ are regulated through the ubiquitin proteasome pathway by different stimuli such as TRAIL and TNF-α.

1.5.2.3 Regulation of FLIP by P53 Signaling Pathways

Studies have shown that p53 expression decreased $FLIP_L$ protein content in colon cancer cells through activation of the ubiquitin proteasome pathway (Fukazawa, Fujiwara et al. 2001). It has also been suggested that p53 level (Fukazawa, Fujiwara et al. 2001; Chandrasekaran and Richburg 2005) or phosphorylation status (Zhou, Yu et al. 2005; Chandrasekaran, McKee et al. 2006) are correlated with FLIP down-regulation in ovarian, colon and hepatocellular carcinoma cells. Taken together, FLIP appears to be regulated through the ubiquitin proteasome pathway and p53 may facilitate this phenomenon, although the mechanism by which p53 facilitates FLIP degradation is not clear.

1.5.2.4 Akt Pathway and FLIP Expression

The involvement of Akt/PKB in the regulation of apoptotic proteins and chemosensitivity has been intensively studied. These studies demonstrated that Akt is a key determinant of chemoresistant in numerous cancer cells, including those of the ovary (Fraser, Leung et al. 2003; Yang, Fraser et al. 2006; Fraser, Bai et al. 2008; Yang, Fraser et al. 2008). Indeed, over-expression of activated Akt rendered chemosensitive ovarian cancer cells resistant to CDDP, and down-regulation of its function in their chemoresistant counterpart by dominant negative Akt (DN-Akt) facilitated CDDP-induced apoptosis (Fraser, Leung et al. 2003; Fraser, Bai et al. 2008). It has also been shown that PI3-kinase/Akt signaling pathway plays

an important regulatory role in FLIP expression in normal and transformed cells, thereby inhibiting apoptosis induced by FasL/TRAIL through the extrinsic death pathway (Suhara, Mano et al. 2001; Nam, Jung et al. 2003; Alladina, Song et al. 2005; Starck, Scholz et al. 2005; Moriyama and Yonehara 2007). In this context, down-regulation of PI3K/Akt by dominant negative (DN)-Akt and the PI3K inhibitor LY294002 decreased FLIP mRNA and protein abundance in different tumor cells (Panka, Mano et al. 2001). Moreover, activation of the PI3-kinase/Akt pathway increased FLIP protein levels in endothelial cell (Suhara, Mano et al. 2001) and human gastric cancer and dephosphorylation of phospho-Akt also negatively regulates FLIP protein level (Nam, Jung et al. 2003). Taken together, the PI3K/Akt pathway is important in the regulation of FLIP expression in human cancers. However, precisely how the PI3-kinase/Akt pathway regulates FLIP, thereby CDDP sensitivity remains to be determined.

Chapter 2 - Objectives and Hypotheses

Despite efforts in improving ovarian cancer treatment outcome during the past decades, chemoresistance remains a major therapeutic hurdle for this gynecologic malignancy. Although it has been demonstrated that different pathways are involved in the regulation of drug-induced apoptosis and chemosensitivity, it seems that the interaction between them are key determinants in cell fate regulation. Moreover, it has been shown that FLIP is important in the regulation of apoptotic cell death by modulating the cell death receptor pathway through suppression caspase-8 activation; however, recent data suggests that this anti-apoptotic protein may be regulated by a number of fundamental cell survival or death pathways, such as those of PI3K/Akt and p53, respectively. It has also shown that p53 tumor suppressor is a determinant in the induction of apoptosis by chemotherapeutic agents; however this pro-apoptotic protein is also regulated by other anti-apoptotic proteins, such as MDM2 and Akt. Understanding the mechanisms of interaction between these pathways will improve our knowledge on the mechanisms underlying chemoresistance.

Although the recent finding suggest that Akt and p53 may be involved in the regulation of FLIP, it should be noted that our understanding of the precise mechanisms by which these events occur is not complete. Indeed, while Akt appears to attenuate the effects of CDDP on p53 up-regulation and on FLIP down-regulation, precisely how Akt elicit its action is unclear. Moreover, CDDP induces phosphorylation and stabilization of p53 and down-regulates FLIP content in sensitive but resistant ovarian cancer cells. Cisplatin can induce these changes through alteration of ubiquitination and proteasomal degradation of these proteins. One possibility is that some responses to cisplatin in chemosensitive cells may be altered in chemoresistant cells. For example, due the inability of cisplatin to down-

regulate Akt content or activity in resistant cells, this cell survival factor may influence the stability of one or both of these proteins via altered ubiquitination pathways (Figure 8).

2.1 Overall Objectives

The overall objective of the current study is to improve our understanding of the cellular and molecular mechanisms of chemoresistance in ovarian cancer cells. In particular, we are interested in elucidating the mechanisms regulating apoptosis in these cells, and in establishing if and how the aberrant regulation of apoptosis contributes to the phenomenon of CDDP resistance. A better understanding of these mechanisms may ultimately lead to improved outcomes in the treatment of human ovarian cancer.

2.2 Specific Hypotheses

We hypothesize that:

(a) Chemoresistance is in part a consequence of reduced CDDP-induced apoptosis,

(b) FLIP modulates chemosensitivity by attenuating CDDP-induced caspase activation and apoptosis

(c) CDDP down-regulates FLIP through the proteasome pathway

(d) Itch is required for CDDP-induced FLIP ubiquitination and its proteasomal degradation in chemosensitive ovarian cancer cells,

(e) p53 is required for CDDP-induced, Itch-mediated FLIP ubiquitination, proteasomal degradation and apoptosis in chemosensitive ovarian cancer cells,

(f) Akt confers CDDP resistance in ovarian cancer cells in part through attenuation of p53-mediated, Itch-dependant FLIP degradation.

2.1.1 Specific Objectives

Specific Objectives of my studies are as follow:

a. To establish the role of FLIP in ovarian cancer chemoresistance. Specifically, we wish to investigate the role of FLIP in the regulation of apoptosis in these cells by comparing the effects of CDDP on FLIP content in chemosensitive ovarian cancer cells and their resistant counterparts, and to determine if and how FLIP is involved in the regulation of CDDP sensitivity by manipulation of FLIP content in these cells and assess its influence on CDDP sensitivity.

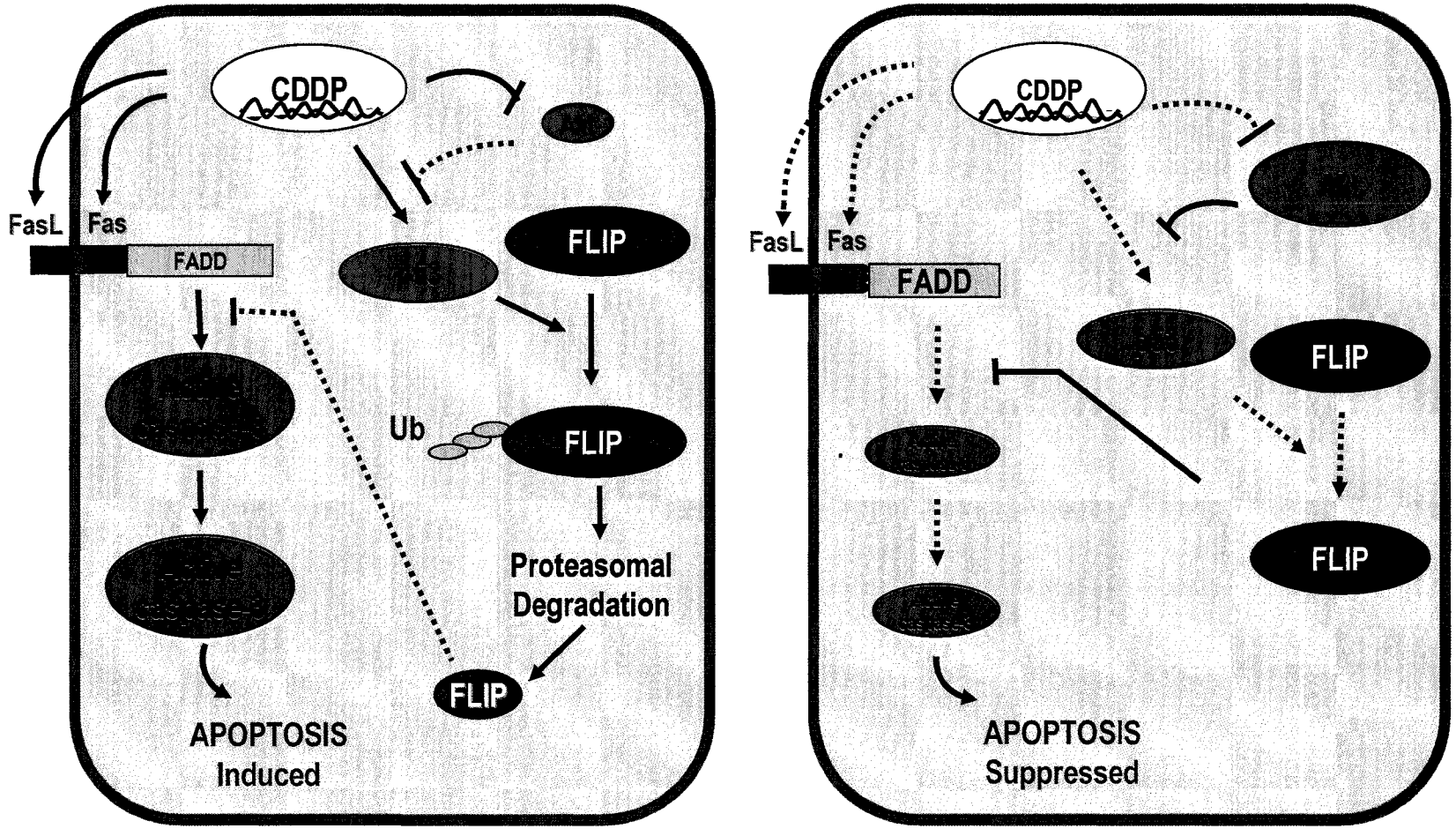
b. To establish the mechanisms by which CDDP-induced FLIP degradation in ovarian cancer cells, including the involvement of FLIP ubiquitination in CDDP sensitivity, and to examine the importance of p53 and Itch in the regulation of FLIP ubiquitination and its proteasomal degradation as well as the mechanisms by which these cell death mediators facilitate CDDP-induced apoptosis.

c. To elucidate the mechanisms implicated in the dysregulation of CDDP-induced FLIP ubiquitination and its proteasomal degradation in chemoresistant cells. Specifically, we are interested in the regulation of FLIP by additional cell fate determinant such as Akt and in determining how this survival factor regulates FLIP, thereby CDDP-induced apoptosis in ovarian cancer cells.

Figure 1: Hypothetical model illustrating the regulation of CDDP-induced FLIP degradation degradation in the control of apoptosis in chemosensitive and chemoresistant ovarian cancer cells.

In chemosensitive cells, CDDP up-regulates p53, induces FLIP degradation through proteasome pathway, CDDP then activates caspase-8 and caspase-3, and induces apoptosis. In resistant cells, Akt prevents CDDP-induced p53-mediated FLIP degradation, thereby suppressing caspase activation and apoptosis.

Hypothetical model illustrating role and regulation of FLIP in OVCA cells



Sensitive cell **Resistant cell**

Figure 1

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Possible role of FLICE-like inhibitory protein (FLIP) in chemoresistant ovarian cancer cells *in vitro*

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Abbreviations

DED, death effector domain; DMSO, dimethyl sulfoxide; FADD, Fas-associated death domain; FasL, Fas ligand; FBS, fetal bovine serum; FLICE, Fas-associated death domain-like interleukin-1b-converting enzyme; FLIP, FLICE-like inhibitory protein; $FLIP_L$, long isoform of FLICE-like inhibitory protein; $FLIP_S$, short isoform of FLICE-like inhibitory protein; GFP, green florescent protein; RPMI-1640, Roswell Park Memorial Institute 1640; siRNA, small interfering RNA; XIAP, X-linked inhibitor of apoptosis protein.

Contribution of Co-Authors

All studies were carried out under the supervision of Dr. Benjamin Tsang. All experimental work was conducted by Mohammad Reza Abedini, unless otherwise noted.
Drs. Xiaojuan Yan and Qing Qiu were involved in the experimental design, consulted on the writing and editing of the manuscript, and also contributed to the editing of the final manuscript.

Abstract

Chemoresistance is a major therapeutic problem and the current knowledge on cellular mechanisms involved is incomplete. In the present study, we have investigated the possible involvement of Fas-associated death domain-like interleukin-1β-converting enzyme (FLICE)-like inhibitory protein (FLIP) in ovarian cancer resistance by comparing chemosensitive (OV2008) and chemoresistant (C13*) ovarian cancer cells treated with cisplatin in vitro, and/ or transfected with FLIP sense cDNA or FLIP small interfering RNA (siRNA) and determining FLIP protein content, cleavage of caspase-8 and caspase-3 and apoptosis.

Cisplatin significantly decreased FLIP protein level, induced cleavage of caspase-8 and caspase-3 and apoptosis in a concentration-dependent manner in cisplatin-sensitive but not -resistant cells. While overexpression of FLIP-attenuated cisplatin-induced cleavage of caspase-8 and caspase-3 and apoptosis in chemosensitive cells, downregulation of FLIP in chemoresistant cells by siRNA increased apoptosis induced by cisplatin.

These results suggest that FLIP plays a significant role in the regulation of apoptosis in human ovarian cancer cells and their sensitivity to cisplatin. This cell survival factor may be an important determinant in chemoresistance in ovarian cancer and may serve as a molecular target for the development of novel therapy for chemoresistant ovarian cancer.

Introduction

Human ovarian epithelial cancer originates from a simple epithelium covering the surface of the ovary and accounts for approximately 90% of all human ovarian malignancies. It is the most lethal cause of the gynecological malignancies in the Western world. Although

platinum derivatives are the first-line chemotherapeutic agents for the treatment of ovarian cancer, chemoresistance remains a major therapeutic problem and the cellular mechanisms involved are poorly understood. Overcoming drug resistance is a key to successful treatment of ovarian cancer (Cheng et al., 2002).

Recent studies have demonstrated that chemoresistance results in part from an inability of the cells to undergo apoptosis in response to a therapeutic agent, and suppressed apoptosis by intracellular survival factors is important in the development of chemoresistance (Sasaki et al., 2000; Li et al., 2001; Hu et al., 2002; Yuan et al., 2003). A defect in the activation of the caspase cascade is an important etiological factor in the resistance of cancer cells to cytotoxic agents (Arts et al., 2000).

The Fas/Fas ligand (FasL) system has been recognized as an important pathway in drug-induced apoptosis and its deficient activation has been implicated in the development of chemoresistance (Schneiderman et al., 1999). Fas-associated death domain-like interleukin-1b-converting enzyme (FLICE)-like inhibitory protein (FLIP) is a Fas-associated death domain (FADD)-binding suppressor of apoptosis (Irmler et al., 1997). It exists in two spliced isoforms, derived from the c-FLIP gene. Its long isoform, long isoform of FLICE-like inhibitory protein ($FLIP_L$), is a 55-kDa protein containing two N-terminal death effectors domains (DED) and an inactive C-terminal caspase-like domain, whereas its short isoform ($FLIP_S$) contains only two DEDs (Irmler et al., 1997).

FLIP is an intracellular survival factor structurally similar to caspase-8, and modulates cell surface receptor-mediated cell death processes by inhibiting the activation of caspase-8 through DED–DED interaction (Kinoshita et al., 2000; Vignati et al., 2002; Kim et al., 2003; Wajant, 2003; Xiao et al., 2003). It has also been shown that $FLIP_L$ is

phosphorylated in resistant glioma cells and the phosphorylated $FLIP_L$ is recruited to DISC to inhibit TRAIL-induced apoptosis (Yang et al., 2003). Moreover, cisplatin decreased $FLIP_S$ content, inhibited $FLIP_L$ phosphorylation and facilitated TRAIL-mediated cell death in human melanoma cells (Song et al., 2003). These studies suggest that FLIP may play a role in the regulation of apoptosis in different cell types. Although the function of FLIP has been studied in different systems, its role in the chemoresistance in ovarian cancer remains to be determined.

In the present study, we have used a pair of chemosensitive and chemoresistant ovarian cancer cell lines as an *in vitro* model to examine the regulation of FLIP in cisplatin-mediated apoptosis and its possible role in cisplatin resistance in human ovarian epithelial cancer.

Results

Cisplatin-induced apoptosis in ovarian cancer cells is associated with decreased FLIP protein content and activation of caspase-8 and caspase-3

To compare the effects of different cisplatin concentrations (0, 2.5, 5, and 10 μM) on FLIP content and apoptosis in cisplatin-sensitive and cisplatin-resistant ovarian cancer cells, $FLIP_L$ and $FLIP_S$ protein content and cell nuclear morphology in OV2008 and C13* cells were assessed following cisplatin treatment by Western analysis and Hoechst nuclear staining, respectively. Ovarian cancer cells express both long ($FLIP_L$) and short ($FLIP_S$) isoforms of FLIP that migrate as a 55 and 28 kDa immunoreactive protein, respectively (Figure 1a). Cisplatin treatment also resulted in a concentration-dependent decrease in the protein content of both isoforms in cisplatin-sensitive but not -resistant cells ($P<0.001$; Figure 1a and b). In addition, the presence of cisplatin induced morphological features of

apoptosis including decreased cell volume, chromatin condensation, and nuclear fragmentation in chemosensitive cells (OV2008). The increase in apoptotic cell number in response to cisplatin was concentration dependent and significant at 2.5 μM (15 vs 4%; $P<0.001$; Figure 1c). In contrast, its resistant variant, C13*, did not undergo cellular or nuclear morphological changes indicative of apoptosis in response to cisplatin ($P>0.05$; Figure 1c). To determine if the observed downregulation of FLIP content by cisplatin was associated with the activation of caspase-8 and caspase-3, extracts of the ovarian cancer cells (OV2008, C13*) cultured in the absence and presence of cisplatin were assessed for caspase cleavage by Western blotting (Figure 1d). Cisplatin treatment decreased the intact caspase-8 and caspase-3 contents and increased their respective cleavage fragments in sensitive (OV2008) but not resistant (C13*) cells. Two way ANOVA on the changes in the cleavage index of caspase-8 (ratio of intensity of cleavage fragments to that of the intact enzyme) indicates a significant cell effect ($P<0.001$), concentration effect ($P<0.001$), as well as an interaction between these two factors ($P<0.001$) brought about by a marked decrease in the intact enzyme and increase in the cleavage fragments in a concentration-dependent manner in the sensitive but not resistant cells (Figure 1d). Similarly, ANOVA of the cleavage indices for caspase-3 (Figure 1d) also showed a significant cell effect ($P<0.001$), concentration effect ($P<0.001$) and interaction between cell and concentration ($P<0.001$). Taken together, these results are consistent with the hypothesis that cisplatin induces apoptosis in sensitive ovarian cancer cells by decreasing FLIP content and that FLIP may play an important role in regulating cisplatin sensitivity in ovarian cancer cells.

Figure 1: Cisplatin decreases FLIP protein content, activates caspase-8 and caspase-3, and induces apoptosis in cisplatin sensitive ovarian cancer cells.

OV2008 and C13* cells (4.8 ×105) were cultured for 24 h with increasing concentrations of cisplatin (2.5–10 μM; DMSO as control). (a) Representative Western blot showing the effect of cisplatin on FLIPL and FLIPS content. (b) Quantification of FLIPL and FLIPS content relative to GAPDH (expressed as fold of internal control); *** $P<0.001$ (relative to C13* at respective cisplatin concentration). (c) Apoptotic response to cisplatin (expressed as % total number of cells assessed); *** $P<0.001$ (relative to C13* at respective cisplatin concentration). (d) Representative Western blots showing cleavage of caspase-8 and caspase-3. The membrane from panel a was reprobed and the relative protein loading onto each lane is indicated by the GAPDH immunoblot shown in panel a. Intensity of both intact and cleavage fragment of caspase-8 and caspase-3 were quantified and the differences in the extent of cleavage in each experimental group was evaluated by comparing the cleavage index of each group (defined as ratio of the level of cleavage fragments to that of the intact enzyme). ** $P<0.01$, *** $P<0.001$ (relative to C13* at respective cisplatin concentration; three replicate experiments).

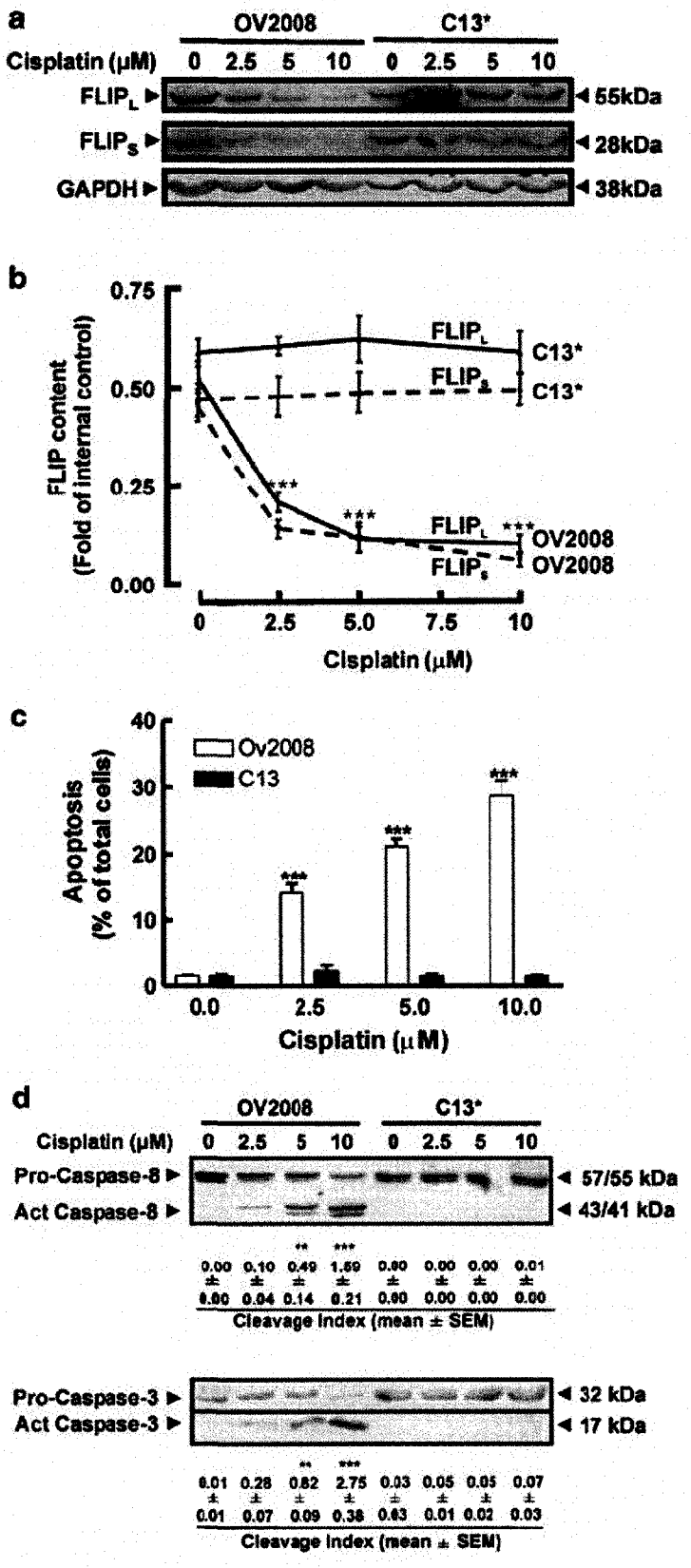
a
OV2008
C13*
Cisplatin (μM)
0 2.5 5 10 0 2.5 5 10
FLIP$_L$
FLIP$_S$
GAPDH
55kDa
28kDa
38kDa
b
FLIP content
(Fold of internal control)
0.75
0.50
0.25
0.00
FLIP$_L$ C13*
FLIP$_S$ C13*
FLIP$_L$ OV2008
FLIP$_S$ OV2008
0 2.5 5.0 7.5 10
Cisplatin (μM)
c
Apoptosis
(% of total cells)
40
30
20
10
0
Ov2008
C13
0.0 2.5 5.0 10.0
Cisplatin (μM)
d
OV2008
C13*
Cisplatin (μM)
0 2.5 5 10 0 2.5 5 10
Pro-Caspase-8
Act Caspase-8
57/55 kDa
43/41 kDa
0.00 ± 0.00
0.10 ± 0.04
0.49 ± 0.14
1.59 ± 0.21
0.00 ± 0.00
0.00 ± 0.00
0.00 ± 0.00
0.01 ± 0.00
Cleavage Index (mean ± SEM)
Pro-Caspase-3
Act Caspase-3
32 kDa
17 kDa
0.01 ± 0.01
0.28 ± 0.07
0.82 ± 0.09
2.75 ± 0.36
0.03 ± 0.03
0.05 ± 0.01
0.05 ± 0.02
0.07 ± 0.03
Cleavage Index (mean ± SEM)

Figure 1

Overexpression of FLIP induces resistance of ovarian cancer cells to cisplatin

To determine if $FLIP_L$ and $FLIP_S$ indeed play a role in the regulation of cisplatin sensitivity, we investigated the effect of cisplatin on apoptosis in cisplatin-sensitive human ovarian cancer cells following $FLIP_L$ and $FLIP_S$ overexpression. OV2008 cells were transiently transfected with $FLIP_L$ and $FLIP_S$ cDNA (1 μg) or LacZ and green florescent protein (GFP) (as respective control) and treated 24 h thereafter with cisplatin (2.5, 5, and 10 μM; 24 h) or dimethyl sulfoxide (DMSO) (vehicle control). Although cisplatin reduced $FLIP_L$ protein content in OV2008 cells transfected with LacZ, overexpression of the protein by cDNA attenuated cisplatin-induced apoptosis ($P<0.001$; Figure 2a). Likewise, addition of cisplatin to OV2008 activated both caspase-8 and caspase-3, as evident by a concentration-dependent increase in the cellular content of their respective cleaved fragments (i.e. 43, 41, 18, and 17 kDa, respectively), and $FLIP_L$ overexpression markedly attenuated these responses compared to LacZ control. Two-way ANOVA of the cleavage indices for both caspase-8 and caspase-3 (Figure 2b) shows significant effects of $FLIP_L$ sense cDNA ($P<0.001$), cisplatin concentration ($P<0.001$), and interactions between the two factors ($P<0.001$). Since cisplatin significantly decreased $FLIP_S$ protein level in chemosensitive cells (OV2008) but not in its resistant counterpart (C13*), we have further investigated the role of $FLIP_S$ in ovarian cancer chemoresistance by assessing the influence of cisplatin on apoptosis in OV2008 human ovarian cancer cells after $FLIP_S$ transfection. Like $FLIP_L$, transient transfection of OV2008 with $FLIP_S$ cDNA increased its protein content relative to the vector expressing GFP (control; $P<0.001$) and attenuated cisplatin-induced apoptosis ($P<0.001$; Figure 2c). Compared to GFP control, $FLIP_S$ overexpression was accompanied by a marked decrease in cisplatin-induced activation of caspase-8 and caspase-3, with significant changes

Figure 2: Overexpression of FLIP induces resistance of ovarian cancer cells (OV2008) to cisplatin.

(a) FLIPL content and apoptotic response in chemosensitive ovarian cancer cells (OV2008) to cisplatin ((2.5–10 μM) for 24 h (DMSO as control)) following transient transfection with pEF6/V5-His vector (1 μg, 24-h) containing FLIPL cDNA or LacZ (control); *P<0.05, **P<0.01, ***P<0.001 (relative to LacZ at respective cisplatin concentration). FLIPL overexpression was confirmed by Western blot. (b) Representative Western blot showing cleavage of caspase-8 and caspase-3. Intensity of both intact and cleavage fragments of caspase-8 and caspase-3 were quantified and differences in the extent of cleavage in each experimental group was evaluated as described in Figure 1d. *P<0.05, ***P<0.001 (relative to LacZ at respective cisplatin concentration; three replicate experiments). (c) FLIPS content and apoptotic response in chemosensitive ovarian cancer cells (OV2008) to cisplatin ((2.5–10 μM) for 24 h (DMSO as control)) following transient transfection with pcDNA3.1 vector (1 μg, 24-h) containing FLIPS cDNA or GFP (control); **P<0.01, ***P<0.001 (compared with GFP at respective cisplatin concentration). FLIPS overexpression was confirmed by Western blot. (d) Caspase-8 and caspase-3 cleavage as assessed by Western blot **P<0.01, ***P<.001 (relative to GFP at respective cisplatin concentration; three replicate experiments). The membranes from panels a and c were reprobed and the relative protein loading onto each lane in panels b and d is indicated by the GAPDH immunoblot shown in panels a and c, respectively.

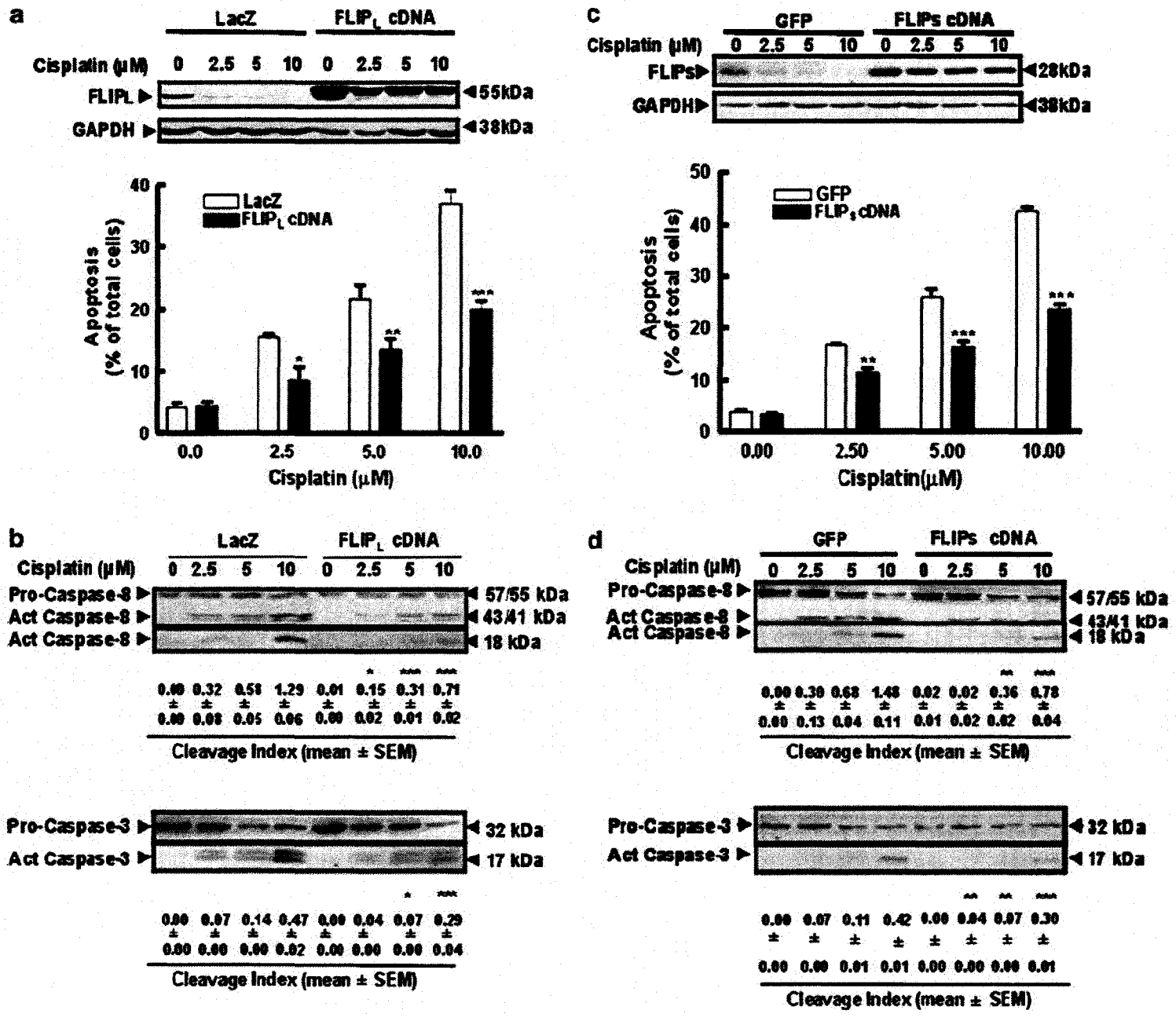
a
LacZ
FLIP$_L$ cDNA
Cisplatin (μM) 0 2.5 5 10 0 2.5 5 10
FLIPL
GAPDH
55kDa
38kDa
Apoptosis (% of total cells)
LacZ
FLIP$_L$ cDNA
Cisplatin (μM)
c
GFP
FLIPs cDNA
Cisplatin (μM) 0 2.5 5 10 0 2.5 5 10
FLIPs
GAPDH
28kDa
38kDa
Apoptosis (% of total cells)
GFP
FLIP$_S$ cDNA
Cisplatin(μM)
b
LacZ
FLIP$_L$ cDNA
Cisplatin (μM) 0 2.5 5 10 0 2.5 5 10
Pro-Caspase-8
Act Caspase-8
Act Caspase-8
57/55 kDa
43/41 kDa
18 kDa
0.00 ± 0.00 0.32 ± 0.08 0.58 ± 0.05 1.29 ± 0.06 0.01 ± 0.00 0.15 ± 0.02 0.31 ± 0.01 0.71 ± 0.02
Cleavage Index (mean ± SEM)
Pro-Caspase-3
Act Caspase-3
32 kDa
17 kDa
0.00 ± 0.00 0.07 ± 0.00 0.14 ± 0.00 0.47 ± 0.02 0.00 ± 0.00 0.04 ± 0.00 0.07 ± 0.00 0.29 ± 0.04
Cleavage Index (mean ± SEM)
d
GFP
FLIPs cDNA
Cisplatin (μM) 0 2.5 5 10 0 2.5 5 10
Pro-Caspase-8
Act Caspase-8
Act Caspase-8
57/55 kDa
43/41 kDa
18 kDa
0.00 ± 0.00 0.30 ± 0.13 0.68 ± 0.04 1.48 ± 0.11 0.02 ± 0.01 0.02 ± 0.02 0.36 ± 0.02 0.78 ± 0.04
Cleavage Index (mean ± SEM)
Pro-Caspase-3
Act Caspase-3
32 kDa
17 kDa
0.00 ± 0.00 0.07 ± 0.00 0.11 ± 0.01 0.42 ± 0.01 0.00 ± 0.00 0.04 ± 0.00 0.07 ± 0.00 0.30 ± 0.01
Cleavage Index (mean ± SEM)

Figure 2

in cleavage indices ($P<0.001$; Figure 2d) for both caspases (effects of $FLIP_S$ sense cDNA and cisplatin concentration, and interactions between the two factors). Taken together, these findings suggest that FLIP may play an important role in regulating the sensitivity of ovarian cancer cells to cisplatin treatment.

Downregulation of FLIP sensitizes chemoresistant ovarian cancer cells to cisplatin

If FLIP is indeed a determinant of cisplatin resistance in ovarian cancer cells, it is expected that FLIP downregulation should sensitize the cisplatin-resistant ovarian cells C13* to the cytotoxic action of the chemotherapeutic agent. Cisplatin-resistant ovarian cancer cells (C13*) were transfected with either $FLIP_L$ small interfering RNA (siRNA) (0, 12.5, 25, 50, and 100 nM) or its scrambled sequence (control) and treated 24 h thereafter with cisplatin (10 μM) or DMSO (vehicle). ANOVA indicates a significant effect of siRNA ($P<0.001$) and concentration ($P<0.001$) as well as a significant interaction between the two factors ($P<0.001$; Figure 3a). Transfection of $FLIP_L$ siRNA significantly decreased $FLIP_L$ content ($P<0.001$; 50 and 100 nM) but had no effect on $FLIP_S$ levels ($P>0.05$). Expression of the scrambled sequence also failed to influence the protein content of both the long and short forms of FLIP. As observed in its effect on $FLIP_L$ content, $FLIP_L$ siRNA significantly increased apoptotic cell death in a concentration-dependent manner ($P<0.001$; Figure 3b). While a significant increase in apoptosis was noted at 12.5 nM of the siRNA ($P<0.001$), only 10% of the total cells had undergone apoptosis at the highest concentration tested (100 nM). To further examine the involvement of $FLIP_S$ in the regulation of apoptosis in ovarian cancer cells, we extended our investigation to assess the effect of cisplatin on apoptosis in cisplatin-resistant human ovarian cancer cells following $FLIP_S$ downregulation. Since cDNA for

Figure3: Downregulation of FLIP sensitizes chemoresistant ovarian cancer cells to cisplatin.

Chemoresistant ovarian cancer cells C13* were transfected with $FLIP_L$ siRNA (sequences described in Materials and methods Section) or a scrambled sequence (0–100 nM; 24 h), and then treated with cisplatin (10 μM, 24 h; a and b). (a) Top panel shows representative Western blot confirming downregulation of $FLIP_L$ but not $FLIP_S$; bottom panel shows quantification of $FLIP_L$ content relative to GAPDH (expressed as fold of internal control); ***P<0.001 (relative to a scrambled sequence at the respective concentration). (b) Cisplatin-mediated apoptotic response to siRNA (expressed as % total number of cells assessed), ***P<0.001, compared with a scrambled sequence at the respective concentration. In addition, C13* cells were transfected with $FLIP_L$ or $FLIP_{L+S}$ siRNA (sequences described in Materials and methods Section) or a scrambled sequence (100 nM; 48 h), and then treated with different concentrations of cisplatin (10–40 μM (DMSO as control), 24 h; c and d). (c) Top panel shows representative Western blot depicting changes in $FLIP_L$ and $FLIP_S$ content following siRNA transfection; bottom panel shows quantification of $FLIP_L$ and $FLIP_S$ content relative to GAPDH (expressed as fold of internal control); $^{++}$P<0.01 compared with $FLIP_L$ siRNA; **P<0.01, ***P<0.001, compared with a scrambled sequence. (d) Cisplatin mediated apoptotic response to siRNA (expressed as % total number of cells assessed); ***P<0.001 (compared with a scrambled sequence at the respective concentration of cisplatin); $^{++}$P<0.01 (compared with $FLIP_L$ siRNA at 40 μM cisplatin).

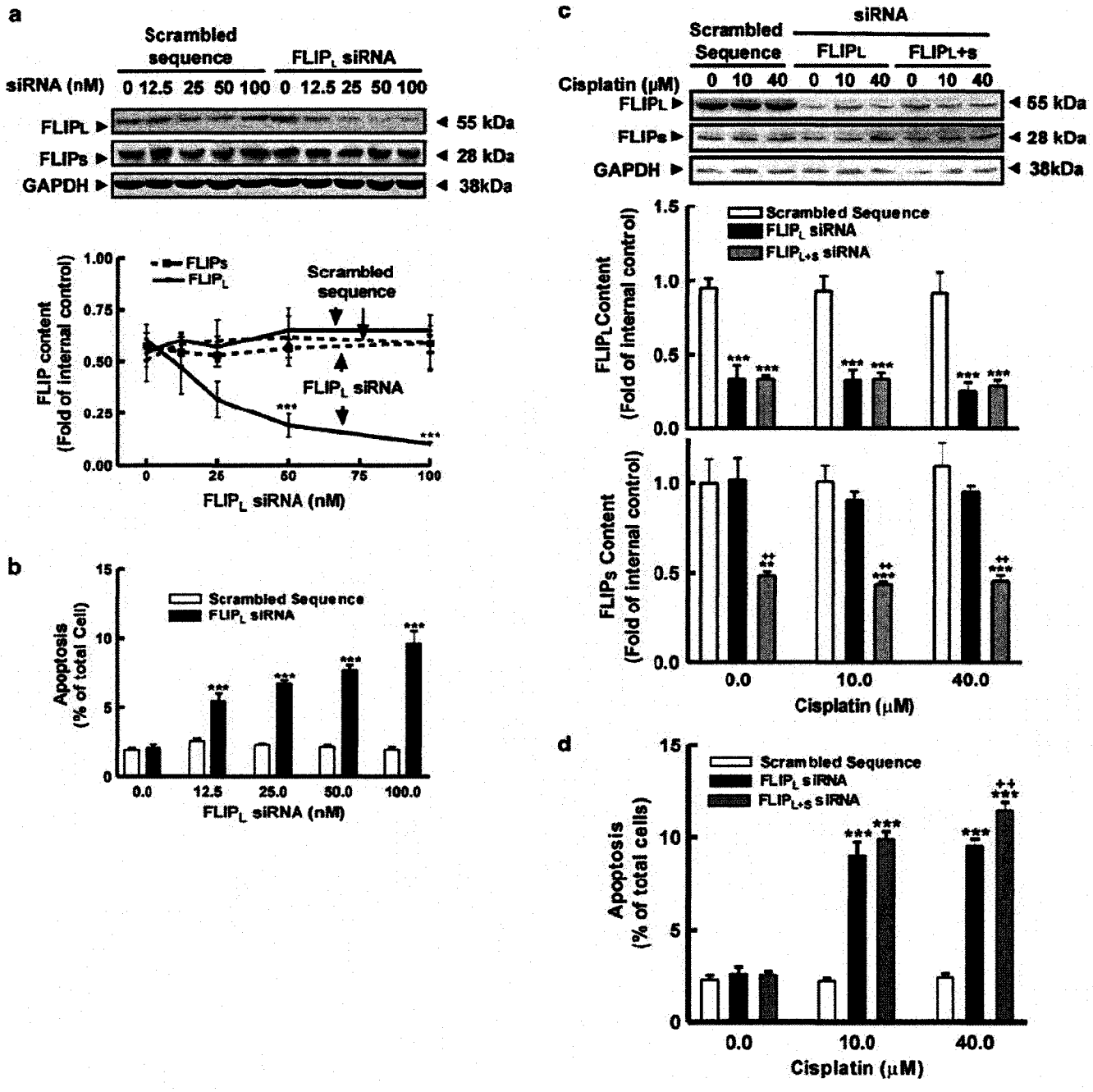
a
Scrambled sequence
FLIP$_L$ siRNA
siRNA (nM) 0 12.5 25 50 100 0 12.5 25 50 100
FLIPL
FLIPs
GAPDH
55 kDa
28 kDa
38kDa
FLIPs
FLIP$_L$
Scrambled sequence
FLIP$_L$ siRNA
FLIP content (Fold of internal control)
FLIP$_L$ siRNA (nM)
b
Scrambled Sequence
FLIP$_L$ siRNA
Apoptosis (% of total Cell)
FLIP$_L$ siRNA (nM)
c
siRNA
Scrambled Sequence
FLIPL
FLIPL+s
Cisplatin (μM) 0 10 40 0 10 40 0 10 40
FLIPL
FLIPs
GAPDH
55 kDa
28 kDa
38kDa
Scrambled Sequence
FLIP$_L$ siRNA
FLIP$_{L+S}$ siRNA
FLIP$_L$Content (Fold of internal control)
FLIPs Content (Fold of internal control)
Cisplatin (μM)
d
Scrambled Sequence
FLIP$_L$ siRNA
FLIP$_{L+S}$ siRNA
Apoptosis (% of total cells)
Cisplatin (μM)

Figure 3

$FLIP_S$ share considerable homology (~75%) with $FLIP_L$ cDNA, it was technically difficult to design an siRNA for $FLIP_S$ that does not interfere with the expression of the long isoform. Thus, in the present study, cisplatin-resistant ovarian cancer cells (C13*) were transfected with $FLIP_L$ siRNA, $FLIP_{L+S}$ siRNA, or scrambled sequence as control (100 nM) and treated 48 h thereafter with cisplatin (0, 10, and 40 μM; 24 h; Figure 3c and d). Relative to the scrambled sequence, $FLIP_L$ siRNA decreased protein content of $FLIP_L$ ($P<0.001$ (vs basal and cisplatin at 10 and 40 μM); Figure 3c) but not of $FLIP_S$ ($P>0.05$; Figure 3c) and significantly increased cisplatin-induced apoptosis ($P<0.001$; Figure 3d). $FLIP_{L+S}$ siRNA decreased protein content of $FLIP_L$ ($P<0.001$) and $FLIP_S$ ($P<0.001$) and induced apoptosis to a significantly but only slightly greater extent than $FLIP_L$ siRNA at 40 μM cisplatin (12% vs. 9%; Figure 3d; $P<0.01$). Taken together, these findings suggest that FLIP is an important factor in cisplatin chemoresistance, although the long isoform may have a relatively more prominent role.

Discussion

In the present study, we have shown that $FLIP_L$ and $FLIP_S$ are expressed in human ovarian epithelial cancer cell lines and that cisplatin decreases $FLIP_L$ and $FLIP_S$ protein content, activates caspase-8 and caspase-3, and induces apoptosis in chemosensitive ovarian cancer cells (OV2008) but not in its resistant variant (C13*). We have also demonstrated that overexpression of $FLIP_L$ and $FLIP_S$ effectively attenuates cisplatin-induced apoptosis in the sensitive cells, and that downregulation of FLIP in the chemoresistant cells by siRNA sensitizes the cells to cisplatin-induced apoptosis. These findings demonstrate a key

regulatory role of FLIP on cisplatin-induced apoptosis in ovarian cancer cells and suggest that FLIP may be an important determinant in cisplatin resistance.

FLIP is an intracellular apoptosis suppressor protein. Although the inhibitory effects of FLIP on apoptosis induced by a variety of stimuli have been demonstrated (Kinoshita et al., 2000; Wang et al., 2000; Xiao et al., 2003), its role in the regulation of cisplatin sensitivity in ovarian cancer is unknown. Previous studies have shown that FLIP interrupts apoptotic signaling by interaction with FADD and caspase-8, thus blocking the activity of caspase-8 (Irmler et al., 1997; Kinoshita et al., 2000; Vignati et al., 2002; Wajant, 2003; Xiao et al., 2003), suggesting that the intracellular level of FLIP may determine the sensitivity of tumor cells to a variety of proapoptotic stimuli such as TNFa, FasL, and TRAIL. Our present studies have shown that cisplatin decreases $FLIP_L$ and $FLIP_S$ contents and induces cell death in chemosensitive cells but not in its resistant counterpart. This effect of cisplatin is associated with the activation of caspase-8 and caspase-3. $FLIP_L$ and $FLIP_S$ overexpression not only suppresses cisplatin-induced apoptosis but also inhibits caspase-8 and caspase-3 activation, suggesting that FLIP may be an important determinant in cisplatin sensitivity in part by preventing caspase activation. In this context, $FLIP_L$ overexpression in B-cell lines (A20) has been shown to confer resistance to FasL-mediated cell kills (Wang et al., 2000). Moreover, $FLIP_L$ is constitutively and more intensely expressed in colon cancer than in normal colon tissues, possibly providing a mechanism by which tumor cells escape apoptotic cell death during chemotherapy (Ryu et al., 2001). However, unlike the present studies that examine both $FLIP_L$ and $FLIP_S$, these studies have only studied the role and regulation of the long isoform as the antiapoptotic protein and the significance of $FLIP_S$ remains to be determined.

Chemoresistance observed in C13* cells seems to be FLIP dependent. The failure of cisplatin to downregulate FLIP and induce apoptosis in the cisplatin-resistant cells suggests that this survival factor may be etiologically relevant to chemoresistance; however, how FLIP is involved in the phenomenon is not clear. Recent studies have shown that phenoxodiol-induced apoptosis in ovarian cancer cells is associated with caspase-8 activation and a reduction in FLIP protein content, suggesting that phenoxodiol enhances the sensitivity of ovarian cancer cells to Fas-induced apoptosis in part by removal of FLIP action and activation of caspase-8 (Kamsteeg et al., 2003). However, while these findings provided an interesting correlation between the downregulation of FLIP and the induction of apoptosis, no direct evidence on the possible involvement of FLIP in chemoresistance in ovarian cancer was presented. The present studies provide the first evidence for a role of FLIP in the regulation of chemosensitivity in ovarian cancer. We have shown that FLIP downregulation in the resistant ovarian cancer cells by $FLIP_L$ and $FLIP_{L+S}$ siRNA expression sensitizes the cells to the cytotoxic action of cisplatin in a concentration-dependent manner. In addition, we have also observed that overexpression of $FLIP_L$ and $FLIP_S$ in chemosensitive cells by sense cDNA rendered them resistant to cisplatin-induced apoptosis. Our findings are supported by the observations that downregulation of FLIP by antisense oligonucleotides or siRNA sensitizes activated mast cells (Yoshikawa et al., 2000), cisplatin-resistant HeLa cells (Kamarajan et al., 2003), and osteosarcoma cells (MG-63) (Kinoshita et al., 2000) to cisplatin- or Fas-mediated apoptosis, although the relative importance of the two FLIP isoforms were not examined in these reports. Taken together, these findings suggest that FLIP is an important determinant in cisplatin resistance.

While some studies indicated that $FLIP_S$ is a more potent inhibitor of apoptosis than $FLIP_L$ in various systems (Kirchhoff et al., 2000; Krueger et al., 2001; Bin et al., 2002), others reported that $FLIP_L$ plays a more important role in the regulation of apoptosis in other cells by different stimuli, including B-cell line and colon cancer cells (Wang et al., 2000; Ryu et al., 2001). In the present study, since cisplatin decreased both $FLIP_L$ and $FLIP_S$ content and their overexpression markedly attenuated cisplatin-induced apoptosis and caspase activation, it is difficult to determine which FLIP isoform plays a more important role in regulating cisplatin sensitivity in ovarian cancer cells. As $FLIP_{L+S}$ siRNA induced apoptosis to a significantly but only slightly greater extent than $FLIP_L$ siRNA at 40 μM cisplatin (12 vs 9%; Figure 3d; $P<0.01$), it is possible that the relatively lower incidence of apoptosis could be due to the relatively less effective $FLIP_S$ downregulation (50–60% ($FLIP_S$) vs ~75% ($FLIP_L$), and that a minimum threshold (~75% decrease) must be reached in order to detect additional apoptotic response. Alternatively, cisplatin has multiple effects in the induction of cell death, and FLIP may account for only one component of the intracellular mechanisms leading to cisplatin resistance in ovarian cancer cells. For example, in addition to suppressing $FLIP_L$ and $FLIP_S$ expression, cisplatin has been shown to upregulate the expression of a number of apoptosis inducers including p53 (Fraser et al., 2003), Fas and FasL (Schneiderman et al., 1999), and to downregulate antiapoptotic proteins such as X-linked inhibitor of apoptosis protein (XIAP) (Li et al., 2001) and protein kinase B/Akt (Asselin et al., 2001). Indeed, whereas overexpression of XIAP or Akt rendered chemosensitive ovarian cancer cells resistant to cisplatin, downregulation of these proteins in chemoresistant counterparts by antisense or dominant-negative expression facilitated cisplatin-induced apoptosis (Asselin et al., 2001; Li et al., 2001; Fraser et al., 2003), suggesting that these cell

survival factors may also be involved in the regulation of cisplatin sensitivity. Since XIAP is acting downstream of FLIP in the caspase-mediated death pathway, the possibility exists that XIAP and Akt are key determinants of cisplatin resistance, and that FLIP-mediated resistance may represent a minor component and is independent of XIAP and Akt. Alternately, the relatively low incidence of apoptosis could be due to insufficient FLIP downregulation, as the siRNA was only able to suppress FLIP content by about 60% as compared to ~90% by cisplatin.

In the present study, we have demonstrated that cisplatin decreases FLIP protein content in human ovarian cancer cells, although precisely how cisplatin elicits this response is not known. The decrease in $FLIP_L$ and $FLIP_S$ levels did not appear to be due to a general cytotoxic effect of the anticancer agent, since earlier studies under identical experimental conditions indicate that cisplatin treatment increases Fas and FasL expression (Schneiderman et al., 1999) and activates downstream caspases (Asselin et al., 2001). Moreover, DNA damage-inducible genes, such as gadd153, gadd45, p21, and c-jun, have also been shown to be upregulated by cisplatin in this cell system (Delmastro et al., 1997). The action of cisplatin is associated with the formation of DNA adducts (Fichtinger-Schepman et al., 1985; Eastman, 1986) and its ability to trigger apoptosis. Apoptosis induced by cisplatin can be inhibited by cycloheximide, suggesting that synthesis of new proteins may be required (Barry et al., 1990). FLIP, with a consensus sequence of a caspase-3-mediated cleavage site (DXXD), is a substrate of caspase-3 and co-treatment of cisplatin and TRAIL in head and neck squamous cell carcinoma decreased $FLIP_S$ content through caspase-3-mediated cleavage and –induced apoptosis (Kim et al., 2003). The latter response was inhibited by $FLIP_S$ overexpression. Fas-mediated $FLIP_L$ cleavage has also been demonstrated in B cells

(Hennino et al., 2000). Alternatively, it is possible that the decrease in FLIP content subsequent to cisplatin challenge may be a consequence of proteasome-mediated processing, initially involving ubiquitination and subsequently degradation of the FLIP molecule (Fukazawa et al., 2001). The regulation of FLIP in human ovarian cancer cells remains to be investigated. Moreover, previous studies have demonstrated that cisplatin decreased $FLIP_S$ expression in melanoma cells (Song et al., 2003). Whether the downregulation of FLIP content in ovarian cancer cells by cisplatin involves suppressed gene transcription and/or post-translational processing via caspase cleavage or proteasome degradation, remains to be determined.

In summary, we have tested the hypothesis that the inability of cisplatin to downregulate FLIP may in part be a contributing factor for chemoresistance in human ovarian cancer. We hereby report that: (1) cisplatin decreases FLIP content and induces apoptosis in the cisplatin-sensitive cells but not in the –resistant counterpart, (2) overexpression of FLIP by cDNA transfection is effective in attenuating cisplatin-induced apoptosis in chemosensitive cells, and (3) FLIP siRNA expression facilitates apoptotic cell death in chemoresistant counterpart induced by cisplatin. These findings suggest that downregulation of FLIP may increase the sensitivity of chemoresistant cells to cisplatin and may be a potential therapeutic strategy for cisplatin-resistant ovarian cancer associated with FLIP overexpression.

Materials and methods

Reagents

Roswell Park Memorial Institute 1640 (RPMI 1640) medium supplemented with 10% (vol/vol) fetal bovine serum (FBS), streptomycin (100 mg/ml), penicillin (100 units/ml), and fungizone (0.625 mg/ml) were used for cell cultures (all from Life Technologies Inc., Burlinton, Ontario, Canada). Cisplatin and DMSO were from Sigma. pcDNA3.1/containing GFP expression vector and pEF6/V5-His containing LacZ were from Invitrogen Corporation (Carlsbad, CA, USA). siRNA for $FLIP_L$ and $FLIP_{L+S}$ were supplied by Dharmacon (Dallas, TX, USA). Primary antibodies used were anti-FLIP mouse monoclonal IgG (ALX-804-428, Clone NF6; Alexis Biochemicals, San Diego, CA, USA), anti-caspase-8 mouse monoclonal IgG (9746, Clone 1C12; Cell signaling, Beverly, MA, USA), anti-caspase-3 rabbit polyclonal IgG (556425; BD Pharmingen, San Diego, CA, USA), and anti-GAPDH mouse monoclonal IgG (ab8245, Clone 6C5; Abcam, Cambridge, MA, USA).

Cell culture

Cisplatin-sensitive (OV2008) and its -resistant variant (C13*) cell lines (4.8×10^5 cells/well) were plated on six-well plates for 20 h in RPMI 1640 with 10% FBS (Asselin et al., 2001).

Preparing of plasmid cDNA

The cDNA fragment ending the open-reading frame of human $FLIP_L$ was amplified by PCR reaction using a set of primers: 5′-TGTGTAGGAGAGGATAAGTTTC-3′ (forward) and 5′-GAGTAGGATGGCTG CTG AAG-3′ (reverse) and the MGC: 2044 clone containing the full length of $FLIP_L$ cDNA as a template. The primers were designed based on the human $FLIP_L$

sequences (GeneBank Accession no. U97074) and the PCR product was subcloned into pEF6/V5-His-TOPO expression vector using pEF6/V5-His-TOPOs TA cloning kit according to the manufacturer's instruction (Invitrogen, Carlsbad, CA, USA). The sense $hFLIP_L$–pEF6/V5-His constructs were verified by automated sequence analysis. The cDNA fragment ending the open-reading frame of human FLIPs was prepared previously (Xiao et al., 2003).

Transient transfection

OV2008 cells (2.4×10^5) were seeded in six-well plates and transfected the following day with 0.1 mg of pcDNA3.1/CT-GFP vector alone, pEF6/V5-His vector alone, or pcDNA3.1/CT-GFP vectors containing $FLIP_S$ and pEF6/V5-His vector containing $FLIP_L$ cDNA, using the Lipofectamine Plus Transfection reagents (Invitrogen, Carlsbad, CA, USA). At 24 h after transfection, cells were treated with cisplatin (2.5, 5, and 10 μM) or DMSO for another 24 h and then harvested for analysis. The overall transfection efficiency for $FLIP_S$ and $FLIP_L$ assessed by GFP expression and a 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside-staining assay against LacZ construct transfected cells, was 70 and 40%, respectively.

siRNA transfection

The scrambled sequences (controls) and siRNAs (supplied by Dharmacon RNA Technologies, Dallas, TX, USA) used in the present study include: (a) smart pool siRNA for $FLIP_L$ ($FLIP_L$ siRNA) containing four individual smart pools specific for the nucleotides 1093–1111, 1103–1120, 1206–1224, and 1455–1473 of $FLIP_L$ gene (Accession no. U97074), and (b) smartpool siRNA for $FLIP_{L+S}$ ($FLIP_{L+S}$ siRNA) specific for nucleotides 411–429, 704–722, 819–837, and 864–882 of $FLIP_L$ gene (Accession no. U97074) and nucleotides

322–340, 615–633, 730–748, 775–793 of $FLIP_S$ gene (Accession no. U97075). C13* cells ($1.8–2.4\times10^5$/well) were seeded in six-well plates and transfected on the following day with 0–100 nM $FLIP_L$ and $FLIP_{L+S}$ siRNA, using RiboJuice siRNA Transfection reagents (Novagen, San Diego, CA, USA). At 24–48 h thereafter, cells were treated for 24 h with cisplatin (10, 40 μM) or DMSO (vehicle) and harvested for analysis.

Determination of apoptosis

At the end of the culture period, cells attached to the growth surface were removed by trypsin treatment (trypsin (0.05%), EDTA (0.53 mM); 37 °C, 1 min). Attached and detached cells were pooled, pelleted, and resuspended in neutral-buffered formalin (10%) containing Hoechst 33258 dye (6.25 ng/ml). At 24 h thereafter, cells were spotted onto slides and assessed for typical apoptotic nuclear morphology (nuclear shrinkage, condensation, and fragmentation) under a fluorescence microscope with appropriate filter combination (DAPI filter). At least 200 cells/treatment group were counted and selected fields and blinded slides assessed randomly to avoid experimental bias (Sasaki et al., 2000).

Protein extraction and Western blotting

Cells were sonicated in lysis buffer containing 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 0.1% NP-40, PBS, 1mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, and 1mM $Na_3(VO_4)$. Equivalent amounts of total protein (50–80 μg) were loaded onto 12% acrylamide gels, separated by electrophoresis and electrotransferred onto nitrocellulose membranes (Xiao et al., 2003). Loading between lanes was assessed by Ponca-S staining. Membranes were blocked in Blotto (5% skim milk in Tris-buffered saline-Tween; 1 h) and

subsequently incubated with primary antibodies (anti-FLIP, 1:500; anti-caspase-8, 1:1000; anti-caspase-3 1:2000, for 0.5 h at room temperature with anti-GAPDH, 1:20 000) in Blotto for 16–20 h at 4 °C. Primary antibodies were detected with horseradish peroxidase-conjugated goat IgG raised against the corresponding species (goat anti-rabbit for anti-caspase-3 (1 : 4000) and goat anti-mouse for anti-FLIP (1 : 1000), anticaspase-8 (1 : 2000), and anti-GAPDH (1 : 20 000)) in Blotto (1 h, RT; Schneiderman et al., 1999). Peroxidase activity was detected by the Enhanced Chemiluminescence Detection Kit, recorded on Hyper Film MP (both from Amersham Pharmacia Biotech Arlington, IL, USA) and analysed (Scion Image software, Scion Inc). To quantify changes in FLIP protein content due to treatment, the ratio of the signal of FLIP and that of GAPDH of the corresponding lane was calculated and expressed as fold of the value determined from an internal standard (interassay pool) to correct for loading differences between lanes and variability between replicate experiments, respectively.

Statistical analysis

Results are expressed as the mean ± s.e.m. of at least three independent experiments. Data were analysed using one- or two-way ANOVA and with Bonferoni post-test to assess differences between experimental groups (PRISM 4.0; Graph-Pad Software Inc.). Statistical significance was inferred at ($P<0.05$).

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Cisplatin Induces p53-dependent FLICE-like Inhibitory Protein Ubiquitination in Ovarian Cancer Cells

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Running Title: Regulation of FLIP Ubiquitination by p53 and Itch

Keywords: FLIP; p53; CDDP; Itch; ubiquitin-proteasome pathway; chemoresistance; ovarian cancer

Abbreviations: CDDP, cis-diaminedichloroplatinum; DED, death effector domain; DMSO, dimethyl sulfoxide; FADD, Fas-associated death domain; FBS, fetal bovine serum; FLICE, Fas-associated death domain-like interleukin-1β-converting enzyme; FLIP, FLICE-like inhibitory protein; $FLIP_L$, long isoform of FLIP; $FLIP_S$, short isoform of FLIP; GFP, green florescent protein; RPMI-1640, Roswell Park Memorial Institute 1640; siRNA, small interfering RNA; XIAP, X-linked inhibitor of apoptosis protein. DMEM, Dulbecco's modified Eagle medium; GAPDH, glyceraldehyde phosphate dehydrogenase; MDM2, murine double minute-2; PMSF, phenylmethylsulfonyl fluoride; RT-PCR, reverse transcriptase polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TP53, tumor protein p53.

Contribution of Co-Authors

All studies were carried out under the supervision of Dr. Benjamin Tsang. All experimental work was conducted by Mohammad Reza Abedini, unless otherwise noted.

Dr. Emilie J. Muller was involved in the immunocytochemistry and confocal microscopy (Fig. 4) and Dr. Jan Brun was consulted in the optimizing experiments for detection of V5-FLIP ubiquitination (Fig. 2). Both of them were consulted on the writing and editing of the manuscript, and also contributed to the editing of the final manuscript. Dr. Richard Bergeron and Dr. Douglas A. Gray provided support for experimental design and data interpretation, as well as in editing the final manuscript.

Abstract

Understanding the mechanism of cisplatin (CDDP) action may improve therapeutic strategy for ovarian cancer. Although p53 and FLICE-like inhibitory protein (FLIP) are determinants of CDDP sensitivity in ovarian cancer, the interaction between p53 and FLIP remains poorly understood. Here, using two chemosensitive ovarian cancer cell lines and various molecular and cellular approaches, we show that CDDP induces p53-dependent FLIP ubiquitination and degradation, and apoptosis in vitro. Moreover, we demonstrated that Itch (an E3 ligase), forms a complex with FLIP and p53 upon CDDP treatment. These results suggest that p53 facilitates FLIP down-regulation by CDDP-induced FLIP ubiquitination and proteasomal degradation.

Introduction

Cisplatin (CDDP) is the first-line anti-cancer agent for human ovarian cancer and is known to act in part by induction of apoptosis (1). In CDDP sensitive cells, it also decreases cellular levels of Flice-like inhibitory protein (FLIP), a FADD-binding suppressor of apoptosis, which is present as two splice variants: $FLIP_L$ (55 kDa) and $FLIP_S$ (28 kDa). FLIP contains two N-terminal death effector domains (DED), which prevent caspase-8 activation through DED-DED interaction (2). Although FLIP down-regulation is an important factor in CDDP-mediated apoptosis, its mechanism remained unclear (3).

The ubiquitin-proteasome pathway (UPP) could be involved in regulating FLIP. UPP is a major regulatory mechanism for intracellular protein level. This process is mediated by an E1 (Ub activating enzyme), E2 (Ub conjugase) and E3 (Ub ligase) complex. Typically, proteins modified by polyubiquitin chains are recognized and degraded by the proteasome

(4). Importantly, Itch, a member of the HECT family of E3 ligases, interacts with FLIP and is believed to mediate its degradation as well as TNFα-induced apoptosis (5).

p53 is a transcription factor which regulates cell cycle progression, DNA repair and apoptosis. p53 is maintained at low levels by its negative regulator, MDM2, which ubiquitinates p53, targeting it for proteasomal degradation (6). *TP53* mutations are frequently observed in human ovarian cancer cells (7) and associated with decreased chemo-responsiveness (8).

In the present study, we investigated the involvement of Itch and p53 in CDDP-induced FLIP down-regulation. We demonstrated that CDDP enhances FLIP-p53-Itch interaction, inducing FLIP ubiquitination and degradation in a p53- and Itch-dependent manner. These results suggest that the modulation of FLIP content may be an effective strategy to overcome chemoresistance in ovarian cancer.

Materials and Methods

Reagents. MG132, Lactacystin and Epoxomicin were from Calbiochem (Ab-1, San Diego, CA). Cell Signaling Inc. (Danvers, MA), Ambion (Austin, TX) and Dharmacon, Inc. (Chicago, IL) provided siRNA for p53, Itch and control, respectively. Ribojuice and Lipofectamine Plus were from Novagen (San Diego, CA) and Invitrogen (Carlsbad, CA), respectively. HA-tagged ubiquitin was provided by Dr. Qiao Li (University of Ottawa, Canada). Adenoviral cDNAs were synthesized at the University of Ottawa Neuroscience Research Institute (Ottawa, Canada). Primary antibodies for immunoblots were mouse monoclonal anti-p53 (DO-1; Santa Cruz Biotechnologies, San Diego, CA), anti-FLIP (NF6; Alexis, Ab-1, San Diego, CA) and anti-Itch (BD Bioscience, San Diego, CA), anti-GAPDH

(ab8245; Abcam, Cambridge, UK), anti-V5 (Invitrogen). Antibodies for precipitation were goat polyclonal anti-HA and anti-V5 (Bethyl Laboratories, Montgomery, TX) and anti-p53 (C-19, Santa Cruz Biotechnologies) and for immunofluorescence were rabbit polyclonal anti-FLIP (Cell Signaling Technology) and anti-p53 (C-19, Santa Cruz Biotechnologies), and mouse monoclonal anti-Itch (BD Bioscience). Donkey secondary antibodies were from Jackson Immunoresearch (West Grove, PA).

Cell Culture and Adenovirus Infection. Chemosensitive ovarian cancer cells (OV2008 and A2780s) were cultured as previously reported (8). Cells were infected with appropriate adenoviral constructs as indicated in the text. LacZ adenovirus was used to normalize each treatment group to same total adenoviral concentration. Adenovirus infection efficiency (MOI = 5; 24 h) was > 90% (8).

FLIP Ubiquitination Analysis. Cells were transfected with HA-ubiquitin (3). After 24 h, spent medium was replaced with fresh RPMI 1640 or DMEM F12 containing CDDP (0-10 μM; 0-9 h) and Epoxomicin. Cells were harvested for FLIP ubiquitination analyses (9). The cell pellet was resuspended in boiling 1% SDS in PBS, heated (100 °C, 5 min) and suspended in 1% Triton X-100 in PBS (1:10). DNA was sheared by sonication, and after centrifugation (14000×g, 15 min), the supernatant [diluted with 1% Triton X-100 and 0.5% bovine serum albumin in PBS (1:1)] was incubated (overnight, 4°C) with primary antibody. The beads were washed (6 times with 1% Triton X-100 in PBS), and the precipitated proteins were immunoblotted.

RNA Interference. Cells transfected for 24 h with p53 siRNA (100 nmol/L), Itch siRNA (100 nmol/L) or control siRNA (100 nmol/L) (3), were treated with CDDP and harvested for subsequent analysis.

Western Blotting. Western blotting was done as previously described (3). Membranes were incubated with anti-FLIP (NF60, 1:500), anti-GAPDH (1:20,000), anti-p53 (1:1,000), anti-Itch (1:500) or anti–V5 (1:5,000) [overnight; 4°C], and with horseradish peroxidase–conjugated anti-rabbit or anti-mouse secondary antibody (1:1,000-10,000) [1 h, room temperature]. Peroxidase activity was visualized with the enhanced chemiluminescent kit (Amersham Biosciences, Piscataway, NJ) and analyzed (Scion Image software, Scion, Inc., Frederick, MD).

FLIP mRNA Analysis. Relative differences in FLIP mRNA levels in experimental groups were determined semi-quantitatively by RT-PCR. Total RNA was reverse transcribed, followed by polymerase chain reaction (10).

Assessment of Apoptosis. Apoptosis was determined morphologically, using Hoechst 33258 nuclear stain (3). The counter was "blinded" to avoid experimental bias.

Immunoprecipitation (IP). IP was performed on whole cell lysates using anti-V5 and -p53 antibodies (11) and immunoblotted for p53, Itch and V5.

Immunocytochemistry and Confocal Microscopy. Cultured OV2008 cells were fixed with methanol (10 min, -20°C). Non-specific binding was blocked with 0.8% (w/v) serum albumin and 1% gelatin in PBS (30 min, room temperature (12). Cells were then incubated with anti-p53 (1:50), anti-FLIP (1:25) and anti-Itch (1:25) (overnight, 4°C) and subsequently with secondary donkey conjugated antibodies: anti-goat (Cy5), anti-mouse (Cy3) and anti-rabbit (FITC) [1:25; 2 h, room temperature]. Cells were mounted with Vectashield (Vector, Burlingame, CA, USA). Cells incubated without primary antibodies served as negative controls. Fluorescence images (1024*1024 pixels) were acquired using a LSM 510 confocal laser-scanning microscope (Zeiss, Germany) with a 63X oil-immersion objective (numerical aperture 1.4) (13). Channel images were merged using Adobe Photoshop 7.01. Immunofluorescence profiles (arbitrary unit) were obtained using the Zeiss LSM 510 software (Zeiss, Germany) (14).

Statistical analyses. All results are expressed as mean ± SE of at least three independent experiments. Data were analyzed by two-way ANOVA and Bonferroni posttest (PRISM software version 3.0, Graph Pad, San Diego, CA). Statistical significance was inferred at $P < 0.05$.

Results and discussion

CDDP down-regulates FLIP protein content via FLIP proteasomal degradation. Our recent study shows that CDDP down-regulates FLIP content and induces apoptosis in ovarian cancer cells (3). To better understand how CDDP decreases FLIP level in ovarian cancer cells, two ovarian cancer cell lines (OV2008 and A2780s) were cultured with CDDP (0-10

μM, 24 h) and FLIP content determined. CDDP decreases $FLIP_L$ and $FLIP_S$ levels in a concentration-dependent manner (Fig.1A). FLIP down-regulation was associated with increased apoptosis (Fig. 1 A). The down-regulation of FLIP by CDDP (0-10 μM; 0-24 h) does not appear to be associated with any change in $FLIP_L$ and $FLIP_S$ mRNA abundance (Fig. 1B, upper panel).

To determine whether proteasomal degradation could be involved in CDDP-induced FLIP down-regulation, OV2008 cells were pretreated for 30 min with proteasome inhibitors MG132 (5 μM), Lactacystin (10 μM) or Epoxomicin (25 nM), and cultured with CDDP for an additional 12 h. Although the proteasome inhibitors displayed no effect on basal FLIP content, they significantly attenuated the decrease in $FLIP_L$ and $FLIP_S$ level induced by CDDP (Fig. 1B, lower panel), suggesting that proteasomal degradation may be responsible for the decreased FLIP content following CDDP challenge. Similarly, CDDP induces the proteasomal degradation of the anti-apoptotic protein Xiap without affecting its mRNA abundance (15).

CDDP enhances FLIP-Itch interaction and FLIP Ubiquitination. Since proteasomal degradation of most proteins is preceded by ubiquitination, we examined whether CDDP induces $FLIP_L$ and $FLIP_S$ ubiquitination. OV2008 and A2780s cells were transfected with HA-ubiquitin (2μg; 24 h), infected (MOI = 25; 24h) with adenoviral V5- $FLIP_L$, V5- $FLIP_S$ or LacZ (as control), and treated with CDDP (0-10 μM) in the presence of Epoxomicin (25 nM) for 1.5 and 3 h, respectively. Ubiquitinated FLIP was immunoprecipitated with anti-HA-ubiquitin and immunoblotted with anti-V5-FL or anti-V5-FS). While FLIP ubiquitination was not detectable with non-specific IgG or in cells infected with LacZ or HA-ub alone, CDDP

Figure 1: CDDP down-regulates FLIP through Itch-dependent proteasomal degradation.

(A) CDDP decreased $FLIP_L$ and $FLIP_S$ contents and induced apoptosis in a concentration-dependent manner in OV2008 and A2780s cells (***, $P < 0.001$ vs. control; $n = 3$). OV2008 and A2780s cells were cultured with CDDP (0-10 μM; 24 h) and assessed for FLIP and GAPDH contents by Western blotting (WB; upper panel), and for apoptosis by Hoechst 33258 nuclear staining (lower panel). GAPDH served as a protein loading control. (B) CDDP, at concentration which decreased FLIP protein contents (10 μM), failed to elicit a significant influence on FLIP mRNA abundance ($P > 0.05$, n=3; upper panel). OV2008 and A2780s cells were cultured for different duration (0-24 h) with CDDP (0-10 μM). FLIP mRNA abundance was determined by RT-PCR (n=3); Western blot analysis indicating that the CDDP-induced $FLIP_L$ and $FLIP_S$ down-regulation was attenuated by the presence of proteasome inhibitors (***, $P < 0.001$, $n = 3$; lower panel). OV2008 cells were pretreated with the proteasome inhibitors MG132 (5 μM), Lactacystin (10 μM) and Epoxomicin (25 nM), and then treated for 12 h with CDDP or DMSO (control). (C & D) CDDP-induced FLIP ubiquitination is dependent on Itch (n=3). OV2008 and A2780s transfected with HA-ubiquitin (2μg; 24 h), infected (MOI = 25; 24h) with adenoviral V5-$FLIP_L$ (V5-F_L, C), V5-$FLIP_S$ (V5-F_S, D) or LacZ (as control) in the absence or presence of Itch siRNA (100 nM; 24 h), and then treated with CDDP (0-10 μM) and Epoxomicin (25 nM, to prevent proteasomal degradation of ubiquitinated FLIP). Cells only infected with LacZ (Lane 2) or transfected with HA-ub (Lane 3) are indicated. At the end of 1.5 h and 3 h, cells were harvested for assessment of Itch and GAPDH contents (C & D, upper panels; Western blotting) as well as V5-$FLIP_L$ and V5-$FLIP_S$ ubiquitination, [C & D, lower panels respectively;

immunoprecipitation (IP: anti-HA-ubiquitin, IgG as control) and Western blotting (WB: V5-F_L or V5-F_S)].

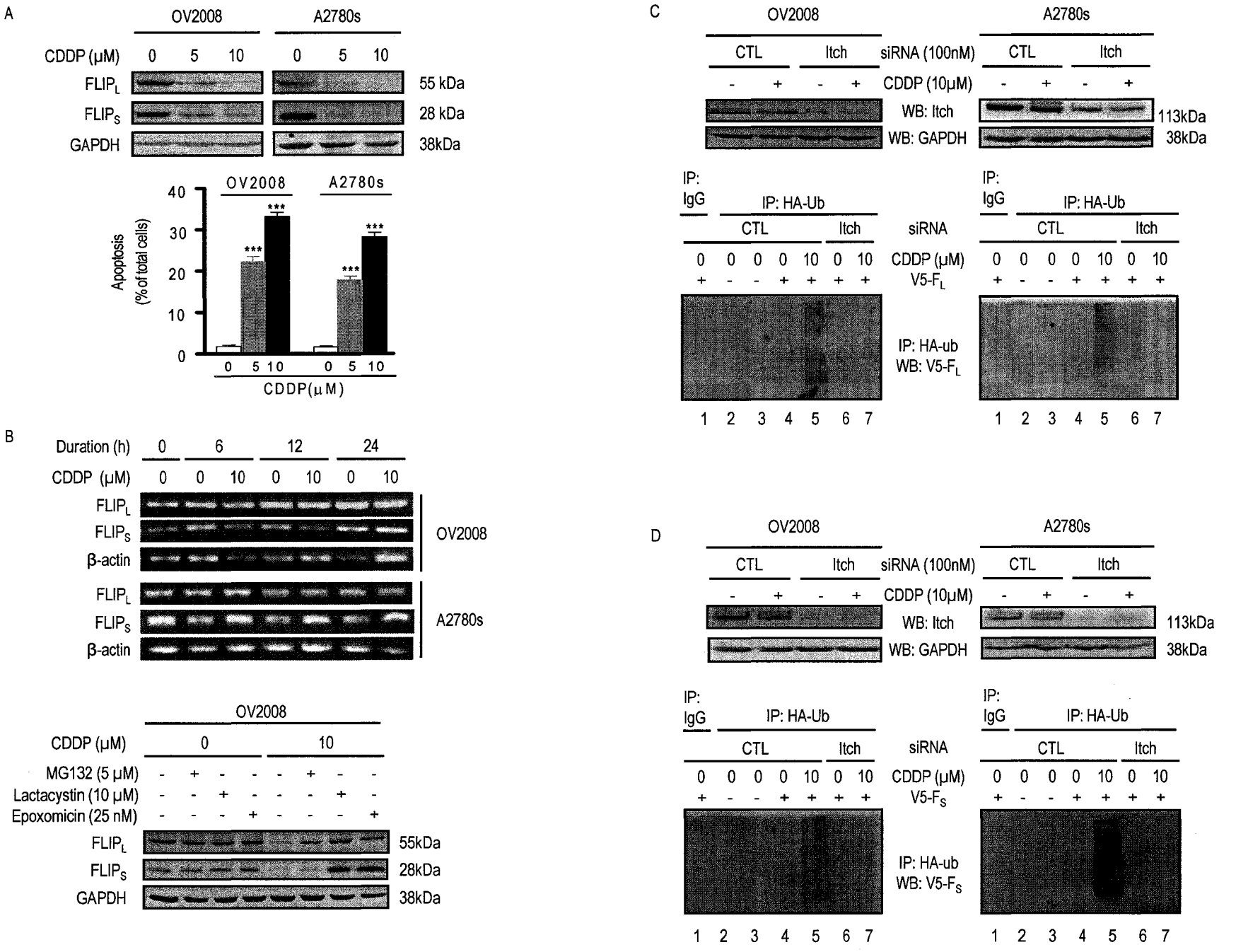
A
OV2008
A2780s
CDDP (μM) 0 5 10 0 5 10
FLIP$_L$
FLIP$_S$
GAPDH
55 kDa
28 kDa
38kDa
Apoptosis (% of total cells)
CDDP(μM)
B
Duration (h) 0 6 12 24
CDDP (μM) 0 0 10 0 10 0 10
β-actin
OV2008
A2780s
MG132 (5 μM)
Lactacystin (10 μM)
Epoxomicin (25 nM)
55kDa
28kDa
38kDa
C
CTL
Itch
siRNA (100nM)
CDDP (10μM)
WB: Itch
WB: GAPDH
113kDa
IP: IgG
IP: HA-Ub
siRNA
CDDP (μM)
V5-F$_L$
IP: HA-ub
WB: V5-F$_L$
D
V5-F$_S$
IP: HA-ub
WB: V5-F$_S$

Figure 1

enhanced $FLIP_L$ and $FLIP_S$ ubiquitination (Fig. 1C-D, lower panel), demonstrating that CDDP-induced FLIP degradation in ovarian cancer cells is associated with FLIP ubiquitination. Since Itch possesses E3 ubiquitin ligase activity and is known to be involved in protein ubiquitination (5), we determined whether Itch plays a role in CDDP-induced FLIP ubiquitination by targeting Itch with siRNA (Fig. 1C-D upper panels). While CDDP increased $FLIP_L$ and $FLIP_S$ ubiquitination in cells transfected with control siRNA, these responses were markedly suppressed by Itch depletion (Fig, 1C-D, lower panels), indicating a requirement for Itch in CDDP-induced FLIP ubiquitination. Although Itch has been shown to facilitate $FLIP_L$ degradation in hepatocytes (5), $FLIP_S$ ubiquitination was not examined nor was the effect of CDDP. The present study is the first to show that Itch plays a vital role in the ubiquitination of both $FLIP_L$ and $FLIP_S$ after CDDP treatment.

To determine whether Itch directly interacts with FLIP and if such an interaction leads to CDDP-dependent FLIP decrease, co-precipitation studies were carried out with OV2008 and A2780s cells transfected with HA-ubiquitin (2μg; 24 h) and subsequently infected (MOI = 25; 24h) with adenoviral V5-$FLIP_L$, V5- $FLIP_S$ or LacZ, and treated with CDDP (0-10 μM) in presence of Epoxomicin (25 nM). As shown in Fig. 2A & C, FLIP-Itch interaction was not detected with non-specific IgG or in cells infected with LacZ, and was slightly detectable in absence of CDDP. This response was enhanced by CDDP after 1.5 h for FLIPS (Fig. 2C, upper panel) and 3 h for $FLIP_L$ (Fig. 2A, upper panel) and associated with an enhancement in CDDP-induced $FLIP_L$ and $FLIP_S$ ubiquitination (Fig. 2, B & D). These results suggest that FLIP-Itch binding may be crucial for FLIP regulation, and that CDDP decreases FLIP content by enhancing FLIP-Itch interaction, FLIP ubiquitination and proteasomal degradation. Our findings are consistent with previous findings that $FLIP_S$

Figure 2: CDDP enhances p53-FLIP-Itch interaction and FLIP ubiquitination

FLIP-p53-Itch interaction and FLIP ubiquitination were enhanced by CDDP (lane 5) after 1.5 h for $FLIP_S$ (figure 2C, upper panel) and 3 h for $FLIP_L$ (figure 2A, upper panel). OV2008 and A2780s transfected with HA-ubiquitin (HA, 2 μg; 24 h), subsequently infected (MOI = 25; 24 h) with either adenoviral V5- $FLIP_L$, (A-B), V5-$FLIP_S$ (C-D) or LacZ (as control), and cultured for different duration with CDDP (0-10 μM) and Epoxomicin (25 nM). Cells only transfected with HA-ub (Lane 1) or infected with adenoviral LacZ (Lane 3) are indicated. Protein-protein interaction was determined by IP-Western. p53, FLIP and ubiquitin immunoprecipitates were immunoblotted [IP: p53, WB: V5 and Itch; IP: V5-tagged $FLIP_L$ or $FLIP_S$, WB: p53 and Itch (A&C); IP: HA-tagged ubiquitin (B&D), WB: V5- $FLIP_L$ or $FLIP_S$ (n=3)].

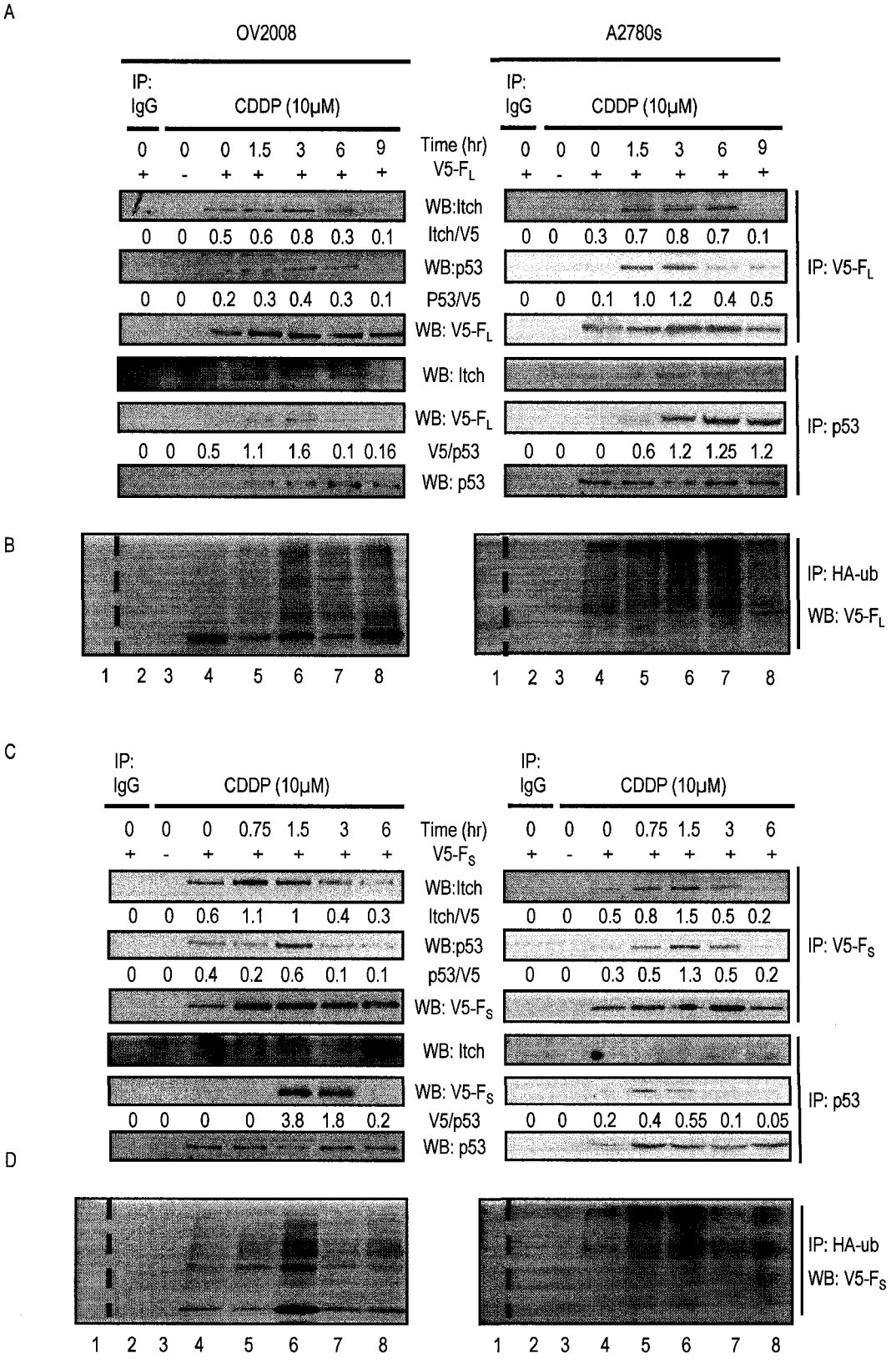
A
OV2008
A2780s
IP: IgG
CDDP (10μM)
Time (hr)
V5-F_L
WB:Itch
Itch/V5
WB:p53
P53/V5
WB: V5-F_L
WB: Itch
V5/p53
WB: p53
IP: V5-F_L
IP: p53
B
IP: HA-ub
WB: V5-F_L
C
V5-F_S
p53/V5
IP: V5-F_S
D

Figure 2

possesses a shorter half-life than $FLIP_L$ due to the presence of a unique C-terminus tail (Lys 193 and Lys195) (9). While FLIP ubiquitination has been detected in several cell subtypes (16-18) the precise mechanism underlying FLIP ubiquitination was not determined. In the present study, we show for the first time that Itch binds to $FLIP_S$ and $FLIP_L$ to facilitate FLIP ubiquitination in a time-dependent manner.

p53 is required for CDDP-induced FLIP ubiquitination

Although some studies suggest p53 could control FLIP protein levels via the regulation of FLIP proteasomal degradation (19), direct evidence for such a conclusion was lacking. We thus investigated whether p53 is involved in the regulation of CDDP-induced FLIP degradation and apoptosis by transfecting ovarian cancer cells with p53 siRNA (0-100 nM; 24 h) prior to CDDP treatment (10 μM; 24 h). As expected, CDDP up-regulated p53, decreased $FLIP_L$ and $FLIP_S$ protein content and induced apoptosis in cells transfected with control siRNA. In contrast, all these responses were attenuated in cells depleted of p53 (Fig. 3A). These results strongly suggest that p53 is an important regulator of FLIP and is required for CDDP-induced FLIP down-regulation and apoptosis. Our studies extend the observations from several studies demonstrating a direct correlation between p53 content and FLIP down-regulation (19-20).

To examine if p53 interacts with FLIP in response to CDDP, we investigated FLIP-p53 interaction by IP (p53 and V5) and WB (V5 and p53, respectively). OV2008 and A2780s cells exhibited lower basal p53 levels, which were up-regulated by CDDP in a time-dependent manner (Fig. 2A and 2C). Furthermore, while FLIP-p53 interaction was barely detectable in the absence of CDDP, this response was increased by CDDP at 1.5 h for $FLIP_S$

Figure 3: p53 is required for CDDP-induced FLIP ubiquitination and apoptosis

(A) p53 silencing attenuated CDDP-induced FLIP degradation and apoptosis. OV2008 and A2780s cells were transfected with p53 siRNA or control siRNA (100 nM; 24 h) and cultured with CDDP (0-10 μM; 24 h). FLIP, p53, and GAPDH contents were assessed by Western blotting (upper panel), and apoptosis was determined by Hoechst 33258 staining (Lower panel; **, $P < 0.001$ *vs.* control; n=3). (B-C) FLIP-p53 interaction and FLIP ubiquitination were enhanced by CDDP (lane 5) after 1.5 h for $FLIP_S$ (figure 3C) and 3 h for $FLIP_L$ (figure 3A) and attenuated in the presence of p53 siRNA (lane 7). OV2008 and A2780s transfected with HA-ubiquitin (2 μg; 24 h), infected (MOI = 25; 24h) with either adenoviral: V5- $FLIP_L$, (B), V5-$FLIP_S$ (C) or LacZ (as control), transfected with p53 or control siRNA (100 nM; 24 h), and then treated with CDDP (0-10 μM) and Epoxomicin (25 nM). Cells only infected with LacZ (Lane 2) or transfected with HA-ub (Lane 3) are indicated. At the end of 3 h and 1.5 h, cells were harvested for assessment of p53-$FLIP_L$ and p53- $FLIP_S$ binding, respectively, as well as ubiquitination of $FLIP_L$ and $FLIP_S$ respectively, as described in Fig 2 (n=3).

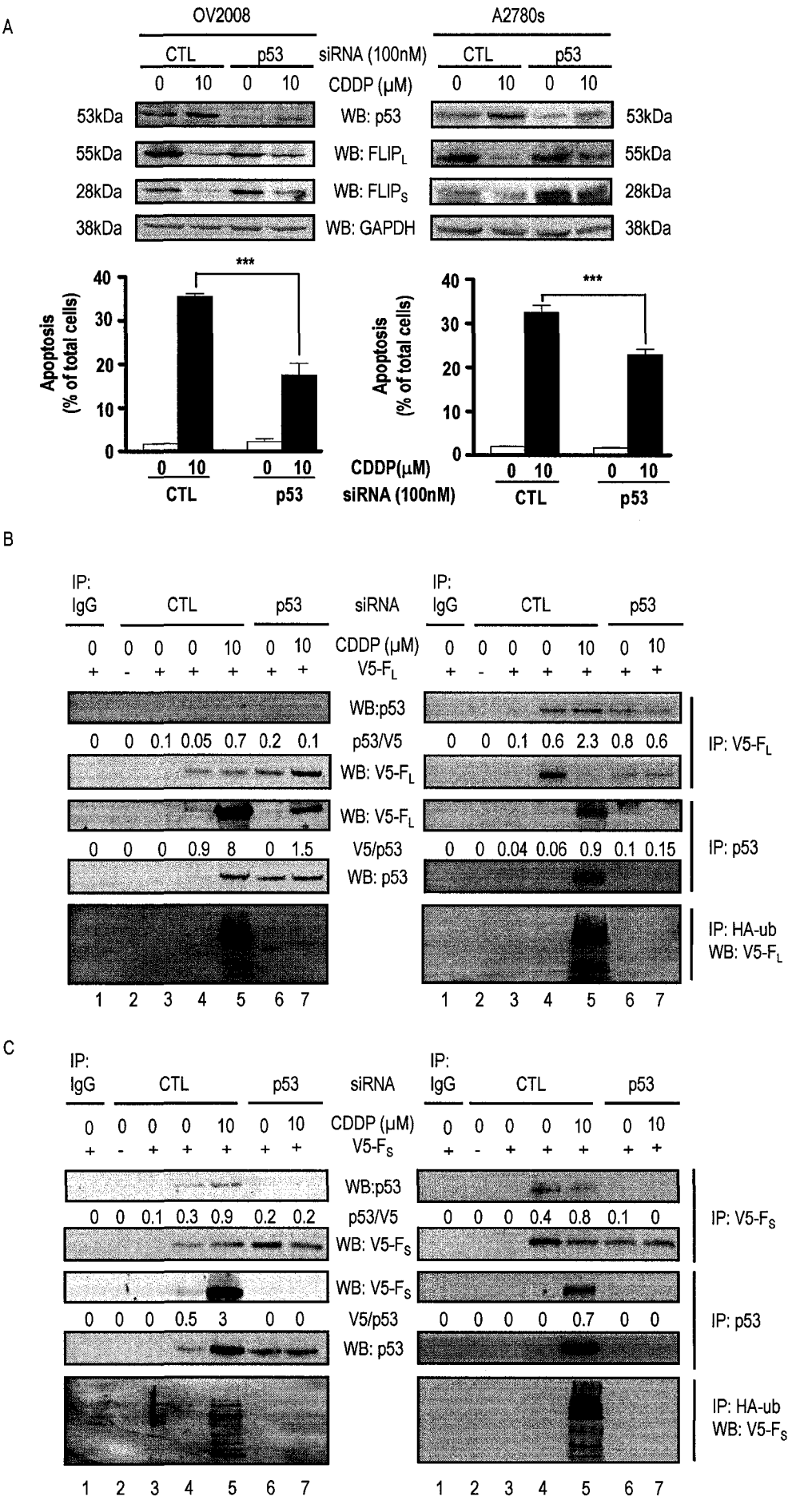
A
OV2008
A2780s
CTL
p53
siRNA (100nM)
CDDP (μM)
0 10 0 10
53kDa
WB: p53
55kDa
WB: FLIP$_L$
28kDa
WB: FLIP$_S$
38kDa
WB: GAPDH
***
Apoptosis (% of total cells)
40
30
20
10
0
CDDP(μM)
siRNA (100nM)
B
IP: IgG
CTL
p53
siRNA
CDDP (μM)
V5-F$_L$
WB:p53
p53/V5
0 0 0.1 0.05 0.7 0.2 0.1
0 0 0.1 0.6 2.3 0.8 0.6
WB: V5-F$_L$
IP: V5-F$_L$
V5/p53
0 0 0 0.9 8 0 1.5
0 0 0.04 0.06 0.9 0.1 0.15
WB: p53
IP: p53
IP: HA-ub
WB: V5-F$_L$
1 2 3 4 5 6 7
C
V5-F$_S$
0 0 0.1 0.3 0.9 0.2 0.2
0 0 0 0.4 0.8 0.1 0
WB: V5-F$_S$
IP: V5-F$_S$
0 0 0 0.5 3 0 0
0 0 0 0 0.7 0 0
IP: HA-ub
WB: V5-F$_S$

Figure 3

(Fig. 2C) and 3 h for $FLIP_L$ (Fig. 2A). CDDP also enhanced p53-Itch binding in a time-dependent manner (Fig. 2A & C). These responses were associated with increased $FLIP_L$ and $FLIP_S$ ubiquitination in response to CDDP (Fig. 2B & D). Our results demonstrate that CDDP increases p53-FLIP-Itch interaction, which may be important for FLIP ubiquitination and degradation.

We further elucidated whether CDDP-induced FLIP ubiquitination is p53-dependent by depleting p53. Our co-precipitation studies demonstrate that CDDP enhances p53-FLIP interaction (Fig. 3B-C). These responses were associated with increased CDDP-induced $FLIP_L$ and $FLIP_S$ ubiquitination, but attenuated after p53 down-regulation by p53 siRNA (100 nM; 24 h; Fig. 3B-C). Interestingly, p53 has been shown to facilitate $FLIP_L$ degradation and ubiquitination in colon cancer cells (19) and temperature-sensitive Germ cell-2 (20). These studies are consistent with our contention that, in response to CDDP, p53 binds to FLIP and Itch to facilitate FLIP ubiquitination and proteasomal degradation.

Our results clearly show that p53 plays an important role in CDDP-induced FLIP ubiquitination and proteasomal degradation, a phenomenon that involves Itch. To further test the above hypothesis, we examined FLIP-p53-Itch co-localization by triple immunolabeling on OV2008 cells treated with or without CDDP (0 - 2.5 μM, 2 h; Fig. 4). In control cells (DMSO; Fig. 4), FLIP-, Itch- and p53-immunoreactivities (-IR) mostly displayed diffuse staining throughout the cytoplasm and at the cell membrane. Individual clusters of FLIP, Itch and p53 along with a few triple co-localizations were observed in the cytoplasm. In contrast, cells treated with CDDP exhibited numerous FLIP-, Itch- and p53 clusters at the cell membrane, with frequent triple co-localizations. Interestingly, CDDP induces p53 nucleus localization at 6 h, but not earlier (data not shown). Our findings are consistent with the

contention that FLIP is recruited by Itch at the cell membrane in a p53-dependent manner. A similar recruitment phenomenon for FLIP by FADD at the Fas receptor has been reported and is believed to suppress caspase-8 activation (2). These results strongly argue in favor of a mechanism involving FLIP-p53-Itch co-localization following CDDP treatment. Moreover, p53, as a docking protein, could facilitate FLIP-Itch interaction, FLIP ubiquitination and degradation, and ultimately apoptosis.

In summary, we report that (1) CDDP down-regulates FLIP protein content via FLIP proteasomal degradation, (2) CDDP enhances FLIP-Itch interaction and FLIP ubiquitination, and (3) p53 and Itch are required for CDDP-induced FLIP ubiquitination. These results are of high relevance for the modulation of FLIP as a possible modality in overcoming chemoresistance in ovarian cancer.

Acknowledgements

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Figure 4: CDDP induces FLIP-p53-Itch triple co-localization at OV2008 cell membrane.

Itch-, FLIP- and p53-IR displayed both clusters and diffuse staining in control cells (A). Clusters were restricted to the cytoplasm and rarely co-localized (arrowhead). In contrast, CDDP treatment (C) resulted in Itch-, FLIP- and p53 clusters at the cell membrane, which were mostly co-localized (arrowheads). Fluorescence intensity profiles (B, D) obtained from OV2008 cell membrane magnifications of A8 (control) and C8 (CDDP-treated) are representatives of diffuse and clustered expression pattern, respectively for Itch, FLIP and p53. While diffuse pattern is represented by constant fluorescence intensity along the membrane of control cells (B1-4), clustered pattern is indicated by hot spots of fluorescence in CDDP-treated cells (D1-4), which also exhibited similar Itch, FLIP and p53 fluorescence profiles. OV2008 cells were treated with DMSO (control, A & B) or CDDP (0-2.5 μM, 2 h; C & D). Protein-protein co-localization was assessed by immunocytochemistry and confocal microscopy. (A & C) Immunofluorescent confocal sections of OV2008 cells treated with DMSO or CDDP and triple labeled for Itch (A1, C1), FLIP (A2, C2), p53 (A3, C3) and merged images (A5-8 and C5-8). White arrow on A8 and C8 indicates the direction of intensity profile measurement (B1-4 and D1-4, respectively). Scale bars in A1 and C1 apply to A1-7 and C1-7, respectively (representative of total of 75 cells for each treatment group, n=3).

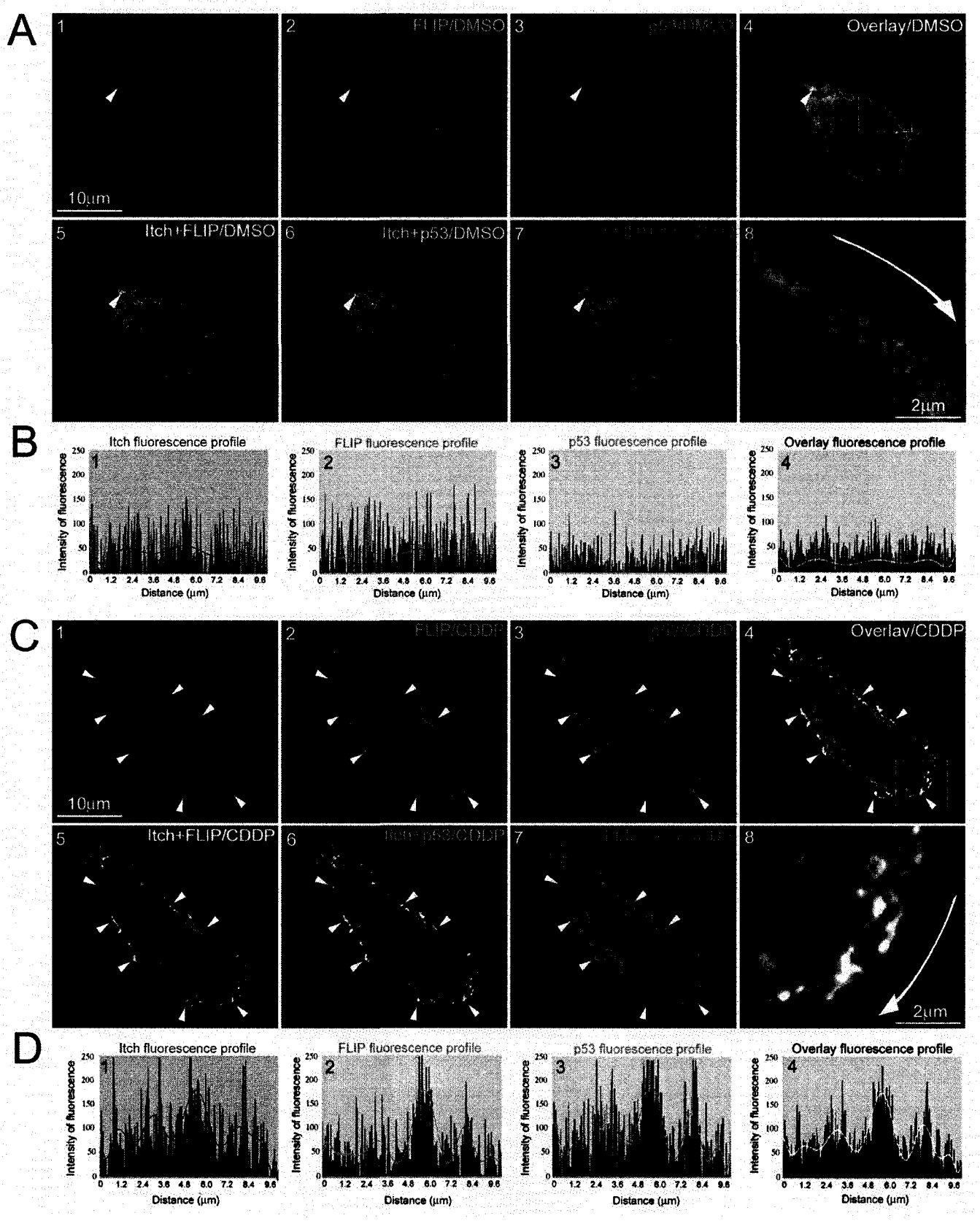
A
1
2
FLIP/DMSO
3
4
Overlay/DMSO
10μm
5
Itch+FLIP/DMSO
6
Itch+p53/DMSO
7
8
2μm
B
Itch fluorescence profile
FLIP fluorescence profile
p53 fluorescence profile
Overlay fluorescence profile
Intensity of fluorescence
Distance (μm)
C
Overlay/CDDP
Itch+FLIP/CDDP
D

Figure 4

Chapter 5 - Manuscript Submitted for Publication

Akt Promotes Chemoresistance in Human Ovarian Cancer Cells by Modulating Cisplatin-induced, p53-dependent Ubiquitination of Flice-Like Inhibitory Protein

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Running Title: Dysregulation of FLIP Ubiquitination by Akt in chemoresistant ovarian cancer

Keywords: FLIP; Akt; p53; CDDP; Itch; ubiquitin-proteasome pathway; chemoresistance; ovarian cancer

Abbreviations: CDDP, cis-diaminedichloroplatinum; DED, death effector domain; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethyl sulfoxide; DN-Akt, dominant-negative Akt; FADD, Fas-associated death domain; FBS, fetal bovine serum; FLICE, Fas-associated death domain-like interleukin-1ß-converting enzyme; FLIP, FLICE-like inhibitory protein; $FLIP_L$, long isoform of FLIP; $FLIP_S$, short isoform of FLIP; GAPDH, glyceraldehyde phosphate dehydrogenase; GFP, green fluorescent protein; MDM2, murine double minute-2; PI3K, phosphatidylinositol-3-OH-kinase; PMSF, phenylmethylsulfonyl fluoride; RPMI-1640, Roswell Park Memorial Institute 1640; siRNA, small interfering RNA; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TP53, tumor suppressor protein 53; XIAP, X-linked inhibitor of apoptosis protein.

Contribution of Co-Authors

All studies were carried out under the supervision of Dr. Benjamin Tsang. All experimental work was conducted by Mohammad Reza Abedini, unless otherwise noted.

Dr. Emilie J. Muller was involved in the immunocytochemistry and confocal microscopy, and performed the quantitation of protein cluster distribution (Fig. 4B-C). She was consulted on the writing and editing of the manuscript, and also contributed to the editing of the final manuscript. Dr. Richard Bergeron and Dr. Douglas A. Gray provided support for experimental design and data interpretation, as well as in editing the final manuscript.

Abstract

Although Akt is a determinant of cisplatin (CDDP) resistance in ovarian cancer cells, which is related in part to its inhibitory action on p53 activation, precisely how Akt confers CDDP resistance is unclear. In the present study, we demonstrate that CDDP induces p53-dependent FLICE-like Inhibitory Protein (FLIP) degradation in chemosensitive ovarian cancer cells but not their resistant counterpart. CDDP induced FLIP-p53-Itch interaction, co-localization and FLIP ubiquitination in chemosensitive but not chemoresistant ovarian cancer cells. Moreover, while activated Akt inhibited CDDP-induced FLIP degradation and apoptosis in sensitive cells, these responses were facilitated by dominant-negative Akt (DN-Akt) expression in chemoresistant cells. Inhibition of Akt function also facilitated p53-FLIP interaction and FLIP ubiquitination, which were attenuated by p53 silencing. These results suggest that Akt confers resistance, in part, by modulating CDDP-induced, p53-dependent FLIP ubiquitination. Understanding the precise etiology of chemoresistance may improve treatment for ovarian cancer.

Introduction

Although cisplatin (CDDP)-centered chemotherapy is the first-line anti-cancer agent for human ovarian cancer, chemoresistance remains a major hurdle to successful treatment. While elevated gene expression, altered drug transport, modified drug target, increased DNA repair or decreased drug-induced DNA damage (1), are believed to be responsible for chemoresistance, recent evidence indicates that the inability of the cells to undergo apoptosis is a key determinant of CDDP resistance (2). In this context, dysregulation of gene products

in various pro-apoptotic [e.g. Fas, caspases and p53; (3-4)] and anti-apoptotic [e.g. Akt, Xiap, FLIP; (5-9)] pathways has been demonstrated in chemoresistant cells.

Fas-associated death domain-like interleukin-1β-converting enzyme (FLICE)-like inhibitory protein (FLIP) is a Fas-associated death domain (FADD)-binding suppressor of apoptosis. It is present in two splice variants, $FLIP_L$ (55 kDa) and $FLIP_S$ (28 kDa), and contains two N-terminal death effector domains (DED), which prevent caspase-8 activation through DED-DED interaction (10-13). We have recently demonstrated that FLIP is a determinant of ovarian cancer chemoresistance and that CDDP decreases FLIP content in chemosensitive ovarian cancer cells but not in their resistant variants (5). Moreover, CDDP induces FLIP ubiquitination and proteasomal degradation in a p53-dependent manner (14). Recent studies have also shown that p53 enhances FLIP degradation in colon cancer cells via an ubiquitin-proteasome pathway (15), while activation of the PI-3K/Akt pathway increases FLIP mRNA and/or protein expression in human umbilical vein endothelial cells (16) and human cancer cells (16-19).

The tumor suppressor protein p53 is a transcription factor regulating cell cycle, DNA repair and apoptosis, and is rapidly up-regulated by DNA-damaging agents, including CDDP (20). TP53 mutation is a frequent event in human ovarian cancer cells (21-22) and is often associated with decreased chemo-responsiveness (7, 23-25). Its content is maintained low by its negative regulator, MDM2, which ubiquitinates p53, targeting it for proteasomal degradation (26).

Akt/PKB (protein kinase B) is a serine/threonine kinase which is normally activated by growth factor in a PI3-kinase (PI-3K)-dependent manner (27-28). Akt/PI3K is activated or over-expressed in ovarian cancer cells (6-7). Indeed, Akt promotes ovarian cell survival and

malignant transformation (7, 29-30). It is also a determinant of CDDP resistance and suppresses X-linked IAP (XIAP) proteasomal degradation (6), p53 levels and function (3, 7), as well as mitochondrial release of the second mitochondria-derived activator of caspases (Smac) (31) and apoptosis inducing factor (32). Our recent studies indicate that CDDP significantly decreases $FLIP_L$ and $FLIP_S$ contents through a transcription-independent mechanism and induces apoptosis in CDDP sensitive but not resistant ovarian cancer cells (5). We also show that CDDP induces FLIP ubiquitination in a p53- and Itch-dependent manner (14). However, whether and how Akt regulates CDDP-induced FLIP down-regulation and apoptosis in chemoresistant cells is not clear.

In the current study, we show that p53 facilitates p53-FLIP-Itch interactions and FLIP ubiquitination and degradation in chemosensitive cells but not in their resistant variants. However, in chemoresistant cells, Akt inhibits FLIP-p53 interaction, FLIP ubiquitination and apoptosis. These results suggest that modulation of Akt may be an effective means to overcome chemoresistance in human ovarian cancer.

Materials and Methods

Reagents

MG132, Lactacystin and Epoxomicin were from Calbiochem (Ab-1, San Diego, CA). Cell Signaling Inc. (Danvers, MA), Ambion (Austin, TX) and Dharmacon, Inc. (Chicago, IL) provided siRNA for p53, Itch and control, respectively. Ribojuice and Lipofectamine Plus were from Novagen (San Diego, CA) and Invitrogen (Carlsbad, CA), respectively. HA-tagged ubiquitin was provided by Dr. Qiao Li (University of Ottawa, Canada). Adenoviral hemagglutinin (HA)-tagged, triple-A mutated (K179A, T308A, and S473A), kinase-dead

dominant negative Akt (DN-Akt) were a generous gift from Dr. Kenneth Walsh (Cardiovascular Research, St. Elizabeth's Medical Centre, Boston, MA). Adenoviral $FLIP_L$, $FLIP_S$, myristoylated Akt1 (AAkt1) and LacZ were synthesized at the University of Ottawa Neuroscience Research Institute (Ottawa, Canada). Primary antibodies for immunoblots are mouse monoclonal anti-p53 (DO-1; Santa Cruz Biotechnologies, San Diego, CA), anti-FLIP (NF6; Alexis, Ab-1, San Diego, CA) and anti-Itch (BD Bioscience, San Diego, CA), anti-GAPDH (ab8245; Abcam, Cambridge, UK), anti-V5 (Invitrogen) and anti-HA (clone 3F10, Roche, Laval, Quebec, Canada). Antibodies for precipitation were goat polyclonal anti-HA and anti-V5 (Bethyl Laboratories, Montgomery, TX) and anti-p53 (C-19, Santa Cruz Biotechnologies) and for immunofluorescence were rabbit polyclonal anti-FLIP (Cell Signaling Technology) and anti-p53 (C-19, Santa Cruz Biotechnologies), and mouse monoclonal anti-Itch (BD Bioscience). Donkey secondary antibodies were from Jackson Immunoresearch (West Grove, PA).

Cell Culture and Adenovirus Infection

Chemosensitive (OV2008 and A2780s) and resistant (C13* and A2780cp, respectively) ovarian cancer cells were cultured as previously reported (7). A2780s-AAkt2 and A2780s-PHM6 cells (generously provided by Dr. Jin Cheng, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL), stably transfected with pcDNA3 vector (Invitrogen, Burlington, Ontario, Canada), containing constitutively active HA-tagged, myristoylated Akt2 or pcDNA3 alone, were cultured as previously reported (31, 33). Cells were cultured in RPMI 1640 or DMEM/F12 with or without G418 (250 μg/mL), infected with appropriate adenoviral $FLIP_L$, $FLIP_S$, AAkt1 and DN-Akt constructs, and LacZ

adenovirus was added to insure that the total concentration of the virus was the same in all treatment groups, as previously described (8, 31). Adenovirus infection efficiency (MOI = 5; 24 h) was > 90% (7).

FLIP Ubiquitination Analysis

Ovarian cancer cells were transfected (5) with HA-ubiquitin. After 24 h, the culture medium was replaced with fresh RPMI 1640 or DMEM F12 containing CDDP (0-10 μM) and Epoxomicin and the cells were cultured for additional 0-3 h. Cells were harvested for FLIP ubiquitination analyses (34). The cell pellet was resuspended in boiling 1% SDS in PBS, heated (100 °C, 5 min) and suspended in 1% Triton X-100 in PBS (1:10). DNA was sheared by sonication, and after centrifugation (1000 x g, 15 min), the supernatant [diluted with 1% Triton X-100 and 0.5% bovine serum albumin in PBS (1:1)] was incubated (overnight, 4°C) with primary antibody. The beads were washed (6 times with 1% Triton X-100 in PBS), and the precipitated proteins were immunoblotted.

RNA Interference

Ovarian cancer cells were transfected with p53 siRNA (100 nmol/L), Itch siRNA (100 nmol/L) or control siRNA (100 nmol/L) for 24 h using Ribu-Juice transfection reagent (Novagen, San Diego, CA) and then treated with CDDP (5). The culture media was removed and the cells were washed once with phosphate-buffered saline and the siRNA mixture was added to each well. After 6 h incubation, the media was removed and replaced with fresh, culture media containing 10% serum for the duration of the culture (24–48 hr). Downregulation was confirmed by western blot analysis.

Western Blotting

Western blotting was carried out as previously described (5). Membranes were incubated overnight at 4°C with anti-FLIP (NF6, 1:500), anti-GAPDH (1:20,000), anti-p53 (1:1,000), anti-Itch (1:500), anti-V5 (1:5,000) or anti-HA (1:1,000) primary antibodies and subsequently in horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody (1:1,000-10,000) 1 h at room temperature. Peroxidase activity was visualized with the enhanced chemiluminescent kit (Amersham Biosciences, Piscataway, NJ). Results were scanned and analyzed using Scion Image software (Scion, Inc., Frederick, MD).

Assessment of Apoptosis

At the end of the culture period, attached cells to the growth surface were removed by trypsin treatment. Floating and attached cells were pooled, centrifuged, and the pellet were resuspended in phosphate buffered formalin (10%) containing Hoechst 33258 (12.5 ng/ml). Cells were spotted onto slides and changes in nuclear morphology was observed using a Zeiss fluorescence microscope (magnification 400X). Cells with typical apoptotic nuclear morphology (nuclear shrinkage, fragmentation and condensation) were identified and counted in different fields, as previously reported (5). At least 200 cells were counted in each treatment group. The counter was "blinded" to sample identity to avoid experimental bias.

Immunoprecipitation (IP)

Cultured cells transfected or infected with cDNA and treated with CDDP were lysed in standard lysis buffer (1 h, 0°C), centrifuged (14,000 x g, 20 min). The supernatants were analyzed for total protein content, and 500 μg of total protein was incubated with the primary antibodies as indicated at Reagent Section and/or 30 μl of Sepharose beads in a final volume of 500 μl. Immunoprecipitation was carried out with gentle rocking (overnight, 4°C). The agarose beads were centrifuged (500 x g, 2 min), and washed 6 times. After the final wash, the beads were resuspended with 30 μl of SDS sample buffer, boiled and then loaded onto 10% SDS-PAGE gels. Following protein transfer to nitrocellulose membrane, anti-p53, anti-Itch and anti-V5 antibodies were used for immunoblotting of p53, Itch and exogenous V5, respectively (3).

Immunocytochemistry and Confocal Microscopy

Cultured OV2008 and C13* cells were fixed with methanol (10 min, -20°C). Non-specific binding was blocked with 0.8% (w/v) serum albumin and 1% gelatin in PBS for 30 min at room temperature (35). Cells were then incubated with anti-p53 (1:50), anti-FLIP (1:25) and anti-Itch (1:25) primary antibodies overnight at 4°C and subsequently with donkey anti-goat Cy5- (p53), anti-mouse Cy3- (Itch) and anti-rabbit FITC-conjugated (FLIP) secondary antibodies (1:25) for 2 h at room temperature. Cells were mounted with Vectashield (Vector, Burlingame, CA, USA). Cells were incubated with corresponding normal IgG instead of primary antibodies as a negative control. Fluorescence images (1024*1024 pixels) were acquired using a LSM 510 confocal laser-scanning microscope (Zeiss, Germany) with a 63x oil-immersion objective (36). Channel images were merged

using Adobe Photoshop 7.01 (Adobe, Ottawa, Canada). Immunofluorescence profiles (arbitrary unit) from cell membranes were obtained from merged images using the Zeiss LSM 510 software (Zeiss, Germany) (37). Quantifications were performed manually. A cluster was defined by a sharp increase in fluorescence (36). Given the numerical aperture (NA) of the objective used (1.4), the size of one pixel was set to 0.057 μm (Pixel size =[(0.46 x wavelength emission)/NA)]/3, according to Nyquist theorem). The minimum number of pixels forming a cluster was set to 10, corresponding to a minimum area of 0.0325 μm2. The criterion to define cluster co-localization was a superimposition of at least five pixels of each cluster. Data were from two different set of cells for each condition.

Statistical analyses

All results are expressed as mean ± SEM of at least three independent experiments. Data were analyzed by two- and three-way ANOVA and the differences between multiple experimental groups were determined by the Bonferroni post-hoc tests (PRISM software version 3.0 or 4.0, Graph Pad, San Diego, CA). Statistical significance was inferred at $P < 0.05$.

Results

CDDP-induced p53-FLIP interaction and FLIP ubiquitination are not observed in chemoresistant ovarian cancer cells. We have previously shown that CDDP down-regulates FLIP content and induces apoptosis in the chemosensitive ovarian cancer cell line (OV2008 & A2780s) but not in their resistant variants (C13* & A2780cp, respectively) (14). Moreover, we have observed that CDDP induces FLIP-p53-Itch interaction and FLIP

ubiquitination in chemosensitive cells (14). We examined whether the inability of CDDP to down-regulate FLIP protein content in resistant cells is due to the failure of CDDP to induce FLIP-p53-Itch interaction and FLIP ubiquitination. Therefore, chemosensitive cells (OV2008 and A2780s) and their resistant counterparts (C13* and A2780cp) were transfected with HA-ubiquitin (2μg; 24 h), infected (MOI = 25; 24h) with either adenoviral V5- $FLIP_L$, V5- $FLIP_S$ or LacZ (as control), and treated with CDDP (0-10 μM) in the presence of Epoxomicin (25 nM) for 1.5 and 3h for the cells infected with $FLIP_S$ and $FLIP_L$, respectively. Protein-protein interaction and FLIP ubiquitination were determined by immunoprecipitation and Western blot (IP-Western). p53, FLIP and ubiquitin immunoprecipitates were immunoblotted [IP: p53, WB: V5 and Itch; IP: V5-tagged $FLIP_L$ or $FLIP_S$, WB: p53 and Itch (A&C); IP: HA-tagged ubiquitin (B&D), WB: V5-$FLIP_L$ or $FLIP_S$ (n=3)]. As shown in Fig. 1A & 1C, while p53-FLIP-Itch interaction was low with IgG and in cells infected with LacZ, it was enhanced in cells treated with CDDP and associated with increased $FLIP_L$ and $FLIP_S$ ubiquitination (Fig. 1B &1D). However, these responses were not observed in the resistant cells suggesting that the failure of CDDP to down-regulate FLIP protein content in chemoresistant cells may be due to its inability to induce FLIP-p53-Itch interaction and FLIP ubiquitination.

CDDP induces FLIP-p53-Itch co-localization in chemosensitive cells but not resistant counterparts. Our previous results show that CDDP induces FLIP-p53-Itch interaction and membranous co-localization in chemosensitive cells upon CDDP challenge (14). To further investigate this phenomenon, we examined FLIP-p53-Itch co-localization by triple immunolabeling on chemosensitive cells (OV2008) and its resistant counterpart (C13*) treated with or without CDDP (0 - 2.5 μM, 2 h; Fig. 2). In OV2008 cells treated with DMSO

Figure 1: CDDP enhances p53-FLIP-Itch interaction and FLIP ubiquitination in chemosensitive ovarian cancer cells but not their resistant counterparts.

FLIP-p53-Itch interaction (A and C, lanes 4 vs. 8) and FLIP ubiquitination (B and D; lanes 5 vs. 10) were enhanced by CDDP for $FLIP_S$ (C and D) and $FLIP_L$ (A and B) in chemosensitive but not resistant cells. OV2008 and A2780s and their resistant variant C13* and A2780cp were transfected with HA-ubiquitin (2 μg; 24 h), subsequently infected (MOI = 25; 24 h) with either adenoviral V5- $FLIP_L$, (A-B), V5-$FLIP_S$ (C-D) or LacZ (as control), and cultured with CDDP (0-10 μM) and Epoxomicin (25 nM). Cells transfected with only HA-ub (B and D, lanes 3 and 8) are indicated. Cell lysates were immunoprecipitated with IgG control (A and C, lanes 1 and 5; B and D, lanes 1 and 6) or without antibody (A and C, lanes 2 and 6; B and D, lanes 2 and 7). Cells transfected only with HA-ub (B and D, lanes 3 and 8) or infected with adenoviral LacZ (A and C, lanes 2 and 6; B and D, lanes 2 and 7) are indicated. Protein-protein interaction was determined by IP-Western. p53, FLIP and ubiquitin immunoprecipitates were immunoblotted [IP: p53, WB: V5 and Itch; IP: V5-tagged $FLIP_L$ or $FLIP_S$, WB: p53 and Itch (A&C); IP: HA-tagged ubiquitin (B&D), WB: V5- $FLIP_L$ or $FLIP_S$]. Results are from three independent experiments.

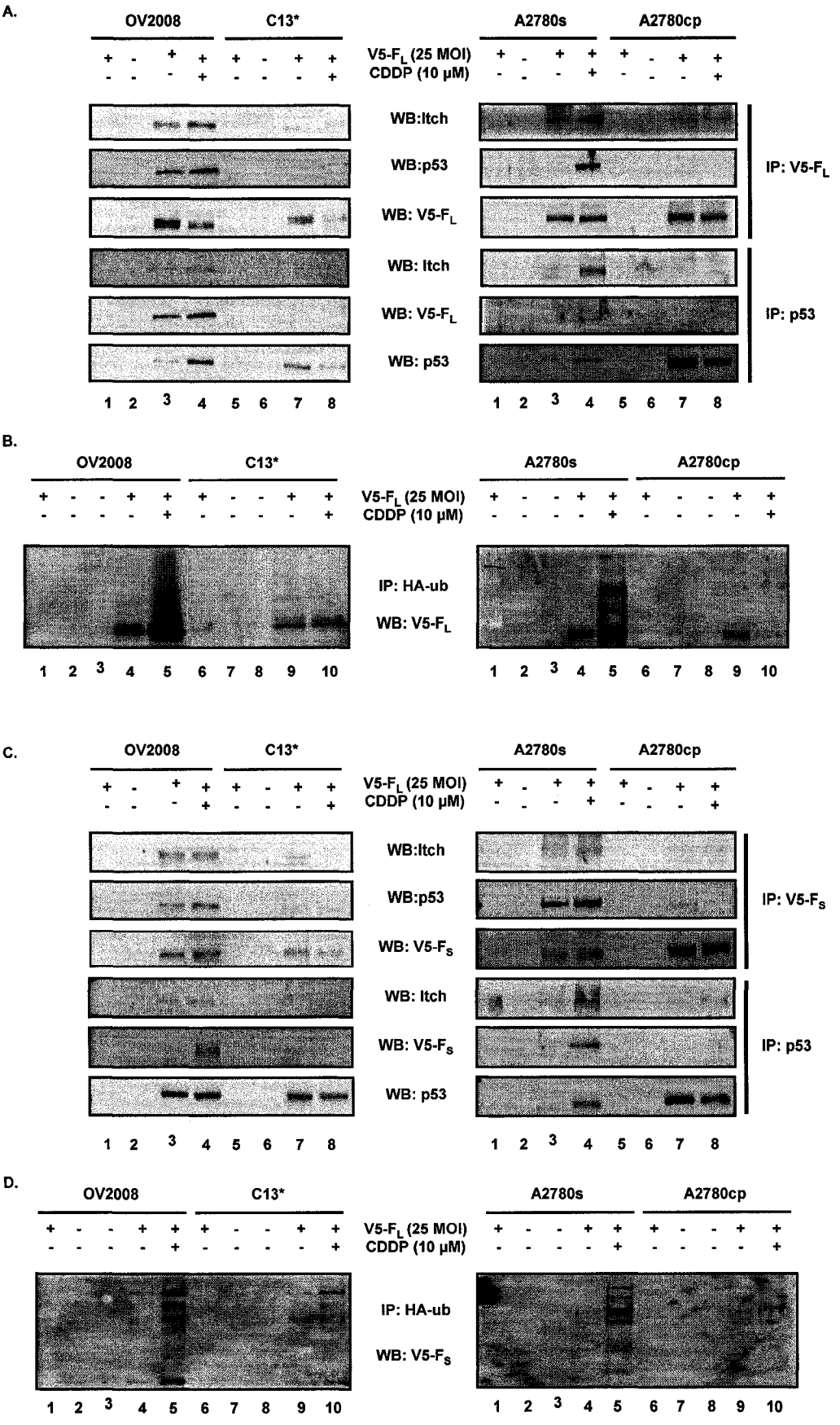
A.
OV2008
C13*
A2780s
A2780cp
V5-F$_L$ (25 MOI)
CDDP (10 μM)
WB:Itch
WB:p53
WB: V5-F$_L$
WB: Itch
WB: V5-F$_L$
WB: p53
IP: V5-F$_L$
IP: p53
1 2 3 4 5 6 7 8
B.
OV2008
C13*
A2780s
A2780cp
V5-F$_L$ (25 MOI)
CDDP (10 μM)
IP: HA-ub
WB: V5-F$_L$
1 2 3 4 5 6 7 8 9 10
C.
OV2008
C13*
A2780s
A2780cp
V5-F$_L$ (25 MOI)
CDDP (10 μM)
WB:Itch
WB:p53
WB: V5-F$_S$
WB: Itch
WB: V5-F$_S$
WB: p53
IP: V5-F$_S$
IP: p53
1 2 3 4 5 6 7 8
D.
OV2008
C13*
A2780s
A2780cp
V5-F$_L$ (25 MOI)
CDDP (10 μM)
IP: HA-ub
WB: V5-F$_S$
1 2 3 4 5 6 7 8 9 10

Figure 1

(control; Fig. 2A1-4), FLIP-, Itch- and p53-immunoreactivities (-IR) displayed clustered and diffuse staining throughout the cytoplasm and few triple co-localizations of FLIP, p53 and Itch clusters were observed. As previously shown (14), OV2008 cells treated with CDDP exhibited a marked increase in the density of triple membrane co-localizations at the cell membrane only (Fig. 2A5-8). Quantifications confirmed this observation, as the proportions of FLIP, p53 and Itch clusters detected at the cell membrane significantly increased ($P < 0.001$) while those detected in other intracellular compartments significantly decreased ($P < 0.001$) (Fig. 2C). Moreover, the proportions of FLIP-p53 and FLIP-p53-Itch co-clusters were significantly increased ($P < 0.001$) in cells treated with CDDP, further arguing in favor of a recruitment of FLIP at the cell membrane in a FLIP-p53-Itch triple complex. Interestingly, in C13* chemoresistant cells treated with CDDP, no difference in the intracellular *vs.* membranous distribution of FLIP, p53 and Itch clusters, nor an increase in the proportion of FLIP co-aggregated with p53 or p53-itch co-clusters, was observed (Fig. 2D). Rather, C13* cells showed similar distribution pattern of FLIP, p53 and Itch individual or co-localized clusters in both intracellular and membranous compartment (Fig. 2D). These data indicate that chemoresistant cells display an intrinsic mechanism preventing FLIP to be recruited at the cell membrane.

Figure 2: CDDP induces FLIP-p53-Itch triple co-localization at OV2008 but not C13* cell membrane.

(A) Triple detection of Itch-, FLIP- and p53-IR in OV2008 cells treated with or without CDDP. Itch-, FLIP- and p53-IR displayed both clusters and diffuse staining in OV2008 (A1-8) and C13* cells (A9-16). In OV2008 cells, CDDP treatment resulted in Itch-, FLIP- and p53 clusters triply co-localized at the cell membrane. In C13* cells, FLIP and p53 clusters were mainly distributed in cytosol and nucleus in the presence (A13-16) or the absence (A9-12) of CDDP. (B) Fluorescence intensity profiles obtained from OV2008 (B1-2) and C13* (B3-4) cell membrane magnifications of A4, 8 (DMSO) and A12, 16 (CDDP) are representatives of diffuse and clustered Itch, FLIP and p53 expression pattern. While diffuse pattern is represented by constant fluorescence intensity along the membrane of control cells (B1, 3-4), clustered pattern is indicated by hot spots of fluorescence in CDDP-treated OV2008 cells (B2). White arrow on A16 indicates the direction of intensity profile measurement (B1-4). Scale bar in A16 applies to A1-16. (C) Quantitative analysis of FLIP, p53 and Itch cluster distribution pattern in cytosol, nucleus and membrane of OV2008 (N = 40 to 55) and C13* cells (N = 37 to 40), treated with or without CDDP. Note the strong increase in the proportion of membranous clusters in OV2008 cells treated with CDDP. Asterisks indicate significant difference in the cell treated with CDDP compared to control (D) Quantitative analysis of FLIP, p53 and Itch cluster co-localization pattern in cytosol, nucleus and membrane of OV2008 and C13* cells, treated or not with CDDP. Note that the proportions of membranous p53-FLIP and p53-FLIP-Itch co-localized clusters strongly increased in OV2008 cells treated with CDDP, although they remained similar in C13* cells under the same treatment.

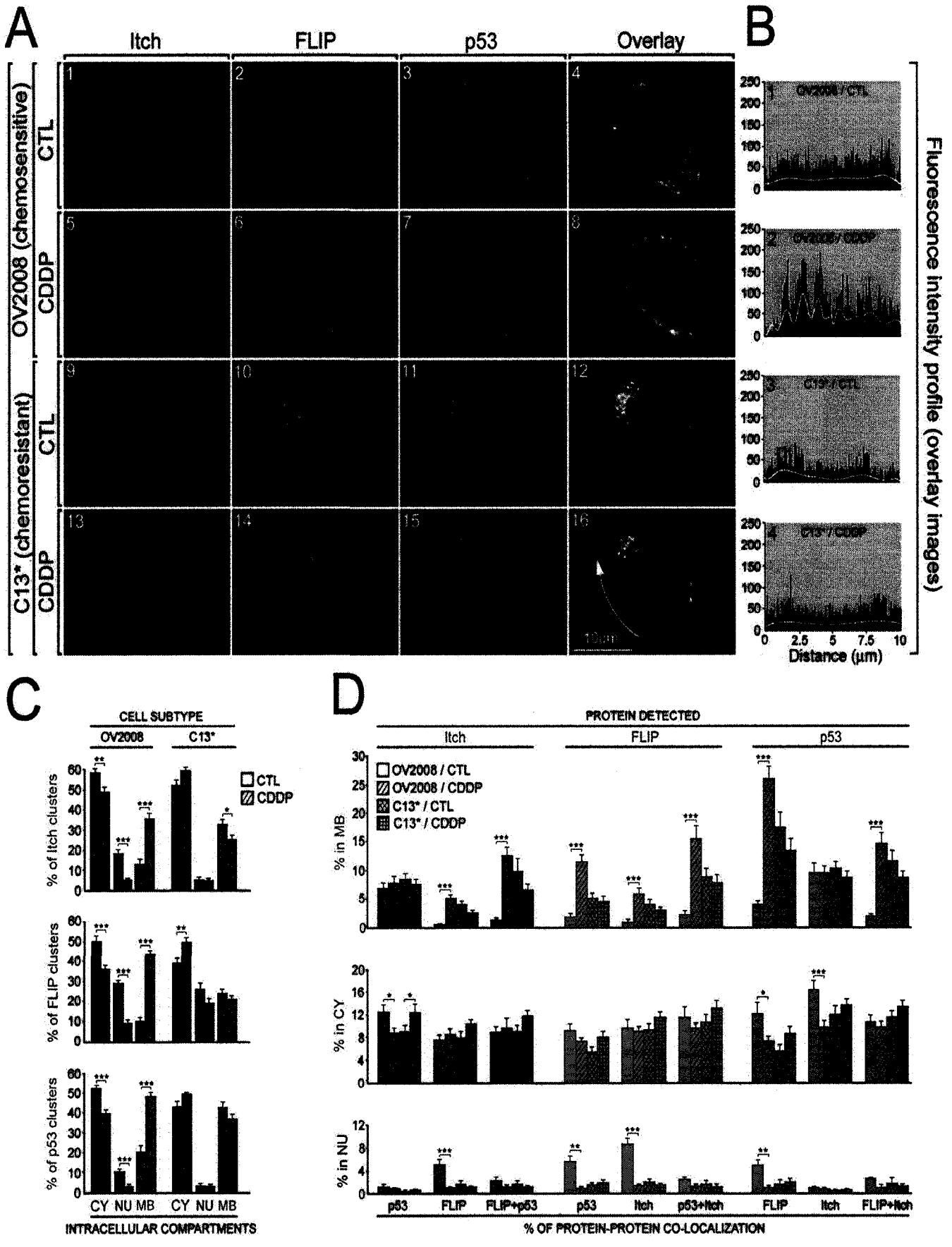
A
Itch
FLIP
p53
Overlay
OV2008 (chemosensitive)
CTL
CDDP
C13* (chemoresistant)
CTL
CDDP
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
B
Fluorescence intensity profile (overlay images)
Distance (µm)
C
CELL SUBTYPE
OV2008
C13*
CTL
CDDP
% of Itch clusters
% of FLIP clusters
% of p53 clusters
CY NU MB CY NU MB
INTRACELLULAR COMPARTMENTS
D
PROTEIN DETECTED
Itch
FLIP
p53
OV2008 / CTL
OV2008 / CDDP
C13* / CTL
C13* / CDDP
% in MB
% in CY
% in NU
p53
FLIP
FLIP+p53
p53
Itch
p53+Itch
FLIP
Itch
FLIP+Itch
% OF PROTEIN-PROTEIN CO-LOCALIZATION

Figure 2

Akt inhibits CDDP-induced FLIP down-regulation and apoptosis in ovarian cancer cells. Akt is a determinant of chemoresistance in ovarian cancer cells (7). To examine the influence of CDDP on Akt content and activation, Chemosensitive ovarian cancer cells (OV2008 and A2780s) and their resistant counterpart (C13* and A2780cp, respectively) were cultured with CDDP (0-10 μM, 24 h). Akt and P(Ser473)-Akt contents were assessed by Western blotting. In OV2008 cells, CDDP down-regulated Akt and P-Akt contents in a time-dependent manner but failed to decrease either response in the resistant variants, C13* cells (Fig. 3 A). CDDP down-regulated Akt content in A2780s cells but not in their resistant variants (A2780cp cells). While CDDP up-regulated P-Akt content in A2780s cells during the first two days of culture, this response was back to control level by 72 h. In resistant cells (A2780cp) treated with CDDP, P-Akt levels remained elevated throughout the 72 h culture period.

While FLIP expression and content has been shown to be up-regulated by Akt in cancer cells (16, 18-19), whether and how Akt regulates CDDP-induced FLIP down-regulation and chemosensitivity is not known. To investigate the role of Akt in CDDP-induced FLIP degradation and chemosensitivity, chemosensitive ovarian cancer cells (OV2008) infected with adenoviral HA-tagged activated Akt1 (AAkt1) or LacZ (MOI=0-10; 24 h) were incubated with CDDP (0-10 μM; 24 h). FLIP content and successful expression of the activated Akt1 were assessed by Western blot, using FLIP and anti-HA antibodies, respectively. As shown in Fig. 4A, while active Akt1 adenovirus had no effect on basal $FLIP_L$ and $FLIP_S$ content (upper panel) and apoptosis (lower panel), it significantly inhibited these responses to CDDP in a concentration-dependent manner ($p<0.001$). Moreover, OV2008 cells transfected with activated Akt2 (AAkt2; 2μg; 24 h) also exhibited decreased

Figure 3: CDDP down-regulates Akt and P-Akt contents in chemosensitive ovarian cancer cells but not in their chemoresistant variants.

Chemosensitive ovarian cancer cells lines (OV2008 and A2780s) and their chemoresistant variants (C13 and A2780cp) were treated with CDDP (0-10 μM) for different duration (24-72 h). **A.** CDDP down-regulated Akt content and P-Akt in chemosensitive cells (OV2008) but not in their resistant counterparts (C13*). B. CDDP also down-regulated Akt content in A2780s cells but not in their resistant variants (A2780cp cells). While CDDP up-regulated P-Akt content in both A2780s and A2780cp cells during the first two days of culture, this response was back to control level by 72 h in A2780s but not in resistant variants (A2780cp).

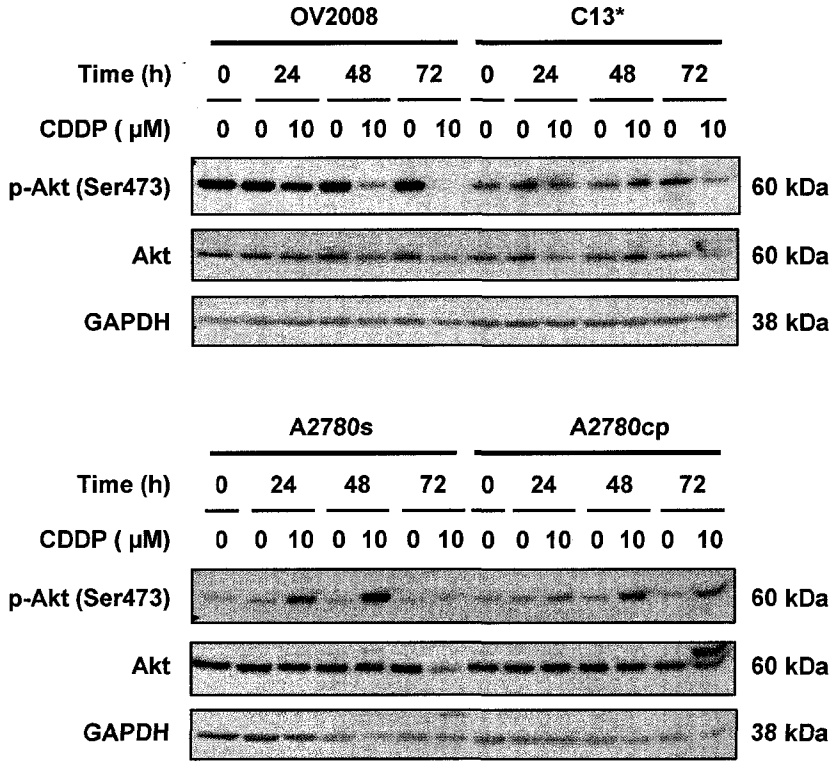
OV2008
C13*
Time (h)
0 24 48 72 0 24 48 72
CDDP (μM)
0 0 10 0 10 0 10 0 0 10 0 10 0 10
p-Akt (Ser473)
60 kDa
Akt
60 kDa
GAPDH
38 kDa
A2780s
A2780cp
Time (h)
0 24 48 72 0 24 48 72
CDDP (μM)
0 0 10 0 10 0 10 0 0 10 0 10 0 10
p-Akt (Ser473)
60 kDa
Akt
60 kDa
GAPDH
38 kDa

Figure **3**

OV2008 cells transfected with activated Akt2 (AAkt2; 2μg; 24 h) also exhibited decreased CDDP-induced $FLIP_L$ and $FLIP_S$ down-regulation (Fig. 4B, upper panel) and apoptosis (Fig. 4B, lower panel), suggesting that Akt activation suppresses CDDP-induced FLIP down-regulation and apoptosis, regardless of its isoform expressed.

To determine whether the effects of Akt on CDDP-induced FLIP down-regulation and apoptosis was cell line-specific, chemosensitive ovarian cancer cells A2780s [stably transfected with constitutively active Akt2 (A2780s-AAkt2) or the empty vector (A2780s-PMH6)] were treated with CDDP (0-10 μM; 0-24 h) and assessed for FLIP down-regulation. While CDDP decreased FLIP protein content and induced apoptosis in A2780s-PMH6 cells, CDDP-induced FLIP down-regulation (Fig. 4C, upper panel) and apoptosis (Fig. 4C, lower panel) in A2780s-AAkt2 cells were completely abrogated. In addition, A2780s infected with adenoviral activated Akt1 (MOI=0-10; 24 h) and treated with CDDP (0-10 μM; 24 h; Fig. 4D), also showed significantly lower CDDP-induced FLIP down-regulation (upper panel) and apoptosis (lower panel).

To further investigate the role of Akt in the regulation of FLIP down-regulation in CDDP-induced apoptosis, C13* cells (chemoresistant, wt p53) were infected with adenoviral HA-tagged dominant negative (DN)-Akt or LacZ (MOI, 0-80; 48 h) and treated with CDDP (10-40 μM; 24 hours). Western blot analyses show that whereas CDDP failed to reduce $FLIP_L$ and $FLIP_S$ content (Fig. 5A, upper panel) and to induce apoptosis in C13* cells (Fig. 5A, lower panel), these responses were facilitated by DN-Akt expression, suggesting that Akt plays an important role in the regulation of CDDP-induced FLIP down-regulation and apoptosis, and may confer CDDP resistance in ovarian cancer cells. Moreover, while CDDP

Figure 4: Akt inhibits CDDP-induced FLIP down-regulation and apoptosis in wt-p53 ovarian cancer cells.

(A) Overexpression of activated Akt1 (AAkt1) inhibits CDDP-induced FLIP degradation and apoptosis ($^{+++}$, $p< 0.001$). OV2008 cells were infected with adenoviral AAkt1 or LacZ (MOI= 0-10; 24 h) and cultured with CDDP (0-10 μM; 24 h). $FLIP_L$, $FLIP_S$, HA-AAkt1 and GAPDH contents were assessed by Western blotting (upper panel). Apoptosis was determined by Hoechst 33258 staining (Lower panel; ***, $P < 0.001$ vs. control). (B) Expression of activated Akt2 (AAkt2) also inhibits CDDP-induced FLIP degradation and apoptosis ($^{+++}$, $p< 0.001$). OV2008 cells were transfected with sense cDNA of AAkt2 or PCMV6 as control (2 μg; 24 h) and cultured with CDDP (0-10 μM; 24 h). $FLIP_L$, $FLIP_S$, and GAPDH contents were assessed by Western blotting (upper panel), and apoptosis (Lower panel; ***, $P < 0.001$ vs. control). (C) Overexpression of activated Akt2 inhibits CDDP-induced FLIP degradation and apoptosis ($^{+++}$, $p< 0.001$). A2780s-PMH6 (control) and A2780s-AAkt2 (active Akt2) cells were treated with CDDP (0-10 μM; 24 h). Compared to A2780s-PMH6 cells, A2780s-AAkt2 cells exhibited an attenuated CDDP-induced FLIP degradation (upper panel) and apoptosis (lower panel; ***, $P < 0.001$). (D) Expression of AAkt1 inhibited CDDP-induced FLIP degradation and apoptosis ($^{+++}$, $p< 0.001$). OV2008 cells were infected with adenoviral AAkt1 or LacZ (MOI= 10; 24 h) and cultured with CDDP (0-10 μM; 24 h). FLIPL, FLIPS, HA-AAkt1 and GAPDH contents were assessed by Western blotting (upper panel), and apoptosis was determined by Hoechst 33258 staining (Lower panel; ***, $P < 0.001$ vs. control). Results are from three independent experiments.

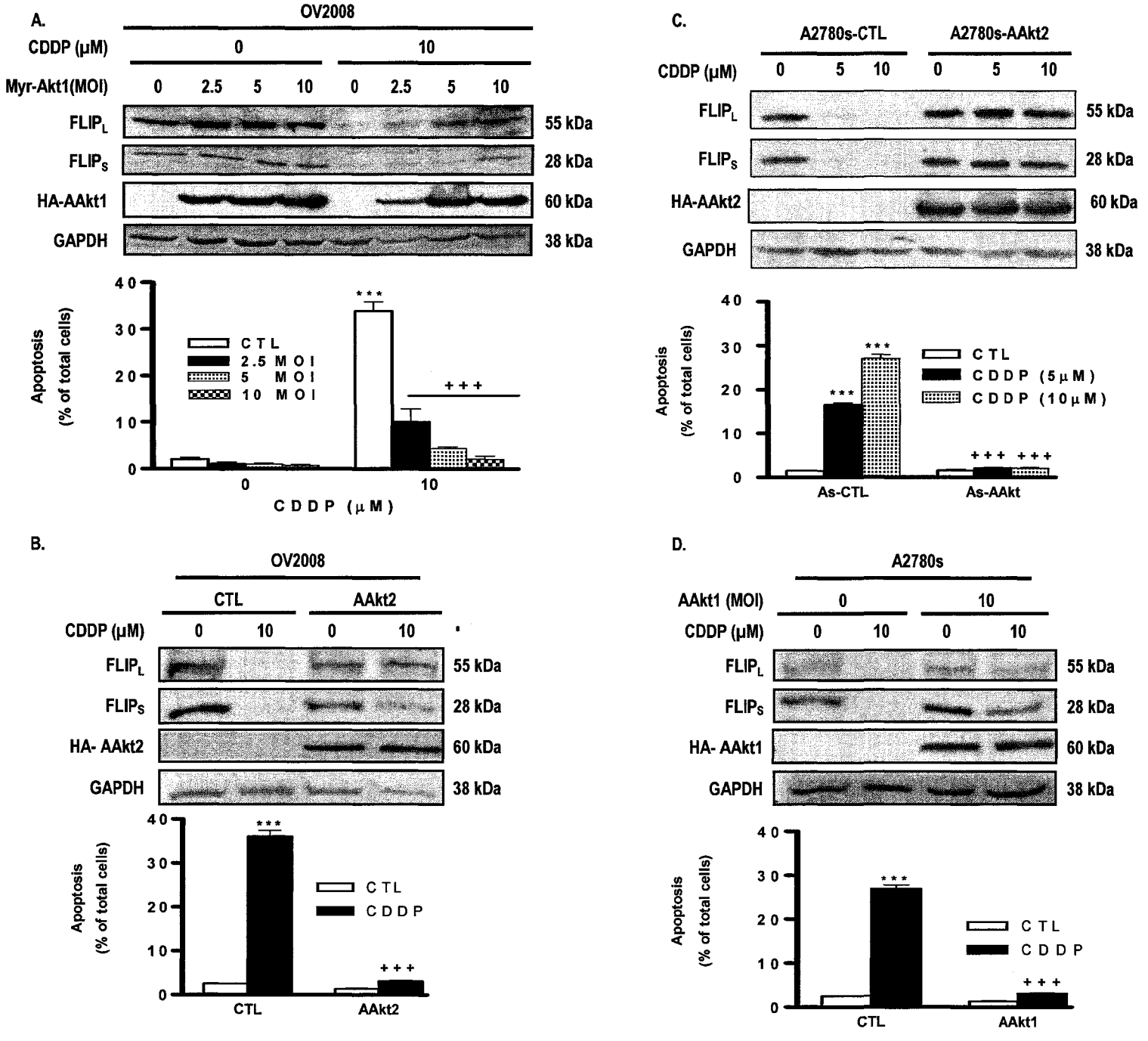
A.
OV2008
CDDP (μM)
0
10
Myr-Akt1(MOI)
0 2.5 5 10 0 2.5 5 10
FLIPL
FLIPS
HA-AAkt1
GAPDH
55 kDa
28 kDa
60 kDa
38 kDa
Apoptosis (% of total cells)
CTL
2.5 MOI
5 MOI
10 MOI
***
+++
CDDP (μM)
B.
OV2008
CTL
AAkt2
CDDP (μM)
0 10 0 10
HA- AAkt2
CTL
AAkt2
C.
A2780s-CTL
A2780s-AAkt2
CDDP (μM)
0 5 10 0 5 10
HA-AAkt2
CDDP (5μM)
CDDP (10μM)
As-CTL
As-AAkt
D.
A2780s
AAkt1 (MOI)
0
10
HA- AAkt1
AAkt1

Figure 4

had no effect on basal FLIP content in A2780cp cells (chemoresistant, mutant p53); DN-Akt expression facilitated CDDP-induced FLIP down-regulation (Fig. 5B, upper panel). The effect of DN-Akt on CDDP-induced FLIP down-regulation in both resistant cell lines was inhibited by Epoxomicin (10nM; Fig. 5C). Taken together these findings suggest that Akt inhibits CDDP-induced FLIP down-regulation by inhibiting its proteasomal degradation. Interestingly, in contrast to that observed in CDDP-resistant C13* cells (wt p53), expression of DN-Akt failed to sensitize A2780cp p53 mutant cells to CDDP-induced apoptosis (Fig. 5B, lower panel).

To further examine the hypothesis that CDDP-induced apoptosis requires p53 function, we have extended the experiment with C13* and A2780cp cells to include additional cell lines with wt- and mutated p53. HEY and OVCA433 cells (wt-p53) infected with DN-Akt (MOI = 0-80, 48 h), transfected with p53 or control siRNA (100 nM; 24 h) and then cultured with CDDP (0-10 μM) for an additional 24 h. Expression of DN-Akt sensitized HEY and OVCA433 cells to CDDP-induced apoptosis ($P < 0.001$), and p53 siRNA markedly attenuated this response ($P < 0.001$). Moreover, OCC1 and OVCAR-3 cells (p53-mutant) were infected with adenoviral DN-Akt or LacZ (MOI = 0-80; 48 h), adenoviral p53 (MOI = 0-10, 24 h), and treated for 24 h with CDDP (0-10 μM). While DN-Akt expression failed to sensitize the cells to CDDP-induced apoptosis ($P > 0.05$), co-expression of wt-p53 sensitized the cells to CDDP ($P < 0.001$) (Fig. 6).

Figure 5: Down-regulation of Akt function facilitates CDDP-induced FLIP degradation in chemoresistant wt- and mutant-p53 ovarian cancer cells but apoptosis in only wt-p53 cells.

(A) C13* infected with DN-Akt (MOI=0-80, 48 h)] and treated with CDDP (0-10 μM; 24 h); CDDP-induced FLIP degradation was facilitated by DN-Akt expression. Down-regulation of Akt also sensitized C13* cells to CDDP-induced apoptosis (***, $P < 0.001$). (B) A2780cp cells were infected with different MOI of adenoviral DN-Akt1 or LacZ (MOI= 0-80; 48 h) and cultured with CDDP (0-40 μM; 24 h). Although DN-Akt expression facilitated basal and CDDP-induced FLIP degradation (B, upper panel), it failed to sensitize the cells to CDDP-induced apoptosis (B, lower panel $P > 0.05$). (C) Akt prevents CDDP-induced FLIP proteasomal degradation. C13* and A2780cp cell were infected with adenoviral DN-Akt or LacZ (MOI= 0-40; 48 h) and treated with CDDP (0-10 μM, 24 h) in the absence and presence of Epoxomicin (25 nM). DN-Akt expression facilitated basal and CDDP-induced FLIP degradation (C), this response was inhibited in the presence of the proteasome inhibitor Epoxomicin.

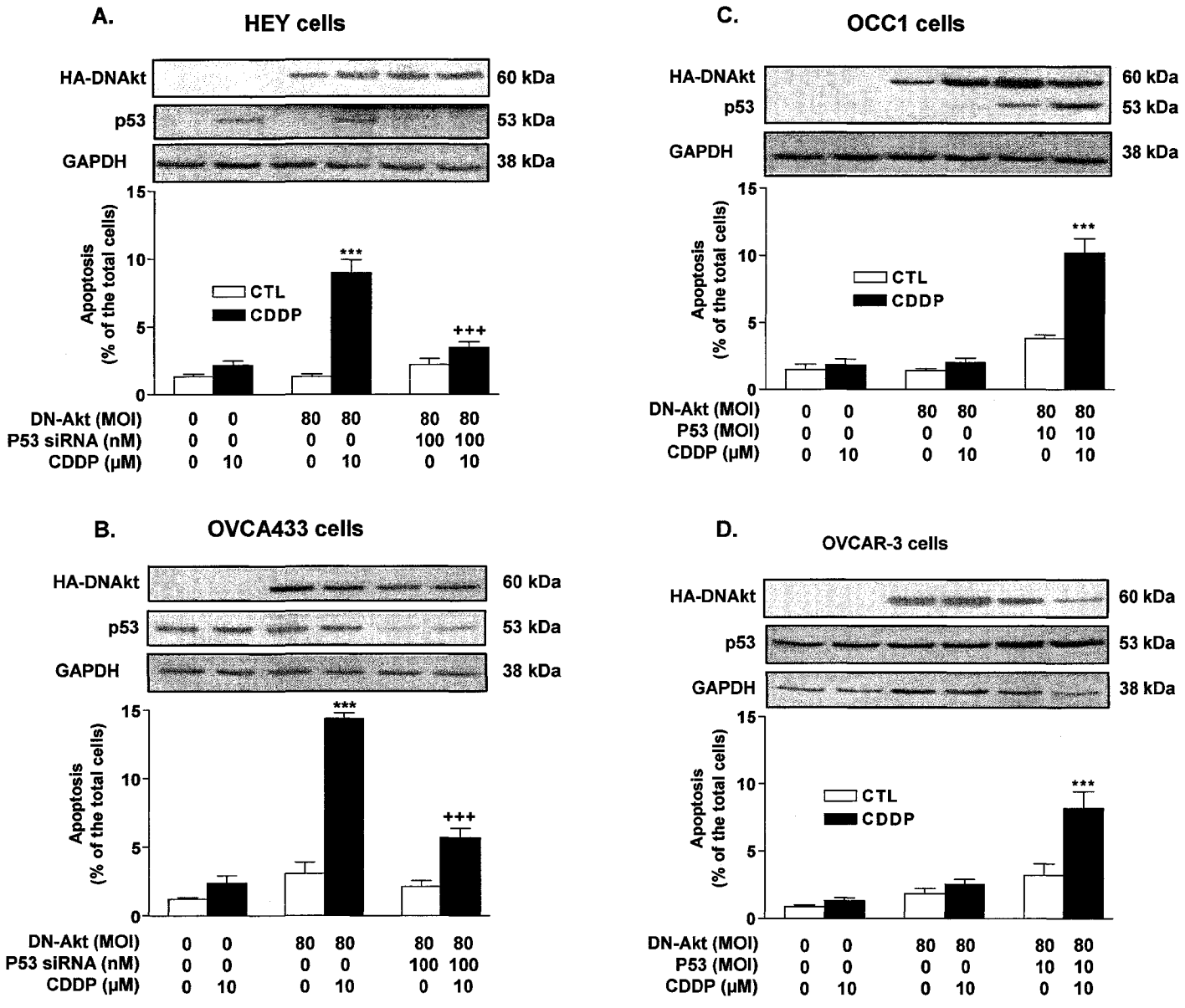
A.
HEY cells
HA-DNAkt
p53
GAPDH
60 kDa
53 kDa
38 kDa
Apoptosis (% of the total cells)
CTL
CDDP
***
+++
DN-Akt (MOI) 0 0 80 80 80 80
P53 siRNA (nM) 0 0 0 0 100 100
CDDP (μM) 0 10 0 10 0 10
B.
OVCA433 cells
C.
OCC1 cells
P53 (MOI) 0 0 0 0 10 10
D.
OVCAR-3 cells

Figure 5

Figure 6: Down-regulation of Akt function facilitates CDDP-induced apoptosis in wt- but not mutant-p53 chemoresistant ovarian cancer cells.

(A-B) DN-Akt expression facilitates CDDP-induced apoptosis in wt-p53 chemoresistant cells. HEY and OVCA433 cells infected with DN-Akt (MOI = 0-80, 48 h), transfected with p53 or control siRNA (100 nM; 24 h) and then treated with CDDP (0-10 μM, 24 h). Expression of DN-Akt sensitized HEY and OVCA433 cells to CDDP-induced apoptosis (***, $P < 0.001$), and p53 siRNA attenuated this response ($^{+++}$, $P < 0.001$). (C-D) DN-Akt-facilitated CDDP-induced apoptosis requires a functional p53. OCC1 and OVCAR-3 cells (p53-mutant) were infected with adenoviral DN-Akt or LacZ (MOI = 0-80; 48 h), adenoviral p53 (MOI = 0-10, 24 h), and treated with CDDP (0-10 μM; 24 h). DN-Akt expression failed to sensitize the cells to CDDP-induced apoptosis ($P > 0.05$), unless the cells were co-expression with wt-p53 (***, $P < 0.001$).

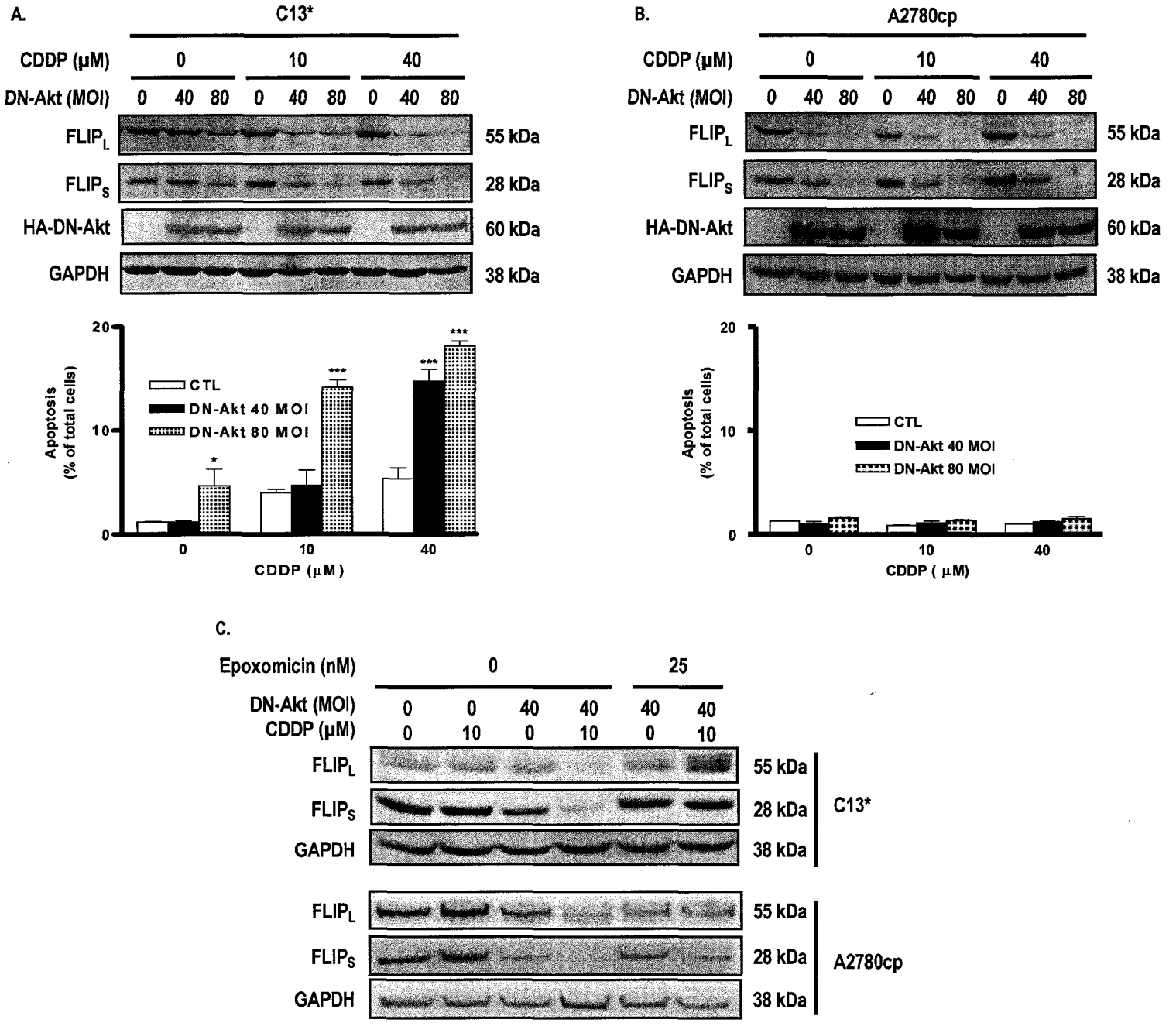
A.
C13*
CDDP (μM)
0
10
40
DN-Akt (MOI)
0 40 80 0 40 80 0 40 80
FLIP$_L$
FLIP$_S$
HA-DN-Akt
GAPDH
55 kDa
28 kDa
60 kDa
38 kDa
Apoptosis (% of total cells)
CTL
DN-Akt 40 MOI
DN-Akt 80 MOI
CDDP (μM)
B.
A2780cp
C.
Epoxomicin (nM)
0
25
DN-Akt (MOI)
0 0 40 40 40 40
CDDP (μM)
0 10 0 10 0 10
C13*
A2780cp

Figure 6

Akt suppresses p53-FLIP interaction and FLIP ubiquitination. To examine whether Akt regulates p53-FLIP binding and FLIP ubiquitination, CDDP resistant cells (C13* & A2780cp), were infected with adenoviral DN-Akt (MOI=80; 48 h), transfected with HA-ubiquitin (2μg; 24 h) and infected (MOI = 25; 24h) with either adenoviral V5- $FLIP_L$, V5-$FLIP_S$ or LacZ (as control). The cells were then transfected with control siRNA or p53 siRNA (100 nM; 24 h) and treated with CDDP (0-10 μM; 1.5-3 h) in the presence of Epoxomicin (10 nM). Protein-protein interaction and FLIP ubiquitination were determined as above. As shown in Fig. 7A & 7C, FLIP-p53 interaction was not detected with non-specific IgG, or in cells only infected with LacZ or only transfected with HA-ub. In the absence of DN-Akt, CDDP had no effect on p53-FLIP interaction and FLIP ubiquitination, but expression of DN-Akt increased p53-FLIP binding (Fig. 7A & 7C) and FLIP ubiquitination (Fig. 7B & 7D). These responses were attenuated by p53 silencing (Fig. 7A-D,), suggesting the possibility that Akt prevents CDDP-induced FLIP down-regulation by inhibiting p53-FLIP binding and FLIP ubiquitination in chemoresistant ovarian cancer cells.

Discussion

In the present study, we have shown that (a) CDDP-induced FLIP ubiquitination and degradation is associated with chemosensitivity in ovarian cancer cells; (b) p53 can directly interact with FLIP and Itch and induce FLIP ubiquitination and its proteasomal degradation in sensitive but not in resistant cells; (c) the CDDP-induced responses were regulated by Akt; and (d) apoptotic response to CDDP was evident in wt-p53 but not mutant p53 chemoresistant cells following Akt down-regulation. These findings suggest that Akt

Figure 7: Akt attenuates CDDP-induced p53-FLIP interaction and FLIP ubiquitination in a p53-dependent manner.

(A) DN-Akt expression facilitated CDDP-induced FLIP-p53 interaction and FLIP ubiquitination (lane 7 and lane 6) after 1.5 h for $FLIP_S$ (C and D) and 3 h for $FLIP_L$ (A & B) responses, which were attenuated in the presence of p53 siRNA (lane 8 and line 9). C13* and A2780cp cells transfected with HA-ubiquitin (2 μg; 24 h), infected with adenoviral DN-Akt (LacZ as control; MOI = 80; 48h), and either: V5- $FLIP_L$ (A and B), V5-$FLIP_S$ (C and D) (MOI = 25; 24h), The cells were then transfected with p53 or control siRNA (100 nM; 24 h) and then treated with CDDP (0-10 μM) and Epoxomicin (25 nM). Cell lysates were immunoprecipitated with IgG control (lanes 1) or without antibody (Lane 2). Cells infected only with LacZ (A and C, Lane 3) or only transfected with HA-ub (B and D, Lane 3) are indicated. At the end of 1.5 h and 3 h, cells were harvested for assessment of p53- $FLIP_L$ and p53-$FLIP_S$ binding, respectively, as well as ubiquitination of $FLIP_L$ and $FLIP_S$ respectively, as described in Fig 1. Results are from three independent experiments.

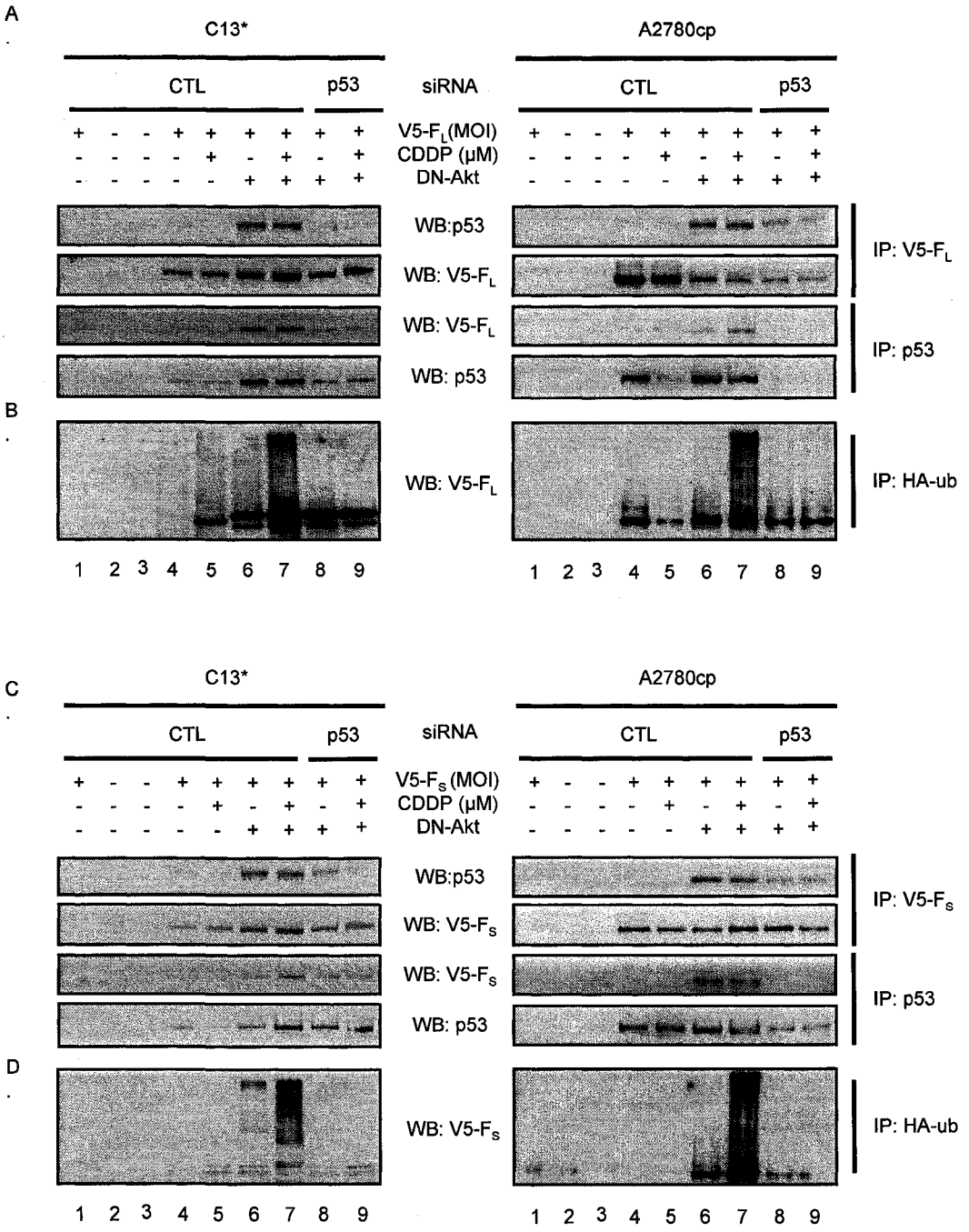
A
C13*
A2780cp
CTL
p53
siRNA
V5-F$_L$(MOI)
CDDP (μM)
DN-Akt
WB:p53
WB: V5-F$_L$
IP: V5-F$_L$
IP: p53
B
IP: HA-ub
1 2 3 4 5 6 7 8 9
C
V5-F$_S$(MOI)
WB: V5-F$_S$
D

Figure 7

promotes chemoresistance, in part, by modulating CDDP-induced, p53-dependent FLIP ubiquitination.

We have previously demonstrated that CDDP induces apoptosis (3, 5-7, 9, 31) and FLIP degradation (5) in chemosensitive ovarian cancer cells but not in their resistant counterparts. We have further shown that CDDP induces FLIP-p53-Itch interaction and co-localization to the cell membrane, and FLIP ubiquitination and proteasomal degradation (14). In the present study, we have observed that the inability of CDDP to down-regulate FLIP in wt-p53 chemoresistant cells is associated with dysregulation of CDDP-induced FLIP ubiquitination and proteasomal degradation, and apoptosis, suggesting that FLIP ubiquitination may be a key determinant of CDDP-induced apoptosis. Moreover, we showed that Itch, an E3 ligase, interacts with both $FLIP_S$ and $FLIP_L$, a response temporally associated with increased FLIP ubiquitination and that CDDP-induced FLIP ubiquitination is dysregulated in chemoresistant cells, suggesting that attenuated FLIP ubiquitination may, in part, contribute to chemoresistance. While FLIP ubiquitination has been demonstrated in several cell lines (38-40), the precise mechanism underlying FLIP ubiquitination was not determined. To our knowledge, this represents the first finding of aberrant regulation of FLIP ubiquitination in chemoresistant ovarian cancer cells.

We have recently demonstrated that Itch is required for CDDP-induced FLIP ubiquitination (14). Moreover, we have shown that Itch directly interacts with FLIP and this response was enhanced by CDDP. The latter response is associated with FLIP-Itch co-localization to the cell membrane and increased CDDP-induced $FLIP_L$ and $FLIP_S$ ubiquitination (14). These results suggest that FLIP-Itch binding may be crucial for FLIP regulation, and that CDDP decreases FLIP content by enhancing FLIP-Itch interaction, FLIP

ubiquitination and proteasomal degradation. In the present studies, we have demonstrated that FLIP-Itch co-localization is associated with FLIP ubiquitination and chemosensitivity in ovarian cancer cells. We have also shown for the first time that Itch binds to $FLIP_S$ and $FLIP_L$ to facilitate FLIP ubiquitination in chemosensitive but not their resistant counterparts. These findings suggest that inability of CDDP to induce FLIP-Itch interaction and subsequently FLIP ubiquitination could contribute to CDDP resistance. In this context, while $FLIP_L$-Itch interaction (14) and FLIP ubiquitination has been detected in several cell subtypes (14, 38-40), the contribution of these phenomenon in chemoresistance has not been determined.

p53, a crucial apoptotic cell death mediator in ovarian cancer cells (7), facilitates FLIP ubiquitination and its proteasomal degradation in colon cancer cells (15). We previously showed that p53 and Itch are required for CDDP-induced FLIP ubiquitination and proteasomal degradation (14). In the present study, we showed that CDDP induces FLIP-p53-Itch interaction and co-localization at the cell membrane in chemosensitive cells but not in their resistant counterparts. Interestingly, CDDP increased p53-FLIP but not p53-Itch co-localization at the cell membrane suggesting that p53-FLIP interaction could occur prior to Itch recruitment. It may be an obligatory step in p53-FLIP-Itch triple co-localization and FLIP ubiquitination.

Our results show a higher basal expression level of p53 in C13* and A2780cp chemoresistant cells when compared to chemosensitive counterparts. p53 expression level was not changed in response to CDDP, according to previous reports (3, 31). Our results show that the density of p53-FLIP-Itch co-clusters at the cell membrane was significantly higher in resistant than in sensitive cells in the absence of CDDP. As expected, CDDP

induces p53 nucleus localization at 6-12 h, but interestingly not earlier (data not shown). It seems that p53-FLIP-Itch and FLIP ubiquitination precedes the nuclear accumulation of p53 (2 h vs. 6-12 h). However, while CDDP failed to alter Itch-p53-FLIP interaction in resistant cells, the proportion of triple co-localization in sensitive cells was significantly increased in response to the chemotherapeutic agent, at a level considerably higher than that observed in its resistant variant. This difference suggests that the lower CDDP-induced FLIP-p53 binding is a more important factor than altered total p53 content in conferring chemoresistance. This represents, to our knowledge, the first demonstration of a pathologic condition under which p53-FLIP-Itch interaction is differentially regulated. Although it has been shown that p53 level is negatively associated with FLIP content (15, 41), our results demonstrate a higher proportion of p53-FLIP interaction in chemosensitive cells treated with CDDP, supporting our hypothesis that p53 serves as a docking protein in facilitating FLIP-Itch interaction and Itch-mediated FLIP ubiquitination (14). Furthermore, the lower proportion of p53, FLIP and Itch cross-interaction in chemoresistant cells when compared to chemosensitive cells could explain the abrogation of CDDP-induced FLIP degradation and apoptosis.

Akt is a determinant of CDDP resistance in ovarian cancer (6, 7, 31). Akt/PI3K modulates the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) - and Fas-mediated cell death by up-regulating FLIP mRNA and/or protein content in cancer cells, hepatocytes and endothelial cells (17-19, 42-47). Here we show that Akt activation inhibited CDDP-induced FLIP down-regulation and apoptosis in chemosensitive ovarian cancer cells (C13*). Moreover, inhibition of Akt function facilitated CDDP-induced FLIP down-regulation in mutant and wt-p53 chemoresistant cells. We further observed that down-regulation of Akt function facilitates $FLIP_L$ and $FLIP_S$ degradation through proteasome

pathway. These findings not only confirm the role of Akt in CDDP resistance in ovarian cancer cells, but also support our contention that modulation of FLIP down-regulation could be one mechanism by which Akt confers chemoresistance.

We observed that suppression of Akt function sensitizes wt-p53 (C13*, HEY & OVCA433), but not p53-mutant chemoresistant cells (A2780cp, OCC1 & OVCAR-3) to CDDP-induced apoptosis, suggesting that Akt-mediated chemoresistance may be mediated, in part, via suppression of p53 function. Although down-regulation of Akt function facilitated FLIP ubiquitination and its degradation in this p53 mutant resistant cell line, CDDP-induced apoptosis was not evident, suggesting that FLIP down-regulation in response to CDDP is necessary but not sufficient to induce apoptosis, and requires the presence of functional p53. This observation is consistent with our previous finding that DN-Akt expression is not able to facilitate CDDP-induced apoptosis in A2780cp (p53 mutant) cells unless reconstituted with wt-p53 (3, 7, 31), and also significantly attenuated in the presence of pifithrin-α-hydrobromide (PFT), a specific inhibitor of p53 function (Fraser et al., 2003). We have also demonstrated that p53 silencing attenuates CDDP-induced mitochondrial Smac release and apoptosis in C13* cells stably transfected with DN-Akt2 compared with control (31). In this context, p53 contains mutations of Val to Phe (codon 172; exon 5) and Arg to Ser (codon 260; exon 8) in A2780cp (Fraser et al., 2008).

Inhibition of Akt function promotes CDDP-induced FLIP-p53 interaction, suggesting that Akt may regulate FLIP ubiquitination and its proteasomal degradation. Thus, Akt may inhibit apoptosis, in part, by attenuating p53-mediated FLIP ubiquitination. Whereas p53 facilitates FLIP down-regulation (15) and p53 level and function could be correlated with FLIP down-regulation in response to various cellular stimuli (15, 41), the present report

provides the first direct evidence for a role of p53 in FLIP degradation. Indeed, Akt has been shown to up-regulate FLIP content (16-19, 42-47) and/or to alter p53 content by activating MDM2 (48-50). Our results provide strong evidence that Akt may have a wide-ranging anti-apoptotic role, which includes interfering with the FLIP-p53 binding and FLIP ubiquitination. Since p53-FLIP interaction is associated or correlated with p53-induced apoptosis (14), this suggests that prevention of FLIP-p53 binding by Akt may be a mechanism by which Akt inhibits apoptosis and confers chemoresistance.

In summary, our studies clearly establish that FLIP ubiquitination is an important contributor to CDDP-induced FLIP degradation and apoptosis and show that chemoresistance is, in part, mediated through the ability of Akt to attenuate this p53-dependent process. While FLIP ubiquitination does not require functional p53, CDDP-induced apoptosis is dependent to p53 status. Furthermore, FLIP ubiquitination can be triggered by FLIP-p53 interaction in an Itch-dependent manner, as down-regulation of Itch by siRNA attenuates CDDP-induced FLIP ubiquitination in chemosensitive ovarian cancer cells (14). Since FLIP-p53 interaction is attenuated in chemoresistant cells in response to CDDP and is restored by down-regulation of Akt function, this phenomenon is likely to be a critical step in CDDP-induced apoptosis. Based on these findings, and to facilitate future investigations into the cellular mechanisms of CDDP resistant in ovarian cancer, a hypothetical model is proposed (Fig. 6). Our results contribute to a better understanding of the mechanism(s) involved in CDDP resistance, which may improve treatment outcomes for human ovarian cancer.

Figure 8: Hypothetical model illustrating the regulation of CDDP-induced p53-FLIP-Itch interaction and FLIP ubiquitination and degradation in the control of apoptosis by Akt in chemosensitive and chemoresistant ovarian cancer cells.

In chemosensitive cells, CDDP up-regulates FLIP-p53-Itch interaction and co-localization to cell membrane, and induces FLIP ubiquitination and degradation. Moreover, CDDP activates caspase-8 and caspase-3, and induces apoptosis. In resistant cells, Akt blocks CDDP-induced FLIP-p53-Itch interaction and co-localization, thereby blocking FLIP ubiquitination and its proteasomal degradation, caspase activation and apoptosis.

Dysregulation of FLIP in chemoresistant OVCA cells

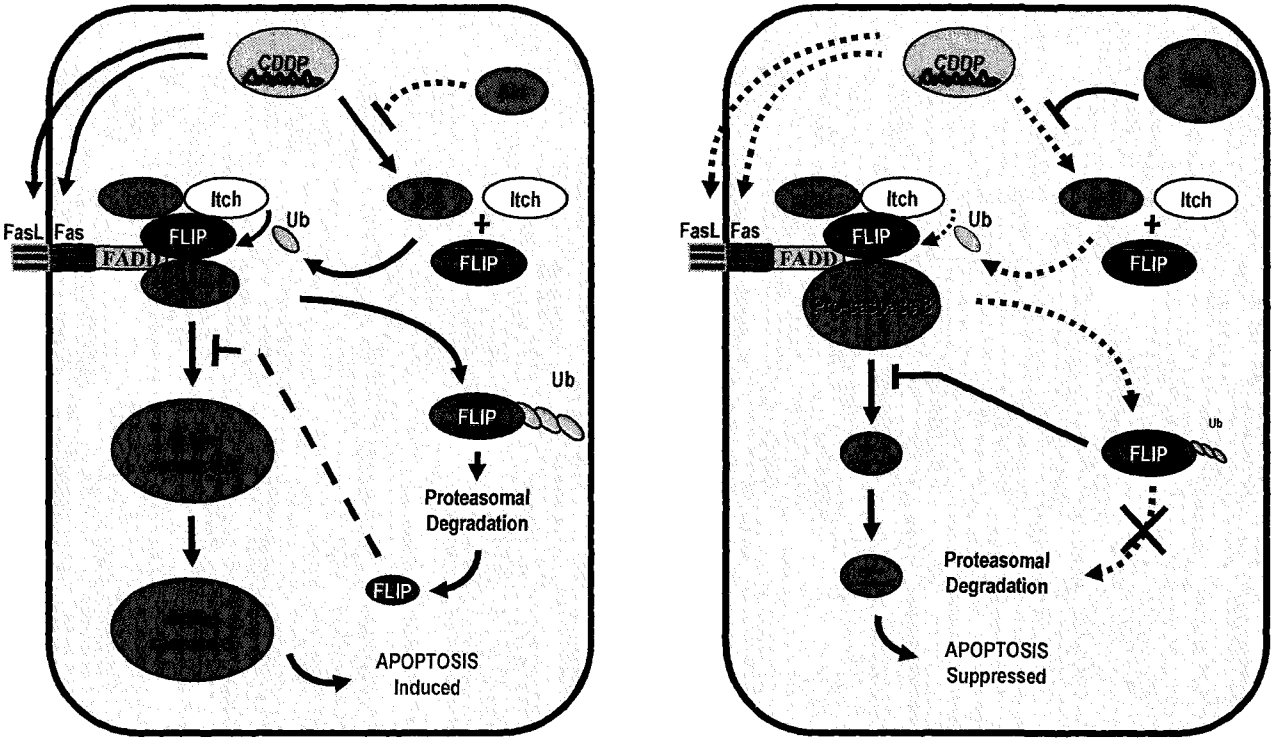

Figure 8

Acknowledgements

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Chapter 6 - General Discussion

6.1 Overview and Significance

Ovarian cancer is the most lethal gynecological malignancy, a condition due in part to the development of resistance to chemotherapy. Chemoresistance is a multi-factorial phenomenon; however, dysregulation of apoptosis signaling pathways is usually observed in chemoresistant cancer and is often associated with poor prognosis. A better understanding of the precise molecular mechanisms implicated in the induction of apoptosis in ovarian cancer cells may ultimately improve our knowledge and understanding of the physiopathology of chemoresistance.

In this thesis, we address the mechanisms of cell fate regulation in human ovarian cancer cells by examining if and how intracellular intermediates such as FLIP, p53, Akt, and the proteasome pathway contribute to the regulation of apoptosis in ovarian cancer cells. Specifically, we have determined if and how they interact to facilitate CDDP sensitivity in ovarian cancer cells and how dysregulation of these processes influences the responsiveness of the cells to CDDP. Understanding the cellular and molecular mechanisms underlying chemoresistance in ovarian cancer cells will provide important clues for the development of new treatment strategies and ultimately improve clinical outcomes for ovarian cancer patients.

6.2 Apoptosis as a Determinant of CDDP Sensitivity

Apoptosis is a critical response to chemotherapeutic agents, such as CDDP, in human ovarian tumors and cancer cells, and is highly correlated to the sensitivity of ovarian cancer cells to CDDP *in vitro* (Gibb, Taylor et al. 1997; Anderson, Lawson et al. 2004). In addition, dysregulation of apoptosis signaling pathways are often associated with poor

clinical response to these drugs *in vitro* and *in vivo* (Skirnisdottir, Seidal et al. 2002; Kupryjanczyk, Szymanska et al. 2003; Murata, Haisa et al. 2004). Thus, the ability to modulate the apoptosis cascade may be a critical determinant in overcoming chemoresistance in human ovarian cancer and understanding the precise mechanisms by which apoptosis in ovarian cancer cells is regulated is highly important.

We have examined the apoptotic response of ovarian cancer cells to CDDP *in vitro* (Li, Sasaki et al. 2000; Sasaki, Sheng et al. 2000; Asselin, Mills et al. 2001; Fraser, Leung et al. 2003) and demonstrated that CDDP induces apoptosis in chemosensitive ovarian cancer cells, but not in their chemoresistant counterparts (Chapters 3 and 5). These findings suggest that the induction of apoptosis is a critical determinant of CDDP sensitivity in ovarian cancer cells.

6.3 Experimental Value and Advantage of Matched Pair Ovarian Cancer Cell Lines

The use of matched pairs of chemosensitive parental cell lines (OV2008 & A2780s) and CDDP resistant variants (C13* & A2780cp) which were developed through prolonged culture in the presence of increasing concentrations of CDDP, have greatly facilitated investigations of the molecular mechanisms of chemoresistance in ovarian cancer cells. These cell lines were established from cells from a serous cystoadenocarcinoma (Brown, Clugston et al. 1993; Mamenta, Poma et al. 1994). The chemoresistant cell variants are genetically identical to the parental lines except for those factors that determine chemosensitivity, and subsequent manipulations of these cells take place on identical genetic backgrounds. This is greatly preferable for the selection of random chemosensitive and chemoresistant cell lines without any confounding difference on their response to the drug.

These paired cell lines allow us to make stronger conclusions on the observed differences between chemosensitive and chemoresistant cells which are related to this particular difference and not simply from the result of random differences between these cell types. The data presented in this thesis has been obtained using paired cell lines, in order to maximize its applicability and relevance. Although we have used one pair ovarian cancer cells (OV2008 and C13*) in chapter three, we have extended these studies to include another pair of chemosensitive and resistant ovarian cancer cells in chapters 4 and 5 of the thesis. Moreover, to examine whether functional p53 requires for CDDP sensitivity, we have used two extra wt-p53 (HEY and OVCA433) and mutant p53 (OCC1 and OVCAR-3) cell lines in chapter 5.

We have assessed apoptosis morphologically (nuclear shrinkage, fragmentation and condensation), using Hoechst nuclear staining. This is a well established assay and have been used extensively to determine apoptosis in our laboratory (Schneiderman, Kim et al. 1999; Sasaki, Sheng et al. 2000; Asselin, Mills et al. 2001; Fraser, Leung et al. 2003; Abedini, Qiu et al. 2004; Dan, Sun et al. 2004; Fraser, Chan et al. 2006; Yan, Fraser et al. 2006; Yang, Fraser et al. 2006; Abedini, Muller et al. 2008; Fraser, Bai et al. 2008; Yang, Fraser et al. 2008) and in those of others (Potter and Hanson 2000; Sima, Wang et al. 2007; Yu, Wang et al. 2007; Zhang, Wu et al. 2007; Nagahama, Ishimaru et al. 2008; Xu, Wang et al. 2008). In addition, the counters in our laboratory were "blinded" to the sample identity to avoid experimental bias.

Moreover, we also determined caspase-8 and caspase-3 activation (evident by caspase cleavage) in chemosensitive ovarian cancer cells treated with CDDP (Abedini, Qiu et al. 2004). As expected, CDDP-induced FLIP down-regulation, caspase-8 and caspase-3 activation and apoptosis (assessed by Hoechst staining) in chemosensitive cells, CDDP were

not able to induce FLIP degradation, caspase activation, nor apoptosis in the chemoresistant variant.

6.4 FLIP as a Determinant of Chemoresistance in Ovarian Cancer Cells

Data from our laboratory and other groups suggest that FLIP dysregulation is an important phenomenon in the development of CDDP resistance in ovarian cancer (Xiao, Yan et al. 2003). Indeed, we have demonstrated that over-expression of FLIP confers resistance to TNFα- and CDDP-induced apoptosis, while FLIP down-regulation sensitized their resistant counterparts to CDDP. Similar observations have been reported by other groups (Kinoshita, Yoshikawa et al. 2000; Kamsteeg, Rutherford et al. 2003; Kim, Ajaz et al. 2003).

In this context, FLIP is amplified or overexpressed in different cancers, including human ovarian cancer (Kamarajan, Sun et al. 2003; Kamsteeg, Rutherford et al. 2003; Mezzanzanica, Balladore et al. 2004; Horak, Pils et al. 2005). Importantly, dysregulation of FLIP expression is often associated with chemoresistance in human ovarian cancer (Kamarajan, Sun et al. 2003; Abedini, Qiu et al. 2004), suggesting that FLIP is implicated in chemoresistance and may regulate chemosensitivity.

Taken together, FLIP is an important regulator of apoptosis and plays a critical role in the regulation of CDDP sensitivity in human ovarian cancer cells. Modulation of FLIP may serve as a potential therapeutic strategy for chemoresistant ovarian cancer.

6.4.1 Regulation of CDDP-Induced FLIP Down-Regulation

We have demonstrated that CDDP down-regulated FLIP protein content in chemosensitive ovarian cancer cells but not in their resistant counterparts (Abedini, Qiu et al.

2004). While FLIP is an important regulator of CDDP-induced apoptosis, the mechanisms by which chemotherapy regulates FLIP in ovarian cancer cells remain largely unknown. Our studies demonstrate that CDDP does not affect FLIP mRNA content (Abedini, Muller et al. 2008), suggesting that CDDP regulates FLIP protein content at the post-translational level. Thus, the inability of CDDP to down-regulate FLIP and maintenance of high levels of FLIP may be one mechanism by which chemosensitivity is dysregulated in chemoresistant cells. CDDP-induced FLIP down-regulation in ovarian cancer cells is blocked by inhibitors of the 26S proteasome, MG132, Lactacystin and Epoximicin, suggesting that CDDP down-regulates FLIP through the proteasome pathway (Abedini, Muller et al. 2008). Furthermore, a specific caspase-3 inhibitor did not block CDDP-induced FLIP down-regulation [Chapter 8, Figure 1, suggesting that the caspase-mediated FLIP processing known to occur during Fas-induced apoptosis (Hennino, Berard et al. 2000; Kim, Ajaz et al. 2003) is not involved in the action of CDDP in the ovarian cancer cells.

6.4.2 Involvement Of Ubiquitin-Proteasome Pathway in FLIP Regulation

While previous studies demonstrate that CDDP induces FLIP ubiquitination and down-regulates FLIP content, the precise mechanisms by which CDDP brings about these responses is not clear. Our findings offer important insight on a novel role for p53 in the propagation of CDDP-induced FLIP degradation (Abedini, Muller et al. 2008). This implies that additional mechanisms, independent of p53 mutational status, may influence CDDP sensitivity in the induction of FLIP degradation. In support of this hypothesis, we demonstrated that CDDP enhances FLIP-p53 interaction and facilitates FLIP ubiquitination and FLIP degradation in chemosensitive ovarian cancer cells (Abedini, Muller et al. 2008).

While CDDP up-regulates p53 content, promotes FLIP ubiquitination, down-regulates FLIP protein content and induces apoptosis in chemosensitive cells, these responses were attenuates by p53 silencing (Abedini, Muller et al. 2008), confirming our hypothesis that CDDP-induced FLIP down-regulation is p53-dependent.

While p53 is required for FLIP ubiquitination, it does not have E3 ligase activity, suggesting that p53 may play a different role in CDDP-induced, p53-dependent FLIP ubiquitination. In this context, our findings clearly showed that Itch (a member of Hect super family) interacts with FLIP and p53 in response to CDDP in chemosensitive cells, and facilitates FLIP ubiquitination. Moreover, our studies suggest that both Itch and p53 are required for CDDP-induced FLIP ubiquitination. These findings strongly suggest, in spite of the E3 ligase activity of Itch, that p53 is needed for FLIP ubiquitination and may serve as a docking protein to facilitate Itch-FLIP interaction and the transfer of ubiquitin to FLIP. This notion is supported by our confocal microscopic findings that FLIP, p53, Itch are mainly diffusedly localized in the cytoplasm in chemosensitive ovarian cancer cells, and that CDDP treatment *in vitro* results in clusters of the three proteins at the cell membrane, a response which is not evident in its chemoresistant counterpart (Abedini, Muller et al. 2008).

Taken together, our studies provide the first evidence in support of the notion that FLIP-p53-Itch interaction and co-localization at the cell membrane may be an integral part of the cellular mechanisms by which CDDP induces FLIP ubiquitination and proteasomal degradation in chemosensitive ovarian cancer cells. However, the importance of this complex and its precise involvement in the regulation of CDDP-induced FLIP ubiquitination in ovarian cancer remains to be determined.

We have demonstrated that CDDP induced $FLIP_S$ and $FLIP_L$ ubiquitination at 1.5 and 3 h, respectively (Chapter 4, Fig. 3). These responses are consistent with the CDDP-induced FLIP protein content as early as 6 h for $FLIP_S$ (Data no shown) and 12 h for $FLIP_L$ (Chapter 4, Fig. 1). In this context, previous studies shown that lysine 192 and 195 in C-terminal to the death effector domains are the principal ubiquitin acceptors in $FLIP_S$ but not in $FLIP_L$. In addition, the $FLIP_S$ specific tail of 19 amino acids, adjacent to the two target lysines, is a key element determining the isoform-specific instability of $FLIP_S$. Site-directed mutagenesis and molecular modeling studies demonstrated that the C-terminal tail is required for correct positioning and subsequent ubiquitination of the target lysines, possibly leading to a better availability of the ubiquitin target Lys192 and Lys195 in $FLIP_S$. Furthermore the structure of $FLIP_L$ is considerably longer at the C-terminus, and other structural features (e.g. protein folding) may function to block access to the lysines and inhibit ubiquitination of these sites, thereby delay its ubiquitination (Poukkula, Kaunisto et al. 2005). It seems the stability of $FLIP_S$ is directly correlated with its ubiquitination, since FLIPs has a shorter half life than $FLIP_L$ (20 min. *vs.* 2 h) (Poukkula, Kaunisto et al. 2005), this may explain why $FLIP_S$ ubiquitination occurs earlier and does not last as long as $FLIP_L$ ubiquitination. Moreover, whether this due the faster deubiquitination of $FLIP_S$ in comparison to $FLIP_L$ by deubiquitin enzyme is not known and requires furthur elucidation. .

6.5 Implication of Altered FLIP Ubiquitination in Chemoresistance

While CDDP down-regulates FLIP protein content in chemosensitive ovarian cancer, it is ineffective in their resistant variants (Abedini, Qiu et al. 2004), suggesting that the inability of CDDP to down-regulate FLIP may be a contributing factor to CDDP

resistance in ovarian cancer. Our results on chemoresistant cells show that CDDP likewise could not induce FLIP ubiquitination (Chapter 5, Figure 1), Taken together, these findings support our hypothesis that dysregulation of FLIP ubiquitination and its proteasomal degradation are critical events through which apoptosis is suppressed and CDDP resistance is conferred in p53-wt ovarian cancer cells.

Compared to their chemosensitive parental cells (OV2008 and A2780s), the chemoresistant C13* and A2780cp cells exhibit high p53 content in the absence of CDDP, despite the fact that CDDP does not induce FLIP-Itch and p53-Itch interactions, and FLIP-p53-Itch co-localization to cell membrane, FLIP ubiquitination and apoptosis in these cells (Figure 1 & 2, Chapter 5) . These observations strongly support the hypothesis that FLIP ubiquitination is important for CDDP-induced FLIP down-regulation and that interaction of p53 and FLIP is necessary for this process.

To our knowledge, our studies offer the first demonstration that Itch, FLIP and p53 interact at the cell membrane following CDDP treatment and that Itch and p53 are required for CDDP-induced FLIP ubiquitination. The triple co-localization of these proteins provides an opportunity for Itch to ubiquitinate FLIP in a p53-dependent manner.

6.6 Akt as a determinant of Chemoresistance in ovarian cancer

The PI3K/Akt pathway is highly activated or over-expressed in cancers, including ovarian cancer (Cheng, Godwin et al. 1992; Bellacosa, de Feo et al. 1995; Yuan, Sun et al. 2000; Sun, Wang et al. 2001), and it is important in the regulation of chemosensitivity. Constitutively activated PI3K (p110) stably transfected in human ovarian cancer cells (DOV-13) conferred taxol resistance *in vitro* and in a xenograft model (Hu, Hofmann et al. 2002). The PI3K inhibitor

LY294002 rendered tumors sensitive to taxol *in vivo*, thereby increasing the median survival time of the mice inoculated with activated p110 cells.

Our laboratory has previously demonstrated that ovarian cancer cells stably transfected with activated Akt2 (A2780s-Akt2) fail to undergo CDDP-induced apoptosis. Suppression of Akt function by dominant-negative Akt (DN-Akt) expression also sensitizes chemoresistant cells to CDDP as evident by increased apoptotic response (Fraser, Leung et al. 2003). Activation of Akt by amino terminus myristoylation facilitates Akt recruitment to the cell membrane (Andjelkovic, Alessi et al. 1997), markedly enhances Akt kinase activity, and confers CDDP resistance in chemosensitive ovarian cancer cells (Yuan, Feldman et al. 2003; Dan, Sun et al. 2004).

Taken together, these results suggest that Akt activation, a frequent (~%30) occurrence event in human ovarian cancer, confers resistance to CDDP and that suppression of Akt function is an effective means of overcoming CDDP resistance.

We have demonstrated that suppression of Akt function facilitates CDDP-induced FLIP down-regulation and sensitized chemoresistant cells to CDDP, and that expression of activated Akt inhibits FLIP down-regulation and apoptosis induced by CDDP (Chapter 5, Figure 43), suggesting a functional relationship between Akt and FLIP. Importantly, while suppression of Akt function leads to FLIP down-regulation by CDDP, this response is attenuated by proteasome inhibition (Chapter 5, Figure 45). These results are consistent with the findings from previous studies (Panka, Mano et al. 2001; Suhara, Mano et al. 2001) and suggest that Akt confers resistance to CDDP in ovarian cancer cells in part by suppressing FLIP down-regulation through the proteasome pathway.

In the present studies, we have demonstrated that FLIP ubiquitination is required for CDDP-induced, proteasomal-dependent FLIP down-regulation by CDDP. Moreover, we have provided evidence which implicates for the first time Akt in the regulation of CDDP-induced FLIP ubiquitination in ovarian cancer cells (Chapter 5, Figure 5). While FLIP ubiquitination is not detected in control cells, DN-Akt expression enhances basal and CDDP-induced FLIP ubiquitination. Thus, our results clearly indicate that Akt is involved in the regulation of CDDP-induced FLIP ubiquitination and its proteasomal degradation, although the mechanism (s) involved is not known and awaits further investigations. It is possible that CDDP is not able to inhibit Akt activation or content in chemoresistant cells compared to their sensitive counterparts. Our laboratory has previously demonstrated that CDDP induces the caspase-3-mediated cleavage of Akt in chemosensitive ovarian cancer cells (Asselin, Mills et al. 2001; Jahani-Asl, Basak et al. 2007). Moreover, since FLIP does not have Akt phosphorylation consensus site (RXXRXS/T), its ubiquitination is unlikely associated with being an Akt substrate and thus negatively regulated by Akt directly, as has been demonstrated with another caspase inhibitor XIAP (Dan, Sun et al. 2004).

Our laboratory has previously demonstrated that inhibition of Akt function in chemoresistant cells facilitates CDDP-induced apoptosis in a p53-dependent manner, suggesting the existence of a functional relationship between Akt and p53 (Fraser, Leung et al. 2003; Fraser, Chan et al. 2006; Fraser, Bai et al. 2008; Yang, Fraser et al. 2008). The present studies show that inhibition of Akt function enhances FLIP-p53 interaction and induces FLIP ubiquitination in wild type p53 chemoresistant ovarian cancer cells C13* (Chapter 5; Figure 57). Importantly, these responses are markedly attenuated by p53 silencing; suggesting that Akt may confer resistance to CDDP by suppressing p53-FLIP binding, thereby FLIP ubiquitination and its proteasomal

degradation. Similar responses were observed in the mutant p53 chemoresistant ovarian cancer cells, A2780cp (Chapter 5, Figure 57), suggesting that CDDP-induced FLIP ubiquitination is unlikely dependent on p53 function. However, since these cells do not undergo apoptosis under the same condition, these findings raise the possibility that FLIP down-regulation is necessary but not sufficient for p53-mediated apoptosis and that a functional p53 is required. Our laboratories have evaluated the *TP53* sequence in A2780cp by direct sequencing of genomic DNA codons 171-173 and demonstrated that p53 contains mutations of Val to Phe (codon 172; exon 5) and Arg to Ser (codon 260; exon 8) in A2780cp (Fraser, Bai et al. 2008). Moreover, these two cell line are from different patient and different ovarian cell types.

While the precise mechanism by which Akt specifically inhibits p53-FLIP interaction remains to be investigated in detail, the involvement of p53 in FLIP ubiquitination is of significance. It is well established that p53 plays an important role as a transcription factor in cell cycle, DNA repair and apoptosis regulation in the nucleus (Lane 1992; Buttitta, Marchetti et al. 1997). In addition, our studies also show that while CDDP increases p53 phosphorylation in chemosensitive cells (but not in resistant cells and is attenuated by Akt activation), p53 targets the mitochondria in the induction of the release of cytochrome C, Smac and AIF, as well as of apoptosis (Yang, Fraser et al. 2006; Yang, Fraser et al. 2008), demonstrating an extra-nuclear action of p53 and its regulation by Akt in the pathobiology of CDDP resistance in ovarian cancer. The present investigations suggest a third and novel mechanism through which p53 regulates apoptosis in ovarian cancer cells by facilitating the FLIP-Itch interaction through formation of FLIP-Itch-p53 clusters at the cell membrane and FLIP ubiquitination, p53 enables CDDP to exert its pro-apoptotic action. Importantly, the

interaction of these three proteins is down-regulated by Akt activation and the FLIP-Itch-p53 clusters at the cell membrane and subsequent FLIP ubiquitination are absence in CDDP-treated chemoresistant ovarian cancer cells, suggesting another important function of p53 in CDDP-induced apoptosis and its dysregulation in chemoresistant ovarian cancer.

6.6.1 Akt as a Therapeutic Target

Recent studies suggest that modulation of the Akt pathway could be a therapeutic modality for the cancer patients including those with ovarian cancer. Studies on specific kinases such PDGF, ErbB2, and Bcl-Abl as therapeutic targets (Buchdunger, Zimmermann et al. 1996; Druker, Tamura et al. 1996; Traxler, Furet et al. 1996; Baselga, Norton et al. 1998; Pegram, Lipton et al. 1998; Shaheen, Davis et al. 1999) provide evidence that modulation of the Akt pathway or its components may be an important therapeutic strategy. While monoclonal antibody-based therapy is another clinical strategy, such as anti-ErbB2 monoclonal antibody (Trastuzamab), for treatment of breast cancer which displays ErbB2 elevation (reviewed in Plosker and Keam 2006), the recombinant monoclonal anti-Akt antibodies inhibit the activation of all three Akt family members, attenuates the activity of constitutively active Akt and the development of xenograft tumors (Shin, Edl et al. 2005) (Shin, Edl et al. 2005). Application of this antibody may be useful for the treatment of human cancer, such as ovarian cancer, which often depends on Akt activation for their growth.

It has recently been reported that three synthetic Akt-selective inhibitors (Breitenlechner, Wegge et al. 2004; Breitenlechner, Friebe et al. 2005) could be used as a novel chemotherapeutics for the treatment of Akt-dependent human tumors. API-2, a specific Akt inhibitor (Yang, Dan et al. 2004), is highly selective for Akt, induces apoptosis in cultured human

cancer cells and inhibits tumor growth in nude mice with xenografts derived from cells transfected with constitutive activated Akt.

Since almost 30% of ovarian cancers are associated with Akt activation, API-2 could be used as a supplement for existing chemotherapeutics for this disease. TCN-P, an API-2-derived compound, demonstrated anti-tumor activity in phase I and phase II clinical trials (Schilcher, Haas et al. 1986; Feun, Blessing et al. 1993); however, its effects were limited due the significant hepatotoxicity. These findings suggest that the modulation of Akt pathway may be a candidate for novel chemotherapeutics, and it may represent an important novel treatment modality for cancers in which Akt activation plays an important role.

6.7 Future Directions

The current studies provide significant evidence that FLIP and Akt are important determinants of CDDP resistance in human ovarian cancer cells. Moreover, Akt-mediated chemoresistance may proceed through suppression of p53-mediated apoptosis, and that this may involve down-regulation of CDDP-induced FLIP-p53-Itch interaction and p53-mediated FLIP ubiquitination. Furthermore, the current studies provide evidence that the ubiquitin proteasome pathway is implicated in the regulation of FLIP degradation by CDDP, and this may also be related to CDDP-induced, p53-mediated FLIP ubiquitination in an Itch dependent manner, thereby CDDP-induced apoptosis.

As such, in addition to the future experiments proposed in the above sections, the following approaches could be considered as future directions.

6.7.1 Xenograft Models

Although the use of cultured cell lines may be convenient and informative, it might not be representative of the cancer cells' behavior *in vivo.* Therefore, the above studies should be extended to evaluate the important pertinent endpoints by using ovarian cancer xenograft models. Xenografts of all chemosensitive and -resistant ovarian cancer cell lines could be used to examine the regulation of Akt and its role on FLIP regulation and CDDP sensitivity. CDDP (at clinical dose equivalent) will be administered intraperitoneally (i.p.) and changes in tumor volume, apoptosis (TUNEL and DNA ladder), and FLIP content and/or ubiquitination (ICC) will be determined.

For additional molecular manipulation of appropriate endpoints, the Tet-On system could be used to generate cell lines stably expressing the reverse tetracycline-controlled transactivator (rtTA) under control of the CMV promoter (e.g. DN-Akt, FLIP, and p53) *in vitro.* The gene of interest can be induced by the addition of doxycyline. Under this system, gene expression can be induced *only* in the appropriate cells, and can be maintained over time through the administration of doxycyline. To demonstrate the functional role of p53 and Akt in the regulation of CDDP-induced FLIP down-regulation and chemosensitivity *in vivo,* chemoresistant cell lines A2780cp and A2780cp-DN-Akt (A2780cp stably expressing DN-Akt via G418 resistance) could be engineered using the above system and xenografts of these cell lines will be established as above, p53 and/or FLIP expression induced through the addition of doxycycline in the drinking water. CDDP will be injected and endpoints will be assessed as above.

6.7.2 Assessment of Clinical Samples

While established cell lines provide an informative and convenient model for the study of molecular mechanisms involved in tumor progression and chemoresistance, their clinical relevance and applicability to the *in vivo* human situation should be established. Specifically, continuous passage of cell lines may change their features such that they no longer are representative of those found in cells present in the original tumors.

The above studies will be extended to include primary cultured human ovarian cancer cells from ascites fluids before and after chemotherapy and solid tumors from patients with stage III/IV ovarian cancer after surgical debulking. These cells will be treated with CDDP *in vitro,* and FLIP, p53 content, total and phospho-Akt will be evaluated. Additionally, the effects of adenoviral DN-Akt expression and activated Akt on CDDP-induced apoptosis, p53-FLIP interaction and FLIP ubiquitination in primary ovarian cancer cells will be examined (as above).

The above studies will demonstrate if the observations on ovarian cancer cell lines are clinically relevant, and also examine the regulation of FLIP, p53 and Akt activation in ovarian tumor chemosensitivity. While these studies will be correlative, they will provide important clinical relevance for the above findings.

6.8 Conclusions

This thesis provides significant insights into the cellular and molecular mechanism of chemoresistance in human ovarian cancer cells. Specifically, we have demonstrated that FLIP is a critical mediator of chemoresistance, and that FLIP confers CDDP resistance in part through inhibiting CDDP-induced apoptosis. CDDP induces FLIP down-regulation through proteasome pathway in chemosensitive ovarian cancer

cells. This is involves FLIP-p53-Itch co-localization, and CDDP-induced, p53- and Itch-dependent FLIP ubiquitination. Additionally, we have provided evidence that Akt is a key determinant of chemoresistance, and that this is related to the regulation of p53-dependent FLIP down-regulation. Specifically, Akt attenuates FLIP down-regulation and apoptosis, while down-regulation of Akt function enhances FLIP-p53 interaction, FLIP ubiquitination in a p53-dependent manner, and sensitizes ovarian cancer cells to CDDP.

Taken together, these findings improve our understanding of cell fate regulation in ovarian cancer cells, and provide evidence which is implicated in the etiology of chemoresistance. A better understanding of the cellular and molecular mechanisms of chemoresistance may ultimately improve treatment modalities for this disease.

Chapter 7 - References

7.1 General Introduction

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7.6 Appendices references

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Chapter 8 - Appendices (Unpublished Data)

CDDP down-regulates FLIP protein content via FLIP proteasomal degradation

Our recent study demonstrated that CDDP down-regulates $FLIP_L$ and $FLIP_S$ content and induces apoptosis in ovarian cancer cells (Abedini, Qiu et al. 2004); however, these responses do not appear to be associated with alterations in $FLIP_L$ and $FLIP_S$ mRNA abundance (Abedini, Muller et al. 2008). To determine if the down-regulation of FLIP contents by CDDP was associated with caspase-3-mediated processing, chemosensitive ovarian cancer cells (OV2008) were pretreated for 30 min with different concentration of caspase-3 inhibitor DEVD (0-25 μM) and cultured with CDDP (0-10 μM, 24 h). FLIP and GAPDH contents (as a loading control) were determined by Western blot. While CDDP decreases $FLIP_L$ and $FLIP_S$ levels, these responses are not affected by the presence of DEVD (Figure 1). Although, DEVD increases basal level of $FLIP_S$ in a concentration-dependent manner, it failed to influence CDDP-induced $FLIP_S$ down-regulation (Figure 1). These results suggest that CDDP may down-regulate FLIP content through other mechanism(s).

Figure 1: CDDP failed to down-regulate FLIP by caspase3-mediated processing

CDDP-induced $FLIP_L$ and $FLIP_S$ down-regulation are not affected by inhibition of caspase-3 activation in OV2008 cells ($P > 0.05$ vs. control; $n = 3$). FLIP and GAPDH contents were assessed by Western blotting. GAPDH served as a protein loading control. While CDDP decreases $FLIP_L$ and $FLIP_S$ contents, these responses were not affected by the presence of caspase-3 inhibitors ($P > 0.05$, $n = 3$). OV2008 cells were pretreated for 30 min with different concentration of caspase-3 inhibitor DEVD (0-25 μm) and cultured with CDDP (0-10 μM, 24 h).

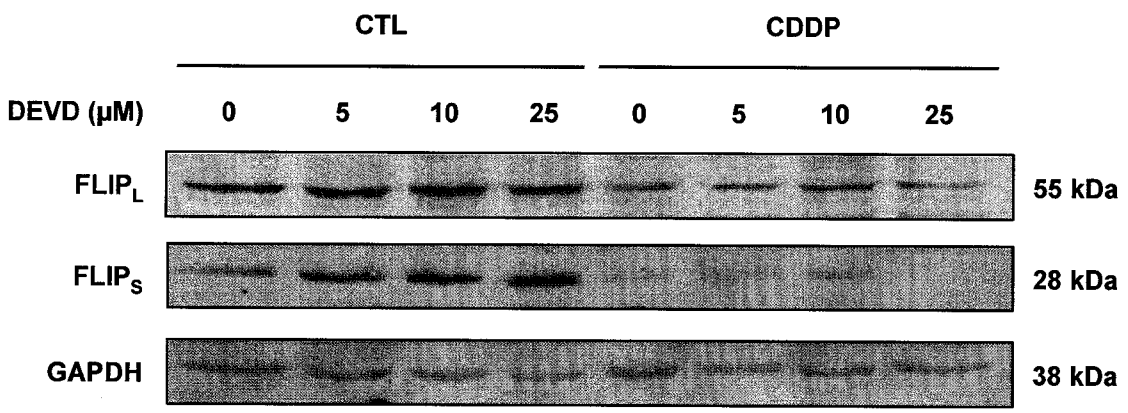
CTL
CDDP
DEVD (μM)
0
5
10
25
0
5
10
25
FLIP$_L$
55 kDa
FLIP$_S$
28 kDa
GAPDH
38 kDa

Figure 1

To determine whether proteasomal degradation is involved in CDDP-induced FLIP down-regulation, OV2008 cells were pretreated for 30 min with proteasome inhibitors Lactacystin (0-25 μM) and MG132 (0-25 μM), and cultured with CDDP for an additional 12 h. Although the presence of Lactacystin had no effect on basal $FLIP_L$ content, it slightly increased that of $FLIP_S$ at 5 μM or higher, and significantly attenuated CDDP-induced $FLIP_L$ ($\geq$ 0.5 μM) and $FLIP_S$ down-regulation (5-10 μM) (Figure 2, n=3).

Moreover, MG132 decreased basal $FLIP_L$ level and slightly increased the basal level of $FLIP_S$ content. MG132 also attenuated CDDP-induced $FLIP_L$ and $FLIP_S$ down-regulation ($\geq$ 2.5 μM) (Figure 3, n=3). To further examine the hypothesis, the above experiment was extended to include the proteasome inhibitor Epoxomicin (0-50 nM). Epoxomicin (10 nM) attenuates CDDP-induced $FLIP_L$ and $FLIP_S$ protein degradation (Figure 4, n=3). Taken together, these findings suggest that FLIP down-regulation in chemosensitive OVCA cells by CDDP involves its proteasomal degradation and proteasome pathway plays an important role in intracellular FLIP protein turnover.

Figure 2: CDDP down-regulates FLIP through proteasomal degradation

CDDP-induced $FLIP_L$ and $FLIP_S$ down-regulation were attenuated by the presence of proteasome inhibitor MG132 ($P < 0.001$, n = 3). FLIP and GAPDH contents were assessed by Western blot. OV2008 cells were pretreated for 30 min with MG132 (0-25 μM) and then treated for 12 h with CDDP or DMSO (control).

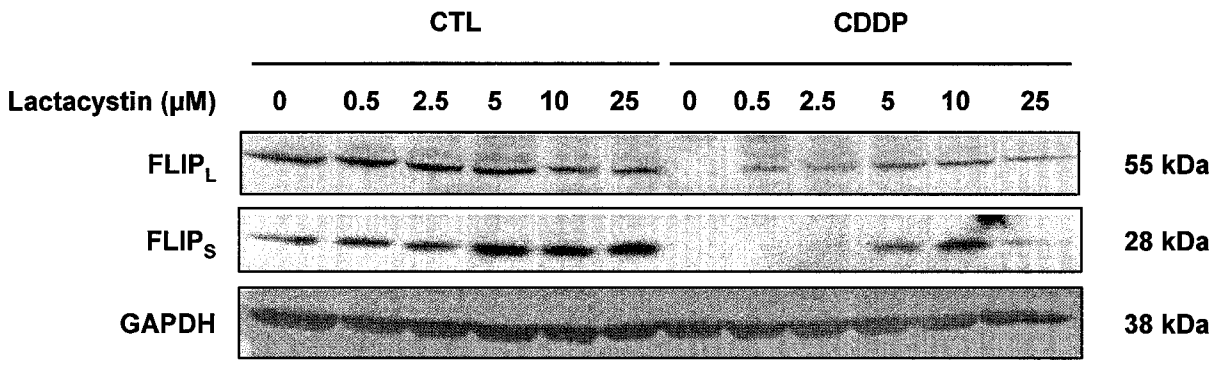
CTL
CDDP
Lactacystin (μM)
0 0.5 2.5 5 10 25 0 0.5 2.5 5 10 25
FLIP$_L$
FLIP$_S$
GAPDH
55 kDa
28 kDa
38 kDa

Figure 2

Figure 3: CDDP down-regulates FLIP through proteasomal degradation

CDDP-induced $FLIP_L$ and $FLIP_S$ down-regulation were attenuated by the presence of the proteasome inhibitor Lactacystin ($P < 0.001$, $n = 3$). OV2008 cells were pretreated for 30 min with Lactacystin (0-25 μM) and then treated for 12 h with CDDP or DMSO (control).

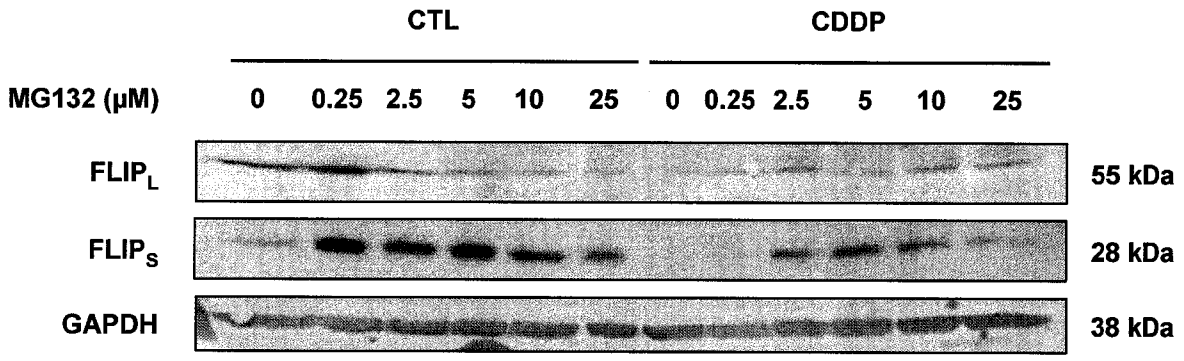
CTL
CDDP
MG132 (μM)
0 0.25 2.5 5 10 25 0 0.25 2.5 5 10 25
FLIP$_L$
FLIP$_S$
GAPDH
55 kDa
28 kDa
38 kDa

Figure 3

Figure 4: CDDP down-regulates FLIP through proteasomal degradation

CDDP-induced $FLIP_L$ and $FLIP_S$ down-regulation were attenuated by the presence of the proteasome inhibitor Epoxomicin ($P < 0.001$, $n = 3$). OV2008 cells were pretreated for 30 min with Epoxomicin (0-50 nM) and then treated for 12 h with CDDP or DMSO (control).

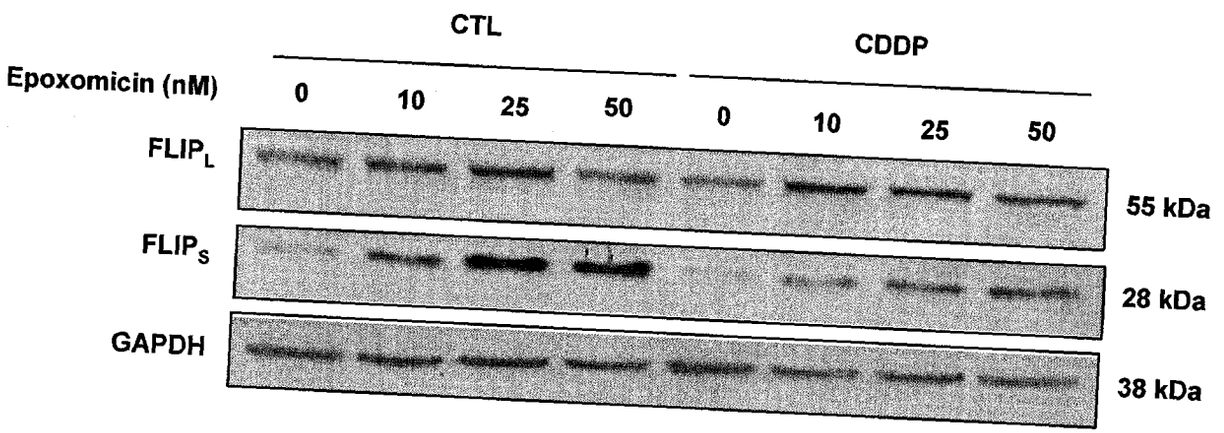
CTL
CDDP
Epoxomicin (nM)
0
10
25
50
0
10
25
50
FLIP$_L$
FLIP$_S$
GAPDH
55 kDa
28 kDa
38 kDa

Figure 4

Our current studies also demonstrate that CDDP-induced FLIP degradation in ovarian cancer cells is associated with $FLIP_S$ and $FLIP_L$ ubiquitination as early as 1.5h and, 3h, respectively (Abedini, Muller et al. 2008). It has been also reported that TRAF-2 binds with FLIP (Park, Kim et al. 2001) and possesses E3 ubiquitin ligase activity (Sun and Chen 2004). To examine whether TRAF-2 directly interacts with FLIP and if such an interaction is affected by CDDP, OV2008 cells were transfected with HA-ubiquitin (2μg; 24 h), infected (MOI = 25; 24h) with adenoviral V5-$FLIP_L$, V5-$FLIP_S$ or LacZ (as control), and treated with CDDP (0-10 μM) in the presence of Epoxomicin (25 nM) for different durations (0-9 h) as indicated in figure 4. FLIP-TRAF-2 interaction was assessed by IP (V5-FLIP) and Western blotting (TRAF-2).

As shown in Figure 12, FLIP-TRAF-2 interaction is not detected with non-specific IgG or in the cells infected with LacZ (lane 2), and was slightly detectable in absence of CDDP. This response was enhanced by CDDP after 3 h for $FLIP_S$ (Figure 4B) and 6 h for $FLIP_L$ (Figure 4A). Since $FLIP_S$ and $FLIP_L$ ubiquitination were induced by CDDP as early as 1.5h and 3h, respectively (Abedini, Muller et al. 2008), it seems that FLIP-TRAF-2 binding is unlikely to be important for the initiation of FLIP ubiquitination.

To examine if p53 interacts with TRAF-2 in response to CDDP, we investigated p53-TRAF-2 interaction by IP (p53) and WB (TRAF-2). OV2008 cells exhibited lower basal p53 levels, which were up-regulated by CDDP in a time-dependent manner (Figure 4A-B). Furthermore, while p53-TRAF-2 interaction was detectable in the absence of CDDP, this response was attenuated by CDDP for $FLIP_L$ (Figure 4A) and $FLIP_S$ (Figure 4B). Our results demonstrate that CDDP decreases p53-TRAF-2 interaction, suggesting that this phenomenon may not be important for FLIP ubiquitination and subsequent degradation.

Figure 5: CDDP enhances FLIP-TRAF-2 and decreases p53-TRAF-2 interactions

FLIP-TRAF-2 interaction was enhanced by CDDP (lane 6) after 3 h for $FLIP_S$ (figure 5B) and 6 h for $FLIP_L$ (figure 5A), but was attenuated with CDDP (Figure 5A-B). OV2008 cells infected (MOI = 25; 24 h) with either adenoviral V5- $FLIP_L$, (A), V5-$FLIP_S$ (B) or LacZ (as control, lane 2), and cultured for different duration with CDDP (0-10 μM) and Epoxomicin (25 nM). Cells only infected with adenoviral LacZ (Lane 2) are indicated. Protein-protein interaction was determined by IP-Western. p53 and FLIP immunoprecipitates were immunoblotted [IP: p53, WB: TRAF-2; IP: V5-tagged $FLIP_L$ or $FLIP_S$, WB: TRAF-2 (A-B), (n=3)].

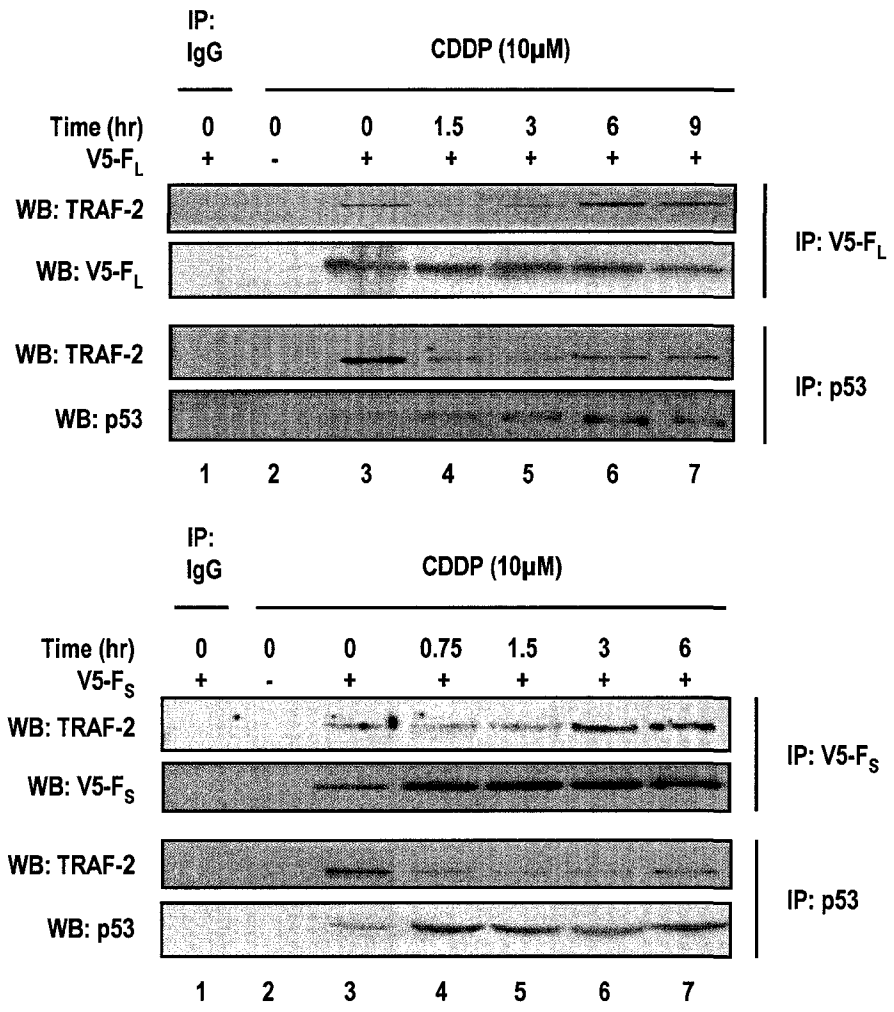
IP:
IgG
CDDP (10μM)
Time (hr) 0 0 0 1.5 3 6 9
V5-F_L + - + + + + +
WB: TRAF-2
WB: V5-F_L
IP: V5-F_L
WB: TRAF-2
WB: p53
IP: p53
1 2 3 4 5 6 7
IP:
IgG
CDDP (10μM)
Time (hr) 0 0 0 0.75 1.5 3 6
V5-F_S + - + + + + +
WB: TRAF-2
WB: V5-F_S
IP: V5-F_S
WB: TRAF-2
WB: p53
IP: p53
1 2 3 4 5 6 7

Figure 5

There are a few mistakes identified in chapters 3 and 4 of this thesis and some areas require clarifications. Since these two chapters have already been published, it is no longer possible to make these corrections/clarifications. However, the following corrections and/or explanations are offered in this appendix.

Page 73 lane 2 chemoresistance remains a major therapeutic problem and our understanding of the cellular mechanisms involved are still far from complete.

Page 74 lane 16 We have demonstrated that CDDP induced apoptosis in a concentration-dependent manner in chemosensitive ovarian cancer cells but not in their resistant counterparts. While we have not assessed the effects of CDDP on cell proliferation and total cell numbers, at least 200 cells in different fields were counted and the percentage of apoptotic cells calculated accordingly. In this context, it has been shown that CDDP caused G2/M arrest, associated with inhibition of cell proliferation, increased expression of p53 protein and $p21^{Cip1/WAF1}$ in cisplatin treated A2780 cells (Horvath, Soucek et al. 2007). It seems that CDDP increased the actual number of the apoptotic cells.

Page 75 lane 21 We have demonstrated that CDDP induced 30-40% cell death in chemosensitive ovarian cancer cells. Ovarian cancers are characterized by diverse heterogenicity and many determinants are implicated in the chemoresistance. Since dysregulation of apoptosis machinery depends

on different pathways, it seems in some cells, survival pathways including Akt, *KRAS* and *BRAF* are activated/overexpressed, and some of the cells may harbor mutation in tumor suppressors PTEN and p53. Therefore different group of the cells are depend on each or several of the determinant and the treatment can not kill all the cells.

Page 83 lane 16	Our findings supported the observations that downregulation of FLIP by antisense oligonucleotides .....
Page 87 lane 16	OV2008 and C13* cell lines were established from cells obtained from a serous cystoadenocarcinoma patient (Brown, Clugston et al. 1993; Mamenta, Poma et al. 1994).
Page 94 lane 2	"strategy" should read "strategies"
Page 94 lane 19	"remained" should read "remains"
Page 96 lane 16	Epoxomicin (25 nM)
Page 98 lane 7	Cells incubated with corresponding normal IgG or without .....
Page 100 lane 7	at a concentration
Page 102 lane 16	in the presence
Page 104 lane 11	by transfecting wt-p53 ovarian cancer cells
Page 105 lane 15	(n=3 independent experiments)

Chapter 9 - Curriculum Vitae

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EDUCATION

05/2004-present	Ph.D. Candidate, Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, Ontario, Canada
09/2002-04/2004	M.Sc. Candidate, Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, Ontario, Canada (Satisfied requirements for transfer into Ph.D. program)
1999-2002	Master in Medical Education, Shaheed Beheshti University of Medical Sciences Tehran, Iran. Thesis "Peer Assisted Learning System (PALS) versus Lecture-Based Learning (LBL) in a course of basic Pharmacology: A controlled and randomized Study"
1984-1989	Pharm.D. School of Pharmacy, Mashhad University of Medical Sciences Mashhad, Iran. Thesis "Photochemical Analysis of Medical Plants in the Ferdos region".

WORK EXPERIENCE

09/2002-present	Graduate Student, Reproductive Biology Unit, Department of Obstetrics & Gynecology and Cellular & Molecular Medicine, University of Ottawa, Chronic Disease Program, Ottawa Health Research Institute, Ottawa, Ontario, Canada
1991-2002	Lecturer of Pharmacology Courses, Birjand University of Medical Sciences (BUMS), Birjand, Iran.

ACADEMIC MEMBERSHIPS AND OTHER RELEVANT ASSOCIATIONS

2006-2008	Member, European Association for Cancer Research\

2006-2008	Associate Member, American Association for Cancer Research
2006-2008	Member, Canadian Fertility and Andrology Society
2000- 2002	Member, Iranian Societies of Medical Education
1997- 2002	Member, Research Council of BUMS
1994- 2002	Member, Community Oriented Medical Education Council (COME) of BUMS
1991- 2002	Member, Iranian Society of Physiology & Pharmacology

AWARDS AND DISTINCTIONS

Iranian Graduate Scholarship Doctoral Research Award	Ministry of Health and Medical Education, Government of IRAN	2002-2006
Graduate (Doctoral) Admission Scholarship	Ministry of Health and Medical Education, Government of IRAN	2002-2006
Entrance Award	Department of Cellular and Molecular Medicine, University of Ottawa	2002

PUBLICATIONS

1. **Abedini M.R.**, Muller E.J., Bruan J., Bergeron R., Gray D.A., Tsang B.K (2008) Cisplatin Induces p53-dependent FLICE-like Inhibitory Protein Ubiquitination in Ovarian Cancer Cells. Cancer Research. 68: 4511-17.

2. Yang X., Fraser M., **Abedini M.R.**, Bai T., and Tsang B.K. (2008) Regulation of apoptosis-inducing factor-mediated, cisplatin-induced apoptosis by Akt. Bri. J Cancer. 98: 803-808.

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MANUSCRIPTS SUBMITTED

1. Abedini M.R., Muller E.J., Bergeron R., Gray D.A., Tsang B.K (2008) Akt promotes Chemoresistance in Human Ovarian Cancer Cells by Modulating Cisplatin-induced, p53 dependent FLIP Ubiquitination.

INVITED TALKS

1. **Abedini M.R.**, Muller E.J., Bruan J., Bergeron R., Gray D.A., Tsang B.K (2008) Cisplatin Induces Itch- and p53-dependent FLICE-Like Inhibitory Protein (FLIP) Ubiquitination and Proteasomal Degradation: A Determinant for Chemosensitivity in Ovarian Cancer. 2008 Montreal 4th Canadian Conference on Ovarian Cancer Research, Montreal QC.

2. **Abedini M.R.** and Tsang B.K. (2007) Cisplatin Induces FLICE-like inhibitory protein (FLIP) Ubiquitination in Ovarian Cancer Cells. 2007 Ottawa Reproductive Biology Workshop, Ottawa, ON.

3. **Abedini M.R.** and Tsang B.K. (2006) Regulation of CDDP-Induced FLIP Down-regulation by Akt and p53. 2006 Ottawa Health Research Institute Research Day, Ottawa, ON.

4. **Abedini M.R.** and Tsang B.K. (2006) CDDP-Induced FLIP Down-regulation in Ovarian Cancer Cells in vitro: Possible Role of Proteasome Pathway and Akt Signaling. Canadian Fertility and Andrology Society, 2006 Annual Meeting, Ottawa, ON,

PUBLISHED ABSTRACTS

1. **Abedini M.R.**, Muller E.J., Bruan J., Bergeron R., Gray D.A., Tsang B.K (2008) Dysregulation of CDDP-induced, Itch and p53-meciated FLIP Ubiquitination and Degradation by Akt. 2008 Reproductive Biology Workshop, Ottawa, ON.

2. Kobayashi N., **Abedini M.R.**, Sakuragi N., and Tsang B.K. PRIMA-1 Increases CDDP Sensitivity in p53 Mutated Chemoresistant Ovarian Cancer Cells: Dependence on Akt Down-Regulation, (2008) Reproductive Biology Workshop, Ottawa, ON.

3. Ali A., Abedini M.R., and Tsang B.K. The Role and Regulation of Checkpoint Kinase 1 in Ovarian Cancer Cells, (2008) Reproductive Biology Workshop, Ottawa, ON.

4. Kobayashi N., **Abedini M.R.**, Sakuragi N., and Tsang B.K. (2008) PRIMA-1 Increases CDDP Sensitivity in p53 Mutated Chemoresistant Ovarian Cancer Cells:

Dependence on Akt Down-Regulation. 2008 Montreal 4th Canadian Conference on Ovarian Cancer Research, Montreal QC.

5. **Abedini M.R.** and Tsang B.K. (2007) Akt inhibits CDDP-induced FLIP ubiquitination and degradation in a p53-dependent manner in ovarian cancer cells, 2007 4th Canada-Japan Bilateral Workshop on Human Reproduction & Reproductive Biology, Hirosaki, Japan 2007

6. **Abedini M.R.** and Tsang B.K. (2007) Akt modulates CDDP-induced FLIP ubiquitination and degradation in a p53-dependent manner in ovarian cancer cells, 4th Annual Cell Signaling Symposium, Dundee UK 2007

7. **Abedini M.R.** and Tsang B.K. (2007) Regulation of FLIP by Cisplatin in Human Ovarian Cancer Cells: Possible Involvement of Akt and p53, Ottawa Reproductive Biology Workshop, Ottawa, ON. 2007

8. **Abedini M.R.** and Tsang B.K. (2006) CDDP-Induced FLIP Down-regulation in Ovarian Cancer Cells in vitro: Possible Role of Proteasome Pathway and Akt Signaling. 97th Annual American Association for Cancer Research, 2006 Annual Meeting, Washington DC., NY 2006

9. **Abedini M.R.** and Tsang B.K. (2005) Regulation of FLIP by Cisplatin in Human Ovarian Cancer Cells: Possible Involvement of Akt and p53. Ottawa Health Research Institute Research Day, Ottawa, ON 2005

10. **Abedini M.R.** and Tsang B.K. (2005) Regulation of FLIP by Cisplatin in Human Ovarian Cancer Cells: Possible Involvement of Akt and p53, Ottawa Reproductive Biology Workshop, Ottawa, ON 2005

11. Fraser M., Yang X., **Abedini M.R.**., Yan X., Jahani-asl A., Boulay H., Wang H., and Tsang B.K. Program on Ovarian Cancer Biology: Mechanisms of Chemoresistance. 3rd Canada-Japan Bilateral Workshop on Human Reproduction & Reproductive Biology, Ottawa, ON, 2004.

12. **Abedini M.R.,** Qiu Q., Yan X., and Tsang B.K. (2004) Possible Role of FLICE-Like Inhibitory Protein (FLIP) in Chemoresistant Ovarian Cancer Cells in vitro. Canadian Conference on Ovarian Cancer, Ottawa, ON, 2004.

13. **Abedini M.R.,** Qiu Q., Yan X., and Tsang B.K. (2004) Possible Role of FLICE-Like Inhibitory Protein (FLIP) in Chemoresistant Ovarian Cancer Cells in vitro. 95th Annual American Association for Cancer Research, Annual Meeting, Orlando, FL. 2004

14. **Abedini M.R.,** Qiu Q., Yan X., and Tsang B.K. (2003) The Role of FLICE-Like Inhibitory Protein (FLIP) in Chemoresistant Ovarian Cancer Cells in vitro. Ottawa Health Research Institute Research Day, Ottawa, ON 2003

15. **Abedini M.R.,** Qiu Q., Yan X., and Tsang B.K. (2003) The Role of FLICE-Like Inhibitory Protein (FLIP) in Chemoresistant Ovarian Cancer Cells in vitro. Ottawa Reproductive Biology Workshop, Ottawa, ON 2003

ASSIGNED MENTORSHIP

07/2007 - present	Ahmed Ali, Ph.D. candidate, Department of Cellular and Molecular Medicine, University of Ottawa.
09/2005 - 08/2006	Faraz Farooq, Undergraduate Honor's Student, Department of Biology, University of Ottawa