Synergistic growth inhibition and enhancement of cell death by combination of Melanoma Differentiation Associated gene-7 (MDA-7/IL-24) and cisplatin in ovarian cancer cell lines

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Synergistic growth inhibition and enhancement of cell death by combination of Melanoma Differentiation Associated gene-7 (MDA-7/IL-24) and cisplatin in ovarian cancer cell lines

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

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Jul. 2009
Acknowledgements:

I would like to thank my parents and brother for their unending love and support. I would also like to thank Dr. Paul Dent and Dr. Adly Yacoub for their help and direction with this project. Last but not least, I would like to thank Dr. Sarah Spiegel for being my committee member and for helping me with my thesis.
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List of Abbreviations:

Ad.mda-7: adenoviral melanoma differentiation-associated gene-7

AKT1/2: v-akt murine thymoma viral oncogene homolog 1/2

AP-1: activator protein-1

Apaf-1: Apoptotic peptidase activating factor 1

ATF-2: activating transcription factor-2

ATG: autophagy-related genes

ATM: ataxia telangiectasia mutated

ATP: Adenosine triphosphate

ATR: Ataxia telangiectasia and Rad3 related

BAX: BCL2-associated X protein

BAK: Bcl-2 homologous antagonist/killer

Bcl-2: B-cell CLL/lymphoma 2

Bcl-xL: B-cell lymphoma-extra large

BRAF: V-raf murine sarcoma viral oncogene homolog B1

BRCA1/2: breast cancer 1/2

CAR: coxsackie virus and adenovirus receptor

CICD: caspases-independent cell death

Cisplatin/cis-DDP: cis-diamminedichloroplatinum

CMA: chaperone-mediated autophagy

CMV: Cytomegalovirus

DAPK: Death-associated protein kinase

DIABLO: direct IAP binding protein with low pl

DMEM: Dulbecco’s Modified Eagle’s Medium

DMSO: dimethyl sulfoxide

DRP-1: dynamin-related protein

ECGF1: endothelial cell growth factor

EGFR: epidermal growth factor receptor
eIF2α: α-subunit of translational initiation factor 2 in eukaryotes
ER: endoplasmic reticulum
ERBB2: erythroblastic leukemia viral oncogene homolog 2
ERCC1/3: excision repair cross-complementing group 1/3
ERK: extracellular signal-regulated protein kinase
FADD: Fas-associated death domain
FAK: Focal adhesion kinase-1
FasL: Fas ligand
FGF3: Fibroblast growth factor 3
FIGO: Federation of Gynaecology and Obstetrics
FLIP: FLICE-inhibitory protein
GADD: Growth Arrest and DNA Damage
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
GBM: glioblastoma multiforme
GSK: Glycogen synthase kinase
GST-MDA-7: glutathione S-transferase MDA-7
hMSH2: human mismatch-repair protein2
HUVEC: Human Umbilical Vein Endothelial Cells
IAPs: inhibitors of apoptosis
IL-24: Interleukin 24
JAK/STAT: Janus kinases /Signal Transducers and Activators of Transcription
JNK/SAPK: c-Jun N-terminal kinase/stress-activated protein kinase
KRAS: Kirsten rat sarcoma viral oncogene homolog
MAPK: mitogen-activated protein kinase
MAPKK: MAPK kinase
MAPKKK: MAPK kinase kinase
MCD: mitotic cell death
MDA-7: melanoma differentiation gene-7
MDM2: transformed 3T3 cell double minute 2
MEF: mouse embryonic fibroblasts
MEK1: MAP2K1
MSH2: MutS homolog 2
mTOR: mammalian target of rapamycin
NCI: National Cancer Institute
NER: nucleotide excision repair
NFkB: nuclear factor kappa-light-chain-enhancer of activated B cells
NIH: National Institutes of Health
NSCLC: non-small cell lung carcinoma
OVCAR: Human ovarian carcinoma cell line
PARP: Poly (ADP-ribose) polymerase
PBS: Phosphate-buffered saline
PCD: programmed cell death
PERK: protein kinase-like endoplasmic reticulum kinase
PI3K: Phosphoinositide-3 kinase
PKC: Protein kinase C
PKR: RNA-dependent protein kinase
PLC-γ: Phospholipase C γ
PTEN: phosphatase and tensin homolog
Raf-1: murine leukemia viral oncogene homolog 1
RB: retinoblastoma protein
RIPK-1: receptor-interacting protein kinase 1
ROS: reactive oxygen species
RT-PCR: Reverse transcription polymerase chain reaction
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBST: Tris-Buffered Saline Tween-20
TCA: tricarboxylic acid
TNF: tumor necrosis factor
TNF-R1: TNF receptor 1
TRADD: TNFRSF1A-associated via death domain

TRAF1/2: TNF receptor-associated factor 1/2

TRAIL: TNF-related apoptosis-inducing ligand

UPR: unfolded protein response

XPA/F: xeroderma pigmentosum, complementation group A/F
Synergistic growth inhibition and enhancement of cell death by combination of Melanoma Differentiation Associated gene-7 (MDA-7/IL-24) and cisplatin in ovarian cancer cell lines

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Abstract

Ovarian cancer is the most lethal gynecological malignancy among women. The current first-line treatments for ovarian cancer are cisplatin, carboplatin and paclitaxel. However, resistance to these platinum-based drugs occurs in the large majority of initially responsive tumors, resulting in fully chemoresistant, fatal disease. Therefore, the resistance to cisplatin therapy has been a critical hurdle in the management of recurrent ovarian cancer. The mechanisms responsible for cisplatin resistance are not completely understood. In the search for new therapies to overcome/bypass cisplatin resistance, melanoma differentiation gene-7 (MDA-7) IL-24, which is a new cytokine, has anti-cancer efficacy by suppressing cell growth and inducing apoptosis in a broad range of tumor cells and does not induce any toxicity in normal cells, thus, making it a potentially effective therapeutic gene for ovarian cancer. The purpose of this study was to evaluate the potential therapeutic efficacy of MDA-7 to treat ovarian
carcinoma. Since adenoviral-mediated MDA-7 gene therapy has been shown to be well tolerated and showed biological activity in clinical studies in the context of other carcinomas we assessed the anticancer effects of Ad.mda-7 and in combination with cis-platinum on ovarian cancer cells.

Our results show that the purified recombinant MDA-7 protein, GST-MDA-7, and Ad.mda7 virus (5) induced growth arrest and apoptosis in ovarian cancer cells. However, the apoptosis induction was low and directly correlated with infectivity of Ad.mda-7 virus (5). The use of a modified Ad.mda-7 virus type5, Ad.mda-7 virus type(5/3), enhanced infectivity and significantly enhanced ovarian cancer cell killing in human ovarian cancer cell lines in vitro compared to unmodified Ad.mda-7 virus, Ad.mda-7 virus type5. Also Ad-mda7 synergizes with cis-platinum in vitro and enhances ovarian cancer cell death. Taken together, these findings demonstrate that MDA-7 is capable of promoting growth suppression and inducing cell death in ovarian cancer cells, at least OVCAR cells and support the pharmacological interest of the combination of MDA-7 and cis-platinum.
Chapter I: Introduction

In the United States, it has been reported that the second most common gynecological cancer is ovarian cancer (Greenlee et al., 2000). The low cancer detection rate of ovarian cancer contributes to its having the worst prognosis of all gynecological cancers. Usually the first step in treating ovarian cancer in women with stage I and stage II is optimal surgical debulking or optimal cytoreduction. The need for chemotherapy after surgery depends on the disease stage. Patients in stage I A and B usually don’t need further therapy, however, higher risk patients at stage I C and stage I grade 3 need chemotherapy to reduce their risk of relapse. Higher stages require combination therapy including platinum drugs, paclitaxel and radiation. Platinum-based drugs are established in the treatment of cancer, including ovarian, head and neck, and lung cancer. The oldest and most commonly used platinum-based drug is cis-diamminedichloroplatinum (cisplatin), (Cersosimo, 1989; Higby et al., 1974). Cisplatin is the most used chemotherapeutic drug in ovarian-cancer patients whose cancers were shown to progress after alkylating-agent-based chemotherapy (Wiltshaw et al., 1979; Neijit et al., 1987). After more than a quarter century of use in the treatment of ovarian cancer, these platinum drugs are still the cornerstones of both front-line management and treatment of recurrent disease (Markman et al., 2003). Cisplatin is activated via hydrolysis, yielding positively charged, very reactive aquated species that subsequently can form stable DNA-adducts and as a consequence cause cell death. Common problems associated with the clinical use of cisplatin are
cumulative toxicities, and the occurrence of inherent or treatment-induced resistant tumor cell subpopulations limit the therapeutic efficacy of cisplatin (Ozols, 1991). This resistance can be acquired through chronic drug exposure or it can result from the intrinsic factors inside the cancer cells (Siddik, 2003). Reducing the extent of DNA damage can increase resistance and this can occur through changes in drug accumulation, intracellular thiol levels, and/or DNA adduct repair (Siddik, 2003). Generally, resistance is multifactorial, in that multiple mechanisms are involved within the same tumor cell (Eastman et al., 1988; Richon et al., 1987). However, the exact mechanism for chemoresistance remains poorly understood. One of the strategies to reduce the systemic toxicity and resistance to platinum is the use of combination therapy. Cytotoxic drugs are used in combination with cisplatin therapy to enhance survival but relapse still remain a major problem. Finding new strategies are of particular significance, one of the candidate to consider is the melanoma differentiation associated gene-7 (MDA-7), which was originally identified from terminally differentiated human melanoma cells treated with beta-interferon and the protein kinase C activator mezerein (Jiang et al, 1995). This gene displayed potent tumor killing and apoptosis-inducing properties in a wide variety of solid tumors including melanoma, malignant glioma, fibrosarcoma and carcinomas of the breast, cervix, colorectal, liver, lung, ovary and prostate. (Fisher et al., 2002; Fisher et al., 2005; Inoue et al., 2006; Sarkar et el., 2002; Sarkar et al., 2006; Sauane et al., 2003; Yacoub et al., 2004). Since MDA-7 potent anti-tumor activity in many cancer cells was established, it was interesting to test its efficacy on ovarian cancer. This study
aimed to explore its effects on OVCAR ovarian cancer cells. In this study we demonstrate that MDA-7 reduces proliferation and enhances cisplatin toxicity of human ovarian cancer.

Chapter II: Background

2.1. Overview of Ovarian Cancer

Ovarian cancer is the leading cause of death among all gynecological malignancies (Silverberg et al., 1990; Yancik et al., 1993) and the fifth leading cause of cancer death among women in the United States. About 1 in every 68 women in the United States will develop ovarian cancer. The American Cancer Society estimates 21,550 new cases of ovarian cancer being diagnosed, and about 14,600 deaths from ovarian cancer in the United States in 2009 (NCI) booklet 2009 (NIH Publication No. 06-1561). While the surgical removal of one or both ovaries (oophorectomy) certainly reduces the incidence of ovarian cancer, it is only recommended as an option for high-risk patients. With diagnosis and treatment at early stages of the disease, the 5-year survival rate can reach 94%. Nearly 70% of ovarian cancers are diagnosed at advanced stage (McNeil, 1995) as a result the 5-year survival rate can be as low as 28% (Beral, 1987). The high mortality of ovarian cancer is mainly due to its poor diagnosis and its aggressive nature.
2.2. Overview of the Molecular Genetics of Ovarian Cancer

Approximately 10% of all epithelial ovarian carcinomas are associated with autosomal dominant genetic predisposition, primarily by inherited mutations in the BRCA1 or BRCA2 tumor suppressor genes (Boyd 1998). Mutations of these genes are also seen in some sporadic ovarian cancers. Other genetic features tend to relate to specific types of ovarian cancer. In women with a known BRCA1 mutation, the cumulative lifetime risk of developing ovarian cancer has been estimated to be approximately 50% (95%CI 44.9 to 55.0%) compared with an approximate 1.7% lifetime risk in the general US population (Brose et al., 2002). The low-grade tumors are relatively genetically stable and are characterized by mutations in a number of genes. The most well characterized molecular alterations are sequence mutations in KRAS, BRAF and ERBB2 oncogenes. Oncogenic mutations in BRAF, KRAS and ERBB2 result in constitutive activation of the mitogen activated protein kinase (MAPK) signal transduction pathway which plays a critical role in the transmission of growth signals into the nucleus (Vogelstein et al., 2004) and contributes to neoplastic transformation (Singer et al., 2003). The most common molecular genetic alteration in mucinous borderline tumors and carcinomas (Type I tumors) is point mutation of KRAS (Mayr et al., 2006). Other mutations such tumor suppressor, PTEN, occurs in ovarian carcinomas (Saito et al., 2000). The critical role of the genetic changes in PTEN and KRAS is highlighted by a recent report showing that inactivation of PTEN and an activating mutation of KRAS are sufficient to induce the development of ovarian endometrioid carcinoma in a mouse model (Dinulescu et al., 2005). A
\textit{PI3K/Pten pathway has} been reported to be sufficient to induce ovarian carcinoma (Wu et al., 2007). Invasive serous and undifferentiated ovarian carcinomas are characterized by TP53 mutations and TP53 protein accumulation. Loss of genetic material from chromosome 17 is also common. The most common molecular genetic change in Type II tumors was shown to be mutations of \textit{TP53} (Salani et al., 2007). Moreover, mutation of \textit{TP53} occurs very early in the genesis of Type II neoplasms. In fact, mutant \textit{TP53} is observed in intraepithelial neoplasia in the fallopian tube fimbria of \textit{BRCA} patients. Importantly, \textit{TP53} mutations are inherited during cancer evolution and contribute to the transformed state. As a result, the initiating genetic changes are retained in both the primary and recurrent tumors. Other genes involved in the molecular aetiology of ovarian epithelial tumors include, CDKN2A, RB, GATA4, RNASET2, CSF1R, ECGF1, EGFR, MYC, SRC, PI3K, AKT2, FGF3 and MDM2.

\section*{2.3. Ovarian Cancer Classification}

Ovarian tumors can be broadly classified into a benign or malignant. Benign ovarian tumors are also known as of low malignant potential (LMP) according to world organization classification (FIGO, 1971, and Serov et al., 1973). There are more than 30 different types of ovarian cancer, categorized by the type of cell where they originate. The three major types of ovarian tumors are epithelial tumors, germ cell tumors and sex cord-stromal tumors, which are named according to their histogenesis and directions of differentiation (Scully, 1979). Epithelial tumors are derived from the
cells on the surface of the ovary. This is the most common form of ovarian cancer and occurs primarily in adults. They account for about 67% of all ovarian neoplasms and 80-90% of malignant ovarian tumors. Epithelial ovarian tumors are also classified into subtypes based on the type of epithelial differentiation that is present in the tumor. The subtype is serous (40 to 70% of all types); endometrioid tumours are the second most common, (20% to 25% of all cases). Mucinous epithelial tumours are rarer, comprising 5% to 20% of all cases (Kosary et al., 1994). Serous tumors are the most common subtype (Scully, 1987). Germ cell ovarian tumors are derived from the egg producing cells within the body of the ovary. This occurs primarily in children and teens and is rare by comparison to epithelial ovarian tumors. They are sub-classified into teratoma, mature cystic teratoma, immature teratomam, monodermal teratoma, dysgerminoma, yolk sac tumor, embryonal carcinoma, and choriocarcinoma. They constitute the second largest group of ovarian neoplasms that is about 20% (Scully, 1987). Dysgerminoma is the most common germ cell tumor, accounting for 50% of all germ cell tumor cases. Yolk sac tumors (also known as endodermal sinus tumors) are the third most common germ cell tumor (Scully et al., 1998). Less common germ cell tumors are embryonal carcinoma (Kurman et al., 1976), immature teratoma, choriocarcinoma, polyembryomas, and mixed germ cell tumors (Scully, 1987). Sex cord-stromal neoplasms account for about 6% of all ovarian neoplasms. They are composed of various cell types derived from gonadal stroma and sex cords. Sex cord stromal tumors include fibrothecomas, juvenile granulosa cell tumors (JGCT), Sertoli-Leydig cell tumors (SLCT), and unclassified sex cord stromal tumors (Young
Granulosa cell tumors and Sertoli-Leydig cell tumors are the most common. Unlike patients with common epithelial tumors, in which 75% are considered to be at stage III or IV at diagnosis, patients with these tumors are at stage I at diagnosis 70% of the time. Also unlike common epithelial tumors, sex cord-stromal tumors often have more specific symptoms. Granulosa cell tumors are most common in postmenopausal women and may cause vaginal bleeding and an elevated level of the tumor marker inhibin in the blood. Sertoli-Leydig cell tumors are rare. About 33% of these tumors produce signs of virilism (infrequent menstrual periods, cessation of menstrual periods before menopause, hoarse voice, and appearance of facial hair).

2.4. Tumor stages

For cancers such as ovarian cancer, correct staging is important as this can impact on treatment decisions. An international system of staging for ovarian cancer is used, which identifies the spread of the ovarian cancer at the point of diagnosis. The staging system is assessed according to the International Federation of Gynaecology and Obstetrics (FIGO). According to FIGO, at stage 1, the tumor is confined to the ovary/ovaries. When only one ovary is affected by the tumor, the ovary capsule is intact; no tumor is detected on the surface of the ovary; and malignant cells are not detected in ascites or peritoneal washings, the tumor is said to be at stage 1A. When both ovaries are affected by the tumor, the ovary capsule is intact; no tumor is detected on the surface of the ovaries; and malignant cells are not detected in ascites
or peritoneal washings, the tumor is at stage 1B. When the tumor is limited to one or both ovaries, with either ovary capsule ruptured, the tumor detected on the ovary surface, or malignant cells detected in the ascites or peritoneal washings, the tumor is at stage 1C. At stage 2, tumor involves one or both ovaries and has extended into the pelvis. When the tumor has extended into the uterus and/or the fallopian tubes and malignant cells are not found in ascites or peritoneal washings, the tumor is at stage 2A. When the tumor has extended to another organ in the pelvis and malignant cells are not detected in ascites or peritoneal washings, the tumor is at stage 2B. When the tumor is defined as 2A or 2B, but the malignant cells are detected in the ascites or peritoneal washings, the tumor is at stage 2C. At stage 3, the tumor involves one or both ovaries with microscopically confirmed peritoneal metastasis outside the pelvis and/or regional lymph node metastasis. When there is microscopic peritoneal metastasis beyond the pelvis, the tumor is at stage 3A. When there is microscopic peritoneal metastasis beyond the pelvis 2 cm or less in greatest size, the tumor is at stage 3B. When there is microscopic peritoneal metastasis beyond the pelvis 2 cm in greatest dimension and/or regional lymph nodes metastasis, the tumor is considered to be at stage 3C.

## 2.5. General treatments of ovarian cancer

### 2.5.1. Treatments for Stage I and Stage II

Treatment for stage 1 and stage 2 ovarian epithelial cancers may include:
2.5.1.1. Surgery: Surgical debulking procedure is feasible in ovarian carcinoma (Heintz et al., 1986). It usually includes removal of the uterus (total hysterectomy), removal of both ovaries and fallopian tubes (bilateral salpingo-oophorectomy), partial removal of the omentum, the fatty layer that covers and pads organs in the abdomen (omentectomy), and surgical staging of the lymph nodes and other tissues in the pelvis and abdomen. Women in Stage I with the lowest-grade tumors in one ovary may sometimes be treated only with the removal of the diseased ovary and tube in order to preserve fertility (Benedet et al., 2000; Vinatier et al., 1996).

2.5.1.2. Chemotherapy: Patients with stage IA or B disease, grade 1 (or sometimes grade 2), usually do not need further therapy after surgery. However, higher risk patients (stage IC, stage I/grade 3) are usually treated with platinum-based chemotherapy to reduce their risk of subsequent relapse (Markman et al., 2003).

2.5.1.3. Clinical trials with radiation therapy, chemotherapy, or new treatments

2.5.2. Treatment for Stage III and Stage IV Ovarian Cancer

2.5.2.1. Surgery: Removal of the tumor (debulking), total abdominal hysterectomy, bilateral salpingo-oophorectomy, and omentectomy

2.5.2.2. Chemotherapy: Combination chemotherapy with a platinum-based drug and a taxane drug delivered intraperitoneally (through the abdominal cavity) (Albets et al., 1996; Armstrong et al., 2006; Elit et al., 2007; Markman et al., 2001).
2.5.2.3. Clinical trials of biologic drugs (targeted therapy) following combination chemotherapy.

2.6. Treatment for Recurrent Ovarian Cancer

If ovarian cancer returns or persists after treatment, chemotherapy is the mainstay of treatment, although it is not generally curative in the setting of relapsed disease (Herzog T. J., 2009). Clinical trial options include additional surgical debulking, and biologic therapy combined with chemotherapy or radiation therapy. Medically inoperable patients can be treated with localized radiotherapy or combined chemoradiation.

2.7. Chemotherapy treatments

2.7.1. Standard Chemotherapy.

The standard initial chemotherapy uses a combination of: A platinum-based drug, such as carboplatin (Paraplatin) or cisplatin (Platinol). Carboplatin is preferred over cisplatin in the combination. Carboplatin works as well as cisplatin but is less toxic and can be administered in a more convenient way (Herzog T. J., 2009); and a taxane, such as Taxol, paclitaxel (McGuire et al., 1996) or docetaxel (Taxotere). Currently paclitaxel is the drug most often used as initial therapy in combination with a platinum drug (Einzig et al., 1994).
2.7.2. Chemotherapy for Relapsed or Refractory Cancer.

Unfortunately, even in patients who respond, the disease eventually becomes resistant to the first-line drugs, and the cancer returns. Some ovarian tumors are resistant to platinum drugs. Once cancer recurs or continues to progress, the patient may be treated with more cycles of carboplatin and a taxane drug, or a different type of chemotherapy drug may be used in combination treatment (McGuire et al., 1996). Gemcitabine is used in combination with carboplatin for women with advanced ovarian cancer that has relapsed at least 6 months after initial therapy. Other drugs used for recurrent ovarian cancer include doxorubicin (Adriamycin), and etoposide.

2.8. Platinum based chemotherapy

The platinum complex cisplatin (cis-diamminedichloroplatinum [II]) cis-DDP, first described as an antineoplastic agent in 1965 by Rosenberg et al., is among the most frequently used chemotherapeutics, indicated against a broad range of solid tumors, which primarily were testicular, ovarian and bladder neoplasms (Cersosimo, 1989; Higby et al., 1974). Recently the spectrum of indications has been expanded including now endometrium-, cervix-, prostate-carcinomas; osteosarcomas as well as head and neck tumors (Petsko, 1995). Additionally, this substance has shown its efficacy against small cell and non-small cell lung cancer (Eberhardt et al., 1998; Havemann, 1982) and is being used in the therapy of gastrocarcinomas as well (Kath et al., 2000; Konishi et al., 1998). Cisplatin is administered intravenously in 3-5 courses spread over several weeks to months. Individual courses are usually 20-40 mg/m2 (Windebank, 1994).
Concurrently, cisplatin was tested in combination with a variety of different drugs. Platinum-containing regimens have proven superior to regimens that lacked platinum compounds (Markman et al., 2003). In a meta-analysis performed on 37 randomized studies involving 5,667 patients with advanced-stage disease, those patients given cisplatin-containing combination chemotherapy were compared with those treated with regimens that did not include cisplatin (Thigpen et al., 1994). Platinum based chemotherapy was superior to non-platinum based chemotherapy. A trend favored platinum combinations over single agent platinum. In studies of cisplatin containing regimens, several trials have compared cyclophosphamide and cisplatin (CP) with cisplatin, doxorubicin and cyclophosphamide (PAC) (McGuire et al., 1996).

2.9. Platinum Mechanism

2.9.1. Antitumor activity of cisplatin

The anticancer activity of cisplatin is widely attributed to the formation of a cross-link adduct involving two adjacent guanines in DNA. Cisplatin forms covalent bonds with nucleophilic sites on guanine present in all DNA. As cisplatin is a bifunctional agent, it is able to bind to two sites in a DNA strand. This results in the formation of inter- and intra- chain cross-linkings (Eastman, 1987), which interferes with cellular transcription and replication (Jordan, 2000). Regulatory mechanisms detect the abnormal DNA and so activate a chain of responses to correct it. This, ultimately, causes cell death. The anticancer efficacy of cisplatin is also influenced by the
efficiency of cisplatin-DNA adduct removal by the cellular repair machinery, with nucleotide excision repair being a major pathway. The repair of platinum-DNA crosslinks is retarded when the DNA is bound to the histones in a nucleosome core particle (Carte et al., 2000; Wang et al., 2003). In 1996 Mello et al., have shown that the human mismatch-repair protein, hMSH2, also binds specifically to DNA containing cisplatin adducts and displays selectivity for the DNA adducts of therapeutically active platinum complexes. These results suggest a role for hMSH2 in mediating cisplatin toxicity. Various adducts are formed upon interaction of platinum complexes with nucleotides, but contribution of individual adducts to antitumor activity and toxicity of platinum complexes still remains to be examined. Warnke et al., 2001 investigated the formation of adducts following the reaction of cis-diaminedichloroplatinum (II) (cisplatin) with various DNA nucleotides. One of the important pathway activated after Cisplatin treatment is the ATR/ATM (ataxia telangiectasia mutated [ATM] and ATM-and Rad3-related [ATR]) pathway (Pabla et al., 2008), Ras signaling, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated protein kinase (ERK) signaling pathways. It is suggested that both ERK and JNK cascades activate a common downstream factor, activator protein-1 (AP-1), which might be required for DNA repair following cisplatin treatment. AP-1 is composed of activating transcription factor-2 (ATF-2) and c-Jun, which have been shown by chromatin immunoprecipitation assay to be involved in regulating the expression of many crucial genes after cisplatin treatment, such as ERCC1, ERCC3, XPA, MSH2, and RAD50; genes that are related to DNA damage repair. The main mechanism for removing the cisplatin-induced DNA
cross-linking is nucleotide excision repair (NER). NER involves more than 30 proteins and is processed in several steps, including damage recognition, incision of a nucleotide fragment flanking the damaged DNA, resynthesis by using the undamaged strand as the template, and then ligation of the existing DNA and the newly synthesized DNA fragment (Dabholkar et al., 1994). In the process, ERCC1 (excision repair cross-complementing group 1), which forms a heterodimeric complex with XPF (xeroderma pigmentosum, complementation group F), is involved in 5’ end incision of the DNA fragment framing the cisplatin-DNA adduct region, and is the rate-limiting step in the repair system (Dabholkar et al., 1994).

2.9.2. Cisplatinum Resistance

Currently, the precise mechanisms that induce cellular apoptosis is not yet fully understood, however cisplatin resistance involves multiple factors (Jordan, 2000). As a summary, cisplatin resistance can be divided into three pharmacological mechanisms: (i) reduced platinum accumulation, (ii) enhanced platinum detoxification and metabolism, and (iii) enhanced repair of DNA damage; and one molecular mechanism: (iv) increased cellular survival signals and decreased apoptotic potential (Jordan, 2000).

2.10. New Approach therapy

The melanoma differentiation associated gene-7 was identified by subtractive hybridization from a human melanoma cell line, HO-1, from terminally differentiated
human melanoma cells treated with beta-interferon and the protein kinase C activator mezerein resulted in cell morphology changes, modifications in gene expression, alterations in surface antigen expression, and terminal cell differentiation (Fisher et al., 1985; Guarini et al., 1989, 1992; Jiang and Fisher, 1993; Jiang et al., 1993, 1995). The MDA-7/IL-24 is localized on human chromosome 1q32–33, a genomic area spanning 195-kb and containing a family of genes associated with the interleukin (IL)-10 family of cytokines (Kotenko et al., 2002; Pestka et al., 2004). Based on its chromosomal location, structure, and expression profile MDA-7 was confirmed to have cytokine nature, and therefore was re-designated as interleukin-24 (Caudell et al. 2002). The mRNA encoding MDA-7/IL-24 is ~2-kb encoding a polypeptide of ~23.8-kDa (Jiang et al., 1995). The MDA-7/IL-24 gene is composed of 7 exons and 6 introns (Huang et al., 2001). Sequence analysis also reveals the presence of a 49-amino acid signal peptide that allows the molecule to be cleaved and secreted. Sequence analysis of MDA-7/IL-24 reveals 3 putative glycosylation sites at amino acids 95, 109, and 126 resulting in different forms and molecular sizes of secreted MDA-7/IL-24. A large body of data demonstrates that IL-24 results in growth suppression and induction of apoptosis in a broad range of cancer cells (Chada et al., 2004; Cunningham et al., 2005; Fisher et al., 2003; Fisher, 2005; Gopalkrishnan et al., 2004; Lebedeva et al., 2005; Sauane et al., 2003; Tong et al., 2005), but not in normal human cells and its apoptotic effect was independent of classic tumor suppressor genes, such as p53, Rb and p16 and Bax. A Phase I/II clinical trial in patients with advanced carcinomas involving intratumoral administration of Ad-IL-24 has
documented that this gene is safe and well tolerated by patients and a single virus injection elicits apoptosis in most of the tumor (Chen et al. 2003; Saeki et al. 2002; and Sieger et al. 2004).

2.11. Overview of Cell signaling

Currently, chemotherapeutic drugs are widely used to treat cancer. Despite the diverse primary modes of action of those anti-tumor drugs, most anti-tumor agents ultimately exert their cytotoxicity by inducing apoptosis. Cancer cells treated in vitro with different drugs usually die via apoptosis and many studies have shown that apoptosis is a primary means of drug-induced tumor cell death in vivo (Kaufmann et al., 2000; Mesner et al., 1997). Recent studies are mainly focused on the signal transduction pathways and molecular mechanisms that link drug-induced damage to an apoptotic response. A complex network of survival and death pathways are integrated during cellular commitment to apoptosis, or the ability to evade apoptosis in response to damage. Among the pathways regulating cell survival and death, the best-characterized pathways are those mediated by the mitogen-activated protein kinase (MAPK) family (Widmann et al., 1999). MAPKs can transduce a variety of extracellular and intracellular stimuli into alterations in gene expression and cell function. Three distinct MAPK pathways have been characterized, extracellular signal-regulated kinase 1 and 2 (ERK1/2), c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), and p38. The ERK1/2 pathway is activated by growth factors through the Ras-Raf-MEK phosphorylation, and by phorbol esters, via PKC-Raf-MEK pathway and plays a key role in cell proliferation, survival and
differentiation (Cobb et al., 1999). The stress-activated MAPK pathways JNK/SAPK (MEK kinase1,3 –MAPK kinase 4,7 [M KK4,7]-JNK1,2,3) and p38 [MAPK kinase kinase [MAPKKK]-M KK3,6 –p38 a,b,g,d) are activated by environmental and chemical stress, e.g. UV light, osmotic and oxidative stress, and inflammatory cytokines (Ahn et al., 1993; Davis et al., 2000; Wilsbacher et al., 1999; Widmann et al., 1999). The activation of MAPKs requires phosphorylation of conserved tyrosine and threonine residues by dual specificity MAPK kinases (MEK), which in turn are activated by phosphorylation of two serine residues by upstream MAPKKKs (Raf). Phosphorylation of MAPKs results in their translocation to the nucleus, where they activate transcription factors by phosphorylation (Lenormand et al., 1998; Adachi et al., 2000). Activity of MAPK kinase (MEK) and MAPKs is inhibited by de-phosphorylation of the regulatory serine, threonine, and tyrosine residues by serine/threonine, tyrosine, and dual-specificity phosphatases, respectively (Chu et al., 1996; Groom et al., 1996). Other less understood ERK isoforms are ERK3, ERK5 and ERK7. ERK3 isoforms are nearly identical to ERK1 and 2 in the core catalytic domain (Gonzalez et al., 1992). ERK5 was originally cloned as a homolog of ERK1/2 and as a protein that interact with the orphan MEK, MEK5 (Lee et al., 1995; Zhou et al., 1995). ERK5 has been implicated in cell cycle control and cell transformation (English et al., 1999; Kato et al., 1998). ERK7 was shown to be involved in cell proliferation (Abe et al., 1999). Recent data suggest that MAPKs may mediate apoptotic signaling by anti-tumor agents. Upon exposure of cancer cells to structurally and functionally distinct anti-tumor agents, MAPKs activities have been shown to be
altered and modulation of MAPK signaling pathways can affect the apoptotic responses to these drugs.

2.12. Overview of Cell death

Cell death is essential for maintenance of tissue homeostasis as well as embryonic development in multicellular organisms and plays important roles in cellular stress responses. The mechanisms for cell death that follows toxic stress are a major focus of basic research and are also very relevant to translational research in clinical oncology. Especially, cell death following cancer therapy is of great interest to researchers. Recently, eight different types of cell death were delineated (Kroemer et al., 2005) and even as more as 11 pathways were described by some researchers, 10 of which appear to be programmed (Melino et al., 2005). Generally, there are two main groups of cell death pathways, apoptotic and non-apoptotic pathways. Apoptotic cell death refers to classic apoptosis and apoptosis induced by loss of attachment to the substrate or to other cells (anoikis) (Gilmore, 2005). Non-apoptotic cell death includes autophagic cell death, necrosis, mitotic cell death (MCD), and caspases-independent cell death (CICD) triggered by mitochondrial outer membrane permealization (MOMP). CICD does share some features with classical apoptosis such as MOMP induction, diffusion of proteins from intermembrane space of mitochondria and the ensuing DNA fragmentation, however, there is no activation of caspase cascade with
subsequent cleavage of the plethora of defined intracellular caspase substrates in CICD, therefore it is classified as a non-apoptotic form of cell death (Okada et al., 2004).

2.12.1. Apoptosis

Apoptosis, the most well-defined type of cell death pathway, is also referred to as type I programmed cell death. It is a cell death program that occurs in various physiological and pathological situations (Hengartner, 2000). The term apoptosis is based on the morphological characteristics of the dying cells, which include membrane blebbing, cellular shrinkage and reduction of cellular volume, condensation of chromatin, and fragmentation of the nucleus, all of which eventually result in fragmentation into membrane bound apoptosis (Kerr et al., 1972; Ziegler et al., 2004). During apoptosis, the cell membrane becomes asymmetric and phosphatidylserine (PS) becomes exposed on the cell surface. This PS signals and leads to the clearance of apoptotic cells by macrophages, thus apoptosis does not trigger inflammation. Apoptosis is a tightly regulated form of cell death and is governed by several genes, some of which are mutated or dysfunctionally regulated in a variety of human tumors (Brown et al., 2005; Philchenkov et al., 2004; Vogelstein et al., 2004). It can be initiated by two different kinds of signals, intracellular stress signals and extra-cellular ligands. Two major apoptotic pathways have been described in eukaryotic cells, extrinsic and intrinsic. Intracellular stress signals, including DNA
damage, growth factor withdrawal, cytoskeletal damage, oxidative stress or oncogene activation, endoplasmic reticulum stress, loss of adhesion and others, converge on the mitochondria and lead to the permeabilization of the mitochondrial outer membrane, through which the intermembrane space proteins are released into the cytosol. The release of cytochrome c and other pro-apoptotic proteins propagates the apoptotic signal. Cytochrome c here serves a cofactor of Apaf-1 to trigger the formation of the apoptosome and subsequent activation of the initiator and executioner caspases such as caspase-9 and -3 (Chipuk et al., 2006; Kroemer et al., 2000; Spierings et al., 2005).

In the extrinsic signaling pathway, extracellular ligands such as FasL, TNFa or TRAIL bind to cell surface receptors, followed by the recruitment of cytosolic adaptor protein (such as FADD and TRADD), activation of initiator caspases such as caspase-8 and -10 and subsequent activation of the downstream effector caspases including caspases-3, -6 and -7 (Nagata, 1999). However, the extrinsic and intrinsic apoptotic pathways cross at the level of the mitochondria since caspase-8 can also cleave the protein Bid into its active form tBid, which is a pro-apoptotic member of the Bcl-2 family of proteins and can induce Bax/Bak-dependent permeabilization of the outer mitochondrial membrane (MOMP) and release of cytochrome c (Li et al., 1998). Cytochrome c together with Apaf-1 activates the executioner caspase-9 that in turn activates effector caspases such as caspase-3. Both apoptotic pathways eventually results in activation of executioner caspases, caspase-3, -6 and -7, the major proteases that degrade the cell. IAPs (inhibitors of apoptosis) can inhibit their activity, while IAPs themselves are inhibited by the proteins SMAC/DIABLO, which can
promote the activation of caspases and the progression of apoptosis (Du et al., 2000; Eckelman et al., 2006; Roy et al., 1997; Verhagen et al., 2000). For tumor cells, they must circumvent apoptosis to survive and proliferate (Hanahan et al., 2000), therefore tumors usually acquire resistance to apoptosis. For example, loss of function mutations of p53 tumor suppressor protein is often observed. Because p53 can promote apoptosis in the context of DNA damage (Vousden et al., 2007), the mutated p53 is frequently related to a failure to induce apoptosis after cellular stress. Other examples include loss of functional pro-apoptotic Bax and Bak or over expression of anti-apoptotic proteins (Kondo et al., 2000; Rampino et al., 1997). What’s more, modifications in the death receptor pathways can also play a role in apoptosis resistance such as the reduction or loss of Fas receptor expression in colon carcinomas (Moller et al., 1994). During tumorigenesis, the genetic and functional alterations that pro- and anti-apoptotic regulators undergo in cells can significantly impede the effective execution of an apoptotic program, resulting in apoptosis resistance, even when these cells are exposed to certain stress (Johnstone et al., 2002).

In order to kill tumor cells, diverse cytotoxic approaches such as gama-irradiation, anticancer drugs, suicide genes, or immunotherapy have been employed and have been shown to induce apoptosis in target cells (Debatin, 1999; Herr et al., 2001; Kaufmann et al., 2000; Lowe et al., 2000; Solary et al., 2000). The underlying mechanism for initiation of an apoptosis response in tumor cells upon cytotoxic therapy may vary for different stimuli and is only partially understood. The damage to DNA or to other critical molecules and/or subcellular structures induced by cancer...
therapy seems to be the initiator of cellular stress response in tumor cells (Rich et al., 2000). Many stress-inducible molecules such as INK, NFkB, ceramide or MAPK/ERK, may have a significant impact on apoptosis pathways (Davis et al., 2000; Herr et al., 2001; Mayo et al., 2000). Most signaling pathways activated by anticancer drugs eventually result in activation of caspases, the proteases that act as common death effector molecules in various forms of cell death (Earnshaw et al., 1999; Degen et al., 2000; Slee et al., 1999; Utz et al., 2000). The ability of anticancer agents to trigger caspase activation appears to be a critical determinant of sensitivity or resistance to cytotoxic therapies (Herr et al., 2001; Kaufmann et al., 2000). As a result, inhibition of caspase activation may be an important factor in chemoresistance (Herr et al., 2001; Kaufmann et al., 2000). Expression levels of individual caspases may affect their overall activity, since deficient expression levels of caspases may impair activation of caspases (Joseph et al., 1999; Janicke et al., 1998; Yang et al., 2001). For example, there is lack of caspase-3 expression in MCF-7 breast carcinoma cells due to a frameshift mutation in the caspase-3 gene (Janicke et al., 1998); and caspase-8 expression was found to be low in a variety of tumor cells derived from malignant brain tumors, small lung cell carcinoma, and neuroblastoma (Fulda et al., 2001; Teitz et al., 2000). In contrast, enhanced transcription of caspase genes in response to cytotoxic treatment may increase expression levels of caspase proteins, which triggers the pro-apoptotic signaling in cancer therapy (Droin et al., 1998). Since Bcl-2 family proteins comprise both anti-apoptotic members such as Bcl-2, Bcl-XI and Mcl-1; pro-apoptotic molecules such as Bax, Bek, Bad and BH3
domain-only molecules such as Bid, Bim, and Noxa which link the extrinsic apoptotic pathway to the intrinsic apoptotic pathway (Antonsson et al., 2000), when Bel-2 family proteins are mutated or their expression levels are altered, the drug response in experimental systems can be altered drastically (Minn et al., 1995). For example, reduced Bax levels cause poor responses to chemotherapy and shorter overall survival in breast or colorectal carcinoma while enhanced levels of Bax promote the response to chemotherapy in vivo (Sturm et al., 1999; Sturm et al., 2001). Some recent studies suggested that there was inhibition of apoptosis by inhibitor of apoptosis proteins (IAPs) in response to cytotoxic therapy. For instance, cIAP1 or cIAP2 can suppress apoptosis in vitro following treatment with cisplatin, cytarabine, or TRAIL or after γ-irradiation (Datta et al., 2000; Suliman et al., 2000). The transcription factor NFkB is activated in response to a number of stimuli such as cellular stress and anticancer drugs and is able to suppress apoptosis by promoting the transcription of genes for several anti-apoptotic proteins including TRAF1, TRAF 2, cIAP1, cIAP2, Bel-XI, and FLIP, which is thought to confer resistance to cytotoxic therapies (Mayo et al., 2000). It has been reported that inhibition of NFkB in combination with chemotherapy enhanced the cytotoxic effect of chemotherapy dramatically when certain types of anticancer treatments result in induction of NFkB transcriptional activity, therefore NFkB is likely to be involved in inducible chemoresistance and inhibition of NFkB may be one of the promising adjuvant approaches to chemotherapy (Mayo et al., 2000). To conclude, alterations in components of the apoptotic machinery have an impact on sensitivity of tumor cells toward cytotoxic
therapy, which suggests a potential way to strengthen the effect of chemotherapy.

2.12.2. Autophagy

Cellular homeostasis requires a balance between biosynthetic and catabolic processes. Eukaryotic cells primarily use two different mechanisms for large-scale degradation, the proteasome and autophagy, with autophagy being the one capable of degrading entire organelles. Autophagy refers to a degrading and recycling process of cellular constituents that plays a role in the bioenergetic management of starvation as well as an important physiological role in human health. It usually occurs at basal levels in most tissues and contributes to the routine turnover of cytoplasmic components. However, it can also be induced by a change of environmental conditions such as nutrient depletion. Besides turnover of cellular components, autophagy is involved in development, differentiation, and tissue remodeling in various organisms (Levine et al., 2004) and is implicated in certain human diseases as well. Three main morphologies of programmed cell death (PCD) have been described in the context of embryonic development (Clarke et al., 2002; Schweichel et al., 1973) and autophagy is called type II PCD which is characterized by the accumulation of autophagic vesicles in side the dying cell and is often observed when massive cell alimination is demanded or when phagocytes do not have easy access to the dying cells. Autophagy is different from apoptosis in that autophagy uses dying cells’ endogenous lysosomal machinery for most of the dying cells’ degradation whereas apoptotic cells use phagocytic cell lysosomes for this process. There are three forms
of autophagy, macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA) (Yang et al., 2005; Yorimitsu et al., 2005). These processes differ in the mode of delivery to lysosomes. Macroautophagy involves the sequestration of unnecessary and dysfunctional organelles and proteins in a double membrane-bounded vesicle called autophagosome. In microautophagy cytosolic components are sequestrated directly by lysosomes. During CMA, a cytosolic and lysosomal chaperone protein, hsc70, assists unfolded proteins to translocate into lysosomes. Among the three types, macroautophagy is the most prevalent form of autophagy (Klionsky et al., 2007; Levine et al., 2004; Mizushima et al., 2007). After formation of autophagosomes in macroautophagy, the autophagosomes undergo a maturation process in which they fuse with lysosomes to form a single membrane-surrounded vesicle called autophagolysosome or autolysosome. The outer membrane of the autophagosome fuses with lysosome (or endosome) membrane while the inner sac enters the lysosome where its membrane and its contents are degraded by hydrolyses such as cathepsins (Eskelinen, 2005; Klionsky et al., 2000; Levine et al., 2004; Mizushima et al., 2002; Mizushima et al., 1998). The resulting products of degradation are reused to maintain basal macromolecular synthesis or are oxidized inside mitochondria to maintain bioenergetics. Autophagic processes have been well characterized in yeast and more than 30 autophagy-related genes (ATG) that encode the proteins executing autophagy have been described in yeast (Klionsky et al., 2003) and in some cases mammalian orthologs have also been identified (Codogno et al., 2005). A concise description of the function of autophagy is recycling, reducing waste and yielding
resources. When damaged organelles and misfolded proteins accumulate in senescent cells or cells under various stresses such as oxidative stress and infection, autophagic machinery degrades and reduces this cellular waste. Basal autophagy is constitutively observed in post-mitotic cells and may play an important role in quality control of cellular components (Komatsu et al., 2007). The amino acids and fatty acids generated from self-digested components by autophagic degradation are used by tricarboxylic acid (TCA) cycle to produce ATP, which provides energy for various cellular events. Recent studies suggested a variety of physiological and pathological roles of autophagy including development, aging, host defense system, neurodegenerative diseases, muscle and cardiac diseases and cancer (Levine et al., 2008; Mizushima et al., 2008). The pro-survival function of autophagy has been very well characterized under circumstances of nutrient starvation when cells can recycle their macromolecules through autophagy and so try to help cells with compromised bioenergetics go through stress (Klionsky et al., 2000). Deletion of autophagy genes increases cell death under nutrient deprivation while in normal conditions knock-out of autophagic genes does not affect survival (Kuma et al., 2004; Takeshige et al., 1992). The role of autophagy in maintaining mammalian cellular homeostasis and survival has been observed at cellular level and the level of entire organism (Kuma et al., 2004; Komatsu et al., 2005). When denying autophagy to cells with defects in apoptosis, the cells are not able to tolerate metabolic stress, reducing cellular fitness and activating a necrotic pathway to cell death (Jin et al., 2007). Autophagy also occurs to maintain cellular energy homeostasis and bioenergetics
where there is growth factor deprivation. In this case, even though the cells are exposed to plenty of nutrients in their extracellular environment, growth factor withdrawal leads to the loss of their ability to uptake the nutrients (Lum et al., 2005). This phenomenon has been observed in apoptosis-deficient cells. In normal cells, serum starvation usually induces apoptosis and it is difficult to see autophagy (Lum et al., 2005). Though apoptosis-deficient cells under stress in the presence of Atg genes are able to perform autophagy to recover themselves, they eventually undergo atrophy and finally die of self-consumption and bioenergetic failure. In apoptosis-competent cells, when the cells cannot be saved by autophagy, then they undergo apoptosis and where to go depends on the kind of stimulus (Boya et al., 2005). It has also been reported that anoxia-reoxygenation induces apoptosis, that the autophagy inhibitor 3-MA increases cell death (Dosenko et al., 2006) and that autophagy plays a protective role in chronic ischemia (Yan et al., 2006). These results suggest that autophagy acts as a cell-survival mechanism. However, in some apoptotic-deficient systems, autophagy seems to be and alternative death mechanism (Debnath et al., 2005; Gozuacik et al., 2004). A role for autophagy in initiating cell death has been reported in cells’s response to ER stress, hypoxia, chemotherapeutic agent, toxins and virus infections (Daido et al., 2004; Talloczy et al., 2002). When apoptosis is blocked by zVAD, many different cell lines die with autophagic features which can be prevented by 3-MA (autophagy inhibitor), siRNA of Beclin1 and Atg7 (genes mediating autophagy), and Wortamannin (autophagy inhibitor) (Yu et al., 2004). The role of autophagy as a cell death mechanism is mainly dependent on the type of
cell line and the stress stimulus and might require particular signaling molecules (Shimizu et al., 2004). When apoptosis fails under certain conditions, cells choose to die via autophagy. Interestingly, it has been found that even apoptotic-competent cells can die through autophagy (Guillon-Munos et al., 2006). An overlap between autophagic and apoptotic pathways has been reported in some studies in which it has been observed that apoptotic factors such as TRAIL, FADD, ceramide, and the kinases DAPK and DRP-1 regulate autophagy (Mills et al., 2004; Pyo et al., 2005). However, the relationship between autophagy and apoptosis is still under debate. Increasing evidence suggests that autophagy plays a cytoprotective role in cancer cells under metabolic stress (Amaravadi et al., 2007; Degenhardt et al., 2006; Mathew et al., 2007; Qadir et al., 2008) and it may contribute to tumor cell survival and tumor formation, however, the molecular mechanism underlying the acquisition of vigorous autophagic activity in cancer cells remains a mystery. A recent study, however, presented that autophagy is also associated with tumor suppression. It showed that Beclin1, a mammalian ortholog of ATG6, interacted with class III PI3-kinase, Vps34, and this interaction was crucial for the induction of autophagy and suppression of the growth of the xenografted breast cancer cell lines (Furuya et al., 2005; Liang et al., 1999; Zeng et al., 2006;) and Beclin1 was identified as a candidate of tumor suppressor. During investigations of autophagy in mammalian cells, a couple of signaling pathways have been revealed to regulate autophagy. The mammalian target of rapamycin (mTOR) is a key molecule for regulating cancer cell proliferation. Rapamycin inhibits mTOR function followed by autophagy induction (Lum et al.,
2005; Ravikumar et al., 2004). Molecules known to suppress mTOR, including PTEN and TSC, both of which are considered as tumor-suppressor gene products, can induce autophagy (Arico et al., 2001; Feng et al., 2005). Interestingly, mTOR-activating molecules such as class I PI3K and Akt, which are frequently activated in various cancer cells, inhibit autophagy (Lum et al., 2005; Takeuchi et al., 2005). All of these findings indicate a tumor-suppression role of autophagy. To conclude, both the gain and loss of autophagy may play crucial roles in tumor formation and growth. When autophagy is activated, it can supply nutrients to tumor cells under metabolic stress; when it is inactive, tumor cells that are supposed to die by autophagy would survive. Recent studies using transplanted autophagy-deficient tumor cells revealed that DNA damage and inflammation can be caused by the loss of autophagy and therefore enhance tumorigenesis (Degenhardt et al., 2006).

2.12.3. Necrosis

Necrosis is defined as a type of cell death that lacks the characteristics of apoptosis and autophagy, and has been considered as an uncontrolled form of cell death. It is characterized by loss of membrane integrity, vacuolization of the cytoplasm and cellular swelling. Upon necrosis, inflammatory response can be induced probably through the release of intracellular components that alert the innate immune system (Edinger et al., 2004; Festjens et al., 2006; Zitvogel et al., 2004). This brisk inflammatory response and immune amplification of the damage signal is in sharp contrast to apoptotic cells that are silently cleared by tissue macrophages. Therefore,
necrosis was viewed as strictly a pathological form of cell death that is not a physiologically programmed process. Necrosis is often associated with unwarranted cell loss due to pathological traumas such as infection or ischemia, however, it can be triggered by Fas or TNFa ligand via their respective receptors (Vercammen et al., 1998), which suggests that necrosis may not be such an uncontrolled form of cell death as initially considered. Despite the significant effects of necrosis under pathological conditions, the molecular mechanisms underlying necrotic cell death are poorly understood. Recent research shows that its occurrence and course might be tightly regulated (Golstein et al., 2006) and that there exists not only accidental necrosis, but also normal physiological and programmed necrosis (Proskuryakov et al., 2002; Syntichake et al., 2002). Increasing evidence suggests that, much like apoptosis, specific genes have evolved to regulate necrotic cell death (Festjens et al., 2006; Golstein et al., 2007). Genetic studies have identified death receptor adaptors, such as receptor-interacting protein kinase 1 (RIPK1) and the tumor necrosis factor (TNF) receptor-associated factor 2 (TRAF2) as essential regulator of death receptor-induced necrotic cell death (Holler et al., 2000; Lin et al., 2004). Cells deficient in RIPK1 are protected from necrotic cell death when treated with Fas ligation and caspase inhibitors or hydrogen peroxide alone (Shen et al., 2004). In a model where L929 mouse fibrosarcoma cell line treated with TNFa was used, cells initiated a complex multi-step signal transduction pathway in which Fas-associated death domain (FADD) is recruited to the TNF receptor 1 (TNF-R1) and induced necrosis, possibly through its death domain (Festjens et al., 2006). The TNFa-induced necrosis here was
accompanied by a rapid burst of mitochondrial ROS (reactive oxygen species) production that could be inhibited by rotenone, an inhibitor of the respiratory chain complex I (Schulze-Osthoff et al., 1993). Furthermore, Bcl-2 could reduce TNF-induced necrosis, presumably by maintaining mitochondrial integrity (Festjens et al., 2006). Down-regulation of the expression of RIPK1 prevented the mitochondrial manifestations of TNFa-treated L929 cells (Festjens et al., 2006). RIPK1-deficient Jurkat cells are refractory to the propagation of necrosis induced by the Fas/TNF-R/TRAIL-R pathway (Chan et al., 2003; Holler et al., 2000). In DNA-damage-induced necrosis, Jun N-terminal kinase seems to be essential as a downstream effector of RIPK1 for the permeabilization of mitochondrial membranes (Yu et al., 2006). RIPK1 might also be indispensable for the accumulation of pro-necrotic ceramides (Thon et al., 2005). Thus RIPK1 may be an important regulator of necrosis, acting as an upstream signal. Another mitochondrial matrix protein, cyclophilin D (CypD), has also been shown to be important in the mediation of necrosis. Knockout of the gene encoding CypD induces resistance to necrotic cell death induced by ROS or Ca overload in, for instance, hepatocytes and fibroblasts (Nakagawa et al., 2005; Schinzel et al., 2005). Caspases might inhibit the necrotic pathway, meaning that in some circumstances their inhibition might enhance cell necrosis. For example, following apoptotic stimulus, such as the expression of Bax or treatment with tumor necrosis factor (TNF) or Fas ligand, cells will die even in the presence of caspase inhibitors like zVAD-fmk or anti-apoptotic molecules such as Bel-XL that prevent caspase activation (Jaattela et al., 2003; Lockshin et al., 2004).
Under these conditions, cells that would normally die through apoptosis exhibit all the characteristics of necrosis. Caspase-independent necrotic cell death can be forestalled by treatment with antioxidants or by eliminating the activity of RIPK1. All these results suggest that necrosis could be programmed.

### 2.12.4. Mitotic Cell Death

Another type of cell death, mitotic cell death (MCD), often referred to as mitotic catastrophe, is caused by aberrant mitosis that results in the formation of cells with two or more micronuclei. In mammalian cells and particularly in tumor cells, mitotic catastrophe is mainly associated with deficiencies in cell cycle checkpoints (Roninson et al., 2001; Castedo et al., 2004). It is a major form of tumor cell death after treatments with IR or certain chemotherapeutic agents (Blank et al., 2003; Torres et al., 1998). MCD features enlarged and multinucleated cells, incomplete nuclear condensation, chromosome alignment defects, unequal DNA separation or mitosis in the presence of DNA damage (Eom et al., 2005; Roninson et al., 2001). As the G2/M checkpoint is responsible for blocking mitosis when there is damaged DNA, altered expression of proteins involved in this checkpoint is possibly associated with mitotic catastrophe. For instance, high expression of proteins such as Cdk1 and cyclin B that promote entry of mitosis or inhibition/knockout of proteins that prevent premature mitosis including ATR, ATM, Chk2, and Plk can induce MCD (Brown et al., 2000; Chan et al., 1999; Jin et al., 1998; Niida et al., 2005). Because p53 can induce G2-suppression in case of DNA damage via the Cdk-inhibitor p21, p53 might be
involved in preventing MCD (Bunz et al., 1998). Indeed, p53-mediated arm of the G2/M checkpoint was shown to play an important role in the prevention of MCD in cells with damaged DNA (Chan et al., 2000; Fei et al., 2003). Besides defects in G2/M checkpoint, defective mitotic spindle checkpoints have been associated with MCD. Appropriate spindle functioning depends on proteins involved in the spindle formation such as Mad and Bub as well as on chromosomal passenger proteins such as Survivin and Aurora kinases (Lens et al., 2003; Lens et al., 2006). Knockout of Survivin gene results in disorganized mitotic spindles in early passage cells that eventually die through a process like MCD (Okada et al., 2004). Upon concomitant inhibition of apoptosis and impaired spindle checkpoint function, the cells may proceed through aberrant anaphase to telophase without any sign of cytokinesis, giving rise to multinucleated cells, most of which become non-viable. However, some of the multinucleated cells can survive through interphase restitution, endo-reduplication and activation of DNA repair processes such as homologous recombination (Blank et al., 2007). Cells that survive abnormal mitosis can potentially divide asymmetrically, giving rise to aneuploid cells which are generally more tumorigenic. When cells go into mitosis with damaged DNA, they are more likely to acquire tumorigenic capacity as these cells are genetically unstable. MCD may kill such cells and therefore prevent tumorigenesis. In a colon cancer xenograft model, a dominant-negative mutant survivin significantly induced mitotic catastrophe and apoptosis as well as inhibition of tumor growth (Tu et al., 2005). High expression of Survivin is often found in tumor cells while rare in normal cells (Duffy et al., 2007).
All these results suggest a tumor suppressive role of MCD. One way to cancer therapy
is to induce cell death in tumor cells via MCD by direct inhibition of the G2
checkpoint in combination with DNA damage (Ricci et al., 2006).

2.13. Overview of MDA-7
Melanoma differentiation associated gene, MDA-7/IL-24, is a gene that encodes a
24-KD protein (Pestka et al., 2004; Sauane et al., 2003). In normal cells, the
expression of MDA-7 is restricted to cells of immune system and melanocytes (Huang
et al., 2001; Caudell et al., 2002; Wolk et al., 2002). MDA-7/IL-24 expression is
decreased during pathologic progression of melanocytes to melanomas which can be
demonstrated by both RT-PCR (Jiang et al., 1995) and immunohistochemistry
(Ellerhorst et al., 2002). This correlation between the loss of gene expression and
tumor invasion suggests that MDA-7/IL-24 might function as a tumor suppressor
(Ekmekcioglu et al., 2001; Lebedeva et al., 2002; Jiang et al., 1995). Based on the
results obtained using in vitro studies and in vivo preclinical animal modeling
(Yacoub et al., 2008 a), MDA-7/IL-24 emerged as a promising new and potentially
applicable anti-tumor therapeutic agent, exhibiting direct tumor growth inhibitory
effects as well as ‘bystander anti-tumor’ properties (Lebedeva et al., 2007). Findings
from a completed phase I dose-escalation trial on 22 advanced cancer patients who received intratumoral injection of a non-replicating adenovirus vector carrying the MDA-7/IL-24 transgene (INGN 241) were recently reported (Cunningham et al., 2005; Fisher et al., 2003; Fisher et al., 2006; Gupta et al., 2006; Lebedeva et al., 2002; Tong et al., 2005), and confirmed that the clinical activities of INGN 241 (Ad.mda-7) include apoptotic and anti-tumor activities and systemic immune activation and can generate both direct tumor growth inhibition and ‘bystander anti-tumor’ effects, indicating that MDA-7 is an ideal new candidate for ovarian cancer therapy.

2.13.1. The mechanism by which MDA-7 functions

Within the IL-10 family, IL-19, IL-20 and IL-24 exhibit impressive sharing of receptor complexes; all three are capable of signaling through IL-20R1/IL-20R2, and both IL-20 and IL-24 can also use IL-22R1/ IL-20R2. However, the biological activities of these three cytokines appear quite distinct; since only MDA-7/IL-24 induces tumor-specific apoptosis, and this effect can be receptor-independent (Sauane et al., 2003). The MDA-7/IL-24 protein binds to IL-20 and IL-22 receptor complexes leading to JAK/STAT activation (Dumoutier et al., 2001; Parrish-Novak et al., 2002; Wang et al., 2002). However, treatment with tyrosine kinase-specific inhibitors (Genistein and AG18) or a JAK-selective inhibitor (AG490) does not alter Ad.mda-7-induced apoptosis in diverse cancer cell lines (Sauane et al., 2003). In addition, there is no correlation between the pattern of expression of IL-20R1, IL-20R2, and IL-22R mRNA and susceptibility to Ad.mda-7-induced cell death in different cell lines. Therefore, signaling events leading to Ad.mda-7-induced
apoptosis might be tyrosine kinase independent and can be distinguished from
*MDA*-7/IL-24 cytokine function-related properties mediated by the IL-20/IL-22
receptor complexes that require JAK/STAT kinase activity (Chada et al., 2004;
Sauane et al., 2003; Su et al., 2005). *In vitro* studies were done to address the
mechanism(s) by which over-expression of *MDA*-7/IL-24 induces death selectively in
cancer cells. Despite these efforts, the precise pathways of *MDA*-7/IL-24-induced
apoptosis remain to be clarified. Evidences from earlier studies implied an
involvement of the intrinsic/mitochondrial pathway of apoptosis. Transfection of
breast carcinoma cells with an *MDA*-7/IL-24 cDNA resulted in pro-apoptotic Bax
up-regulation and subsequent apoptosis induction (Su et al., 1998; Madireddi et al.,
2000). The onset of apoptosis was blocked by anti-apoptotic Bcl-2 over-expression.
Further studies in melanoma, glioblastoma multiforme (Yacoub et al., 2003; Yacoub
et al., 2004; Su et al., 2003), renal carcinoma (Yacoub et al., 2004) and prostate
carcinomas (Lebedeva et al., 2003; Su et al., 2006; Saito et al., 2005) confirmed the
involvement of the Bcl-family of proteins in *MDA*-7/IL-24-induced apoptosis. In most
tumor cell contexts, over-expression of MDA-7/IL-24 protein leads to the dramatic
down-regulation of anti-apoptotic (Bcl-2 and Bcl-xL) and/or to the up-regulation of
pro-apoptotic (Bax and Bak) members of the Bcl-family. Interestingly, it has been
found that Ad.mda-7 can induce apoptosis in bax-null DU-145 prostate cancer cells
(Lebedeva et al., 2003); therefore, apoptosis can be mediated by a bax-independent
pathway. Moreover, over-expression of anti-apoptotic Bcl-family members
differentially protects prostate cancer cells from the *MDA*-7-induced apoptosis
Bcl-xL over-expression prevents Ad.mda-7-induced apoptosis in DU-145 and PC-3 prostate carcinoma cells, while Bcl-2 over-expression protects LNCaP cells from MDA-7/IL-24-induced apoptosis. The reasons for this disparity are not completely understood and are currently under investigation. Detailed studies in prostate cancer cells identify reactive oxygen species (ROS) as an active component in MDA-7/IL-24-induced apoptosis (Fig. 1) (Lebedeva et al., 2003; Lebedeva et al., 2005; Yacoub et al., 2003). When overexpressed inside a cancer cell MDA-7/IL-24 protein directly or indirectly affects mitochondria, resulting in mitochondrial dysfunction and the inner mitochondria membrane potential to drop thereby leading to ROS production and to apoptotic death. All of the changes described above are absent after Ad.mda-7 infection of normal prostate epithelial cells. Antioxidants (Nacetyl-L-cysteine and Tiron) and inhibitors of mitochondrial permeability transition (cyclosporine A and bongkrekic acid) inhibit Ad.mda-7-induced mitochondrial dysfunction and apoptosis in prostate cancer cells. By contrast, agents facilitating ROS production (arsenic trioxide, NSC656240, and PK11195) facilitate Ad.mda-7-induced apoptosis. Ectopic expression of Bcl-2 and Bcl-xL inhibits mitochondrial changes, ROS production, and apoptosis, providing additional support for a correlation between mitochondrial dysfunction and Ad.mda-7 action. These studies present definitive evidence that changes in mitochondrial function and ROS production represent key components associated with selective killing of prostate cancer cells by MDA-7/IL-24. The ability of MDA-7/IL-24 to produce ROS may prove extremely important for treatment of pancreatic cancer.
Pancreatic cancer cells are resistant to \textit{MDA-7/IL-24}-induced apoptosis due to a diminished capacity to convert \textit{MDA-7/IL-24} mRNA into protein (Su et al., 2001). This translational block can be reversed by combinational treatment with Ad.mda-7/IL-24 and agents that increase the ROS levels within cells, such as arsenic trioxide, N-(4-hydroxyphenyl) retinamide, or dithiophene (Lebedeva et al., 2005). Induction of apoptosis \textit{in vitro} and suppression of tumorigenesis \textit{in vivo} in nude mice are induced in pancreatic cancers independently of their K-\textit{ras} status upon combinatorial treatment with Ad.mda-7/IL-24 and a ROS-inducing agent. ROS inhibitors, such as N-acetyl-cysteine and Tyron, can block this effect. In addition to modification of mitochondrial function, \textit{MDA-7/IL-24} has also been shown to kill cancer cells by causing endoplasmic reticulum (ER) stress (Gupta et al., 2006; Sauane et al., 2004; Sauane et al., 2006). ER stress may be caused by misfolded protein accumulation followed by the activation of a highly conserved unfolded protein response (UPR). These events lead to apoptosis through the induction of growth suppression and DNA-damage-inducible (\textit{GADD}) genes (Berridge et al., 2000; Berridge MJ, 2002). The hypothesis of a role for \textit{MDA-7/IL-24}-induced ER stress and cancer cell apoptosis is supported by the fact that \textit{MDA-7/IL-24} induces \textit{GADD} genes and further activates p38 MAPK in the context of transformed cells (Gupta et al., 2006; Sarkar et al., 2002). Previous studies document that signaling events leading to Ad.mda-7-induced transformed cell apoptosis are tyrosine kinase-independent (Sauane et al., 2003). These results suggest that \textit{MDA-7/IL-24} cancer cell-specific activity can occur through mechanisms independent of binding to its currently
recognized cognate receptors and might even occur independent of receptor function.

2.13.2. Bystander effects of MDA-7

An adenovirus vector expressing a nonsecreted version of MDA-7/IL-24 protein was generated by deletion of its signal peptide (Sauane et al., 2004). This non-secreted protein was as effective as wild-type secreted MDA-7/IL-24 in inducing apoptosis in prostate carcinoma cell lines and displayed transformed cell specificity and localization of MDA-7/IL-24 in the Golgi/ER compartments. These results indicate that MDA-7/IL-24-mediated apoptosis can be triggered through a combination of intracellular as well as secretory mechanisms and could occur efficiently in the absence of protein secretion. Treatment of susceptible prostate cancer cell lines with Ad.mda-7 as well as Ad.SP-MDA-7 induces killing to a comparable extent through ERK1/2-dependent and JAK/STAT-independent pathways (Sauane et al., 2004). The fact that both secreted and non-secreted forms of MDA-7/IL-24 protein have comparable apoptosis-inducing activity was unanticipated, adding an additional level of complexity in understanding how this novel molecule works. Localization of full-length MDA-7/IL-24 protein in the ER/Golgi compartments is consistent with the signal peptide hypothesis (Lingappa et al., 1980) and the currently known and predicted secreted cytokine nature of the protein (Fisher et al., 2003). Because the signal-peptideless mutant of MDA-7/IL-24 protein does not contain an export signal, it is predicted to remain in the cytosol. We have, however, confirmed through confocal immunofluorescence studies that a significant fraction of this protein is able
to enter the ER and Golgi apparatus and that proteins derived from wild-type and mutant viruses appear to have overlapping patterns of localization within the cell (Sauane et al., 2004). It is not possible to rule out cryptic internalization signals that become active in the absence of the actual signal peptide, as the identity of these cryptic sites is currently hypothetical. Western blot analyses performed on proteinderived cytosolic and extracellular fractions of cells infected with both viruses indicated that only full-length MDA-7/IL-24 was processed and secreted. It is also possible that adenovirus infection produced relatively large amounts of protein that even in the absence of a specific targeting sequence possesses the ability to cross membranes and accumulate in the ER/Golgi because of charge and/or tertiary structure. However, because localization of MDA-7/IL-24 is similar in both normal (P69) and cancer (DU-145) cells, differences in cellular localization of this protein can be excluded as a direct mechanism underlying the differential apoptosis-inducing activity of MDA-7/IL-24 toward cancer cells. There are two possible explanations of differential MDA-7/IL-24-induced killing in tumor and normal cells. One is that enhanced sensitivity may be due to the ‘activated’ or ‘destabilized’ nature of tumor compared to normal cells, which enhances cell death after the ER-stress response is triggered. The other possibility is that MDA-7/IL-24 not only induces the classical ER-stress response that favors apoptosis, but it also induces additional specific pathways that cause apoptosis only in transformed cell lines. We demonstrated that MDA-7/IL-24 localizes to the ER compartment both in normal and cancer cells and is therefore in a position to induce this pathway irrespective of the transformation status.
of the cell (Sauane et al., 2004). Current data provide some support for both hypotheses, however additional studies are required. GADD family gene induction as well as p38 MAPK activation is induced only in transformed and not in normal cells (Gupta et al., 2006; Sarkar et al., 2002) indicating that the disparate response might be due to differential activation, either in strength or in duration of the ER-stress response. However, this does not rule out the activation of additional pathways, specifically in cancer cells. In a microarray-based study, MDA-7/IL-24 was shown to induce expression of ER-stress response genes such as BiP/GRP78, PP2A, HSJ1 and TRA1 in H1299 lung carcinoma cells (Sieger et al., 2004). Nevertheless, with the exception of BiP/GRP78, which is selectively up-regulated in cancer cells (Gupta et al. 2006), no comparative data for most of these genes is available contrasting normal and cancer cells.

### 2.13.3. Pathways involved in MDA-7 functions

Expanding studies in diverse systems reveal that the signal transduction pathways mediating MDA-7/IL-24-induced apoptosis are varied and multiple signaling pathways are activated in different tumor cell lines upon Ad.mda-7 infection or
treatment with recombinant GST-MDA-7 protein and Ad-MDA7 (Sauane et al., 2004; Yacoub et al., 2003, 2004, and 2008). Experiments in melanoma cell lines demonstrated that SB203580, a specific inhibitor of p38 MAPK pathway, protects these cells from Ad-MDA7-induced apoptosis. Ad.mda-7 infection resulted in phosphorylation of p38 MAPK and induction of the GADD family of genes in melanoma cells, but not in normal melanocytes (Sarkar et al., 2002). In general, over-expression of each GADD gene from the family in combination (GADD34, GADD45α, GADD45β, GADD45γ and GADD153) led to synergistic or cooperative antiproliferative effects (54). Ad.mda-7 infection resulted in significant induction of GADD153, GADD45α and GADD34, and a moderate induction of GADD45γ (Sarkar et al., 2002). Both inhibition of the p38 MAPK pathway (either pharmacologically with SB203580 or by an adenovirus expressing a dominant negative p38 MAPK) and inhibition of the GADD family of genes by an antisense approach rescued melanoma cells from Ad.mda-7-induced apoptosis. Activation of the p38 MAPK pathway followed by induction of the GADD family of genes also plays a crucial role in Ad.mda-7-modulated apoptosis in glioblastoma multiforme (Yacoub et al., 2003; 2004), prostate cancer (Gupta et al., 2006; Sauane et al., 2003; Saito et al., 2005), and breast cancer cells. Paradoxically, p38 MAPK phosphorylation by MDA-7/IL-24 in chronic lymphocytic leukemia (CLL)-B-cells and in glioblastoma promoted survival of these malignant cells (Sainz-Perez et al., 2006; Yacoub et al., 2008). Both MDA-7/IL-24 mRNA and protein were overexpressed in the CLL B-cells examined, and p38 MAPK, a downstream MDA-7/IL-24 signaling target, was highly
phosphorylated in all CLL cells, but not in normal B-cells. Obviously, phospho-p38 MAPK has no pro-apoptotic functions in CLL cells and may instead be required for survival, as suggested from these studies. Correspondingly, phosphorylation of p38 MAPK following transfection of CLL cells with *MDA-7/IL-24* promoted CLL cell survival (Sainz-Perez et al., 2006). These studies do not exclude the possibility of *MDA-7/IL-24* signaling through a p38 MAPK-independent mechanism; however, their data suggest a competition between *MDA-7/IL-24* and SB203580 (a specific inhibitor of p38 MAPK) for p38 MAPK activation. In non-small cell lung carcinoma (NSCLC) cells, Ad.mda-7 induced apoptosis via up-regulation of double stranded RNA-dependent protein kinase (PKR). Infection with Ad.mda-7 led to the phosphorylation of PKR and also its downstream targets eIF2α, Tyk2, Stat1, Stat3, and p38 MAPK (Pataer et al., 2002; Emdad et al., 2006). Ad.mda-7 treatment activated caspases 3, 8 and 9 and cleavage of Bid and PARP in NSCLC cells. The activation of PKR appeared to be upstream of caspase activation because pretreatment with caspase inhibitors failed to prevent PKR phosphorylation. Treatment with a serine/threonine kinase inhibitor 2-aminopurine blocked Ad.mda-7-induced apoptosis as well as activation of PKR and eIF2α (Pataer et al., 2002). A recent study by Chada *et al* (Chada et al., 2006) reported PKR up-regulation in breast cancer cell lines upon Ad.mda-7 infection. Further investigation of the interaction between MDA-7/IL-24 and PKR in NSCLC cells suggested a role for posttranslational regulation of PKR by MDA-7/IL-24 (Pataer et al., 2005). A physical interaction of MDA-7/IL-24 and PKR was confirmed by immunofluorescence and coimmunoprecipitation studies. In both
studies (Pataer et al., 2002; Pataer et al., 2005), the authors indicate an inability of Ad.mda-7 to induce apoptosis in PKR null (-/-) mouse embryonic fibroblasts (MEF), but not in PKR wild-type MEFs, providing additional proof of PKR involvement in Ad.mda-7-modulated apoptosis. Nevertheless, this finding requires further independent confirmation given that Ad.mda-7 does not induce apoptosis in a wide array of normal human or rodent cells. Activation of p38 MAPK following Ad.mda-7 infection appears to be a common event linking the PKR and p38 MAPK pathways (Sarkar et al., 2002; Pataer et al., 2002; Emdad et al., 2006). It is possible that in melanoma cells p38 MAPK activation is downstream of PKR activation, although in melanoma cells the post-p38 signal transduction changes seem to be more important in Ad.mda-7-induced apoptosis. Since eIF2α phosphorylation activates the transcription factor ATF4, which in turn activates GADD153 (Fawcett et al., 1999), there is a significant level of cross talk between the PKR and the p38 MAPK signal transduction pathways. Additional studies are required to identify the upstream molecules within PKR and p38 MAPK pathways involved in Ad.mda-7-induced apoptosis. The c-Jun NH2-terminal kinase (JNK) pathway is another important signaling pathway involved in MDA-7/IL-24-induced apoptosis. Infection with Ad.mda-7 radiosensitizes malignant glioma (Su et al., 2003; Yacoub et al., 2003) and prostate cancer cells (Su et al., 2006). A combination of Ad.mda-7 and γ-irradiation activates JNK in glioma and prostate cancer cells, and treatment with a specific JNK inhibitor, SP600125, prevents apoptosis after the combination treatment (Su et al., 2006; Yacoub et al., 2003). JNK activation leads to down-regulation of anti-apoptotic
Bcl-xL and/or Bcl-2 in glioma and prostate carcinoma cells (Yacoub et al., 2004; Su et al., 2006) and to the up-regulation of pro-apoptotic Bax and Bak proteins in prostate carcinoma cells (Su et al., 2006). Blocking of JNK phosphorylation with SP600125 abrogates these changes in the levels of Bcl-family proteins. In NSCLC cells, curcumin (dietary pigment that inhibits JNK activation) prevents phosphorylation of c-jun and radiosensitization by Ad.mda-7 (Kawabe et al., 2002). A potential negative regulation of s-catenin and PI3K/Akt signaling pathways, which are implicated in cell-cell adhesion, cytoskeletal rearrangements, and membrane trafficking in breast and lung cancer cells, by MDA-7/IL-24 was reported (Mhashilkar et al., 2003). Using microarray analysis, increased protein expression was evident from tumor suppressor genes such as E-cadherin, APC, GSK-3β, and PTEN after Ad.mda-7 infection. At the same time, expression of proto-oncogenes involved in s-catenin and PI3K signaling was decreased. Ad.mda-7 treatment led to a redistribution of cellular s-catenin from the nucleus to the plasma membrane. As a result, LEF/TCF transactivation was significantly reduced, and E-cadherin-s-catenin adhesion complex was up-regulated in a tumor cell-specific manner. Furthermore, Ad.mda-7 infection of breast and lung cancer cells down-regulated the expression of PI3K pathway members (p85 PI3K, FAK, ILK-1, Akt, and PLC-γ). Experiments did not indicate if over-expression of s-catenin or activation of the PI3K pathway protects these cells from Ad.mda-7-induced apoptosis. In conclusion, Ad.mda-7 appears to negatively regulate both the PI3K and the s-catenin signaling pathways in breast and lung cancer cells resulting in restoration of apoptosis induction. Moreover, PI3K
activation has been implicated in chemoresistance and radioresistance in tumor cells; thus, Ad.mda-7 treatment should serve as a chemo- and radiosensitizer. Ad.mda-7 regulates multiple members of the s-catenin and PI3K pathways that are considerably redundant and simultaneously produce antiproliferative, pro-apoptotic, and antimetastatic phenotypes. Although it is not clear where these changes are initiated, MDA-7/IL-24 appears to act upstream of PI3K, PLC-γ, and PTEN. It is significant that the s-catenin and PI3K pathways were not altered by MDA-7/IL-24 expression in normal HUVEC cells. Activation of the Fas-FasL signaling pathway occurred in the human ovarian cancer cell line MDAH 2774 following infection with Ad.mda-7, where Ad.mda-7 significantly inhibited cell proliferation and induced apoptosis. Early MDA-7/IL-24-induced activation of the transcription factors c-Jun and activating transcription factor 2 (ATF2) stimulated the transcription of an immediate downstream target, the death-inducer Fas ligand (FasL), and its cognate receptor Fas. The activation of NF-κB and induction of Fas-associated factor 1, FADD, and caspase-8 were associated with the activation of Fas-FasL (Gopalan et al., 2005). However, another study by Emdad et al (Emdad et al., 2006) reported p38 MAPK activation upon adenovirus-mediated MDA-7 infection in several ovarian cancer cell lines. The level of phosphorylated p38 MAPK correlated with the killing effect of MDA-7/IL-24 in these cells, and SB203580 (selective p38 MAPK inhibitor) significantly abolished this killing effect. Thus, activation of p38 MAPK presents a more general effect than induction of the Fas-FasL pathway and might be a key element in MDA-7/IL-24-induced apoptosis in ovarian carcinoma cell lines as well as
in other cancer cell lines (Emdad et al., 2006; Gupta et al., 2006; Sarkar et al., 2002; Yacoub et al., 2003).

**Chapter III. Aims of the studies**

The purpose of this study is to explore the efficacy of MDA-7 on ovarian cancer cells; to find out the mechanism by which MDA-7 functions in ovarian cancer cells; to try a new way of delivering MDA-7 to its target and to see the impact on ovarian cancer cells from its combinational use with cisplatinum.

**Chapter IV. Materials& Methods**

4.1. Cells lines

SKOIII, OV4, OVCAR cells were obtained from the American Type Culture Collection. Clones of SKOIII, OV4, OVCAR cells expressing MDA-7 were obtained by infecting the corresponding cells with Ad.mda-7 (5) or ((5/3)). Western blotting confirmed expression of MDA-7.

4.2. Reagents

DMEM (Dulbecco’s Modified Eagle’s Medium) and Penicillin-Streptomycin were
from Gibco (Life technology, New York). Typan blue solution and dimethyl sulfoxide (DMSO) were all purchased from Sigma Chemical (St. Louis, MO). Phospho-P38 rabbit polyclonal IgG, phospho-JNK rabbit polyclonal IgG, phospho-AKT rabbit polyclonal IgG, caspase3 mouse monoclonal IgG, and anti-caspase9 mouse monoclonal IgG were from Cell Signaling (Beverly, MA). PARP (H-250) rabbit polyclonal IgG, p-PERK (Thr981) rabbit polyclonal IgG, GAPDH (2D4A7) mouse monoclonal IgG, and p-ERK (E-4) mouse monoclonal IgG were from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

4.3. Cell culture

SKOIII, OV4, OVCAR cells were cultured in DMEM supplemented with 5% fetal bovine serum (FBS, Hyclone, Logan, UT) and 2% penicillin/streptomycin. Cells were incubated in either 6cm dishes that are used for extraction of proteins for further western blot analysis or 12-well plates that are used for typan blue assay and all in humidified atmosphere of 5% CO2 at 37 °C. Since both OV4 and OVCAR cells grow fast, usually 200,000 cells are plated in a 6cm dish and 10,000 cells are plated in a well of a 12-well plate. As SKOIII grows much slower than OV4 and OVCAR, 250,000 cells are plated in a 6cm dish and 20,000 cells are plated in a well of a 12-well plate. After 24h of incubation when the cells become attached well to the plates, they are subjected to treatment with virus or some other drugs such as
GST-MDA-7 or Cis-platinum.

4.4. Adeno-virus infection

Ad.mda-7 (5) or (5/3) and control adenoviral vectors were used to infect ovarian cancer cells. The viral titers range from 20moi to 100moi, depending on the cell types and the experimental design. The cells were incubated with purified virus in 1ml of DMEM without FBS at 37 °C in a humidified atmosphere of 5% CO₂ with gentle agitation. After 3~4h, fresh DMEM with 10% FBS was added and the cells were incubated in humidified atmosphere of 5% CO₂ at 37 °C at different time course.

4.5. Assessment of Cell Viability

The cells cultured in the 12-well plates were isolated by trypsinization and recovered by centrifugation. After trypsinization with 300ul of trypsin for some time and cells could be seen detached from the wells under a microscope, 600ul of Phosphate-buffered saline (PBS, PH7.4) from Invitrogen was then added into the wells and all the cells were collected into a 15ml conical tube for centrifugation for 5min at 1400rpm. The supernatant was discarded and pellet kept for typan blue assay. In terms of the cell number estimated, certain amount of typan blue was added into the tube and the cell pellet was resuspended. Cells were counted in all four fields of a hemocytometer. Cell viability was evaluated by assessing trypan blue inclusion/exclusion of isolated cells under light microscopy and scoring the percentage of cells exhibiting blue staining.

4.6. Preparation of cell extracts and western blot analysis

After treatment for 24h, 48h, or 72h, cells in the 6cm plates were lysed with lysis
buffer (2Xlaemml, 120mM Tris HCl pH 6.8, 4% SDS, 20% Glycerol, 10% beta-mercaptoethanol, 0.001% bromophenol blue). The cell lysate was then boiled on a heating block at 95°C for 10min. Protein concentration was determined using a kit from Bio-rad. Aliquots each of which contains 40ug of sample protein were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat milk in TBST (5% nonfat milk in 10mM Tris/HCl, 100mM NaCl, 0.01% Tween-20, pH 7.6) for 2h and then exposed to primary antibodies overnight at 4°C. The primary antibodies that we used were Phospho-P38 rabbit polyclonal IgG, phospho-JNK rabbit polyclonal IgG, phospho-AKT rabbit polyclonal IgG, caspase3 mouse monoclonal IgG, and anti-caspase9 mouse monoclonal IgG from Cell Signaling (Beverly, MA) and PARP(H-250) rabbit polyclonal IgG, p-PERK(Thr981) rabbit polyclonal IgG, GAPDH(2D4A7) mouse monoclonal IgG, and p-ERK(E-4) from Santa Cruz Biotechnology(Santa Cruz, CA) followed by washing with TBST(3x15min), after which the membranes were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibody for 2h, followed by washing with TBST(3x15min). Proteins imprinted on the membranes were visualized by enhanced chemiluminescence and quantified by densitometry.

4.7. Statistical analysis

All of the experiments were performed at least three times. Results are expressed as mean±SEM. A p<0.05 was considered significant.
5.1. GST-MDA7 effects on OVCAR and SKOVIII cell carcinoma

Previously it has been shown that recombinant GST-MDA-7 and Ad.mda-7 reduced proliferation and enhanced cell death in diverse cancer cell lines including non-established human Glioblastoma multiforme (GBM) cells (Cunningham et al., 2005; Fisher et al., 2005; Gupta et al., 2006; Lebedeva et al., 2002; Tong et al., 2005; Yacoub et al., 2004), to investigate whether MDA-7 could be used to treat ovarian cancer cells, we assayed the recombinant GST-MDA-7 on SKOIII and OVCAR ovarian carcinoma cell lines, the most commonly used ovarian cancer cell lines in laboratories; and the cell viability as well as growth suppression was evaluated. To find out the best dose of MDA-7 to treat ovarian carcinoma dose response assays were performed. Cells were treated with GST-MDA-7 at 0nM, 50nM, 100nM, and 200nM for 72 hours or 96 hours, after which cells were collected and subjected to trypan blue staining. For OVCAR, at 72h after treatment with GST-MDA-7, the percentages of live cells are about 100% at 0nM, 80% at 50nM, 63% at 100nM, and 41% at 200nM while the percentages of cell death are about 10% at 0nM, 10.5% at 50nM, 11% at 100nM, and 17.5% at 200nM(Figure 1 A, B); at 96h, the percentages of live cells are about 100% at 0nM, 80% at 50nM, 60% at 100nM, and 30% at 200nM while the percentages of cell death are about 10% at 0nM, 11% at 50nM, 15% at 100nM, and 18% at 200nM(Figure 1 C, D). For SKOVIII, at 72h after treatment with GST-MDA-7, the percentages of live cells are about 100% at 0nM, 98% at 50nM,
95% at 100nM, and 90% at 200nM while the percentages of cell death are about 8% at 0nM, 9% at 50nM, 10% at 100nM, and 16.5% at 200nM (Figure 1 E, F); at 96h, the percentages of live cells are about 100% at 0nM, 90% at 50nM, 87% at 100nM, and 80% at 200nM while the percentages of cell death are about 8.5% at 0nM, 11% at 50nM, 12.5% at 100nM, and 17.5% at 200nM (Figure 1 G, H). These results showed that the number of living cells decreased when increasing the concentration of GST-MDA-7 and the percentage of cell death become higher upon increasing the concentration of GST-MDA-7 in both OVCAR and SKOVIII, but GST-MDA-7 did not cause as much cell growth suppression in SKOVIII cells as it did in OVCAR cells (Figure 1.). GST-MDA-7 caused growth suppression and induced cell death in ovarian cancer cells, with OVCAR being the most responsive one. Currently the Ad.mda-7 virus is widely used on a variety of cancers, and already in a phase I (Cunningham et al., 2005; Gupta et al., 2006; Lebedeva et al., 2002; Tong et al., 2005), so it is important to evaluate the infectivity of MDA-7 viral vectors and their ability to suppress growth and induce cell death in ovarian cancer cells.
Figure 1a. GST-MDA-7 promotes growth suppression and induces cell death in OVCAR and SKOVIII cells. (A, B). Growth suppression in OVCAR cells after treatment with GST-MDA-7 for 72h and 96h. (C, D). GST-MDA-7 induces cell death in OVCAR cells after treatment with GST-MDA-7 for 72h and 96h. Error bars denote standard error.
Figure 1b. GST-MDA-7 promotes growth suppression and induces cell death in OVCAR and SKOVIII cells. (E, F). Growth suppression in SKOVIII cells after treatment with GST-MDA-7 for 72h and 96h. (G, H). Cell death in SKOVIII cells after treatment with GST-MDA-7 for 72h and 96h. Error bars denote standard error.
5.2. Effects of Ad.mda-7-5 on growth and cell death in ovarian cancer cells

The Ad.mda-7 (5) virus, which is able to infect ovarian cancer cells and leads to the over-expression of MDA-7 (Gopalan et al., 2007) and which has been under clinical trials, might have the same effect as GST-MDA-7 did on ovarian cancer cells. OVCAR is the most sensitive ovarian cancer cells to MDA-7. Therefore in this study Ad.mda-7 virus (5) was used to infect OVCAR cells. OVCAR cells were treated with Ad.cmv-5 as negative control and Ad-MDA7-5 at 20moi, 50moi, 80moi, and 150moi. The cells were harvested 48hours after treatment and subjected to trypan blue assay. By counting the number of dead and living cells, the percentage of cell death was obtained. As the concentration of Ad-MDA7 increased, more cell death was observed (Figure 2). In the control group, there was about 9% cell death. When the cells were treated at 20moi, the percentage of cell death is about 17%. At 50moi, it is about 17.5%. At 80moi, it is around 24%, while at 150moi, it went up to 34.5% (Figure 2). In general agreement with other findings in other varieties of cancer cells, Ad.mda-7 (5) was able to infect ovarian cancer cells, OVCAR and enabled the expression of MDA-7, which thereafter induced cell death in OVCAR. Unfortunately, MDA-7 (5) here induced the fraction of cell death that was not statistically or clinically significant at average titration such as 20moi or 50 moi and even at high titration such as 80 moi. Thus it was necessary to find ways to promote the infection or expression efficiency of Ad.mda-7, hopefully better results could be obtained than those from Ad.mda-7 (5).
Figure 2. MDA7-5 dose response in OVCAR cells. OVCAR cells were treated with Ad.cmv-5, and Ad-MDA7-5 at 20moi, 50moi, 80moi, and 150moi for 48h. The percentage of cell death increased as the dose of Ad-MDA7-5 increased. Error bars denote standard error.

5.3. Ad.mda-7 ((5/3)) promotes growth suppression and induces cell death in ovarian cancer cells more effectively than Ad.mda-7 (5)

It has been reported that the modified version of Ad.mda-7 virus, Ad.mda-7 ((5/3)), which was able to infect tumor cells by bypassing CAR receptors, exhibited higher
efficiency in infecting ovarian cancer cells than the older version, Ad.mda-7 (5), we tried this virus to see its effect on our ovarian cancer species, OVCAR. The OVCAR cells were treated with Ad.mda-7 ((5/3)) at 20moi, 50moi, and 80moi. Like what we did above with Ad.mda-7 (5), the cells were harvested 48 hours after treatment and subjected to trypan blue assay. By counting the number of dead and living cells, the percentage of cell death was obtained. As the concentration of Ad-MDA7-(5/3) increased, more cell death was observed (Figure 3). In the control group, there was about 8.5% cell death. When the cells were treated at 20moi, the percentage of cell death is about 32%. At 50moi, it is about 47%. At 80moi, it is around 53% (Figure 3). When compared with Ad.mda-7 (5), Ad.mda-7 ((5/3)) caused more cell death at 20moi, 50moi and 80moi (Figure 4). The percentage of cell death was much higher when the OVCAR cells were treated with Ad.mda-7 ((5/3)) than that with Ad.mda-7 (5) at the same concentration, which is in agreement with previous reports that Ad.mda-7 ((5/3)) is much more effective in infecting ovarian cancer cells than Ad.mda-7 (5). To increase the cell death caused by Ad.mda-7, Ad.mda-7 ((5/3)) has been one of the options. Besides this, there should be some other method to achieve this goal such as the use of Ad.mda-7 in combination with other anti-tumor agents like the commonly used cisplatinum.
**Figure 3.** Ad-MDA7-(5/3) dose response in OVCAR cells. OVCAR cells were treated with Ad.cmV-(5/3), and Ad-MDA7-(5/3) at 20moi, 50moi, and 80moi for 48h. The percentage of cell death increased as the dose of Ad-MDA7-(5/3) increased. Error bars denote standard error.
Figure 4. Dose response comparison between OVCAR cells treated with Ad-MDA7-5 and Ad-MDA7-(5/3). The percentage of dead cells was higher in the groups treated with Ad-MDA7-(5/3) at 20moi, 50moi, and 80moi than those treated with Ad-MDA7-5 at the same concentration. Error bars denote standard error.

5.4. Cis-platinum shows synergy with MDA-7

Chemotherapeutic drugs such as paclitaxel in combination with cis-platinum has long been considered as the first-line chemotherapeutic strategy in clinics for patients with ovarian cancer but recently some types of ovarian cancer have become resistant to cis-platinum. We would like to see first whether OVCAR is resistant to cis-platinum
and then whether cis-platinum works synergistically with MDA-7. From the above studies, Ad.mda-7-5 has been shown to be effective in killing OVCAR ovarian cancer cells, therefore we used Ad.mda-7-5 in combination with cis-platinum to see whether they cooperate to cause cell death in OVCAR cells and therefore whether Ad.mda-7-5 could become one of the partners of cis-platinum in the future treatment of ovarian cancer. The OVCAR cells we used showed dose response to cis-platinum after treatment for 72 hours. In the control group, there was about 17% cell death. At 1uM of cis-platinum, the cell death was about 20%. At 2uM, it was 28. At 3uM, it was around 35%(Figure 5). Cis-platinum was effective in killing OVCAR cells.
Figure 5. Cis-platinum dose response in OVCAR cells. OVCAR cells were treated with Cis-platinum at 0uM, 1uM, 2uM and 3uM for 72h. The percentage of cell death increased as the concentration of Cis-platinum become higher. Error bars denote standard error.

After infecting OVCAR cells with Ad.mda-7, some groups of cells were treated with Cis-platinum at 1uM or 2uM. When the cells were treated with Ad.mda-7 at 20moi, there was about 22% cell death, which was about 8% more than the control. When the
cells were treated with Ad.mda-7 at 20moi in combination with Cis-platinum at 1uM, there was about 24% cell death; while the cells were treated with Ad.mda-7 at 20moi in combination with Cis-platinum at 2uM, there was about 43% cell death. MDA-7 worked synergistically with Cis-platinum in the induction of cell death of OVCAR cells. So far, MDA-7 had been shown to be a promising anti-tumor agent on ovarian cancer cells especially OVCAR cells. But what was the mechanism for its activity?

Figure 6. Ad-MDA7-5 and Cis-platinum showed synergistic effect on OVCAR cells in the induction of cell death. The percentage of cell death was higher in cells treated with both Ad-MDA7-5 and Cis-platinum for 72hours than treatment with MDA7-5 only. Error bars denote standard error.
5.5. The molecular mechanism by which MDA-7 functions

In order to clarify the molecular mechanism as well as the signaling pathways through which MDA-7 increased cell death of OVCAR cells, 20 plates of cells were treated as the following for 48 hours:

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage of Cell Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. CMV(50moi)</td>
<td>7.0%</td>
</tr>
<tr>
<td>2. MDA-7 (5)(50moi)</td>
<td>13.5%</td>
</tr>
<tr>
<td>3. CMV+cisplatinum(3uM)</td>
<td>12.5%</td>
</tr>
<tr>
<td>4. MDA-7 (5)+cisplatinum</td>
<td>27.0%</td>
</tr>
<tr>
<td>5. CMV+dominant-negative caspase-9 (dn9)(50moi)</td>
<td>7.5%</td>
</tr>
<tr>
<td>6. MDA-7 (5)(50moi)+dn9</td>
<td>11.5%</td>
</tr>
<tr>
<td>7. CMV+cisplatinum(3uM)+dn9</td>
<td>12.0%</td>
</tr>
<tr>
<td>8. MDA-7 (5)+cisplatinum+dn9</td>
<td>16.5%</td>
</tr>
<tr>
<td>9. CMV+JNK inhibitor (JIP)(10uM)</td>
<td>7.0%</td>
</tr>
<tr>
<td>10. MDA-7 (5)(50moi)+JIP</td>
<td>12.3%</td>
</tr>
<tr>
<td>11. CMV+cisplatinum(3uM)+JIP</td>
<td>12.0%</td>
</tr>
<tr>
<td>12. MDA-7 (5)+cisplatinum+JIP</td>
<td>19.5%</td>
</tr>
<tr>
<td>13. CMV+dominant-negative p38 (dn38)(50moi)</td>
<td>8.0%</td>
</tr>
<tr>
<td>14. MDA-7 (5)(50moi)+dn38</td>
<td>12.5%</td>
</tr>
<tr>
<td>15. CMV+cisplatinum(3uM)+dn38</td>
<td>12.3%</td>
</tr>
<tr>
<td>16. MDA-7 (5)+cisplatinum+dn38</td>
<td>18.6%</td>
</tr>
<tr>
<td>17. CMV+Bcl-xL virus (50moi)</td>
<td>6.0%</td>
</tr>
<tr>
<td>18. MDA-7 (5)(50moi)+Bcl-xL</td>
<td>9.5%</td>
</tr>
<tr>
<td>19. CMV+cisplatinum(3uM)+Bcl-xL</td>
<td>9.6%</td>
</tr>
<tr>
<td>20. MDA-7 (5)+cisplatinum+Bcl-xL</td>
<td>17.0%</td>
</tr>
</tbody>
</table>

The percentage of cell death was obtained by doing trypan blue assay. MDA-7 only increased cell death by 6.5%. cis-platinum increased cell death by 5.5%. Their combination did it by 20%, which was dramatic increase of cell death and showed their synergy in this context (Figure 7). When the cells were treated in the same way but plus dominant-negative caspase-9, the percentage of cell death remained almost
the same in plate 5, but decreased in plate 6 by 2%, in plate 7 by 0.5%, and in plate 8 by 10.5%. It suggests that caspase-9 was favorably involved in cell death (Figure 7). When the cells were treated in the same way but plus JNK inhibitor, JIP, the percentage of cell death remained the same in plate 9, but decreased in plate 10 by 1.2%, in plate 11 by 0.5%, and in plate 12 by 7.5%. It suggests that JNK was favorably involved in cell death (Figure 7). When the cells were treated in the same way but plus dominant negative p38, the percentage of cell death remained almost the same in plate 13, but decreased in plate 14 by 1%, in plate 15 by 0.2%, and in plate 16 by 8.4%. It suggests that p38 was favorably involved in cell death (Figure 7). When the cells were treated in the same way but plus Bcl-xL, the percentage of cell death decreased in plate 17 by 1%, in plate 18 by 4%, in plate 19 by 2.9%, and in plate 20 by 10%. It suggests that Bcl-xL was favorably involved in cell survival (Figure 7).
Trypan OVCAR CMV MDA7 Cis dn9, JIP, dn38, and BCLxl

Figure 7. Cell death of OVCAR treated with CMV (50moi), MDA-7 (5) (50moi), cis-platinum (3uM), dominant-negative caspase-9, JNK inhibitor, dominant-negative p38, and Bcl-xL.
In order to test what genes were involved in OVCAR cells’ response to MDA-7, cell lysate from cells treated with Ad.cmv or Ad.mda-7 (5) for 0h, 12h, 24h, and 48h were subjected to western blot analysis. PARP Cleavage was seen only 48h after treatment with Ad.mda-7 (Figure 8). P-AKT level went down at 12h, 24h and 48h after treatment with Ad.mda-7 (Figure 8). P-JNK level went up at 12h, 24h and 48h after treatment with Ad.mda-7 (Figure 8). P-38 level went up only obviously at 24h and 48h after treatment with Ad.mda-7 (Figure 8). P-ERK level went down at 12h, and 24h but went up 48h after treatment with Ad.mda-7 (Figure 8). GAPDH, which was loading control for the western blot analysis, remained almost the same. These results suggest that apoptosis occurred in OVCAR at least 48 hours after treatment with Ad.mda-7; and that JNK, AKT, p38and ERK were involved in OVCAR cells’ response to MDA-7 and their variations facilitated apoptotic cell death of OVCAR.
Figure 8: Western blot analysis of pro and antiapoptotic genes in OVCAR after treatment with Ad.cmv or Ad.mda-7.
Chapter VI. Discussion

Although there is recently tremendous advancement in the treatment of ovarian cancer, the overall long-term survival rate of this disease has not been improved as expected. Therefore, here comes the necessity of developing and testing new therapeutic agents and strategies. Previously it has been reported that the human melanoma differentiation associated gene-7 (MDA-7) has strong anti-tumor activity against human and murine cancer cells. So, we expect to see whether MDA-7 could become one of the candidates for ovarian cancer chemotherapy. The above findings show that MDA-7 can be a candidate for ovarian cancer therapy with its capability of inhibiting cell growth and inducing cell death. As an ideal agent for chemotherapy, not only should it be effective in killing the cancer cells but it also should be readily delivered to the target of interest. It is already known that it is difficult to administrate a chemotherapeutic drug that is protein and keep them active upon arrival at their destination for patients with cancer. In this case, it seems impossible to use GST-MDA-7 at clinics. Fortunately, viral vectors have currently become one of the focuses for effectively delivering the chemotherapeutic agents, with Ad.mda-7 virus being one of them. The recombinant virus Ad.mda-7 has been shown to be effective in infecting ovarian cancer cells, suggesting a promising way for delivering MDA-7 to its targets in patients with ovarian cancer. However, the effect of MDA-7 (5) only effects obviously at high titration of the virus and a long time after infection. This could be explained by the fact that there are no CAR receptors on OVCAR or
SKOVIII cell surfaces. It has been reported that CAR serves as a receptor for both subgroup B coxsackieviruses and adenovirus. Many adenovirus serotypes recognize CAR with the notable exception of subgroup B viruses (Ads 3 and 7) (Defer et al. 1990; Roelvink et al. 1998). Ad 5’s entry into tumor cells is mediated by CAR. Possibly because of its capacity of CAR (coxsackievirus and adenovirus)-dependent infection of tumor cells and the lack of CAR on OVCAR cell surface, MDA-7 (5) can induce the fraction of cell death that is not statistically or clinically significant as suggested by the above findings. The modified version of Ad.mda-7 virus, Ad.mda-7 ((5/3)), was reported to be able to infect tumor cells by bypassing CAR receptors, exhibiting higher efficiency in infecting ovarian cancer cells than the older version, Ad.mda-7 (5). We tried this virus to see its effect on our ovarian cancer species, OVCAR. What is more, with this virus, if it is more effective, we can confirm further the mechanism by which MDA-7 exerts its effect as suggested previously. MDA-7 ((5/3)) exhibited apparent effect on the OVCAR cells at relatively low titration (20 moi) of the virus and only 24h after infection. However, there are yet no clinical trials going on for this type of virus. Future work needs to be done to evaluate its chemotherapeutic efficacy both in vitro and in vivo. When the Ad.mda-7 virus is administrated in clinics at high titration like the case in vitro, it may cause side effect through infecting other irrelevant cells whose cell surfaces are rich in CAR receptors, which thus compromises the clinical application of MDA-7 (5) virus, so a modified Ad.mda-7 or some other ways of delivery that can target cancer cells more specifically are in need.
Although cis-platinum alone did not exert much effect on OVCAR because of partial resistance, it is really good news to see that it worked synergistically with MDA-7 (5) and ((5/3)) in vitro. In vivo studies are necessary both in a short time course as done in vitro in the present study or in a long time course to see if resistance emerges and to further clarify the molecular mechanism for it. A following step must be to find ways of eliminating the trigger of resistance. In this case, once MDA-7 virus is used in clinics, we could predict what side effects would occur and what could be prepared to stop them.

In this study, MDA-7 did inhibit cell growth and induced cell death of OVCAR; nevertheless, the mechanism was unknown. Previous studies suggested that MDA-7 was able to cause apoptosis in cancer cells through multiple pathways (Sauane et al., 2004). We tested several genes to see whether they were involved in the action of MDA-7 in OVCAR. From Figure 7 and Figure 8, we could see that Caspase-9, played important roles in cell death caused by MDA-7, indicating the occurrence of apoptosis in OVCAR. JNK and p38 were phosphorylated and activated, possibly promoting apoptosis here. The low level of antiapoptotic gene Akt and the pro-survival effect of another antiapoptotic gene Bcl-xL after MDA-7 treatment further confirmed that OVCAR died through apoptosis.
Chapter VII. Conclusions and implications for further research

This study demonstrated that MDA-7 promoted growth suppression and induced cell death through apoptosis in ovarian cancer cells. However, apoptosis might not be the only type of cell death here and it is necessary to test whether autophagy or MCD occurs in OVCAR after MDA-7 treatment. Although cisplatinum synergized with MDA-7, it is likely that there is some other antitumor drug that can act in the same way or even better. It is natural to test MDA-7’s chemotherapeutic effect in combination with other anti-tumor agents that target different pathways involved in tumorigenesis for better treatment or even cure of ovarian cancer.
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