ABSTRACT

Title of Thesis: METHYLSELENINIC ACID SENSITIZES NOTCH 3-ACTIVATED OVCA429 OVARIAN CANCER CELLS TO CARBOPLATIN

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Ovarian cancer is the deadliest of gynecologic cancers and is usually diagnosed at advanced stage due to invalidated screening test. Although carboplatin has been used for treating ovarian cancer for years, high-grade serous ovarian cancer expressing a constitutively active form of the intracellular domain of Notch 3 develops resistance to this platinum-containing drug. Thus, finding a novel treatment or therapeutic targets are necessary. Here we test the hypothesis that the combinational treatment of methylseleninic acid (MSeA) and carboplatin, two chemicals displaying overlapping effect on DNA damage response, may target Notch 3 for improved efficacy on ovarian cancer treatment. The OVCA429/NICD3 cells expressing an activated form of Notch 3 were resistant to carboplatin, but co-treatment with MSeA synergistically sensitized the cell to an extent similar of that in OVCA429/pCEG control cells. The synergistic effect can be suppressed by the presence of a hydrogen peroxide scavenger N-acetyl
cysteine (NAC) and kinase inhibitors of ATM and DNA-PKcs. In summary, MSeA and carboplatin synergistically sensitize OVCA429/NICD3 cells in a pathway involves oxidative stress, ATM and DNA-PKcs, suggesting a new strategy to improve the efficacy of carboplatin treatment for high-grade ovarian cancer.
METHYLSeleninic Acid Sensitizes Notch 3-Activated OVCA429 Ovarian Cancer Cells to Carboplatin

By

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CHAPTER 1: DNA DAMAGE RESPONSES, CARBOPLATIN, SELENIUM AND CANCER

1.1 DNA CROSSLINKS

The unwinding and separation of the two strands of DNA are crucial for DNA replication and transcription (Deans and West, 2011). DNA crosslinks prohibit DNA double helix from separation by forming covalent bonds in bases in the same strand (called as intrastrand crosslinks), between opposite strands (known as interstrand crosslinks), or between protein and DNA (named as DNA-protein crosslinks). They may be induced by endogenous sources, such as the by-products of lipid peroxidation - acrolein, crotonaldehyde (Kozekov et al., 2003), β-unsaturated aldehydes (Stone et al., 2008), and exogenous sources, including aldehydes, heavy metal ions, ionizing radiation, UV light, and chemotherapeutic agents (Ide et al., 2010). In mammalian cells, it is estimated that 80 events of interstrand crosslinks occur naturally in one cell per day (Bernstein and Bernstein, 1991). Comparing the incidence of interstrand crosslinks to the incidence of other DNA damages, this number is quite small but they are refractory to be repaired.

The formation of different types of DNA crosslinks, which result in contortion of structure (Rabik and Dolan, 2007), depends on factors such as types of inducers, cellular metabolism, cell cycle phase, and environmental toxicants. Based on the clastogens, DNA lesions have various chemical structures, physical
conformations, and biological consequences (Barker et al., 2005). Mixed population of the lesions is formed by DNA crosslinking agents (Gruenert et al., 1985). Interstrand crosslinks have usually smallest percentage of lesions but are the most toxic ones.

DNA damage response pathways can alert cells to initiate an appropriate response to clastogens through DNA repair checkpoint and signal transduction. The adduct causes DNA distortion which is recognized by cellular proteins that involved in DNA repair pathways. Based on the types of damage structure, DNA adducts can be recovered by four major DNA repair pathways: (1) nucleotide-excision repair (NER), (2) base-excision repair (BER), (3) mismatch repair (MMR), and (4) double-strand-break repair (DSB). NER, BER, and MMR are the three possible excision-repair pathways to fix DNA crosslinks. These three DNA repair pathways repair DNA damage through excising damage sites, followed by resynthesizing using the opposite strand as a template.

If the lesions are unable to be repaired, they cause permanent genome instability or cell death (Kelland, 2007; Barker et al., 2005; Muniandy et al., 2010). To control cell growth, differentiation, and stress responses, signal transduction pathways are activated by sensor proteins including ataxia telangiectasia-mutated (ATM), checkpoint kinase 1 (CHK1), checkpoint kinase 2 (CHK2), p53, p21, and the breast cancer associated 1 (BRCA1) (Kelland, 2007). However, DNA crosslinks-induced cell death induction by platinum-based chemotherapeutic reagents is not completely understood. Interstrand crosslinks
can block the processing of the DNA replication fork by inhibiting the separation of double helix. If the cleavage of a DNA replication fork occurs, it can lead to DNA double strand breaks. Distorted structure also prevents binding of DNA-interacting proteins to DNA. Interstrand crosslinks may induce p53-dependent apoptosis or p53-independent mitotic catastrophe (a cell is destroyed during mitosis when apoptosis is not working) in tumor cells (Deans and West, 2011).

1.1.1 **Nucleotide excision repair (NER)**

NER is the major pathway to remove distorted, bulky lesions. Bulky lesions and helix-distorting DNA adducts are usually induced by UV light or bulky DNA adducts. Mutations in the genes controlling NER result in xeroderma pigmentosum (XP), Cockayne’s syndrome (CS), and trichothiodystrophy (TTD) (Costa et al., 2003). Patients with XP have mutations in one of the seven XP genes (*XPA* to *XPG*), while patients with CS carry mutations in one of the two CS genes (*CSA* and *CSB*). All of these genetic diseases with defective NER display photosensitivity and predisposition to sunlight-induced skin cancer (Cleaver *et al.*, 1994).

There are two sub-pathways of NER, transcription-coupled NER (TCR) and global genomic NER (GGR), which differ in the first few steps of DNA repair. In TCR, CS proteins (CSA and CSB) sense the bulky lesions and transduce signals to downstream mediators. On the other hand, in GGR, XPC/HR23B complex is the recognition protein that reaches the impair sites and causes DNA distortion.
Next, CS proteins and a domain on XPC interact with TFIIH and XPG for TCR and GGR, respectively, followed by recruitment of XPA or heterotrimeric replication protein A (RPA). Later, CS or XPC/HR23B damage-recognition proteins dissociate from damaged regions, and then XPA and RPA facilitate the loading of XPD and XPB (Costa et al., 2003; Hoeijmakers, 2001). TFIIH is needed for transcription initiation while helicase XPB and XPD unwind and make a bubble structure of DNA near the unrepaired lesions (Evans et al., 1997). The unwound 24 to 32 bp bubble structure are removed by dual incision by the endonucleases XPG, excision repair cross-complementing-1 (ERCC1), and XPF. Finally, DNA synthesis and nick annealing are complete by DNA polymerase ε/δ and DNA ligase, respectively (Costa et al., 2003).

1.1.2 Base excision repair (BER)

BER can correct non-helix-distorting base lesions after exposure to oxidative radicals, alkylating agents, ionizing radiation, or spontaneous base loss (Hoeijmakers, 2001). These clastogens can lead to single strand breaks (SSBs), small base damage, abasic sites, and 8-hydroxyguanine. The incidence of the above alterations is relatively high, reaching $10^5$ to $10^6$ lesions per cell per day. However, there is no known genetic disorder associated with BER genes mutations possibly due to the essentiality to repair DNA through BER.

BER is categorized into two sub-pathways, short-patch (one nucleotide) and long-patch BER (more than one nucleotide), depending on the number of
impaired nucleotides, the cell cycle stage, and cell preference (Wei and Englander, 2008). However, the exact mechanistic details as to how the cell decides which branch of BER to go through is not completely clear (Hoeijmakers, 2001). Short-patch BER is the major pathway which can only repair one base at one time while long-patch BER can deal 2 to 10 nucleotides (Fortini and Dogliotti, 2006). They partially share the repair steps, such as damaged base removal, cleavage, and DNA resynthesis. DNA glycosylase is the first enzyme responsible for cutting the N-glycosylic bond that links DNA bases to sugar molecules. There are three glycosylases, including uracil-DNA glycosylase (UNG), thymine-DNA glycosylase (TDG), and 8-oxoguanine glycosylase (OGG1) (Darwanto et al., 2009). Glycosidase cleavage generates an intermediate apyrimidinic/apurinic (AP) or abasic site, followed by AP endonuclease 1 (APE1) cleavage of the phosphodiester backbone to expose a 3-OH and a 5’-deoxyribose-5-phosphate (5’-dRp) (Demple et al., 1991; Gellon et al., 2008). Next, APE1 interacts with DNA polymerase β (Pol β) that exhibits AP lyase activity so they can cut off the 5’-dRp residue and synthesize the right nucleotides. The nick is then filled by the XRCC1/DNA Ligase III complex.

Long-patch BER also needs DNA glycosylase, APE1, and DNA Pol β, but they can repair longer impaired nucleotides than short-patch BER does. However, for long-patch BER, DNA Pol β interacts with the proliferating cell nuclear antigen (PCNA) and the replication factor C (RFC), displacing a strand of DNA near the unrepaired lesions and also generating a flap of 2 to 10 nucleotides.
(Matsumoto, 2001). The flap is cleaved by flap endonuclease 1 (FEN1), followed by gap filling through DNA ligase I.

1.1.3 Mismatch repair (MMR)

Mismatch repair corrects DNA mismatches that escape proofreading during DNA replication (Kunkel and Erie, 2005). Insertion/deletion lesions (IDLs) due to template slippage is also recognized and repaired by MMR (Hoeijmakers, 2001). DNA mismatch is first recognized by the MSH2/MSH6 (MutSα) and the MSH2/MSH3 (MutSβ) heterodimers with their respective substrate preferences. MutSα prefers to recognize base-base and small insertion/deletion mismatches while other IDLs are bound by the MutSβ (Jiricny, 2006). Next, MutSα and MutSβ consume one ATP to substitute mismatched bases or IDLs and pull out DNA damage. MutS then forms a complex with MutL that has three forms (MutLα, MutLβ, and MutLγ). MLH1, a subunit of MutLα, contains two interaction domains: one for MutS and another for PMS2, PMS1, or MLH3. When MLH1 interacts with PMS2 to form MutLα, MutLα functions as a matchmaker or facilitator; they mediate events in MMR. The MLH1-associated complex is responsible for the recruitment of proteins needed for excision and repair synthesis. A number of DNA metabolism proteins including PCNA, RPA, RFC, exonuclease I, DNA Pol ε/δ, and endonuclease FEN1 coordinate and excise DNA mismatches, resynthesize correct nucleotides, and ligase the nick (Kunkel and Erie, 2005). Different from BER and NER, MMR can discriminate newly
synthesized DNA strand from the parental strand according to the interaction between MutLα and PCNA (Clark et al., 2000).

Loss of expression in MMR enzymes, such as hMLH1 and hPMS2, causes DNA replication error and insertion/deletion, leading to spontaneous mutations. Particularly, when the errors occur in short repeated nucleotides sequences, it is known as microsatellite instability (Misdraji et al., 2004). Mutations in hMLH1 gene have been shown to be associated with increased risk of developing colorectal cancer. Based on many epidemiologic studies, it has been found that a large proportion of people with hereditary non-polyposis colorectal carcinoma history (HNPCC) have a tendency in MMR genes mutations, over 90 percent of which occur in hMLH1 and hMSH2 (Mitchell et al., 2002).

1.1.4 Double-strand-break repair

Unrepaired DSB may induce apoptosis and permanent cell cycle arrest. Mammalian cells have two major pathways to repair DSB: homologous recombination (HR) and non-homologous end joining (NHEJ). NHEJ is thought to contribute substantially to DSB repair throughout the cell cycle whereas HR is only involved in late S and G2 phases (Rothkamm et al., 2003). Both NHEJ and HR are involved in replication fork-induced DSB and one-ended DSB. However, two-ended DSB is usually reconnected by HR (Lundin et al., 2002).

As a result of DSBs, not all damage termini are complete nucleotides, instead, many are non-conventional end groups. Impaired DNA ends may contain
abnormal 3’ phosphate groups, 5’ hydroxyl groups, damaged backbone sugar residues, and damaged DNA bases. These groups need to be deleted or modified so that they can meet the requirements of DNA ligation – 5’-phosphate and 3’-OH group (Chappell et al., 2002).

In the process of NHEJ, Ku70 and Ku80 form a heterodimer that binds strongly to the ends of the DSB in order to stabilize the damage. The dimer then interacts and forms a complex with DNA-PKcs (the catalytic subunit of DNA-PK). DNA-PKcs is autophosphorylated in order to trigger the kinase activity of DNA-PK complex, which facilitates the recruitment of other repair proteins to the DSB site. If DNA hairpin is formed as intermediates in V(D)J recombination (a genetic recombination in the early stages of immunoglobulin and T cell receptors production) (Sadofsky, 2001), DNA-PK complex recruits the structure-specific Artemis nuclease to DNA ends (Ma et al., 2005). Artemis/DNA-PK complex then functions as an endonuclease for cleaving 3’-ssDNA overhangs after polymerase has the new strand (Budman and Chu, 2005). Once the work is done, the Artemis/DNA-PK complex may fall apart from Ku-DNA complex to allow following repair steps (Lieber et al., 2004). Finally, the XRCC4/DNA ligase IV complex and XRCC4-like factor (XLF) are localized by DNA-PK complex to DNA ends to join two strands of DNA for ligation (O’Driscoll and Jeggo, 2006; Lord et al., 2006). When encountering damaged DNA ends with 3’ phosphate groups or 5’ hydroxyl groups, polynucleotide kinase (PNK), which functions to phosphorylate 5’-OH groups and removes 3’-phosphate ends, is thought to play
an important role in the modification of damaged DNA termini in human cells. XRCC4 interacts with PNK to permit the conversion of DNA ends (Koch et al., 2004). However, if NHEJ is inhibited by inactivation of either XRCC4 or DNA-PKcs, PNK is unable to convert damaged DNA termini (Chappell et al., 2002). This indicates the integral partnership between the above three proteins.

NHEJ is generally considered as a homology-independent DSB repair pathway that directly joins two DNA ends. Therefore, although NHEJ efficiently rejoins the DSB ends, nucleotides may be lost at the sites of damage (McHugh et al., 2000) that lead to mutagenic changes (Lord et al., 2006).

On the contrary, HR rejoins DSB ends using a DNA sequence homologous in the genome (sister chromatids, homologous chromosomes, or repeated regions on the same or opposite chromosomes) as a template so the repair is considered accurate and known as an error-free repair mechanism (Shrivastav et al., 2007). The initial step of HR is to generate single-stranded DNA (ssDNA) in order to allow numerous factors for resynthesizing DNA. The coordination of the C-terminal binding protein interacting protein (CtIP), exonuclease-1, and the MRE11-RAD50-NBS1 (MRN) complex is required for DNA resection. In particular, MRE11 exhibits ssDNA endonuclease and 3’ to 5’ double strand DNA (dsDNA) exonuclease activities for the formation of a 3’ ssDNA overhang (Lewis et al., 2004; Langerak and Russell, 2011; Limbo et al., 2007). A 3’-overhang is protected by RPA so that secondary structures cannot be formed. RAD51 is then localized to displace RPA by the breast cancer associated protein 2 (BRCA2).
Binding of BRCA2 to ssDNA results in the formation of a DNA-protein filament that facilitates the search for homologous templates, the invading for the target homologous sequence, the initiation of the DNA synthesis, and the formation of the crossover X-shape Holliday junction (Hinz, 2010). Next, DNA polymerases can extend the 3’ end of damaged strand while RecQ helicases can unwind the undamaged DNA duplex. After sufficient DNA synthesis, the Holliday junction is resolved by cleavage. The final repair processes require the removal of flaps, filling in the gaps, and ligating the remaining nicks to complete the repair of DSB by HR (Helleday et al., 2007). Because HR rejoins DSB by using sister chromatid as a template, it allows the cells to repair DNA with high fidelity.

1.1.5 Double strand break induced signal transduction pathways

DNA double helix is supposed to be unwound by helicases before DNA polymerase starts replicating it. However, DNA crosslinks prevent DSB from unwinding. Therefore, during S phase, when processing mediators encounter crosslinks at the site of replication fork the replication fork may collapse and DSB are formed as intermediates in an attempt to restart replication (McHugh et al., 2000; McCabe et al., 2009). The DSBs can either be repaired by HR or NHEJ, or induce ATM-dependent cell cycle arrest (Lord et al., 2012). To date, by proteomics analysis, the ATM kinase is known to phosphorylate more than 700 substrates (Matsuoka et al., 2007). ATM is the heart of DSB repair pathway and can also be activated by oxidative stress. The kinase activity of ATM can be
induced throughout the cell cycle (Cortez, 2005). It is involved in the maintenance of genome stability and in the activation of DNA damage checkpoints (O’Driscoll and Jeggo, 2006). People with mutations in the AT gene are characterized by cerebellar ataxia, telangiectasia, immunodeficiency, and a high incidence of malignancy. Cells derived from AT patients show hypersensitivity to ionizing radiation, genome instability, and cell cycle checkpoint deficit (Iijima et al., 2008).

MRN complex is thought to be the first sensor protein that rapidly recognizes and binds to the DSB damage sites. Two units each of Mre11 and Rad50, and one Nbs1 assemble the U-shape flexible MRN complex. When MRN complex binds to the 2-ended DSB site, the Mre11 dimer synapses symmetrically the two ends of DNA. On the contrary, when binding the 1-ended DSB substrate, the Mre11 dimer binds to one single DNA strand and shapes an asymmetric structure. The C terminus of Nbs1 localizes MRN complex to the nucleus (Williams et al., 2010) and recruits ATM to the damaged DNA. Mutations in Nbs1 can result in Nijmegen breakage syndrome, a rare autosomal recessive disorder exhibiting chromosomal instability. In addition, autophosphorylation of ATM on Ser1981 is required for activation of its kinase activity and the dissociation of the kinase-inactive ATM dimers to form the active ATM monomer. MRN complex not only induces but also contributes to ATM kinase activation by increasing the affinity between ATM and its substrates through protein-protein interactions (Lee and Paull, 2005).
Another early step in the response to DSB is the phosphorylation of H2AX histone protein at Ser-139 by ATM or the DNA-PK complex, generating γH2AX. After DSB occurs, γH2AX is phosphorylated within 1 to 3 minutes and marks the damage sites by forming microscopic foci in nucleoli (Lobrich, et al., 2010). γH2AX foci superficially attract DSB repair proteins to the surrounding DNA damage site. γH2AX expression levels shows a linearly correlation with the severity of DNA damage (Paull et al., 2000) and is known as an indicator of DNA breaks. The major function of γH2AX is to help retain activated mediators downstream ATM to the damaged site, including mediators of DNA damage checkpoint 1 (MDC1), tumor protein 53 binding protein 1 (53BP1), BRCA1, and structural maintenance of chromosomes 1 (SMC1) (Podhorecka et al., 2011). H2AX knockout in mice or knockdown in cells exhibited a significant increase in chromosome aberrations (Celeste et al., 2003).

Phosphorylation of CHK2, CHK1, and p53 by ATM occurs at sites of DSB throughout the nucleoplasm in the S phase of the cell cycle (Lord et al., 2012). All these proteins are involved in cell growth control. CHK1 and CHK2 are protein kinases that serve to amplify the DNA damage signal of ATM. After ATM-mediated phosphorylation of CHK1 at Ser-345 and Ser-317, CHK2 at Thr-68, they can phosphorylate their major substrate, cell division cycle-25A (CDC25A) phosphatase, which is considered as an oncogene that promotes progression through S phase (Zou et al., 2001). CDC25A is a target of the E2F family that regulates S phase quiescence and is known to be overexpressed in several tumor
tissues, including breast and head and neck cancer. CDC25A overexpression also indicates a common consequence of being insufficient in suppressing a p53 induced growth arrest (Ruppenthal et al., 2007). In normal cellular progressing, CDC25A activates the cyclin-dependent kinase 2 (CDK2) needed for DNA synthesis, but its expression is decreased in the response to DNA damage or stalled replication (Falck et al., 2001). However, the function of CDC25A is inhibited after extensive phosphorulation at multiple N-terminal sites by CHK1 and CHK2, resulting in cell progression delay in the S phase and apoptotic responses (Uto et al., 2004; Li and Stern, 2005).

The tumor suppressor p53 is the negative regulation of cell progression. When it is directly activated by ATM or CHK2, p53 promotes the transcription of many downstream mediators that either stop cell cycle or induce apoptosis. A number of studies have found that p53 transcription is activated during early S phase and in cells progressing into S phase (Mosner et al., 1995; Reich and Levine, 1984). Interestingly, p53 also plays an important role in the S phase checkpoint. An examination in Swiss3T3 cells treated with the DNA damaging agent camptothecin showed that p53 protein levels increased earlier in S phase than in the progressing into S-phase. p21 is a gatekeeper protein whose expression is activated by p53,. In S phase of the cell with unrepaired DNA damage, p53 induces the activation of p21 that binds to the complex composed of replication factor C, DNA polymerase d, FEN1 and, proliferating cell nuclear antigen, causing the disconnection from replication fork and the blockage of DNA
synthesis phase (Christmann et al., 20105). p21 activation also inhibits cyclin-dependent kinase (CDK) function that causes cell cycle arrest. Consequently, the above actions of p53 prevent accumulation of irreversible genetic damage and maintain genome integrity, which may lead to permanent cellular transformation and carcinogenesis, (Janus et al., 1999; Takahashi et al., 2011). Thus, p53 has been considered as the “guardian of the genome”.

1.1.6 The link between DNA crosslinks and cancer

Cancer cells generally proliferate faster and have less error-correcting mechanisms than healthy cells. Some chemotherapeutic drugs, such as carboplatin, kill cancer cells by inducing DNA crosslinks. However, DNA crosslink is also related to carcinogenesis in normal cells. Several lines of evidence indicate the link between carcinogenesis and DNA crosslinks. DNA crosslinking agents, such as formaldehyde and hexavalent chromium compounds that induce DNA-protein crosslinks, have been found to induce an increase in the population of p53 mutation in people who are exposed to above chemical environments (Shaham et al., 2003; Hanaoka et al., 1997). These results may explain why some positions of physicians, laboratory assistants and technicians, hospital orderlies, and hexavalent chromium workers have high risk of cancer. Also, the positive association between DNA-protein crosslinks and p53 mutations indicates that the number of crosslink is a risk factor (Merk and Speit, 1998). In animal studies, pre-cancer phenotypes, inflammation, hyperplasia, ulceration, and cellular atypia,
were observed in hairless mice that were treated with photoactivatable bifunctional psoralens and exposure to UV light in order to induce crosslinks (Dunnick et al., 1987). Both clinical and animal studies show a strong connection between crosslinks and cancer-related markers.

1.2 NOTCH PATHWAY

Notch is an evolutionarily conserved membrane protein found from drosophila to vertebrates (Artavanis-Tsakonas et al., 1995). Notch is thought to function primarily as a transcriptional activator that plays important roles in controlling cell fate regulation, cell proliferation, and cell death during development and in various organs (Shih and Davidson, 2009). The Notch pathway covers a short-range signaling mechanism between neighboring cells but results in gene expression changes (Kovall, 2008). The Notch pathway is an uncommon signaling pathway, whose activation does not need secondary messengers for amplification (Tien et al., 2009). The three main molecular constitutions in the Notch pathway are DSL ligands (Delta and Serrate in Drosophila, Lag2 in C. elegans), Notch receptors and nuclear effectors. To date, four Notch receptors have been identified. The N-terminal EGF-repeat region of the Notch extracellular domain (ECD) interacts with DSL ligand that changes the conformation of Notch receptor protein, triggering a series of proteolytic cleavages by tumor necrosis factor-α-converting enzyme (TACE), metalloprotease, and γ-secretase (Wang et al., 2008). TACE makes the first
cleavage, separating ECD from Notch protein. ECD then is trans-endocytosed into neighboring cells. The second cleavage is mediated by γ-secretase, releasing the Notch intracellular domain (NICD) into cytoplasm. Subsequently, NICD translocates into the nucleus and interacts with nuclear effectors, the DNA-binding CSL (CBF1, Su(H) and LAG-1) protein. The co-activator Mastermind (Mam) and other transcription factors are recruited to the CSL complex, repressing or activating target genes (Bray, 2006). The Notch pathway is complicated, which resolves equivalent cells into distinct fates due to small or weak differences of signaling amplification (Bray, 2006). However, the exact mechanistic detail as to how the cell decides what signals to amplify is not completely understood.

1.2.1 Notch in cancer development and progression

Dysfunctional Notch guides cells towards malignant transformation. Many observations indicated the link between alternations in the Notch pathway and cancers. Interestingly, Notch can be oncogenic or anti-proliferative in cancer formation, depending on the type of signals (Lobry et al., 2011; Maillard and Pear, 2003). Some studies have shown an anti-proliferative function of Notch in skin cancer, human hepatocellular carcinoma, medullary thyroid, cervical cancer, and small cell lung cancer. For example, p21 expression and the resultant G1/S arrest is upregulated by Notch 1 (Panelos and Massi, 2009). Furthermore, Notch 1 induces caspase-3 activity, resulting in a decrease in proliferation (Okuyama et al., 2004). Several studies reported that deletion of Notch 1 results in spontaneous
epidermal and corneal hyperplasia in old mice. Thick growing skins are sensitive to chemical-induced skin carcinogenesis that is regulated through β-catenin-mediated signaling (Nicolas et al., 2003; Demehri et al., 2009).

On the contrary, data suggest that Notch signaling is pivotal in oncogenic functions due to the cross talk with oncogenic signaling pathways. In solid tumors, the Notch pathway promotes the transcription of Hairy enhance of split family (Hes), nuclear factor-kappa B (NF-κB), vascular growth factor receptor (VEGF), mammalian target of rapamycin (mTOR), p21, p27, protein kinase B (Akt), estrogen receptor (ER), androgen receptor (AR), and epidermal growth factor receptor (EGFR) (Rizzo et al., 2008; Wang et al., 2008; Wang et al., 2010). All these NICD-targeting genes are involved in tumor development and progression. However, in the haematopoietic system, Notch also could be either oncogenic, such as T-cell acute lymphoblastic leukaemia, a disease characterized by Notch 1-activating mutations (Aifantis et al., 2008), or suppressive in chronic myelomonocytic leukaemia by downregulating the Hes1 expression (Klinakis et al., 2011). Constitutively active Notch 3 expression induces tumorigenesis by the sustained expression of pre-T cell receptor (TCR) and the continuing expression of NF-κB in T-cell leukemogenesis (Bellavia et al., 2000). Moreover, high Notch 3 protein expression is observed in ovarian serous carcinoma, indicating a role of this protein in tumorigenesis. Furthermore, inactivation of Notch 3 leads to suppression of cell proliferation and induction of apoptosis (Park et al., 2006).
Therefore, depending on the cell types and Notch-targeted genes, the Notch pathway can be considered as either a tumor suppressor or a tumor promoter.

1.3 CARBOPLATIN

It all starts by the accidental discovery of the chemotherapeutic potential of cis-diamminedichloroplatinum(II), or cisplatin, by Barnett Rosenberg at Michigan State University, East Lansing, United States (Kelland, 2007). When the Rosenberg Lab was analyzing chemicals in order to understand whether electric and magnetic dipole fields are involved in cell division regulating, they found the neutral cis-isomer \([\text{PtII (NH}_3\text{)}_2\text{Cl}_2]\), which turned into cisplatin later. This finding was published in 1965 (Rosenberg et al., 1965) and the following test was done by 1969, showing the anticancer effect in mice (Rosenberg et al., 1969). Based on its chemotherapeutic effect, cisplatin was approved by the US Food and Drug Administration (FDA) in 1979 to be a clinical anticancer drug. However, cisplatin continued to show severe side effects and high toxicity. If the leaving group of platinum-based drug is more stable than chloride, the toxicity of cisplatin becomes lower (Kelland, 2007). Based on the theory, many platinum-based drugs have been designed in an attempt to reduce the side effects of cisplatin. Thus, the most promising second generation platinum analog carboplatin was developed.

The worldwide approved carboplatin has been used clinically since it was introduced in mid-1980s by the collaboration between Johnson Matthey Plc (JM) and the Institute of Cancer Research (ICR) in London (Kelland, 2007).
Carboplatin shares the same mechanism of action with cisplatin but has decreased toxicity (Cruet-Hennequart et al., 2009) due to a slow leaving group, a bidentate dicarboxylate (CBDCA) ligand. Besides, carboplatin has a low protein binding affinity so it has longer half-life of ultrafilterable platinum and a higher cumulative urinary platinum excretion in comparison to cisplatin (van der Vijgh, 1991). Therefore, carboplatin dosage can be given up to 4-fold higher than cisplatin. Although carboplatin functions on lung, colorectal, head and neck cancers, as well as lymphoma and melanoma, it is preferentially used to treat ovarian and testicular cancer (Wheate et al., 2010).

![Carboplatin Structure](image)

**Figure 1.1 Carboplatin Structure.** Carboplatin is a second generation platinum-based chemotherapeutic drug. Carboplatin contains a slow leaving group - bidentate dicarboxylate (CBDCA) ligand instead of the two chloride ligands appeared in cisplatin. This makes it less toxic compared to cisplatin.

1.3.1 **Mechanism of action of carboplatin**

It is crucial to understand the mechanism of action of a drug in order to set a reference for drug design and modification. Based on a drug’s biochemical and physiological impact, effective analogous compounds with less toxicity and side-effects can be synthesized. For the design of anticancer drugs, knowing how the
chemical acts is usually the first step, followed by steps to modify drugs with different functional groups and to find the best working environments.

Carboplatin [\textit{cis-Diammine (1,1-cyclobutanedicarboxylato) platinum(II)}] belongs to the large group of chemotherapy drugs with DNA alkylation function (Kojima \textit{et al}., 1994). During transporting and targeting, carboplatin becomes aquated, losing oxalate ions \([C_2O_4^{2-}]\) and gaining two water molecules. This negatively electrophile carboplatin can interact with nucleophilic molecules such as DNA strands. When attacking DNA, carboplatin primarily binds to the N7 atom of the imidazole ring of guanosine (G) and to a lesser extent to adenosine (A), generating three different forms of DNA lesions, including monoadducts, intrastrand crosslinks and interstrand crosslinks (Cruet-Hennequart \textit{et al}., 2009). Monoadducts are first formed while one water molecule is removed from carboplatin (Rabik and Dolan, 2006). However, greater than 95% of monoadducts will then transform into crosslinks and left only around 2% monoadducts (Table 1.1) (Deans and West, 2011). Intrastrand crosslinks, which involve adjacent bases to one another on the same DNA strand such as GpG 1,2 intrastrand (60 to 65%) and ApG 1,2 intrastrand (20 to 25%), comprise the majority of all adducts. Additional minor product of intrastrand crosslinks include GpXpG 1,3 intrastrand crosslink that sandwiches a base between the two platinated guanosine (approximately 2%) (Rabik and Dolan, 2006; Kelland, 2007; Unger \textit{et al}., 2009). In addition, less than 5% of interstrand crosslinks contain two guanosines from the opposite DNA strands (McHugh \textit{et al}., 2001).
Table 1.1 Percent of three types of carboplatin-induced DNA adducts.

<table>
<thead>
<tr>
<th></th>
<th>DNA Monoadduct</th>
<th>Intrastrand Crosslinks</th>
<th>Interstrand Crosslink</th>
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<tbody>
<tr>
<td>% of DNA adducts</td>
<td>~2%</td>
<td>GpG 1,2 intrastrand crosslinks: 60-65%</td>
<td>G-G interstrand crosslinks: &lt;5%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ApG 1,2 intrastrand crosslinks: 20-25%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GpXpG 1,3 intrastrand crosslinks: ~2%</td>
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</tbody>
</table>

All crosslinks result in DNA strand conformation change such as unwinding or bending toward the grooves. There are different theories underlying the link between cytotoxicity and DNA lesions on the basis of the level of distortion in the DNA strand. Some believe that interstrand crosslinks that can unwind DNA up to 80° are more toxic than other DNA lesions because DNA strand separation is inhibited (Deans and West, 2011). On the other hand, some concern that 1,2 intrastrand crosslinks have high cytotoxicity since the ineffective *trans* isomer of carboplatin is only able to form 1,3 intra- and interstrand crosslinks, owing to
stereochemical constraints (Eastman and Barry, 1987). In conclusion, carboplatin is used as an anticancer drug to kill tumor based on the property to induce the formation of DNA crosslinks.

1.3.2 Adverse effects of Carboplatin

The cytotoxicity for killing cancer results from carboplatin-induced DNA adducts. DNA lesions may lead to DNA replication inhibition, cell cycle arrest, or even apoptosis (Unger et al., 2009). However, though insufficient dose may result in resistance later on, toxicity restricts the dosage for cancer treatment. Cisplatin causes very serious nephrotoxicity and peripheral neuropathy (Donzelli et al., 2004). Nephrotoxicity is primarily due to the platinating agent uptake by proximal tubule cells of the nephron (Yao et al., 2007). Proximal tubular epithelial cells are found to show 5 time higher concentration of cisplatin than in serum level (Arany and Safirstein, 2003). Nephrotoxicity can be controlled by giving diuretics and pre-hydration while there is no way to deal with peripheral neuropathy. By contrast, carboplatin does not show severe side effects on kidney and peripheral neurons but causing myelosuppression (Wahstaff et al., 1989). Myelosuppression which is the chief reason of dose limitation is a decrease in blood cells production, leading to severe thrombocytopenia (a platelet count below 50,000 per μL) and less often leucopenia (the total white cell count drops below 4000 per mm$^3$). Gastrointestinal toxicity also occurs in most patients receiving carboplatin, but nausea and vomiting symptoms are usually delayed for a few hours later or are
mild to moderate than patients receiving cisplatin. When conventional carboplatin
dose is given, 20 to 40% of patients have thrombocytopenia and less than 20% get
leucopenia (Rabik and Dolan, 2006). In addition, high dose carboplatin can result
in life-threatening toxicity.

1.3.3 Carboplatin resistance

After the clinical use of platinum agents, tumor cells may show resistance to
the treatment. There are two possible reasons to explain the carboplatin resistance:
first, insufficient amount of carboplatin reach the target DNA; second, insufficient
formation of carboplatin-induced DNA adducts to induce cell death.

Carboplatin enters cells much more slowly than other classes of small-
molecule anticancer drugs. The uptake of carboplatin is affected by many factors
including intracellular sodium level, potassium level and pH. Recently, the major
copper influx transporter, copper transporter 1 (CTR1), was found to play an
important role in mediating the cellular accumulation of carboplatin. It was found
that Saccharomyces Cerevisiae with yCTR1 gene deletion was associated with a
decrease in the accumulation of platinum chemotherapeutic drugs (Ishida et al.,
2002). In vitro, Ctr−/− mouse embryonic fibroblasts treated with a clinically
achievable concentration of carboplatin (2 μM) showed only about 35% uptake of
carboplatin in comparison to wild-type cells. CTR1 deficiency also led to a 2 to 3
fold increase in carboplatin resistance (Holzer et al., 2006). On the other hand,
increased hCTR1 expression in human small-cell lung cancer cells successfully
enhanced the uptake of carboplatin and made cancer cells more susceptible to carboplatin treatment (Song et al., 2005). In addition, carboplatin resistance is reduced in mice with the mCTR1 alleles deleted (Howell et al., 2008). Taken together, many lines of research have pointed out that CTR1 functions to mediate carboplatin transport and plays a key role in drug resistance.

Carboplatin resistance may result from an increase in DNA repair or an increase in damage tolerance after the formation of carboplatin-induced DNA adducts. Ovarian and testicular cancers are hypersensitive to platinum-induced DNA adducts, which is likely to be attributed to a reduced repair capacity. On the contrary, various carboplatin-resistant cancer cells show an increased efficacy in DNA adducts repair pathways (Johnson et al., 1994; O’Dwyer et al., 2000). Since NER is the major pathway to recover carboplatin-induced intrastrand crosslinks, increased NER repair may result in resistance to platinum treatment. NER needs the XPA complex for loading downstream factors, such as XPF and ERCC1, for damage removal. Moreover, primary testicular cancer cells exhibited low constitutive NER activity due to low expression level of XPA (Koberle et al., 1999), XPF, and ERCC1 (Welsh et al., 2004). Also, cisplatin-resistant ovarian cancer cells with increased NER showed a positive correlation with ERCC1 and XPF expression levels (Ferry et al., 2000). Knockdown of ERCC1 in ovarian cancer cells showed hypersensitivity to cisplatin-induced DNA adducts and a significant decrease in NER efficiency (Chang et al., 2005). Furthermore, patients
with ovarian cancer express increased ERCC1 mRNA levels and show clinical resistance to platinum-based chemotherapeutic drugs (Reed, 2005).

Tolerance to DNA damage also results in drug resistance. Typically, unrepaired DNA damage triggers cellular death (Kelland, 2007). MMR functional loss cannot repair DNA lesions and does not respond to apoptosis signals, subsequently leading to carboplatin and cisplatin resistance. Furthermore, DNA polymerases β and η are found to bypass DNA lesions by translesion synthesis (Bassett et al., 2002).

1.4 SELENIUM

Selenium was discovered in association with tellurium (named for the Earth) in 1817 by Jöns Jakob Berzelius. It was named after the Moon or the Moon goddess Selene. Selenium is a non-metal element and belongs to the group VIA of the periodic table together with oxygen and sulfur. Because its chemistries are similar to sulfur, selenium is usually found in accompany with native sulfide ores. Besides, selenium can substitute for sulfur in compounds or amino acids. Selenium was first notified as a potentially toxic chemical element, using in glassmaking and ceramic manufacturing, where it is used to give a red color to glasses. In addition, selenium is also used as an important veterinary toxin. After knowing that selenium functions in regulating cellular metabolism in animals, the element was classified as an essential trace mineral. Selenium plays important
roles in the maintenance of an away of biological and physiological functions; in particular, it is a component of some antioxidant enzymes in human.

Recommended daily allowance (RDA) of selenium in the US is 55 μg/day for the general healthy adults and 60 to 70 μg/day during pregnancy and lactation (Washington, DC: National Academy Press, 2000). Inorganic forms of selenium are generally present in the soil and can be absorbed by plants. On the other hand, organic forms of selenium are high in certain foods, such as Brazil nuts, seafood, kidney, liver, and dairy products. Selenium deficiency is not common in the US since Americans consume foods grown in selenium-sufficient land and are well-nourished. Estimated selenium intake in the US exceeds RDA value. However, people who live on the land deficient in selenium are at risk, being unable to fully express selenoproteins. In Europe, the intake is as low as 30 μg/day and some New Zealanders consume selenium at a level less than 30 μg per day (Duffield et al., 1999). Besides, in selenium-deficient regions in China, people have insufficient selenium intake (≤10 μg/day) (Moreno-Reyes et al., 1998).

1.4.1 Selenium deficiency

Selenium deficiency may be exacerbated when low selenium intake is in combination with additional stress such as chemical exposure, UV irradiation, and oxidative stress. Deficiency in selenium can lead to impaired immune competence, susceptibility to viral infection and cardiovascular diseases. Besides, body selenium deficiency is associated with depression and other negative mood states,
including anxiety, confusion, and hostility (Hawkes and Hornbostel. 1996; Rayman, 2000).

The two well-known selenium deficient diseases are Keshan disease and Kashin-Beck disease. Keshan disease is named after Keshan County of Heilongjiang province in China, where symptoms were first described in this selenium deficient land. It is characterized by a congestive cardiomyopathy, having heart failure and pulmonary edema. Congestive cardiomyopathy is often fatal; especially for children and women of child-bearing age (Whanger, 1989). The etiology of the disease is demonstrated as the increased susceptibility of heart muscle to Coxsackievirus B3 due to selenium deficiency (Beck et al., 1994). However, the mechanism of viral infection is still unknown. Kashin-Beck disease is a chronic osteoarthropathy, resulting from lack of both selenium and iodine (Moreno-Reyes et al., 1998). Kashin-Beck disease is usually found in young children aged 5 to 15 that are characterized by a disorder of the bones and joints. Although selenium deficiency is also a problem in New Zealand, it is reflected in low blood selenium concentration and urinary outputs. There is no adverse consequences for the health of New Zealanders who consume less than 30 μg selenium per day. Some believe that the resident New Zealanders have adapted to their low selenium intake (Robinson et al., 1985).

When young rats were fed a selenium deficient diet for 13 weeks, they showed a significant decrease in serum selenium level but selenium concentration in the brain remains (Prohaska and Ganther, 1976). This suggests that the brain receives
high priority for retaining this micronutrient under dietary selenium deficiency (Chen and Berry, 2003).

1.4.2 Selenium, human health, and cancer

Selenium is an important trace element to counteract the toxic effect of heavy metals. Together with vitamin E, selenium can detoxify arsenic and mercury. Some of the anti-cancer property of selenium can be attributed to its toxic effect. The link between selenium and cancer has been known for half a century based on both geographic and clinical studies. In 1996, the first interventional trial, the Nutritional Prevention of Cancer (NPC) study, was done by using the selenized yeast at the dose of 200 μg/day (3 to 4 folds greater than RDA). The study was designed to determine the selenium effects on preventing skin cancer but accidentally found the link between selenium and lower mortalities and incidences for prostate, colorectal, and lung cancer (Clark et al., 1996).

One of the major follow up studies, Selenium and Vitamin E Cancer Prevention Trial (SELECT), did not show a role for selenomethionine in prostate cancer suppression. It has been noted that the form of selenium in the SELECT trial is different from the collection of selenium compounds in selenized yeast used in the NPC study. Although selenomethionine accounts for 50 to 80% of total selenium in selenized yeast, other selenium species such as selenocysteine, methylseleninic acid (MSeA) should contribute to the efficacy of selenium chemoprevention observed in the NPC trial (Fritz et al., 2011). However, ample
pre-clinical and cell studies have indicated that MSeA is far more effective than selenomethionine in the suppression of tumorigenesis. An examination in PC-3 (human prostate cancer) cells xenograft in nude mice treated with MSeA suggests the inhibition of tumor growth without apparent genotoxicity (Li et al., 2008).

Also, many human studies have shown that selenium supplementation is effective in increasing plasma glutathione peroxidase (GPx) activity. MSeA, L-selenomethionine, and selenite intake are associated with optimal GPx response. A New Zealand clinical study published in 1999 and the WHO/FAQ/IAEA group published in 1996 mentioned that 40 μg selenium supplementation per day, which is less than the US RDA, is sufficient in achieving two thirds of maximal GPx antioxidant activity (Duffield et al., 1999). GPx activity is an indicator of many diseases or dysfunction of tissues. In addition to prevent the onset of metabolic syndromes, adequate GPx activity shows a decrease in the risk of cardiovascular disease (Hamanishi et al., 2004) and prevents early pregnancy loss (Al-Gubory et al., 2010).

1.4.3 Selenium toxicity

Selenium overdose is toxic to human health and the upper limit is set at 400 μg/day. High selenium intake may result in type 2 diabetes by the induction of insulin resistant. Insulin binds to cell membrane receptors and then triggers hydrogen peroxide as a secondary messenger (Goldstein et al., 2005). However, the major selenoperoxidase, GPx1, decomposes hydrogen peroxide and thus might
impact the normal insulin signaling (Rayman, 2012). In addition, all forms of selenium can be changed directly or indirectly into hydrogen selenide that generates selenium dioxide, superoxide anion, and hydrogen peroxide (Combs, 2004). Those reactive oxygen species (ROS) can interact with critical macromolecules, such as DNA, proteins, and lipids, leading to cell damage response. ATM and DNA-PK kinases activity can be activated to initiate the signal-transduction pathways to deal with the oxidative stress and DNA damage response. We have recently shown that supranutritional doses of selenium can induce ROS and the subsequently DNA damage response to induce early tumorigenesis barriers (Rocourt and Cheng, 2012).

1.4.4 Selenoproteins

Selenium can regulate metabolic pathways through selenium-containing proteins. Because of the similar chemistry property with sulfur, selenium can be incorporated into proteins by non-specific substitution of sulfur in methionine and cysteine or co-translationally incorporated into selenoproteins in the form of the 21st amino acid, selenocysteine (Sec). According to the computational sequence analyses, there are 25 selenoproteins in the human genome and most selenoproteins are directly or indirectly involved in redox regulations (Gregory et al., 2003). To date, twelve selenoproteins are well-characterized as oxidoreductase, including glutathione peroxidases (GPx1-4, GPx6), thioredoxin reductases (TrxR1-3), iodothyronine deiodinases (DIO1-3), and selenophosphate
synthetases 2 (SPS2). The remaining selenoproteins are named in alphabetic order, such as selenoprotein P (SelP) (Papp et al., 2007).

There is a total of five GPxs (GPx1-4, GPx6) that catalyze the decomposition of hydrogen peroxide or lipid hydroperoxide to water and oxygen while oxidizing reduced glutathione. Glutathione peroxidases 1 (GPx1) is the most abundant selenoprotein in liver and the main antioxidant against acute oxidative stress in mice (Cheng et al., 1998). Many diseases are associated with GPx1 expression level. For example, GPx1 expression in Alzheimer’s patients is significantly decreased and is considered as an indicator of disease occurrence (Vural et al., 2010). GPx1 accounts for the effect of dietary selenium in the suppression of the virulence of Coxsackievirus B3 that leads to Keshan disease (Beck et al., 1994).

Since oxidative stress has been linked to neurodegenerative diseases, protecting the brain from ROS damage is critical. TrxRs expression is found to be high in the neuronal cells (Soerensen et al., 2008). However, TrxRs expression is maintained in the brain of mice fed a selenium-deficient diet, indicating a pivotal role of TrxRs in the brain (Schomburg et al., 2003). Moreover, SelP, a selenium transport protein, also plays an important role in the antioxidative defense in neuronal tissues (Steinbrenner and Sies, 2009). SelP is found to co-localize with insoluble amyloid-β (Aβ) plaques and neurofibrillary tangles, which are thought to increase oxidative stress and promote neuronal degeneration in the Alzheimer’s brain (Bellinger et al., 2008). A follow-up study showed that an increased Aβ toxicity and apoptosis are observed in SelP knockdown neuronal N2A cells.
(Takemoto et al., 2010). Furthermore, reduced expression of SelP is known to be linked to certain cancers, such as prostate cancer (Cooper et al., 2008).

1.5 RESEARCH BACKGROUND

Ovarian is the fifth-leading cause of cancer death among women in the United States (Siegel et al., 2012). Since there are no validated or proven screening tests for detecting ovarian cancer, most patients are diagnosed at advanced-stage. Fortunately, carboplatin, which is a second generation of platinum-based anti-ovarian cancer drug, was approved for clinical used in 1989. Carboplatin has lower toxicity than cisplatin due to a slower leaving group. It exhibits lower reactivity to form DNA crosslinks which disturbs cellular replication. However, chemotherapy resistance may develop in ovarian cancer after drug treatment. The Notch pathway is one of the causes of carboplatin resistance. In clinical study, it is found that 22% of high-grade serous ovarian cancer samples from stage-II-IV have altered NOTCH signaling (TCGA Research Network, 2012). Besides, another study has shown that NOTCH3 was overexpressed in 66% of high-grade serous carcinoma and only in 33% of low-grade tumor compared with normal ovarian surface epithelium (Park et al., 2006). Notch 3 receptor has extracellular and intracellular (NICD) domains. After receiving signal outside of cell membrane, NICD domain translocates into nucleus, form complex with CSL and the mastermind-like (MAML) family, and converts CSL into a transcriptional activator that promotes the transcription of genes downstream in the Notch
pathway (Beatus and Lendahl, 1998). NICD activates a few oncogenic pathways, including NF-κB. However, the primary Notch3 ligand that initiates signal transduction in ovarian cancer remains unclear.

Thus, we used OVCA429 is model to study the nature of Notch in ovarian cancer. OVCA429 is originally from serous of late-stage human ovarian adenocarcinoma, which has normal level of Notch 3 and was proven to show cisplatin-resistance. OVCA429 with Notch 3 overexpression is more resistant to platinum-based anti-cancer drugs than control cells. Hence, targeting Notch 3 may find a potential way to improve the efficacy of DNA damaging drugs such as MSeA and carboplatin.

The inverse relationship between selenium status and cancer incidence have been proven by both geographical and clinical studies (Clark et al., 1999; Rayman, 2000). MSeA is far more effective than other forms of selenium on the suppression of tumor in animal and cell studies (Ip et al., 2000; Drake, 2006). MSeA is used as a prooxidant inducing excess ROS level which results in DNA damage. On the other hand, carboplatin kills tumor principally by inducing DNA crosslinks which inhibit DNA replication and cause DNA breaks. Besides, carboplatin can also induce ROS which triggers cells toward apoptosis or even exhibits side effects, such as cardiotoxicity and ototoxicity (Cheng et al., 2008; Chen et al., 2010). Thus, we believe MSeA and carboplatin have overlap efficacy on anti-ovarian tumor. Combining two treatments may improve the efficacy of cancer killing.
In our findings, MSeA and carboplatin have synergistic effects in Notch 3 overexpressed cells. The co-treatment killed Notch 3 overexpressed cells down to a similar level of control cells through p53-independent-induced apoptotic death.
CHAPTER 2: MATERIALS AND METHODS

2.1 CELLS AND CULTURE CONDITIONS

The OVCA429/pCEG human ovarian carcinoma expressing a GFP empty vector and OVCA429/NICD3 expressing the vector carrying a constitutively active form of the intracellular domain of Notch3 were maintained in RPMI 1640 (Mediatech Inc, Herndon, VA) supplemented with 10% heat-inactivated fetal bovine serum and 100 U/ml penicillin and streptomycin at 37°C in a 5% CO₂ incubator. Both cell lines were sorted by fluorescence-activated cell sorter (FASC) for GFP expression that was introduced using lentivirus, and were gifted from Dr. Yangxin Fu’s lab at the University of Alberta, Canada. Methylseleninic acid (MSeA) and N-acetylcysteine (NAC) were obtained from Sigma-Aldrich (St. Louis, MO), and were dissolved in phosphate-buffered saline (PBS). NAC is a free radical scavenger that mainly abolishes hydrogen peroxide (H₂O₂). Carboplatin was purchased from Enzo Life Sciences (Farmingdale, NY), and was dissolved in water. KU 60019 and NU 7026 were purchased from Tocris (Ellisville, MO), and were dissolved in DMSO (dimethyl sulfoxide). KU 60019 is a selective inhibitor of ATM (Golding et al., 2009) while NU 7026 inhibits DNA-PKcs kinase activity (Willmore et al., 2004).

2.2 SULFORHODAMINE B COLORIMETRIC ASSAY
Sulforhodamine B has been used to determine drug-induced cytotoxicity, based on the measurement of cellular protein content for large-scale drug-screening applications. Cells were seeded at a concentration of 10,000 OVCA429 cells per well of 96-well plate and allow for attachment overnight. At the same time, cells were seeded into another plate and allowed for attachment overnight (Day 0). Cells were incubated with RPMI 1460 medium only (negative control), or the medium containing MSeA (0.25-2 μM), carboplatin (1-25 μM), KU 60019 (3 μM), NU 7026 (10 μM), and/or NAC (10 mM) for 48 hours while cells at Day 0 was directly fixed with the following steps. Cells were fixed with 10% trichloroacetic acid (TCA) for 1 hour at 4 °C and the plates were washed 5 times with water and air dried. 0.4% sulforhodamine B in 1% acetic acid was used to stain living cells for 20 minutes at room temperature. After staining, unbound dye was removed by washing 5 times with 1% acetic acid and plates were air dried. Later 200 μL of 10 mM unbuffered TRIS base (pH 10.5) was added to each of the wells and incubated for 30 minutes at 37 °C. Finally, cellular protein content was measured by a plate reader (BMG LabTech, Cary, NC) at 492nm. Percentage of cell viability was calculated using the formula below (Vichai and Kirtikara, 2006).

\[
% \text{ of cell viability} = \frac{\text{mean } OD_{\text{sample}} - \text{mean } OD_{\text{day } 0}}{\text{mean } OD_{\text{neg control}} - \text{mean } OD_{\text{day } 0}} \times 100\%
\]

2.3 IMMUNOFLUORESCENCE

Cells were seeded onto sterilized coverslips and incubated with MSeA, carboplatin, KU 60019 and/or NU 7026 for 24 hours. After treatment, cells were
first washed 3 times (5 minutes per wash) with PBS, fixed in 4% paraformaldehyde for 15 minutes at RT and permeabilized in 90% methanol for 10 minutes at -20 °C. After another 3 times of washing in PBS, cells were then incubated in 0.3% Triton-X for 15 minutes at RT. Cells were then washed 3 times with PBS before being blocked in 3% BSA in PBS for 30 minutes. Next, the coverslips were incubated overnight at 4 °C with anti-pATM S1981 (1:300; Rockland, Gilbertsville, PA), anti- pDNA-PKcs S2056 (1:300, Abcam), and γH2AX (phospho-H2AX on Ser139, 1:500, Abcam). Coverslips were washed in PBS with 0.1% Tween-20 for 5 times and then were incubated with secondary antibodies (Alexa 594 goat anti-mouse IgG, Invitrogen) for 1 hour at RT in dark. After washing in PBS with 0.1% Tween-20 for 5 times, coverslips were mounted onto slides with a drop of ProLong® Gold antifade reagent containing DAPI (Invitrogen, Grand Island, NY). All the images were taken under the same parameters of brightness, contrast, and exposure time by using a Zeiss Axio Observer Z1m fluorescence microscope (Carl Zeiss, Oberkochen, Germany). Photos were processed using deconvolution with the software AxioVision Release 4.7.2.0. The pATM S1981, pDNA-PKcs S2056, and γH2AX focus-positive cells are defined as those containing at least five foci (Camphausen et al., 2004).

2.4 FLOW CYTOMETRY ASSAY
Flow cytometry assay is widely used to determine the ratio of cells in different phases of cell cycle by measuring the amount of DNA content in cells. Propidium iodide (PI) is a commonly used nuclei fluorescent dye which binds to the DNA double helix in the nuclei and requires blue light as the excitation source (e.g., 488 nm argon ion laser). The different parts of the cell cycle are determined based on the intensity of fluorescence of the nuclei.

Cells were cultured in 10 cm² dishes and treated with 2 μM MSεA and 5 μM carboplatin for 24 hours. The cell culture medium was collected before trypsinizing cells from dishes to completely collect G2/M cells. Then the cells were incubated in trypsin/EDTA for 30 min to ensure complete detachment and separation as single cells. Cells were collected into centrifuge tubes, resuspended in ice-cold PBS, and centrifuged at 4°C with a speed of 500×g for 5 min. Cells were washed again with cold PBS and resuspended by pipetting into 100 μl PBS. Each tube was vortexing while adding 5 mL of -20°C 70% ethanol dropwise for fixation. Samples were stored at -20°C overnight. Prior to the analysis, samples were centrifuged at 4°C at a speed of 500×g for 5 min. Fixative ethanol was aspirated, and cells were washed with 5 mL PBS twice at 4°C at a speed of 500×g for 5 min. After supernatant was taken out, cells were vortexed and resuspended in 1 mL propidium iodide solution (25 μg/mL) containing RNaseA (100 μg/mL) for 30 min at room temperature in dark. The DNA was then analyzed by a FACS calibur cytometer with CELLQuest program (Becton Dickinson, San Jose,
CA). ModFit LT (Version 3.0, Verity Software House, Topsham, ME) was applied for cell cycle analysis on overlaid histograms.

2.5 STATISTICAL ANALYSIS

The data were analyzed by using the SAS 9.0 software (SAS Institute Inc., Cary, NC). Two-tailed student’s t-test was applied to determine statistical significance between the treatments and the control. The level of statistical significance was set at $p < 0.05$. 
CHAPTER 3: RESULTS

3.1 SYNERGISTIC EFFECT OF MSEA AND CARBOPLATIN IN THE KILLING OF OVCA429/pCEG AND OVCA429/NICD3 CELLS

Ovarian carcinomas expressing a constitutively active form of the intracellular domain of Notch3 are more resistant to platinum therapeutic agents, such as carboplatin (Park et al., 2006; Park et al., 2010). We have previously shown that MSeA treatment kills HCT116 colorectal, PC-3 prostate and U-2 OS osteosarcoma cells in a manner depending on ROS (Wu et al., 2010; Qi et al., 2010; Cheng et al., 2012). Because ROS could be involved in the Notch3 pathway (Cheng et al., 2008; Chen et al., 2010), we tested the hypothesis that MSeA can improve the desensitization of OVCA429 ovarian cells to carboplatin treatment when Notch 3 is expressed. The cells were treated with pharmacologically achievable concentration of MSeA (0.25-2 μM) (Saifo et al., 2010; Qi et al., 2012), carboplatin (1-25 μM) (Elferink et al., 1987), or combinations of the two reagents. As determined by SRB assays, carboplatin (Figure 3.1.A) or MSeA (Figure 3.1.B) alone killed both OVCA429/pCEG and OVCA429/NICD3 cells in a dose-dependent manner, and the latter cells were more resistant than the former cells to the reagents (Table 3.1). Besides, OVCA429/NICD3 cells treated with 0.5-1 μM MSeA exhibited a significant increase in cell viability. Interestingly, analyses of cell viability in the combinational treatment of a gradient concentration of carboplatin and MSeA (Table 3.1) suggested a synergistic effect
of these two reagents in OVCA429/NICD3 cells. While treatment of
OVCA429/NICD3 cells with MSeA (2 µM) killed 13.9% of the cell, treatment of
the cells with a gradient concentration of carboplatin (0-25 µM) did apparently
sensitize the cell. When the cells were co-treated with MSeA (2 µM) and a
gradient concentration of carboplatin (0-25 µM), an exponential curve of
increased sensitivity was observed as opposed to carboplatin treatment alone
(Figure 3.1.C), suggesting a synergistic effect of MSeA and carboplatin in the
killing of OVCA429/NICD3 cells. KU 60019 or NU 7026 co-treated with MSeA
and carboplatin does not impact on cell viability while NAC co-treatment shows a
significant reverse on cell viability in both cell lines (Figure 3.2 and 3.3). Of note,
the combination of MSeA (2 µM) and carboplatin (25 µM) sensitized the
refractory OVCA429/NICD3 cells to an extent similar to that in OVCA429/pCEG
cells. However, there was no such synergistic effect in OVCA429/pCEG cells.
Table 3.1 Sensitivity of OVCA429/pCEG and OVCA429/NICD3 ovarian cells to MSeA and carboplatin. Cells were cultured in 96-well plates and treated with MSeA and/or carboplatin at the indicated concentration for 2 days. Cell viability was assessed by SRB assay. The optical density in the condition without MSeA or carboplatin treatment was set as 100%. Values are mean ± S.E.M. (n = 3). #, p < 0.05, compared to no MSeA treatment. *, p < 0.05, compared to no carboplatin treatment.

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<th>Carboplatin (μM)</th>
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<td>100</td>
<td>92.5±9.4</td>
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<td>74.4±6.1*</td>
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<td>51.7±3.1*</td>
<td>48.3±6.1*</td>
<td>41.3±4.6*</td>
<td>30.3±3.2**</td>
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<tr>
<th>OVCA429/NICD3 MSeA (μM)</th>
<th>Carboplatin (μM)</th>
<th>0</th>
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<th>2</th>
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<td>105.2±6.8</td>
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<td>117.8±4.8*</td>
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<td>118.3±4.6</td>
<td>104.7±4.9</td>
<td>95.8±6.0*</td>
<td>83.6±9.8*</td>
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<tr>
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<td></td>
<td>109.3±6.5</td>
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<td>98.8±6.6</td>
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<td>2</td>
<td></td>
<td>86.1±6.8</td>
<td>76.9±6.1*</td>
<td>64.2±6.6*</td>
<td>61.2±1.2*</td>
<td>48.2±6.9**</td>
<td>36.2±4.4**</td>
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</table>
Figure 3.1 Synergistic effect of MSeA and carboplatin on OVCA429/NICD3 cells. OVCA429/pCEG and OVCA429/NICD3 cells were treated with a gradient concentration of MSeA (A) or Carboplatin (B) for 2 days. *, p < 0.05, compare to OVCA429/pCEG cells. C, OVCA429/NICD3 cells were treated with carboplatin (0-25 µM) in the absence or presence of MSeA (2 µM) for 2 days. #, p < 0.05, compared to predicted additive effect. Cell viability was assessed as described in Table 3.1 legend.
Figure 3.2 Sensitivity of OVCA429/pCEG and OVCA429/NICD3 cells to protein kinase inhibitors or NAC under the same concentration of carboplatin. OVCA429/pCEG cells were treated with MSeA, carboplatin, and KU 60019 (A), NU 7026 (B), or NAC (C). OVCA429/NICD3 cells were treated with MSeA, carboplatin, and KU 60019 (D), NU 7026 (E), or NAC (F). Values are mean ± S.E.M. (n = 3). *, p < 0.05, compared with NAC treatment.
Figure 3.3 Sensitivity of OVCA429/pCEG and OVCA429/NICD3 cells to protein kinase inhibitors or NAC under the same concentration of MSeA. OVCA429/pCEG cells were treated with MSeA, carboplatin, and KU 60019 (A), NU 7026 (B), or NAC (C). OVCA429/NICD3 cells were treated with MSeA, carboplatin, and KU 60019 (D), NU 7026 (E), or NAC (F). Values are mean ± S.E.M. (n = 3). *, p < 0.05, compared with NAC treatment.
3.2 CELL CYCLE ANALYSIS OF OVCA429/pCEG AND OVCA429/NICD3 CELLS TREATED WITH MSEA AND CARBOPLATIN

To understand the nature of the synergistic effect, we first performed flow cytometric analyses to determine cell cycle distribution of the cells co-treated with MSeA (2 µM) and carboplatin (5 µM). Interestingly, the cell phase percent distributions are different between two cells under no treatment. OVAC429/NICD3 cells intrinsically contained a significantly higher percentage of cells in G2/M phase and a lower percentage in S and G1 phases compared with OVAC429/pCEG cells (Table 3.2), indicating a poor respond to chemotherapy due to a low proliferation (Itamochi et al., 2002; Kwintkiewicz et al., 2012). MSeA and carboplatin co-treatment shows significant effects on cell cycle changes. At day 2, the percent of S phase exhibits a significant decrease in control cells while the incidence of G1 phases exhibit a significant increase and the percent of G2/M phase exhibit a significant decrease in the Notch 3 overexpressed cells. However, even though there is a statistical significance, the biological significance is minimal. Of note, there was a time-dependent induction of sub-G1 cell publication at day 1 and day 2 after the co-treatment in OVAC429/pCEG (2.2±0.4% and 8.9±1.0%) and in OVAC429/NICD3 (3.4±0.3% and 9.1±0.9%) cells. These results suggest that the co-treatment may induce DNA fragmentation in the cells, but this may not account for the resistance of OVAC429/NICD3 cells to the therapeutic treatment.
Table 3.2 Flow cytometric analyses of the percent G1, S, and G2/M OVCA429/pCEG and OVCA429/NICD3 cells treated with MSeA (2 μM) and carboplatin (5 μM) for 1 or 2 days. Values are mean ± S.E.M. (n = 3). *, p < 0.05, compared to OVCA429/NICD3 cells. #, p < 0.05, compared to Day 0.

<table>
<thead>
<tr>
<th></th>
<th>0</th>
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<td><strong>Sub G1, %</strong></td>
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<tr>
<td>OVCA429/pCEG</td>
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<td>2.2±0.4#</td>
<td>8.9±1.0#</td>
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<tr>
<td>OVCA429/NICD3</td>
<td>1.4±0.1</td>
<td>3.4±0.3#</td>
<td>9.1±0.9#</td>
</tr>
<tr>
<td><strong>G1, %</strong></td>
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<td></td>
</tr>
<tr>
<td>OVCA429/pCEG</td>
<td>32.7±1.0*</td>
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<td>34.0±2.7</td>
</tr>
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<td>OVCA429/NICD3</td>
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<td><strong>S, %</strong></td>
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<td>OVCA429/pCEG</td>
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<td>OVCA429/NICD3</td>
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<td><strong>G2/M, %</strong></td>
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<td>OVCA429/pCEG</td>
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3.3 EFFECT OF MSEA AND CARBOPLATIN CO-TREATMENT ON THE FORMATION OF pDNA-PKcs S2056, pATM S1981 and H2AX in OVCA429/pCEG and OVCA429/NICD3 CELLS

We next determined the nature of DNA damage response in the ovarian cancer cells co-treated with MSeA (2 µM) and carboplatin (5 µM) for 24 h. The phosphorylation of ATM on Ser-1981 and DNA-PKcs on Ser-2056 play crucial roles in the cellular response to DNA damage; they in turn phosphorylate their substrates such as H2AX. At 24 h, pDNA-PKcsS2056 level rose significantly in both OVCA429/pCEG and OVCA429/NICD3 cells, and the induction could be reversed in the presence of NU 7026 (Figure 3.4A). Co-treatment of NAC attenuated pDNA-PKcsS2056 expression in OVCA429/pCEG but not in OVCA429/NICD3 cells. On the other hand, the MSeA and carboplatin co-treatment significantly induced the expression of pATMS1981 in OVCA429/pCEG but not in OVCA429/NICD3 cells (Figure 3.4B). The induction of pATMS1981 was inhibited in the presence of KU 60019 or NAC. The MSeA and carboplatin co-treatment did not induce γH2AX formation, and γH2AX level was not differ between OVCA429/pCEG and OVCA429/NICD3 cells before and after the co-treatment or in the presence of NU 7026, KU 60019 or NAC (Figure 3.4C). Consistent with our observation, it has been shown previously that level of γH2AX is intrinsically low in OVCAR-3 human ovarian cancer cells, and carboplatin treatment even at a high dose (100 µM) does not induced γH2AX formation (Qian et al., 2006; Fishel et al., 2007).
Figure 3.4 Immunofluorescent analyses of pDNA-PKcs S2056 (A), pATM S1981 (B) and γH2AX (C) in OVCA429/pCEG and OVCA429/NICD3 cells co-treated with MSeA (2 µM) and carboplatin (5 µM) for 24 h. Cells were co-treated with NU 7026 (10 µM), KU 60019 (10 µM), or NAC (10 mM) Values are mean ± S.E.M. (n = 3). *, p < 0.05, compared to OVCA429/NICD3 cells. #, p < 0.05, compared to Day 0.
3.4 EFFECT OF KU60019, NU7026, AND NAC ON THE SENSITIVITY OF OVCA429/pCEG AND OVCA429/NICD3 CELLS TO THE MSEA AND CARBOPLATIN CO-TREATMENT

Next, we determine whether DNA-PKcs, ATM and ROS are involved in the sensitivity of OVCA429/pCEG and OVCA429/NICD3 cells to MSeA and carboplatin co-treatment. The presence of KU 60019 (3 μM) or NU 7026 (10 μM) decreased the cell viability in both OVCA429/pCEG and OVCA429/NICD3 cells co-treated with MSeA (2 μM) and carboplatin (25 μM) (Figure 3.5). Both MSeA and carboplatin has been shown previously to increase oxidative stress level in both animal and cell studies (Cheng et al., 2008; Chen et al., 2010). Thus, we co-treated NAC with MSeA and carboplatin to determine whether ROS are involved in the sensitivity of ovarian cancer cells to the two agents. Here, we showed that NAC administration (10 mM) resulted in a significant increase of cell viability in both OVCA429/pCEG and OVCA429/NICD3 cells (Figure 3.5). The cell viability was significantly lower in OVCA429/NICD3 cells with MSeA and carboplatin treatment or with KU 60019 administration than in OVCA429/pCEG cells.
Figure 3.5 Effects of NU 7026, KU 60019 and NAC on the sensitivity of OVCA429/pCEG and OVCA429/NICD3 cells to MSeA (2 µM) and carboplatin (25 µM). Cells were for 2 days and cell viability was assessed by SRB assay as described in Table 1. *, p < 0.05, compared to OVCA429/pCEG cells. #, p < 0.05, compared to MSeA and carboplatin treatment only.
CHAPTER 4: DISCUSSION

In this study, we consider that Notch 3 overexpression provides the resistance of human ovarian carcinoma to MSeA and carboplatin-induced DNA damage. Continuing expression of Notch 3 can induce tumorigenesis and is even essential for the survival of human ovarian carcinoma (Rose, 2009). Based on our results, Notch 3 overexpressed human ovarian carcinomas cells are more resistant to MSeA or carboplatin treatment alone than control cells. Interestingly, synergistic effects were observed at dose 2 μM MSeA and 2-25 μM carboplatin in the Notch 3 overexpressed cells, decreasing the cell viability down to a similar level with the control cells. Besides, the cells were treated with pharmacologically achievable concentration of MSeA (0.25-2 μM) (Saifo et al., 2010; Qi et al., 2012), carboplatin (1-25 μM) (Elferink et al., 1987), or combinations of the two reagents. In human patients receiving selenomethionine, a precursor of MSeA, a serum selenium concentration of 15 μM was obtained (Fakih et al., 2008); A 100% peak serum concentration of carboplatin can be up to 39 μg/ml (105μM) (Oguri et al., 1988). Based on our results and previous studies, all indicate that it may be potential to use MSeA to enhance carboplatin antitumor efficacy in Notch 3 overexpressed human ovarian carcinoma cancer.

Cell cycle profiles showed a significant difference on the distribution of cell cycle between the control and Notch 3 overexpressed cells. In OVCA429/NICD3 cells, low percent of S phase indicates a low proliferation and a poor respond to
chemotherapy (Itamochi et al., 2002; Kwintkiewicz et al., 2012), which is an evidence of one reason of drug resistance. Although the percent of G2/M phase is significantly higher in OVCCA429/NICD3 than in OVCA429/pCEG cells, cell cycle arrest was not observed. Defects in the G2/M arrest may allow damaged cells to enter mitosis and even undergo apoptosis (Tyagi et al., 2002). In summary, Notch 3 overexpression may indirectly change the distribution of cell cycle phases.

Both MSeA and carboplatin induce DNA damage response in order to kill cancer cells. The incidence of sub G1 phase showed a time-dependent increase with MSeA and carboplatin co-treatment in both cells. However, MSeA and carboplatin co-treatment does not impact on cell cycle distribution change. These results suggest that the co-treatment may induce DNA fragmentation in the cells, but this may not account for the resistance of OVAC429/NICD3 cells to the therapeutic treatment.

Selenium compounds trigger cell death by inducing ROS-induced DNA breaks while carboplatin kill cells by inducing DNA crosslinks, which may also lead to DNA breaks. Based on the functions of killing cells, they indicate an overlap of antitumor efficacy. ATM and DNA-PKcs are two major DNA damage response protein kinases, which conduct signals to downstream mediators to either repair damage or stop cell cycle. Based on the present results, either ATM inhibitor KU 60019 or DNA-PKcs inhibitor NU 7026 administration significantly decreased the cell survival in both control and Notch 3 overexpressed cells. p53, which
induces cell growth when is upregulated by ATM, is one of the downstream factors of ATM. Thus, the protein kinase inhibitors results indicate that MSeA and carboplatin-induced cytotoxicity was not dependent on p53. This is consistent with other studies which were done with cisplatin to understand the relationship between p53 and OVCA429 cells. It is known that OVCA429 cells have wild-type p53 function (Elbendary et al., 1994). One study has exhibited that cisplatin was relatively more effective against mutant/null p53 cell lines than against the wild-type cell lines (Hagopian et al., 1999). Two-fold dose of cisplatin is needed for killing OVCA429 cells than other mutant/null p53 cells. Besides, in order to significantly induce p53 expression level, up to 5-fold IC_{50} is required in OVCA429 cells (Hagopian et al., 1999). Another study also showed that cisplatin could not induce p53 in resistant cells (Siddik et al., 1998). Therefore, in OVCA429 with wild-type p53, ATM and DNA-PKcs are not the major roles to response to platinum-based-induced anticancer drug – cisplatin and carboplatin. Loss of DNA damage response proteins availabilities makes human ovarian carcinoma easier to be killed.

Although carboplatin-induced DNA damage is p53-independent, DNA-PKcs and ATM can still be activated with MSeA and carboplatin. DNA-PKcs was activated in both cell lines, showing that it may not be the reason to explain the difference of drug resistance of Notch 3 overexpressed cells. On the other hand, ATM activity was exhibited in control cells but not in Notch 3 overexpressed cells treated with MSeA and carboplatin co-treatment, indicating that it may be
the cause of drug resistance of Notch 3. However, the level of γH2AX did not increase with treatments, which may due to the characteristic of OVCA429 cells. In clinical study, it is found that there were sequence alternations in the ATM gene detected in 137 out of 270 Austian hereditary breast and ovarian cancer patients (Thorstenson et al., 2003). It is possible that OVCA429 cells have sequence alternations in the ATM gene though there is no any reference for OVCA429 cells at current stage. Thus, γH2AX inactivation may due to the ATM gene sequence alternations. Moreover, two findings referred that OVCAR-3 human ovarian cancer cells exhibited low expression level of γH2AX even after 100 μM carboplatin treatment (Qian et al., 2006; Fishel et al., 2007). Taken together, an increase of ATM activation may be the reason to explain the sensitivity to MSeA and carboplatin co-treatment in OVCA429/pCEG cells.

Antioxidants have been considered to reduce carboplatin-induced side effects, including ototoxicity (Okur et al., 2007; Moon et al., 2011), nephrotoxicity, and gastrointestinal toxicity (Wu et al., 2004). However, it is still controversial whether it is a good idea suggesting ovarian cancer patients to take NAC ROS scavenger or not. In our results, NAC concurrent significantly increased cell viabilities in both cell lines, exhibiting a reverse effect of NAC to MSeA and carboplatin treated human ovarian cancer cells. Notch 3 does not impact on NAC-reversed cell survival. It is shown that NAC also blocks cisplatin-induced apoptosis in both lung and ovarian cancer cells (Wu et al., 2004). Another in vitro experiment has shown that NAC supports the metabolism of detached tumor cells
and could promote metastasis (Schafer et al., 2009). In summary, taking NAC can not only reduce side effects but promoting tumorigenesis. Finding a way to improve the efficacy of carboplatin as well as decrease the cellular toxicity of carboplatin is still a long way to go.

The drug resistance in ovarian cancer is also associated with BRCA1/2 mutation/deletion, which causes genomic instability due to dysfunctional homologous recombination, and other potential related pathways (Hiss, 2012). In our project, we only focus on Notch 3 characteristics because Notch 3 downstream target genes are associated with oncogenic, such as nuclear factor-kappa B (NF-κB) and vascular growth factor receptor (VEGF). NF-κB and VEGF control the transcription of DNA and the development of new blood vessels for nutrient and oxygen supply in cancer, respectively. We believe that MSeA sensitizes Notch 3-activated OVCA429 ovarian cancer cells to carboplatin, and this effeteness could be even improved if combines with other potential pathways.
CHAPTER 5: CONCLUSION AND PROSPECTS

It is known that ovarian cancer with NICD3 overexpression shows resistance to carboplatin (Park et al., 2006; Park et al., 2010). Here we showed that NICD3 expression can indirectly change cell fate by significantly decreasing the percent of S phase. Low percent S phase is a cause of low proliferation that may lead to drug resistance (Itamochi et al., 2002; Kwintkiewicz et al., 2012). However, NICD3 expression did not apparently impact on DNA fragmentation since there was no difference of the percent of Sub G1 between OVCA429/pCEG and OVCA429/NICD3 cells. Interestingly, the combinational treatment of carboplatin and MSeA sensitized the refractory OVCA429/NICD3 cells to an extent similar to that in OVCA429/pCEG cells in a synergistic manner. In clinical study, Notch 3 was found to be overexpressed in 66% of high-grade serous ovarian cancer (Park et al., 2006). OVCA429 cells are also high-grade and the doses that we treat are both pharmacologically achievable. Thus, it is promising to improve carboplatin treatment of ovarian cancer by additional MSeA administration.

We found that NAC administration significantly increased cell viability in both cells. This result indicates that MSeA (Wu et al., 2010; Qi et al., 2010; Cheng et al., 2012) and carboplatin-induced ROS damage (Cheng et al., 2008; Chen et al., 2010) can be reversed by NAC. Thus, cautious consideration of taking dietary
antioxidants should be taken for ovarian cancer patients to take lower the carboplatin efficacy.

ATM (Goodarzi et al., 2008) and DNA-PKcs (Sadofsky, 2001; Shrivastav et al., 2008) can trigger cellular apoptosis and recruit downstream factors for DNA repair. We found that the presence of ATM or DNA-PKcs inhibitor significantly decreased cell survival in both OVCA429/pCEG and OVCA429/NICD3 cells. pDNA-PKcsS2056 can be induced by MSeA and carboplatin co-treatment in both cell lines. However, the expression of pATMS1981 was significantly induced in OVCA429/pCEG but not in OVAC429/NICD3 cells by MSeA and carboplatin co-treatment. Thus, the cause of drug resistance and high cell viability in OVAC429/NICD3 cells may due to pATMS1981 instead of pDNA-PKcsS2056 expression level.

Our results indicate that Notch 3 could be a therapeutic target for malignant and advanced stages of ovarian adenocarcinoma due to the synergistic effect of carboplatin and MSeA and the change of cell cycle distribution. Targeting the Notch pathway has recently become a subject of intense research for chemotherapy. However, a preclinical study has demonstrated that preventing NICD 3 separation from the extracellular domain could be a new target-based therapy for tumors with Notch activity (McAuliffe et al., 2012). Future study should be proposed to suppress γ-secretase that leaves transmembrane proteins for further suppressing the Notch pathway.
Abbreviation List

1. NER: nucleotide-excision repair
2. BER: base-excision repair
3. MMR: mismatch repair
4. DSB: double-strand-break repair
5. ATM: ataxia telangiectasia-mutated
6. CHK1: checkpoint kinase 1
7. CHK2: checkpoint kinase 2
8. BRCA1: breast cancer associated 1
9. XP: xeroderma pigmentosum
10. CS: Cockayne’s syndrome
11. TTD: trichothiodystrophy
12. TCR: transcription-coupled NER
13. GGR: global genomic NER
14. RPA: replication protein A
15. ERCC1: excision repair cross-complementing-1
16. SSB: single strand break
17. UNG: uracil-DNA glycosylase
18. TDG: thymine-DNA glycosylase
19. OGG1: 8-oxoguanine glycosylase
20. AP: apyrimidinic/apurinic
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<th>Description</th>
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<td>21.</td>
<td>APE1: AP endonuclease 1</td>
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<tr>
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<td>5’-dRp: 5’-deoxyribose-5-phosphate</td>
<td></td>
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<tr>
<td>23.</td>
<td>Pol β: DNA polymerase β</td>
<td></td>
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<tr>
<td>24.</td>
<td>PCNA: proliferating cell nuclear antigen</td>
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<tr>
<td>25.</td>
<td>RFC: replication factor C</td>
<td></td>
</tr>
<tr>
<td>26.</td>
<td>FEN1: flap endonuclease 1</td>
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<tr>
<td>27.</td>
<td>IDLs: Insertion/deletion lesions</td>
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<td>28.</td>
<td>MutSα: MSH2/MSH6</td>
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</tr>
<tr>
<td>29.</td>
<td>MutSβ: MSH2/MSH3</td>
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</tr>
<tr>
<td>30.</td>
<td>HNPCC: hereditary non-polyposis colorectal carcinoma history</td>
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<tr>
<td>31.</td>
<td>HP: homologous recombination</td>
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<td>32.</td>
<td>NHEJ: non-homologous end joining</td>
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<td>33.</td>
<td>the catalytic subunit of DNA-PK: DNA-PKcs</td>
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<td>34.</td>
<td>XLF: XRCC4-like factor</td>
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<tr>
<td>35.</td>
<td>PNK: polynucleotide kinase</td>
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<td>36.</td>
<td>ssDNA: single-stranded DNA</td>
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<tr>
<td>37.</td>
<td>CtIP: C-terminal binding protein interacting protein</td>
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<td>38.</td>
<td>MRN: MRE11-RAD50-NBS1</td>
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<tr>
<td>39.</td>
<td>dsDNA: double strand DNA</td>
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<td>40.</td>
<td>BRCA2: breast cancer associated protein 2</td>
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<td>41.</td>
<td>MDC1: DNA damage checkpoint 1</td>
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<tr>
<td>42.</td>
<td>53BP1: tumor protein 53 binding protein 1</td>
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</tbody>
</table>
43. SMC1: structural maintenance of chromosomes 1
44. Hes: Hairy enhance of split family
45. NF-κB: nuclear factor-kappa B
46. VEGF: vascular growth factor receptor
47. mTOR: mammalian target of rapamycin
48. Akt: protein kinase B
49. ER: estrogen receptor
50. AR: androgen receptor
51. EGFR: epidermal growth factor receptor
52. TCR: T cell receptor
53. FDA: US Food and Drug Administration
54. JM: Johnson Matthey Plc
55. ICR: Institute of Cancer Research
56. CBDCA: bidentate dicarboxylate
57. G: guanosine
58. A: adenosine
59. CTR1: copper transporter 1
60. RDA: recommended daily allowance
61. SELECT: Selenium and Vitamin E Cancer Prevention Trial
62. MSeA: methylseleninic acid
63. ROS: reactive oxygen species
64. Sec: selenocysteine
65. GPx: glutathione peroxidase
66. TrxR: thioredoxin reductase
67. DIO: iodothyronine deiodinases
68. SPS2: selenophosphate synthetases 2
69. SelP: selenoprotein P
70. Aβ: amyloid-β
71. NAC: N-acetylcysteine
72. PBS: phosphate-buffered saline
73. H2O2: hydrogen peroxide
74. TCA: trichloroacetic acid
75. DMSO: dimethyl sulfoxide
76. pATM S1981: ATM autophosphorylation at Ser-1981
77. pDNA-PKcs S2056: pDNA-PKcs autophosphorylation at Ser-2056
78. γH2AX: H2AX phosphorylation at Ser-139
References


