ABSTRACT

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

Tiffany J Tzeng, Master of Science, 2012

Thesis directed by: Assistant Professor Wen-Hsing Cheng
Department of Nutrition and Food Science

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-PK_{cs}. In summary, MSeA and carboplatin synergistically sensitize OVCA429/NICD3 cells in a pathway involves oxidative stress, ATM and DNA-PK_{cs}, 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

Tiffany J Tzeng

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Advisory Committee:

Assistant Professor Wen-Hsing Cheng, Chair

Assistant Professor Seong-Ho Lee

Assistant Professor Zhengguo Xiao

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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 cauese 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 glycosylasese, 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 backcone 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 exhibitsa 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 G_2 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-PK_{cs} (the catalytic subunit of DNA-PK). DNA- PK_{cs} 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 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 *ATM* 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 intraceullular 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 [PtII $(NH_3)_2Cl_2$], 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 decresaed 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).

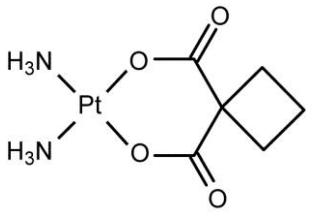


Figure 1.1 Carboplatin Structure. Carboplatin is a second generation platinum-based chemotherapeutic drug. Carboplatin contains a slow leaving group - bidentate dicarboxylate (CBDCA) ligand instead of the two chloride ligands appeared in cisplatin. This makes it less toxic compared to cisplatin.

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 durg design and modification. Based 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 tomodify drugs with different functional groups and to find the best working environments.

Carboplatin [*cis*-Diammine (1,1-cyclobutanedicarboxylato) platinum(II)] belongs to the large group of chemotherapy drugs with DNA alkylation function (Kojima *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 *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 *et al*., 2009). In addition, less than 5% of interstrand crosslinks contain two guanosines from the opposite DNA strands (McHugh *et al.*, 2001).

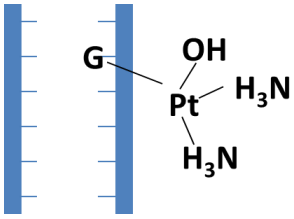
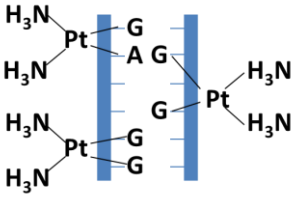
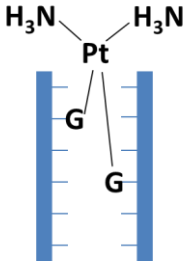
	DNA Monoadduct	Intrastrand Crosslinks	Interstrand Crosslink
			
% of DNA adducts	~2%	GpG 1,2 intrastrand crosslinks: 60-65% ApG 1,2 intrastrand crosslinks: 20-25% GpXpG 1,3 intrastrand crosslinks: ~2%	G-G interstrand crosslinks: <5%

Table 1.1 Percent of three types of carboplatin-induced DNA adducts.

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^{o} 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 toviral 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 base 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, decomposeshydrogen 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 selenoprotiens are directly or indirectly involved in redox regulations (Gregory *et al.*, 2003). To date, twelve selenoporoteins 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℃ in a 5% CO_2 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 (H_2O_2). 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 was 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 ℃ 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 ℃. 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).

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

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 ℃. 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 ℃ 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 lgG, 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 cm^2 dishes and treated with 2 μM MSeA 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^oC with a speed of 500×g for 5 min. Cells were washed again with cold PBS and resuspended by pipetting into100 μl PBS. Each tube was vortexing while adding 5 mL of -20^oC 70% ethanol dropwise for fixation. Samples were stored at -20℃ overnight. Prior to the analysis, samples were centrifuged at 4^oC at a speed of 500×g for 5 min. Fixative ethanol was aspirated, and cells were washed with 5 mL PBS twice at 4^oC 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 FACScalibur 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.

OVCA429/pCEG						
MSeA (μM)	Carboplatin (μM)					
	0	1	2	5	15	25
0	100	92.5±9.4	92.2±7.1	88.8±7.2	74.4±6.1*	68.4±4.3*
0.25	100.2±10.2	90.0±5.4	89.9±7.9	93.5±13.8	75.3±7.2	66.1±11.3
0.5	92.7±4.4	94.1±6.4	89.9±7.5	84.2±8.6	75.2±7.5	63.5±9.6
1	87.1±8.2	82.3±6.5	77.9±6.6	76.5±4.0	61.4±3.6	47.6±0.7$^{\#*}$
2	68.5±3.5$^{\#}$	62.6±6.4	51.7±3.1$^{\#*}$	48.3±6.1$^{\#*}$	41.3±4.6$^{\#*}$	30.3±3.2$^{\#*}$
OVCA429/NICD3						
MSeA (μM)	Carboplatin (μM)					
	0	1	2	5	15	25
0	100	108.5±7.0	106.0±6.4	97.1±5.7	93.2±9.3	91.7±7.5
0.25	104.6±3.5	101.7±5.2	101.8±5.9	105.2±6.8	98.3±5.2	96.5±10.6
0.5	117.8±4.8$^{\#}$	110.3±5.2	118.3±4.6	104.7±4.9	95.8±6.0*	83.6±9.8*
1	109.3±6.5	103.7±4.0	113.3±3.6	98.8±6.6	88.5±5.5	75.4±8.8*
2	86.1±6.8	76.9±6.1$^{\#}$	64.2±6.6$^{\#}$	61.2±1.2$^{\#*}$	48.2±6.9$^{\#*}$	36.2±4.4$^{\#*}$

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.

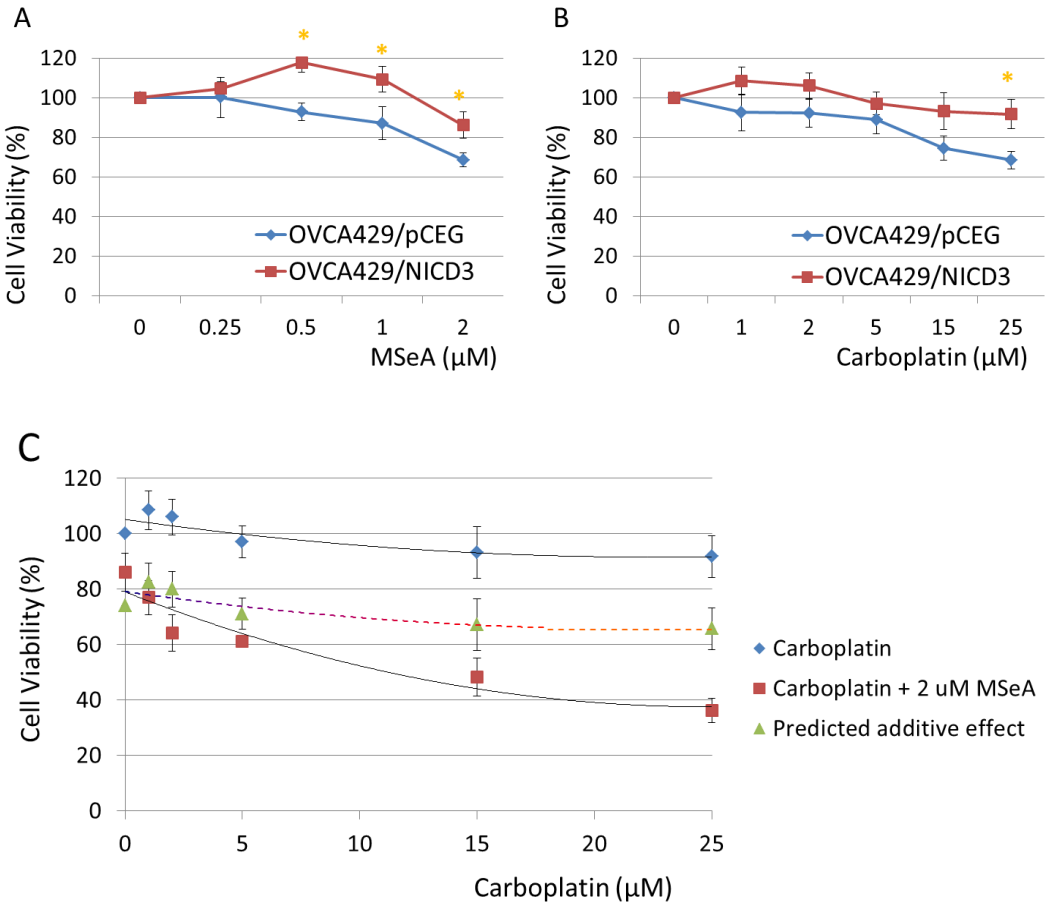


Figure 3.2 Sensitivity of OVCA429/pCEG and OVCA429/NICD3 cells to protein kinase inhibitors or NAC under the same concentration of carboplatin. OVCA429/pCEG cells were treated with MSeA, carboplatin, and KU 60019 (A), NU 7026 (B), or NAC (C). OVCA429/NICD3 cells were treated with MSeA, carboplatin, and KU 60019 (D), NU 7026 (E), or NAC (F). Values are mean ± S.E.M. (n = 3). *, $p < 0.05$, compared with NAC treatment.

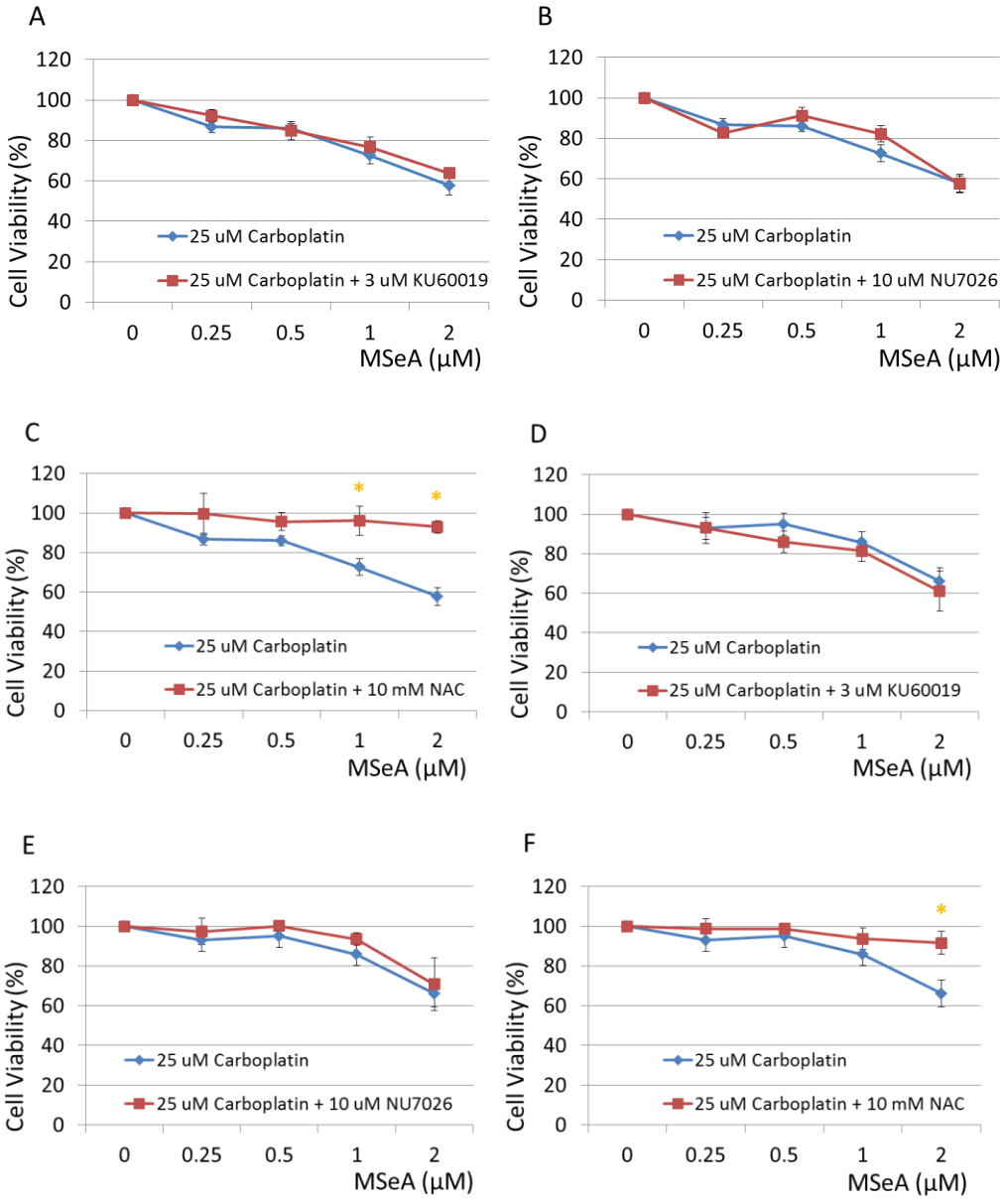
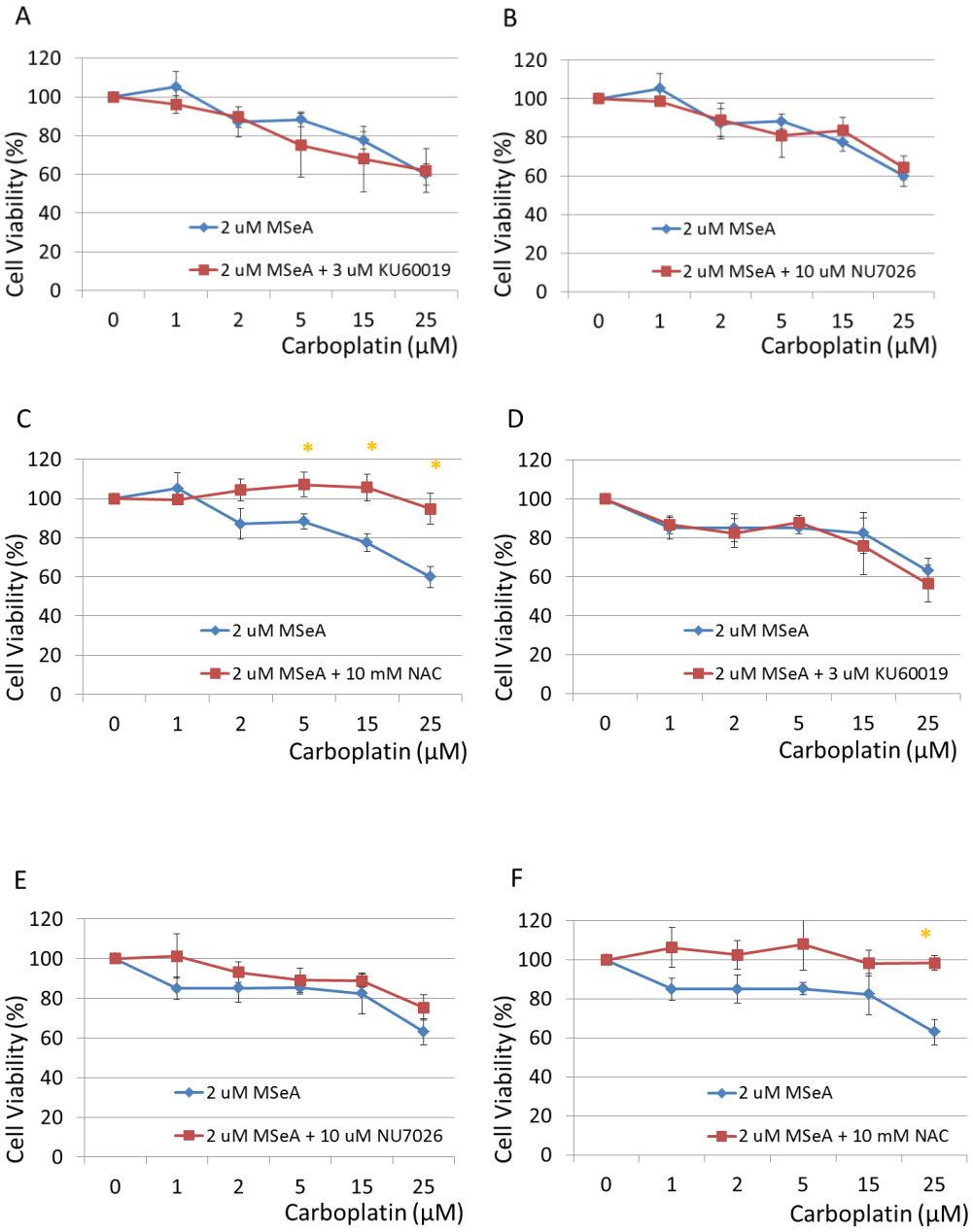


Figure 3.3 Sensitivity of OVCA429/pCEG and OVCA429/NICD3 cells to protein kinase inhibitors or NAC under the same concentration of MSeA. OVCA429/pCEG cells were treated with MSeA, carboplatin, and KU 60019 (A), NU 7026 (B), or NAC (C). OVCA429/NICD3 cells were treated with MSeA, carboplatin, and KU 60019 (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.

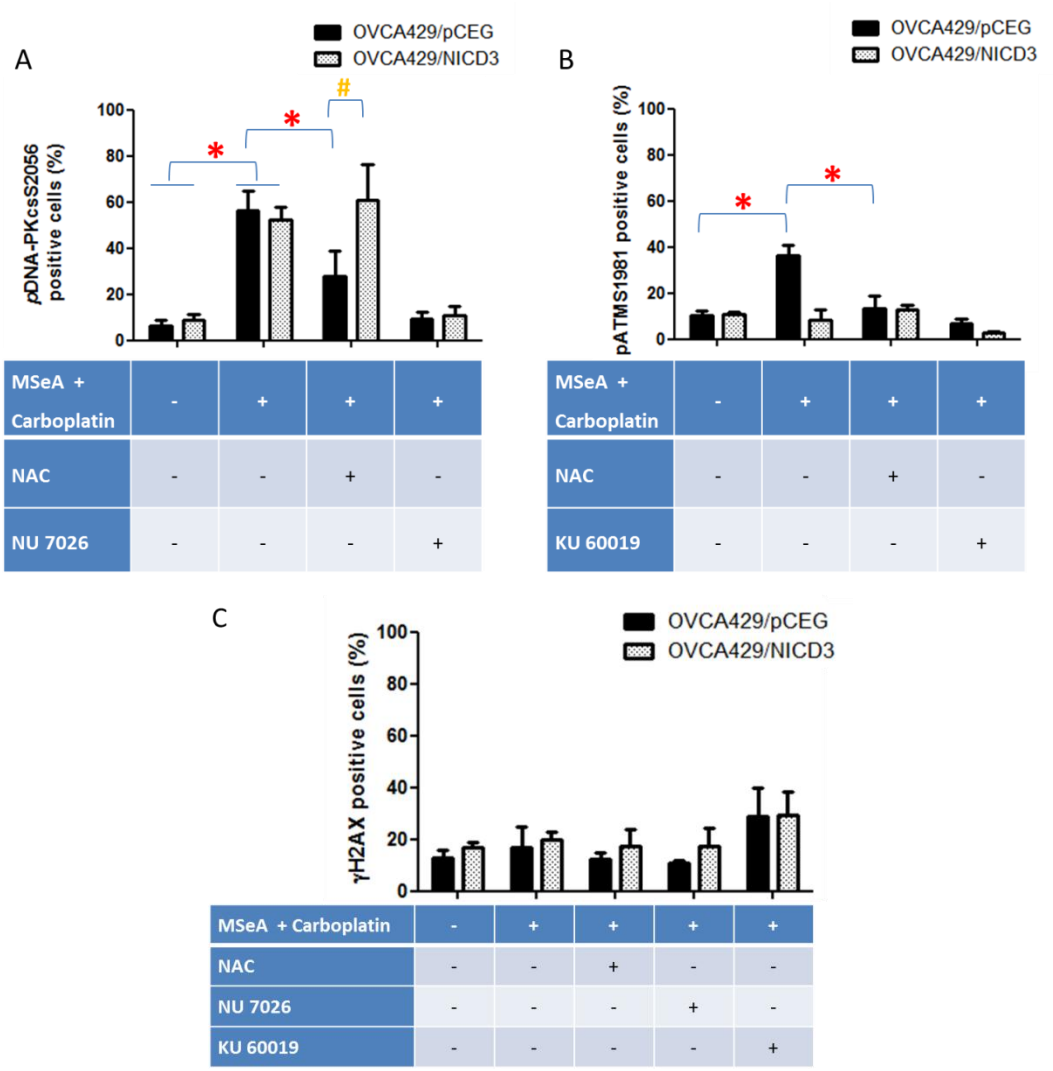
Table 3.2 Flow cytometric analyses of the percent G1, S, and G2/M OVCA429/pCEG and OVCA429/NICD3 cells treated with MSeA (2 μM) and carboplatin (5 μM) for 1 or 2 days. Values are mean ± S.E.M. (n = 3). *, $p < 0.05$, compared to OVCA429/NICD3 cells. #, $p < 0.05$, compared to Day 0.

	Day		
	0	1	2
Sub G1, %			
OVCA429/pCEG	0.7±0.1	2.2±0.4#	8.9±1.0#
OVCA429/NICD3	1.4±0.1	3.4±0.3#	9.1±0.9#
G1, %			
OVCA429/pCEG	32.7±1.0*	34.7±2.7	34.0±2.7
OVCA429/NICD3	13.4±0.6	12.7±0.8	15.7±0.3#
S, %			
OVCA429/pCEG	27.1±3.1*	25.1±2.7	18.2±1.2#
OVCA429/NICD3	12.3±0.6	13.9±0.7	12.4±0.8
G2/M, %			
OVCA429/pCEG	39.6±2.5*	38.0±0.5	38.9±2.5
OVCA429/NICD3	72.9±0.7	70.0±0.8	62.7±1.1#

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-PK_{cs} 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-PK_{cs}S2056 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 OVAC429/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).

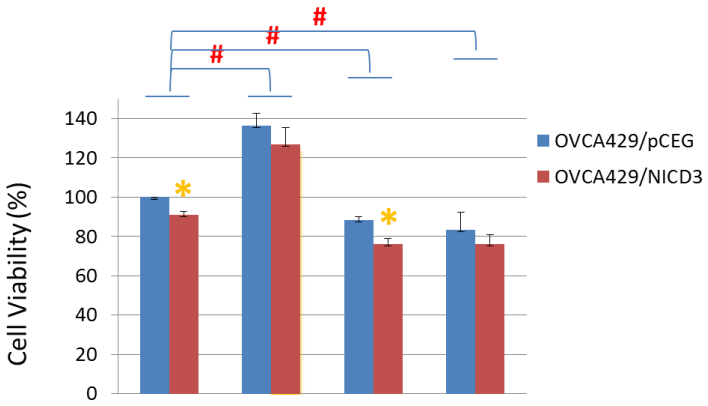
Figure 3.4 Immunofluorescent analyses of pDNA-PK$_{cs}$ S2056 (A), pATM S1981 (B) and γH2AX (C) in OVCA429/pCEG and OVCA429/NICD3 cells co-treated with MSeA (2 μM) and carboplatin (5 μM) for 24 h. Cells were co-treated with NU 7026 (10 μM), KU 60019 (10 μM) , or NAC (10 mM) Values are mean ± S.E.M. (n = 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-PK_{cs}, 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.



MSeA (µM)	2	2	2	2
Carboplatin (µM)	25	25	25	25
NAC (mM)		10		
KU 60019 (µM)	-	-	3	
NU 7026 (µM)	-	-	-	10

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-PK_{cs} (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-PK_{cs} inhibitor significantly decreased cell survival in both OVCA429/pCEG and OVCA429/NICD3 cells. pDNA-PK_{cs}S2056 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-PK_{cs}S2056 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

21. APE1: AP endonuclease 1

22. 5’-dRp: 5’-deoxyribose-5-phosphate

23. Pol β: DNA polymerase β

24. PCNA: proliferating cell nuclear antigen

25. RFC: replication factor C

26. FEN1: flap endonuclease 1

27. IDLs: Insertion/deletion lesions

28. MutSα: MSH2/MSH6

29. MutSβ: MSH2/MSH3

30. HNPCC: hereditary non-polyposis colorectal carcinoma history

31. HP: homologous recombination

32. NHEJ: non-homologous end joining

33. the catalytic subunit of DNA-PK: DNA-PK_{cs}

34. XLF: XRCC4-like factor

35. PNK: polynucleotide kinase

36. ssDNA: single-stranded DNA

37. CtIP: C-terminal binding protein interacting protein

38. MRN: MRE11-RAD50-NBS1

39. dsDNA: double strand DNA

40. BRCA2: breast cancer associated protein 2

41. MDC1: DNA damage checkpoint 1

42. 53BP1: tumor protein 53 binding protein 1

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. H_2O_2: hydrogen peroxide

74. TCA: trichloroacetic acid

75. DMSO: dimethyl sulfoxide

76. pATM S1981: ATM autophosphorylation at Ser-1981

77. pDNA-PK_{cs} S2056: pDNA-PK_{cs} autophosphorylation at Ser-2056

78. γH2AX: H2AX phosphorylation at Ser-139

References

Abraham, R. T. (2001). "Cell cycle checkpoint signaling through the ATM and ATR kinases." Genes Dev 15(17): 2177-2196.

Agarwal, M. L., *et al*. (1998). "A p53-dependent S-phase checkpoint helps to protect cells from DNA damage in response to starvation for pyrimidine nucleotides." Proc Natl Acad Sci U S A 95(25): 14775-14780.

Aifantis, I., *et al*. (2008). "Molecular pathogenesis of T-cell leukaemia and lymphoma." Nat Rev Immunol 8(5): 380-390.

Al-Gubory, K. H., *et al*. (2010). "The roles of cellular reactive oxygen species, oxidative stress and antioxidants in pregnancy outcomes." Int J Biochem Cell Biol 42(10): 1634-1650.

Arany, I. and R. L. Safirstein (2003). "Cisplatin nephrotoxicity." Semin Nephrol 23(5): 460-464.

Artavanis-Tsakonas, S., *et al*. (1995). "Notch signaling." Science 268(5208): 225-232.

Azrak, R. G., *et al*. (2008). "Silencing survivin results in synergy between methylseleninic acid and paclitaxel against skov3 ovarian cancer cells." Cancer Biology & Therapy 7(12): 1901-1908.

Barker, S., *et al*. (2005). "DNA-protein crosslinks: their induction, repair, and biological consequences." Mutat Res 589(2): 111-135.

Bassett, E., *et al*. (2002). "Frameshifts and deletions during in vitro translesion synthesis past Pt-DNA adducts by DNA polymerases beta and eta." DNA Repair (Amst) 1(12): 1003-1016.

Beatus, P. and U. Lendahl (1998). "Notch and neurogenesis." Journal of Neuroscience Research 54(2): 125-136.

Beck, M. A., *et al*. (1994). "Increased virulence of a human enterovirus (coxsackievirus B3) in selenium-deficient mice." J Infect Dis 170(2): 351-357.

Bellavia, D., *et al*. (2000). "Constitutive activation of NF-kappaB and T-cell leukemia/lymphoma in Notch3 transgenic mice." EMBO J 19(13): 3337-3348.

Bellavia, D., *et al*. (2008). "Notch3: from subtle structural differences to functional diversity." Oncogene 27(38): 5092-5098.

Bellinger, F. P., *et al*. (2008). "Association of selenoprotein p with Alzheimer's pathology in human cortex." J Alzheimers Dis 15(3): 465-472.

Bray, S. J. (2006). "Notch signalling: a simple pathway becomes complex." Nat Rev Mol Cell Biol 7(9): 678-689.

Budman, J. and G. Chu (2005). "Processing of DNA for nonhomologous end-joining by cell-free extract." Embo Journal 24(4): 849-860.

Cadron, I., *et al*. (2010). "The impact of enzastaurin (LY317615.HCl) on CA125 biosynthesis and shedding in ovarian cancer cells." Gynecol Oncol 118(1): 64-68.

Camphausen, K., *et al*. (2004). "Flavopiridol enhances human tumor cell radiosensitivity and prolongs expression of gammaH2AX foci." Mol Cancer Ther 3(4): 409-416.

Cao, H. and Y. Wang (2009). "Fragmentation of isomeric intrastrand crosslink lesions of DNA in an ion-trap mass spectrometer." J Am Soc Mass Spectrom 20(4): 611-617.

Cao, L., *et al*. (2006). "ATM-Chk2-p53 activation prevents tumorigenesis at an expense of organ homeostasis upon Brca1 deficiency." EMBO J 25(10): 2167-2177.

Celeste, A., *et al*. (2003). "H2AX haploinsufficiency modifies genomic stability and tumor susceptibility." Cell 114(3): 371-383.

Chang, I. Y., *et al*. (2005). "Small interfering RNA-induced suppression of ERCC1 enhances sensitivity of human cancer cells to cisplatin." Biochem Biophys Res Commun 327(1): 225-233.

Chappell, C., *et al*. (2002). "Involvement of human polynucleotide kinase in double-strand break repair by non-homologous end joining." EMBO J 21(11): 2827-2832.

Chen, H. H., *et al*. (2010). "Pravastatin attenuates carboplatin-induced nephrotoxicity in rodents via peroxisome proliferator-activated receptor alpha-regulated heme oxygenase-1." Mol Pharmacol 78(1): 36-45.

Chen, J. and M. J. Berry (2003). "Selenium and selenoproteins in the brain and brain diseases." J Neurochem 86(1): 1-12.

Cheng, C. F., *et al*. (2008). "Pravastatin attenuates carboplatin-induced cardiotoxicity via inhibition of oxidative stress associated apoptosis." Apoptosis 13(7): 883-894.

Cheng, W. H. (2009). "Impact of inorganic nutrients on maintenance of genomic stability." Environ Mol Mutagen 50(5): 349-360.

Cheng, W. H., *et al*. (1998). "Cellular glutathione peroxidase is the mediator of body selenium to protect against paraquat lethality in transgenic mice." J Nutr 128(7): 1070-1076.

Cheng, W. H., *et al*. (2012). "Targeting Werner syndrome protein sensitizes U-2 OS osteosarcoma cells to selenium-induced DNA damage response and necrotic death." Biochem Biophys Res Commun 420(1): 24-28.

Christmann, M., *et al*. (2005). "Fen1 is induced p53 dependently and involved in the recovery from UV-light-induced replication inhibition." Oncogene 24(56): 8304-8313.

Clark, A. B., *et al*. (2000). "Functional interaction of proliferating cell nuclear antigen with MSH2-MSH6 and MSH2-MSH3 complexes." J Biol Chem 275(47): 36498-36501.

Clark, L. C., *et al*. (1996). "Effects of selenium supplementation for cancer prevention in patients with carcinoma of the skin. A randomized controlled trial. Nutritional Prevention of Cancer Study Group." JAMA 276(24): 1957-1963.

Cleaver, J. E., *et al*. (1994). "Prenatal-Diagnosis of Xeroderma-Pigmentosum and Cockayne-Syndrome." Prenatal Diagnosis 14(10): 921-928.
Combs, G. F., Jr. (2004). "Status of selenium in prostate cancer prevention." Br J Cancer 91(2): 195-199.
Cooper, M. L., *et al*. (2008). "Interaction between single nucleotide polymorphisms in selenoprotein P and mitochondrial superoxide dismutase determines prostate cancer risk." Cancer Res 68(24): 10171-10177.
Cortez, D. (2005). "Unwind and slow down: checkpoint activation by helicase and polymerase uncoupling." Genes Dev 19(9): 1007-1012.
Costa, R. M., *et al*. (2003). "The eukaryotic nucleotide excision repair pathway." Biochimie 85(11): 1083-1099.
Cruet-Hennequart, S., *et al*. (2009). "Characterization of the effects of cisplatin and carboplatin on cell cycle progression and DNA damage response activation in DNA polymerase eta-deficient human cells." Cell Cycle 8(18): 3039-3050.
Darwanto, A., *et al*. (2009). "Mechanisms of base selection by human single-stranded selective monofunctional uracil-DNA glycosylase." J Biol Chem 284(23): 15835-15846.
Davoli, T. and T. de Lange (2011). "The causes and consequences of polyploidy in normal development and cancer." Annu Rev Cell Dev Biol 27: 585-610.
Deans, A. J. and S. C. West (2011). "DNA interstrand crosslink repair and cancer." Nat Rev Cancer 11(7): 467-480.
Demehri, S., *et al*. (2009). "Epidermal Notch1 loss promotes skin tumorigenesis by impacting the stromal microenvironment." Cancer Cell 16(1): 55-66.
Demple, B., *et al*. (1991). "Cloning and expression of APE, the cDNA encoding the major human apurinic endonuclease: definition of a family of DNA repair enzymes." Proc Natl Acad Sci U S A 88(24): 11450-11454.
Donzelli, E., *et al*. (2004). "Neurotoxicity of platinum compounds: comparison of the effects of cisplatin and oxaliplatin on the human neuroblastoma cell line SH-SY5Y." J Neurooncol 67(1-2): 65-73.
Drake, E. N. (2006). "Cancer chemoprevention: selenium as a prooxidant, not an antioxidant." Med Hypotheses 67(2): 318-322.
Dronkert, M. L. and R. Kanaar (2001). "Repair of DNA interstrand cross-links." Mutat Res 486(4): 217-247.
Duffield, A. J., *et al*. (1999). "An estimation of selenium requirements for New Zealanders." Am J Clin Nutr 70(5): 896-903.
Dunnick, J. K., *et al*. (1987). "Toxicity of 8-methoxypsoralen, 5-methoxypsoralen, 3-carbethoxypsoralen, or 5-methylisopsoralen with ultraviolet radiation in the hairless (HRA/Skh) mouse." Toxicol Appl Pharmacol 89(1): 73-80.
Eastman, A. and M. A. Barry (1987). "Interaction of trans-diamminedichloroplatinum(II) with DNA: formation of monofunctional adducts and their reaction with glutathione." Biochemistry 26(12): 3303-3307.

El-Bayoumy, K. (2009). "The negative results of the SELECT study do not necessarily discredit the selenium-cancer prevention hypothesis." Nutr Cancer 61(3): 285-286.

Elbendary, A., *et al*. (1994). "Transforming growth factor beta 1 can induce CIP1/WAF1 expression independent of the p53 pathway in ovarian cancer cells." Cell Growth Differ 5(12): 1301-1307.

Evans, E., *et al*. (1997). "Open complex formation around a lesion during nucleotide excision repair provides a structure for cleavage by human XPG protein." EMBO J 16(3): 625-638.

Fakih, M. G., *et al.* (2008). "A phase I and pharmacokinetic study of selenomethionine in combination with a fixed dose of irinotecan in solid tumors." Cancer Chemother Pharmacol 62(3): 499-508

Falck, J., *et al*. (2005). "Conserved modes of recruitment of ATM, ATR and DNA-PKcs to sites of DNA damage." Nature 434(7033): 605-611.

Falck, J., *et al*. (2001). "The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis." Nature 410(6830): 842-847.

Ferry, K. V., *et al*. (2000). "Increased nucleotide excision repair in cisplatin-resistant ovarian cancer cells: role of ERCC1-XPF." Biochem Pharmacol 60(9): 1305-1313.

Fishel, M. L., *et al*. (2007). "Manipulation of base excision repair to sensitize ovarian cancer cells to alkylating agent temozolomide." Clin Cancer Res 13(1): 260-267.

Forrest, R. A., *et al*. (2012). "Activation of DNA damage response pathways as a consequence of anthracycline-DNA adduct formation." Biochem Pharmacol 83(12): 1602-1612.

Fortini, P. and E. Dogliotti (2007). "Base damage and single-strand break repair: mechanisms and functional significance of short- and long-patch repair subpathways." DNA Repair (Amst) 6(4): 398-409.

Fritz, H., *et al*. (2011). "Selenium and lung cancer: a systematic review and meta analysis." PLoS One 6(11): e26259.

Gao, R. J., *et al*. (2012). "Methylseleninic acid is a novel suppressor of aromatase expression." Journal of Endocrinology 212(2): 199-205.

Gao, Z., *et al*. (2011). "C terminus of Clostridium perfringens enterotoxin downregulates CLDN4 and sensitizes ovarian cancer cells to Taxol and Carboplatin." Clin Cancer Res 17(5): 1065-1074.

Garcia, A. and J. J. Kandel (2012). "Notch: a key regulator of tumor angiogenesis and metastasis." Histol Histopathol 27(2): 151-156.

Gellon, L., *et al*. (2008). "Intrinsic 5 '-deoxyribose-5-phosphate lyase activity in Saccharomyces cerevisiae Trf4 protein with a possible role in base excision DNA repair." DNA Repair (Amst) 7(2): 187-198.

Goldstein, B. J., *et al*. (2005). "Role of insulin-induced reactive oxygen species in the insulin signaling pathway." Antioxid Redox Signal 7(7-8): 1021-1031.

Goodarzi, A. A., *et al*. (2008). "ATM signaling facilitates repair of DNA double-strand breaks associated with heterochromatin." Mol Cell 31(2): 167-177.

Goodarzi, A. A., *et al*. (2008). "ATM signaling facilitates repair of DNA double-strand breaks associated with heterochromatin." Mol Cell 31(2): 167-177.

Gruenert, D. C., *et al*. (1985). "Induction of DNA-DNA cross-link formation in human cells by various psoralen derivatives." Cancer Res 45(11 Pt 1): 5394-5398.

Hagopian, G. S., *et al*. (1999). "Expression of p53 in cisplatin-resistant ovarian cancer cell lines: modulation with the novel platinum analogue (1R, 2R-diaminocyclohexane)(trans-diacetato)(dichloro)-platinum(IV)." Clin Cancer Res 5(3): 655-663.

Hamanishi, T., *et al*. (2004). "Functional variants in the glutathione peroxidase-1 (GPx-1) gene are associated with increased intima-media thickness of carotid arteries and risk of macrovascular diseases in japanese type 2 diabetic patients." Diabetes 53(9): 2455-2460.

Hanaoka, T., *et al*. (1997). "Elevated serum levels of pantropic p53 proteins in chromium workers." Scand J Work Environ Health 23(1): 37-40.

Hawkes, W. C. and L. Hornbostel (1996). "Effects of dietary selenium on mood in healthy men living in a metabolic research unit." Biol Psychiatry 39(2): 121-128.

Helleday, T., *et al*. (2007). "DNA double-strand break repair: from mechanistic understanding to cancer treatment." DNA Repair (Amst) 6(7): 923-935.

High, F. A. and J. A. Epstein (2008). "The multifaceted role of Notch in cardiac development and disease." Nat Rev Genet 9(1): 49-61.

Hinz, J. M. (2010). "Role of homologous recombination in DNA interstrand crosslink repair." Environ Mol Mutagen 51(6): 582-603.

Hiss, D. (2012). "OptimizingMolecular-Targeted Therapies in Ovarian Cancer: The Renewed Surge of Interest in Ovarian Cancer Biomarkers and Cell Signaling Pathways." J Oncol 2012:737981.

Hoeijmakers, J. H. (2001). "Genome maintenance mechanisms for preventing cancer." Nature 411(6835): 366-374.

Holzer, A. K., *et al*. (2006). "Contribution of the major copper influx transporter CTR1 to the cellular accumulation of cisplatin, carboplatin, and oxaliplatin." Mol Pharmacol 70(4): 1390-1394.

Howell, S. B. (2008). "Pharmacologic principles of intraperitoneal chemotherapy for the treatment of ovarian cancer." Int J Gynecol Cancer 18 Suppl 1: 20-25.

Ide, H., *et al*. (2011). "Repair and biochemical effects of DNA-protein crosslinks." Mutat Res 711(1-2): 113-122.

Iijima, K., *et al*. (2008). "Dancing on damaged chromatin: functions of ATM and the RAD50/MRE11/NBS1 complex in cellular responses to DNA damage." J Radiat Res 49(5): 451-464.

Ip, C., *et al*. (2000). "In vitro and in vivo studies of methylseleninic acid: evidence that a monomethylated selenium metabolite is critical for cancer chemoprevention." Cancer Res 60(11): 2882-2886.

Ishida, S., *et al*. (2002). "Uptake of the anticancer drug cisplatin mediated by the copper transporter Ctr1 in yeast and mammals." Proc Natl Acad Sci U S A 99(22): 14298-14302.

Itamochi, H., *et al*. (2002). "Low proliferation activity may be associated with chemoresistance in clear cell carcinoma of the ovary." Obstet Gynecol 100(2): 281-287.

Jacobson, G. K. (2010). "Dietary selenium and selenized yeast." Agro Food Industry Hi-Tech 21(4): 14-16.

Janus, F., *et al*. (1999). "The dual role model for p53 in maintaining genomic integrity." Cell Mol Life Sci 55(1): 12-27.

Jiricny, J. (2006). "The multifaceted mismatch-repair system." Nat Rev Mol Cell Biol 7(5): 335-346.

Johnson, S. W., *et al*. (1994). "Relationship between platinum-DNA adduct formation and removal and cisplatin cytotoxicity in cisplatin-sensitive and -resistant human ovarian cancer cells." Cancer Res 54(22): 5911-5916.

Joutel, A. and E. Tournier-Lasserve (1998). "Notch signalling pathway and human diseases." Semin Cell Dev Biol 9(6): 619-625.

Kanaar, R. and C. Wyman (2008). "DNA repair by the MRN complex: break it to make it." Cell 135(1): 14-16.

Kao, S. C., *et al*. (2011). "Second-line therapy for castrate-resistant prostate cancer: a literature review." Asia Pac J Clin Oncol 7(3): 212-223.

Kelland, L. (2007). "The resurgence of platinum-based cancer chemotherapy." Nat Rev Cancer 7(8): 573-584.

Klinakis, A., *et al*. (2011). "A novel tumour-suppressor function for the Notch pathway in myeloid leukaemia." Nature 473(7346): 230-233.

Koberle, B., *et al*. (1999). "Defective repair of cisplatin-induced DNA damage caused by reduced XPA protein in testicular germ cell tumours." Curr Biol 9(5): 273-276.

Koch, C. A., *et al*. (2004). "Xrcc4 physically links DNA end processing by polynucleotide kinase to DNA ligation by DNA ligase IV." EMBO J 23(19): 3874-3885.

Kojima, M., *et al*. (1994). "Potentiation of cis-diammine(1,1-cyclobutanedicarboxylato)platinum(II) by amphotericin B in BALB/c nude mice bearing human ovarian carcinoma cells." Jpn J Cancer Res 85(11): 1159-1164.

Kovall, R. A. (2008). "More complicated than it looks: assembly of Notch pathway transcription complexes." Oncogene 27(38): 5099-5109.

Kozekov, I. D., *et al*. (2003). "DNA interchain cross-links formed by acrolein and crotonaldehyde." J Am Chem Soc 125(1): 50-61.

Kryukov, G. V., *et al*. (2003). "Characterization of mammalian selenoproteomes." Science 300(5624): 1439-1443.

Kuhne, M., *et al*. (2004). "A double-strand break repair defect in ATM-deficient cells contributes to radiosensitivity." Cancer Res 64(2): 500-508.

Kunkel, T. A. and D. A. Erie (2005). "DNA mismatch repair." Annu Rev Biochem 74: 681-710.

Kwintkiewicz, J., *et al*. (2012). "Metastasis-associated protein 3 (MTA3) regulates G2/M progression in proliferating mouse granulosa cells." Biol Reprod 86(3): 1-8.

Langerak, P. and P. Russell (2011). "Regulatory networks integrating cell cycle control with DNA damage checkpoints and double-strand break repair." Philos Trans R Soc Lond B Biol Sci 366(1584): 3562-3571.

Larson, C. A., *et al*. (2009). "The role of the mammalian copper transporter 1 in the cellular accumulation of platinum-based drugs." Mol Pharmacol 75(2): 324-330.

Ledermann, J. A., *et al*. (2010). "Inhibition of carboplatin-induced DNA interstrand cross-link repair by gemcitabine in patients receiving these drugs for platinum-resistant ovarian cancer." Clin Cancer Res 16(19): 4899-4905.

Lee, J. H. and T. T. Paull (2005). "ATM activation by DNA double-strand breaks through the Mre11-Rad50-Nbs1 complex." Science 308(5721): 551-554.

Lee, K. B., *et al*. (2012). "CYR61 controls p53 and NF-kappaB expression through PI3K/Akt/mTOR pathways in carboplatin-induced ovarian cancer cells." Cancer Lett 315(1): 86-95.

Lewis, L. K., *et al*. (2004). "Role of the nuclease activity of Saccharomyces cerevisiae Mre11 in repair of DNA double-strand breaks in mitotic cells." Genetics 166(4): 1701-1713.

Li, G. X., *et al*. (2008). "Superior in vivo inhibitory efficacy of methylseleninic acid against human prostate cancer over selenomethionine or selenite." Carcinogenesis 29(5): 1005-1012.

Li, J. and D. F. Stern (2005). "DNA damage regulates Chk2 association with chromatin." J Biol Chem 280(45): 37948-37956.

Lieber, M. R., *et al*. (2004). "The mechanism of vertebrate nonhomologous DNA end joining and its role in V(D)J recombination." DNA Repair (Amst) 3(8-9): 817-826.

Limbo, O., *et al*. (2007). "Ctp1 is a cell-cycle-regulated protein that functions with Mre11 complex to control double-strand break repair by homologous recombination." Mol Cell 28(1): 134-146.

Liu, H., *et al*. (2010). "Notch3 is critical for proper angiogenesis and mural cell investment." Circ Res 107(7): 860-870.

Lobrich, M., *et al*. (2010). "gammaH2AX foci analysis for monitoring DNA double-strand break repair: strengths, limitations and optimization." Cell Cycle 9(4): 662-669.

Lobry, C., *et al*. (2011). "Oncogenic and tumor suppressor functions of Notch in cancer: it's NOTCH what you think." J Exp Med 208(10): 1931-1935.

Lord, C. J., *et al*. (2006). "Targeting the double-strand DNA break repair pathway as a therapeutic strategy." Clin Cancer Res 12(15): 4463-4468.

Lundin, C., *et al*. (2002). "Different roles for nonhomologous end joining and homologous recombination following replication arrest in mammalian cells." Mol Cell Biol 22(16): 5869-5878.
Ma, Y., *et al*. (2005). "Repair of double-strand DNA breaks by the human nonhomologous DNA end joining pathway: the iterative processing model." Cell Cycle 4(9): 1193-1200.
Ma, Y., *et al*. (2002). "Hairpin opening and overhang processing by an Artemis/DNA-dependent protein kinase complex in nonhomologous end joining and V(D)J recombination." Cell 108(6): 781-794.
Maillard, I. and W. S. Pear (2003). "Notch and cancer: best to avoid the ups and downs." Cancer Cell 3(3): 203-205.
Malina, J., *et al*. (2007). "Conformation of DNA GG intrastrand cross-link of antitumor oxaliplatin and its enantiomeric analog." Biophys J 93(11): 3950-3962.
Mao, Z., *et al*. (2008). "DNA repair by nonhomologous end joining and homologous recombination during cell cycle in human cells." Cell Cycle 7(18): 2902-2906.
Matsumoto, Y. (2001). "Molecular mechanism of PCNA-dependent base excision repair." Prog Nucleic Acid Res Mol Biol 68: 129-138.
McAuliffe, S. M., *et al*. (2012). "Targeting Notch, a key pathway for ovarian cancer stem cells, sensitizes tumors to platinum therapy." Proc Natl Acad Sci U S A 109(43): E2939-2948.
McCabe, K. M., *et al*. (2009). "DNA interstrand crosslink repair in mammalian cells." J Cell Physiol 220(3): 569-573.
McHugh, P. J., *et al*. (2000). "Repair of intermediate structures produced at DNA interstrand cross-links in Saccharomyces cerevisiae." Mol Cell Biol 20(10): 3425-3433.
McHugh, P. J., *et al*. (2001). "Repair of DNA interstrand crosslinks: molecular mechanisms and clinical relevance." Lancet Oncol 2(8): 483-490.
Merk, O. and G. Speit (1998). "Significance of formaldehyde-induced DNA-protein crosslinks for mutagenesis." Environ Mol Mutagen 32(3): 260-268.
Misdraji, J., *et al*. (2004). "Defective mismatch repair in the pathogenesis of low-grade appendiceal mucinous neoplasms and adenocarcinomas." Mod Pathol 17(12): 1447-1454.
Mitchell, R. J., *et al*. (2002). "Mismatch repair genes hMLH1 and hMSH2 and colorectal cancer: a HuGE review." Am J Epidemiol 156(10): 885-902.
Moon, I. J., *et al*. (2011). "N-acetylcysteine and N-nitroarginine methyl ester attenuate Carboplatin-induced ototoxicity in dissociated spiral ganglion neuron cultures." Clin Exp Otorhinolaryngol 4(1): 11-17.
Moreno-Reyes, R., *et al*. (1998). "Kashin-Beck osteoarthropathy in rural Tibet in relation to selenium and iodine status." N Engl J Med 339(16): 1112-1120.
Mosner, J., *et al*. (1995). "Negative feedback regulation of wild-type p53 biosynthesis." EMBO J 14(18): 4442-4449.

Muniandy, P. A., *et al*. (2010). "DNA interstrand crosslink repair in mammalian cells: step by step." Crit Rev Biochem Mol Biol 45(1): 23-49.
Murray, J. M., *et al*. (2012). "DNA double-strand break repair within heterochromatic regions." Biochem Soc Trans 40(1): 173-178.
Nicolas, M., *et al*. (2003). "Notch1 functions as a tumor suppressor in mouse skin." Nat Genet 33(3): 416-421.
Noll, D. M., *et al*. (2006). "Formation and repair of interstrand cross-links in DNA." Chem Rev 106(2): 277-301.
O'Driscoll, M. and P. A. Jeggo (2006). "The role of double-strand break repair - insights from human genetics." Nat Rev Genet 7(1): 45-54.
O'Dwyer, P. J., *et al*. (2000). "Clinical pharmacokinetics and administration of established platinum drugs." Drugs 59 Suppl 4: 19-27.
Oguri, S., *et al*. (1988). "Clinical pharmacokinetics of carboplatin." J Clin Pharmacol 28: 208-15.
Okur, E., *et al*. (2007). "Effect of N-acetylcysteine on carboplatin-induced ototoxicity and nitric oxide levels in a rat model." Laryngoscope 117(12): 2183-2186.
Okuyama, R., *et al*. (2004). "High commitment of embryonic keratinocytes to terminal differentiation through a Notch1-caspase 3 regulatory mechanism." Dev Cell 6(4): 551-562.
Panelos, J. and D. Massi (2009). "Emerging role of Notch signaling in epidermal differentiation and skin cancer." Cancer Biology & Therapy 8(21): 1986-1993.
Papp, L. V., *et al*. (2007). "From selenium to selenoproteins: synthesis, identity, and their role in human health." Antioxid Redox Signal 9(7): 775-806.
Park, J. T., *et al*. (2010). "Notch3 overexpression is related to the recurrence of ovarian cancer and confers resistance to carboplatin." Am J Pathol 177(3): 1087-1094.
Park, J. T., *et al*. (2006). "Notch3 gene amplification in ovarian cancer." Cancer Res 66(12): 6312-6318.
Paull, T. T., *et al*. (2000). "A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage." Curr Biol 10(15): 886-895.
Podhorecka, M., *et al*. (2010). "H2AX Phosphorylation: Its Role in DNA Damage Response and Cancer Therapy." J Nucleic Acids 2010.
Poklar, N., *et al*. (1996). "Influence of cisplatin intrastrand crosslinking on the conformation, thermal stability, and energetics of a 20-mer DNA duplex." Proc Natl Acad Sci U S A 93(15): 7606-7611.
Prohaska, J. R. and H. E. Ganther (1976). "Selenium and glutathione peroxidase in developing rat brain." J Neurochem 27(6): 1379-1387.
Purow, B. (2012). "Notch inhibition as a promising new approach to cancer therapy." Adv Exp Med Biol 727: 305-319.

Qi, Y., *et al*. (2010). "Selenium compounds activate ATM-dependent DNA damage response via the mismatch repair protein hMLH1 in colorectal cancer cells." J Biol Chem 285(43): 33010-33017.
Qian, X., *et al*. (2006). "Activity of PXD101, a histone deacetylase inhibitor, in preclinical ovarian cancer studies." Mol Cancer Ther 5(8): 2086-2095.
Rabik, C. A. and M. E. Dolan (2007). "Molecular mechanisms of resistance and toxicity associated with platinating agents." Cancer Treat Rev 33(1): 9-23.
Radtke, F. and K. Raj (2003). "The role of Notch in tumorigenesis: oncogene or tumour suppressor?" Nat Rev Cancer 3(10): 756-767.
Rayman, M. P. (2000). "The importance of selenium to human health." Lancet 356(9225): 233-241.
Rayman, M. P. (2012). "Selenium and human health." Lancet 379(9822): 1256-1268.
Rayman, M. P., *et al*. (2009). "Selenium and vitamin E supplementation for cancer prevention." JAMA 301(18): 1876; author reply 1877.
Reed, E. (2005). "ERCC1 and clinical resistance to platinum-based therapy." Clin Cancer Res 11(17): 6100-6102.
Reich, N. C. and A. J. Levine (1984). "Growth regulation of a cellular tumour antigen, p53, in nontransformed cells." Nature 308(5955): 199-201.
Rizvi, I., *et al*. (2010). "Synergistic enhancement of carboplatin efficacy with photodynamic therapy in a three-dimensional model for micrometastatic ovarian cancer." Cancer Res 70(22): 9319-9328.
Rizzo, P., *et al*. (2008). "Rational targeting of Notch signaling in cancer." Oncogene 27(38): 5124-5131.
Robinson, J. R., *et al*. (1985). "Urinary excretion of selenium by New Zealand and North American human subjects on differing intakes." Am J Clin Nutr 41(5): 1023-1031.
Rocourt, C. R., *et al*. (2012). "The catalytic subunit of DNA-dependent protein kinase is downstream of ATM and feeds forward oxidative stress in the selenium-induced senescence response." J Nutr Biochem.
Rogakou, E. P., *et al*. (1999). "Megabase chromatin domains involved in DNA double-strand breaks in vivo." J Cell Biol 146(5): 905-916.
Rose, S. L. (2009). "Notch signaling pathway in ovarian cancer." Int J Gynecol Cancer 19(4): 564-566.
Rosenberg, B., *et al*. (1965). "Inhibition of Cell Division in Escherichia Coli by Electrolysis Products from a Platinum Electrode." Nature 205: 698-699.
Rosenberg, B., *et al*. (1969). "Platinum compounds: a new class of potent antitumour agents." Nature 222(5191): 385-386.
Rothkamm, K., *et al*. (2003). "Pathways of DNA double-strand break repair during the mammalian cell cycle." Mol Cell Biol 23(16): 5706-5715.
Ruppenthal, S. L., *et al*. (2007). "Interference between p53 and cdc25C in cell cycle regulation." Int J Oncol 31(2): 345-352.
Sadofsky, M. J. (2001). "The RAG proteins in V(D)J recombination: more than just a nuclease." Nucleic Acids Res 29(7): 1399-1409.

Schafer, Z. T., *et al*. (2009). "Antioxidant and oncogene rescue of metabolic defects caused by loss of matrix attachment." Nature 461(7260): 109-113.
Schomburg, L., *et al*. (2003). "Gene disruption discloses role of selenoprotein P in selenium delivery to target tissues." Biochem J 370(Pt 2): 397-402.
Schrauzer, G. N. (2000). "Selenomethionine: a review of its nutritional significance, metabolism and toxicity." J Nutr 130(7): 1653-1656.
Shaham, J., *et al*. (2003). "DNA-protein crosslinks and p53 protein expression in relation to occupational exposure to formaldehyde." Occup Environ Med 60(6): 403-409.
Shih Ie, M. and B. Davidson (2009). "Pathogenesis of ovarian cancer: clues from selected overexpressed genes." Future Oncol 5(10): 1641-1657.
Shrivastav, M., *et al*. (2008). "Regulation of DNA double-strand break repair pathway choice." Cell Res 18(1): 134-147.
Siddik, Z. H., *et al*. (1998). "Independent pathways of p53 induction by cisplatin and X-rays in a cisplatin-resistant ovarian tumor cell line." Cancer Res 58(4): 698-703.
Siegel, R., *et al*. (2012). "Cancer Statistics, 2012." Ca-a Cancer Journal for Clinicians 62(1): 10-29.
Soerensen, J., *et al*. (2008). "The role of thioredoxin reductases in brain development." PLoS One 3(3): e1813.
Song, I. S., *et al*. (2005). "Role of human copper transporter Ctr1 in the transport of platinum-based antitumor agents in cisplatin-sensitive and cisplatin-resistant cells. (vol 4, pg 113, 2005)." Mol Cancer Ther 4(5): 864-864.
Steinbrenner, H. and H. Sies (2009). "Protection against reactive oxygen species by selenoproteins." Biochim Biophys Acta 1790(11): 1478-1485.
Stone, M. P., *et al*. (2008). "Interstrand DNA cross-links induced by alpha,beta-unsaturated aldehydes derived from lipid peroxidation and environmental sources." Acc Chem Res 41(7): 793-804.
Suchankova, T., *et al*. (2012). "Platinum-DNA interstrand crosslinks: molecular determinants of bending and unwinding of the double helix." J Inorg Biochem 108: 69-79.
Takahashi, P., *et al*. (2011). "Elevated transcription of the p53 gene in early S-phase leads to a rapid DNA-damage response during S-phase of the cell cycle." Apoptosis 16(9): 950-958.
Takashima, Y., *et al*. (2009). "Dependence of DNA double strand break repair pathways on cell cycle phase in human lymphoblastoid cells." Environ Mol Mutagen 50(9): 815-822.
Takemoto, A. S., *et al*. (2010). "Role of selenoprotein P in Alzheimer's disease." Ethn Dis 20(1 Suppl 1): S1-92-95.
Thorstenson, Y. R., *et al*. (2003). "Contributions of ATM mutations to familial breast and ovarian cancer." Cancer Res 63(12): 3325-3333.
Tien, A. C., *et al*. (2009). "A Notch updated." J Cell Biol 184(5): 621-629.

Tyagi, A. K., *et al.* (2002). "Silibinin strongly synergizes human prostate cancer DU145 cells to doxorubicin-induced growth inhibition, G2-M arrest, and apoptosis." Clin Cancer Res 8(11):3512-9.

Unger, F. T., *et al*. (2009). "DNA damage induced by cis- and carboplatin as indicator for in vitro sensitivity of ovarian carcinoma cells." BMC Cancer 9: 359.

Uto, K., *et al*. (2004). "Chk1, but not Chk2, inhibits Cdc25 phosphatases by a novel common mechanism." EMBO J 23(16): 3386-3396.

van der Vijgh, W. J. (1991). "Clinical pharmacokinetics of carboplatin." Clin Pharmacokinet 21(4): 242-261.

Vichai, V. and K. Kirtikara (2006). "Sulforhodamine B colorimetric assay for cytotoxicity screening." Nat Protoc 1(3): 1112-1116.

Vural, H., *et al*. (2010). "Alterations of plasma magnesium, copper, zinc, iron and selenium concentrations and some related erythrocyte antioxidant enzyme activities in patients with Alzheimer's disease." J Trace Elem Med Biol 24(3): 169-173.

Wagstaff, A. J., *et al*. (1989). "Carboplatin. A preliminary review of its pharmacodynamic and pharmacokinetic properties and therapeutic efficacy in the treatment of cancer." Drugs 37(2): 162-190.

Wang, L., *et al*. (2009). "Methyl-selenium compounds inhibit prostate carcinogenesis in the transgenic adenocarcinoma of mouse prostate model with survival benefit." Cancer Prev Res (Phila) 2(5): 484-495.

Wang, T., *et al*. (2008). "An overview of Notch3 function in vascular smooth muscle cells." Prog Biophys Mol Biol 96(1-3): 499-509.

Wang, Z., *et al*. (2010). "Targeting Notch signaling pathway to overcome drug resistance for cancer therapy." Biochim Biophys Acta 1806(2): 258-267.

Wang, Z., *et al*. (2008). "Exploitation of the Notch signaling pathway as a novel target for cancer therapy." Anticancer Res 28(6A): 3621-3630.

Wei, W. and E. W. Englander (2008). "DNA polymerase beta-catalyzed-PCNA independent long patch base excision repair synthesis: a mechanism for repair of oxidatively damaged DNA ends in post-mitotic brain." J Neurochem 107(3): 734-744.

Welsh, C., *et al*. (2004). "Reduced levels of XPA, ERCC1 and XPF DNA repair proteins in testis tumor cell lines." International Journal of Cancer 110(3): 352-361.

Whanger, P. D. (1989). "China, a country with both selenium deficiency and toxicity: some thoughts and impressions." J Nutr 119(9): 1236-1239.

Wheate, N. J., *et al*. (2010). "The status of platinum anticancer drugs in the clinic and in clinical trials." Dalton Trans 39(35): 8113-8127.

Williams, G. J., *et al*. (2010). "Mre11-Rad50-Nbs1 conformations and the control of sensing, signaling, and effector responses at DNA double-strand breaks." DNA Repair (Amst) 9(12): 1299-1306.

Wu, L., *et al*. (2004). "Oxidative stress is a mediator of glucose toxicity in insulin-secreting pancreatic islet cell lines." J Biol Chem 279(13): 12126-12134.

Wu, M., *et al*. (2010). "Selenium compounds activate early barriers of tumorigenesis." J Biol Chem 285(16): 12055-12062.
Xia, Y., *et al*. (2005). "Effectiveness of selenium supplements in a low-selenium area of China." Am J Clin Nutr 81(4): 829-834.
Yao, X., *et al*. (2007). "Cisplatin nephrotoxicity: a review." Am J Med Sci 334(2): 115-124.
Zeng, Y. and Y. Wang (2006). "Sequence-dependent formation of intrastrand crosslink products from the UVB irradiation of duplex DNA containing a 5-bromo-2'-deoxyuridine or 5-bromo-2'-deoxycytidine." Nucleic Acids Res 34(22): 6521-6529.
Zhou, B. B. and J. Bartek (2004). "Targeting the checkpoint kinases: chemosensitization versus chemoprotection." Nat Rev Cancer 4(3): 216-225.
Zou, X., *et al*. (2001). "The cell cycle-regulatory CDC25A phosphatase inhibits apoptosis signal-regulating kinase 1." Mol Cell Biol 21(14): 4818-4828.