GENE EXPRESSION KINETICS AND PROTEIN DISTRIBUTION OF NUCLEOTIDE EXCISION REPAIR FACTORS IN THE INNER EAR AS A FUNCTION OF *cis*-DIAMMINEDICHLOROPLATINUM-II DNA DAMAGE

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The kinetics of the rate-limiting genes of the molecular DNA repair pathways of nucleotide excision repair (NER) were quantified from the inner ear as a function of *cis*-diamminedichloroplatinum-II (cisplatin) treatment. The distribution of the post-translational products of these genes was evaluated among neurons and sensory hair cells of the inner ear following cisplatin treatment. These NER factors (genes & post-translational products) are only potentiated by DNA damage and are particularly sensitive to cisplatin induced DNA damage. A 2 x 3 x 2 factorial design, consisting of two treatment conditions (saline and cisplatin treated Fischer344 rats), three survival times and two molecular analysis methods (polymerase chain reaction and immunohistochemistry) was employed in this dissertation. The results revealed at least five important findings. **First**, it revealed for the first time that complex DNA repair molecular pathways such as NER exist in the inner ear. **Second**, it revealed for the first time that molecules used by advanced tumor cells to detect and repair damaged DNA from cisplatin genotoxicity also generalize to the inner ear and are stimulated by even small sub-toxic doses of cisplatin. **Third**, it revealed for the first time that NER proteins reside in the cytoplasm of neurons under normal conditions and translocate to the nucleus under conditions of genomic stress. **Fourth**, it revealed for the first time that the basal coil of the mammalian cochlea differs

from the apical coil in the magnitude and latency in which NER molecules translocate from the cytoplasm to the nucleus under conditions of genomic stress. **Fifth**, the current work provides the bases for a new line of hearing research focused on molecular mechanisms of inner ear DNA repair.

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PREFACE

Research on cisplatin ototoxicity has revealed that various inner ear epithelia are largely resistant to cisplatin chemotherapy. For instance, the vestibular epithelia are mostly unaffected and the nonsensory epithelia of the cochlea survive cisplatin treatment. Tumor biology has revealed that complex DNA repair mechanisms contribute to cell survival after cisplatin treatment. Nucleotide excision repair (NER) is the major defense strategy by which cisplatin-resistant tumor cells identify and repair cisplatin induced DNA damage. This suggests, that the inner ear also may use NER as a defense strategy. Gene expression kinetics of the rate-limiting genes of global genomic NER were studied as a function of chronic cisplatin chemotherapy to test this possibility. The specific genes studied were xeroderma pigmentosum (XP) *c* and *a*. These genes are only up-regulated in the presence of DNA damage and cisplatin DNA damage is a particularly potent stimulus for their up-regulation. The results revealed statistically significant up-regulation in gene expression for both *xpc* and *xpa* after cisplatin treatment. This work is significant to hearing research for at least three reasons. **First**, it reveals for the first time that complex DNA repair molecular pathways such as NER respond to DNA injury in the ear. The NER reaction pathway was originally considered to be a means by which organisms removed damaged DNA induced by sun-light (UV radiation). The ear is embedded in the temporal bone shielded from sun-light but the results suggest that the ear has conserved the NER molecular pathway. **Second,** it reveals for the first time that molecules used by advanced tumor cells to detect and repair damaged DNA are normally functional in the ear. Tumor cells that are hard to kill use *xpc* and *xpa* to repair damaged DNA and survive cisplatin antineoplatic therapy. Small (sub-toxic) doses of cisplatin were enough to significantly up-regulate *xpc* and *xpa* in the ear. This suggests, that both genes are highly sensitive to the level of DNA damage in the inner ear.

Third, it provides the bases of a new line of hearing research focused on molecular mechanisms of inner ear DNA repair.

DNA repair may not be equally distributed among cells of the inner ear. For instance, although vestibular abnormalities are rare, high frequency hearing loss is characteristic of cisplatin chemotherapy. Experiments have revealed that the neurosensory epithelium of the cochlea is highly susceptible to cisplatin toxicity. Recent research has revealed the presence of cisplatin DNA adducts among the various cell types in the cochlea. The nonsensory epithelia survive cisplatin toxicity while the neurosensory epithelia suffer the most damage. DNA repair biology indicates that terminally differentiated cells (e.g., neurons) are less proficient at NER than non-terminally differentiated cells. The cells of the nonsensory cochlear epithelia are able to engage in spontaneous mitosis as well as damage induced mitosis, therefore they are non-terminally differentiated. Hair cells and neurons of the cochlear neurosensory epithelium are unable to undergo mitosis or change there phenotype and are considered terminally differentiated. Therefore, cochlear hair cells and neurons may be deficient in NER and thus susceptible to cisplatin DNA damage. Protein expression of XPC and XPA were assessed in the cochlea after cisplatin treatment to test this possibility. The results revealed that cochlear hair cells and neurons expressed both XPC and XPA. This work is significant for at least three reasons. **First**, it may serve as the bases for a paradigm-shift in DNA repair pathobiology. The notion that terminally differentiated cells, such as neurons are deficient in NER is based on a number of excellent *in vitro* experiments. The present *in vivo* work in the mammalian inner ear revealed that XPC and XPA are expressed competently among cochlear hair cells and neurons under normal conditions and when antagonized by cisplatin treatment. This motivates a new line of research aimed at further understanding the significance of NER among cochlear hair cells

and neurons. **Second,** it revealed that although neurons competently express NER proteins, these proteins reside in the cytoplasm under normal conditions and translocate to the nucleus under conditions of genomic stress. This is the first demonstration of cytoplasmic to nuclear translocation of the rate-limiting factors of NER. This translocation phenomenon is characteristic of neurons and not hair cell. **Third**, NER may not fully account for all cell-type vulnerabilities (e.g., basal vs. apical hair cells) to cisplatin ototoxicity. As reviewed in detail in Chapter I, the chemical biology of cisplatin is complex and cell damage may occur from a variety of biomolecular interactions. It is unlikely that one specific molecular pathway (e.g, DNA repair) completely accounts for cisplatin ototoxicity. Molecular mechanisms such as free radical promotion and altered ionic homeostasis also are involved.

The end result of cisplatin ototoxicity is cell death, however, not all cochlear cells show the same degree of susceptibility to cisplatin. For instance the apical outer hair cells (OHCs) and neurons are more resistant than basal OHCs and neurons. Indeed, histopathological studies of human temporal bones and animal specimens reveal a base-to-apex pattern of OHC and neural loss, where the basal OHCs and neurons suffer the greatest damage. Recent research has shown that the basal OHCs are less proficient at glutathione expression compared to the apical OHCs. This is important because glutathione determines cellular availability of sylfhydryls (sulfur) which are needed for DNA repair. Indeed, cancer research has shown that depleted glutathione results in reduced NER capacity. Therefore, NER might be deficient among basal OHCs and neurons compared to apical OHCs and neurons. The expression of the rate-limiting proteins of NER (XPC & XPA) was evaluated in the cochlea to test this possibility. The results revealed two important findings. **First**, the results showed that OHCs at the base and apical turns of the cochlea were competent in XPC and XPA expression. This result is relevant because it suggests

that the difference in succeptability between basal and apical OHCs to cisplatin toxicity may not be due to differences in DNA repair molecular pathways. **Second**, the results showed that spiral ganglion neurons at the basal and apical coils of the cochlea exhibited a difference in XPC and XPA protein expression following cisplatin intoxication. There was a magnitude and latency difference between the basal and apical spiral ganglion cells where basal cells responded later and with less magnitude than apical cells. This difference in latency and magnitude may account for the difference in susceptibility between neurons at the base compared to those at the apex.

I. INTRODUCTION

Cisplatin is an effective anticancer xenobiotic. It is one of the three most widely used chemotherapeutic agents in the world and commands annual U. S. sales of $500 million (Gordon & Hollander, 1993; Weiss & Christian, 1993). It has become a primary agent in the treatment of head and neck squamous cell carcinoma, testicular cancer, small cell lung cancer, ovarian cancer and a variety of childhood neoplasms (Giaccone, 2000). Antineoplastic activity increases with increasing cisplatin dosage. Unfortunately, nephrotoxicity and ototoxicity are dose limiting side effects. Nephrotoxicity can be controlled through hydration therapy but ototoxicity remains a challenge. Cisplatin chemotherapy leads to permanent bilateral high frequency sensorineural hearing loss and tinnitus. The reader is referred to the Appendix for a definition of potentially unfamiliar terms used throughout this text.

The audiometric frequencies that are most affected are $\geq$ 1 kHz and speech recognition is impaired (Forastiere, Takasugi, Baker, Wolf & Kudla-Hatch, 1987; van der Hulst, Dreschler & Urbanus, 1988). The degree of sensorineural hearing loss that results from cisplatin chemotherapy ranges from 40-90 dB HL (Myers, Blakley, Schwan, Rintelmann & Mathog, 1991; Kopelman, Budnick, Sessions, Kramer &Wong, 1988). The slope of the hearing loss increases by 45 dB per octave with increasing dosage (Brock et al., 1990). The reported clinical incidence of cisplatin induced hearing loss varies due to confounding variables such as, influence of other medications, age, general health of patients, prior noise exposure, pre-existing hearing

loss, cumulative dose (Pollera et al, 1988; Schweitzer, 1993a) and the methods used to determine ototoxicity (e.g., conventional audiometry, high frequency audiometry, otoacoustic emission, auditory brainstem response and acoustic reflex threshold). Therefore, the incidence estimates range from 20 to 90% for adults and 40 to 100% for children (Gratton, 1990; McHaney, Thibadoux, Hayes & Green, 1983; Schell et al, 1989; Weatherly, Owens, Catlin & Mahoney, 1991; Park, 1996).

The onset of hearing loss may occur as soon as 48 hours post-treatment or may be delayed (e.g., 4 days post treatment) and clinical recovery shows no consistent pattern (Aguilar-Markulis, Beckley, Priore and Mettlin, 1981; Pollera, et al, 1988). The vestibular system appears to be resistant to permanent damage from cisplatin treatment. There is significant variability in audiologic results among cisplatin treated patients. Generalizing results beyond the sample of patients selected for a particular study remains a challenge. The development of a sensorineural hearing loss that progresses from high to low frequencies with increasing dosage is consistent and well documented among cisplatin treated patients (Forastiere, Takasugi, Baker, Wolf & Kudla-Hatch, 1987; Van der Hulst, Dreschler & Urbanus, 1988). In order to develop future otoprotective strategies to ameliorate cisplatin induced hearing loss, an understanding of the underlying molecular otopathology is required. The hearing research literature has supported two dominant hypotheses regarding the underlying molecular otopathology of cisplatin. One hypothesis suggests that otopathology is due to cisplatin's reactivity with ion channels that alters ionic homeostasis. The other hypothesis suggests that otopathology is due to cisplatin's reactivity with biomolecules that facilitates the generation of reactive oxygen species (ROS). Both hypotheses have empirical support from basic research, however therapeutic approaches

based on these hypotheses have not been successful. This suggests that other reactions are involved and a greater understanding of cisplatin's reactivity is needed.

The reactive site on cisplatin is the transition metal ion, platinum(II). The general coordination chemistry of platinum(II) carries over to the compound cisplatin. For instance, platinum(II) is a Lewis acid class B (soft) metal, which means that it will bind preferentially to sulfur and nitrogen. The cisplatin molecule also binds preferentially to sulfur and nitrogen. This can be demonstrated in tissue after systemic cisplatin treatment and the use of sulfur to titrate cisplatin away from its intracellular binding sites (Guthrie & Balaban, 2004). The sulfur binds with cisplatin to produce a complex that may act as a catalyst and promote electron transfer between nucleophilic and electrophylic molecules. The titration of cisplatin away from its intracellular binding sites reveals that intracellular cisplatin may be low-affinity bound and active. Low-affinity bound transition metals will generate free radicals via the metal mediated Haber-Weiss reaction (Zdolsek, Roberg & Brunk, 1993). Cisplatin treatment is known to generate free radicals *in vivo* (Davis, Nick & Agarwal, 2001). The ability of cisplatin to generate free radicals *in vivo* contributes to its ototoxic effects. The direct binding of cisplatin to biomolecules also may contribute to otoxicity.

The Lewis acid classification (class B) of platinum predicts that it will bind directly to sulfur and nitrogen rich biomolecules. The platinum(II) ion exhibits a kinetic preference for sulfur over nitrogen while the cisplatin molecule exhibits a thermodynamic preference for nitrogen over sulfur. Biochemical research has shown that cisplatin's binding to sulfur bearing biomolecules is unstable and cisplatin bound to sulfur bearing biomolecules do not prevent stable binding to nitrogen rich DNA bases. DNA binding underlie cisplatin cytotoxicity and antineoplasm (Jamieson & Lippard, 1999). In addition to altering ionic homeostasis and

generating ROS, cisplatin induced ototoxicity may results from DNA binding in the cochlea. Recent reseach has revealed significant levels of cisplatin bound to DNA among various cell types in the cochlea after systemic treatment (van Ruijven, de Groot, Hendriksen & Smoorenburg, 2005). This suggests that cisplatin's metabolism from its introduction to the blood stream to its interactions in the cytoplasm prepares it for binding to DNA.

Research on cisplatin metabolism in the blood is limited. The cisplatin molecule is inert but after metabolism in the blood it becomes reactive. Cisplatin may bind to most, if not all plasma proteins through sulfur bonds or nitrogen-termini on amino acids as predicted from its Lewis acid classification (Ivanov et al., 1998). The antineoplastic and cytotoxic activity of cisplatin is independent of whether it is bound or unbound to plasma proteins (Pizzo, Swaim, Roche & Gonias, 1998). Plasma protein binding may determine the rate of tissue distribution and toxicity. Cisplatin easily crosses the blood-labyrinthine barrier but the molecular details of transport are unknown (Gately & Howell, 1993). Further research is needed to better understand the significance of cisplatin's metabolism in the blood and inner ear fluids. Such information may help to explain the heterogeneous distribution of cisplatin DNA adducts in cochlear tissues.

The interaction of cisplatin with the plasma membrane is poorly defined and the mechanisms by which cisplatin enters and leaves the cell are controversial. The level of uptake and the rate of uptake are the net result of a series of binding and releasing from transferring molecules. Cisplatin has its highest affinity for sulfur and nitrogen atoms but it will weakly coordinate with oxygen, phosphates and other anions. Therefore most biomolecules can be targeted. Cisplatin may interact with proteins, peptides, oligosaccharides and cholesterol via electrostatic, covalent and coordination binding. Most of these interactions are temporary and may influence membrane permeability, fluidity, enzymology and channel functioning (Wang,

Lu, Li, 1996). These effects may be amplified and propagated throughout the cell. Cisplatin binds preferentially to sulfur bearing plasma membrane proteins and the levels of such proteins tend to decrease. Binding to any plasma membrane protein could lead to conformational changes and loss of function. The net result is a change in the normal biology of the cell and perfusion of cisplatin across the plasma membrane. Ultimately, cisplatin effectively crosses the plasma membrane and becomes speciated in the cytoplasm which further prepares it for DNA binding.

The majority of research related to the speciation of cisplatin in the cytoplasm has focused on unbound-unspeciated cisplatin (Kartalou & Essigmann, 2001). Only about 10% of cisplatin may exist in this form, because once cisplatin enters the blood stream, about 90% is quickly bound to plasma proteins. Protein-bound cisplatin remains cytotoxic and may determine tissue distribution. Very little is known about the fate of these platinum products. Of interest is how specific protein bound cisplatin molecules cross the plasma membrane and become speciated. The fate of these speciated products may provide new information on antineoplasm and side effects of cisplatin treatment. Unbound-unspeciated cisplatin is transformed in the cytoplasm to aqueous platinum species (Kartalou & Essigmann, 2001). These aqueous platinum species are potent electrophiles that exbibit high affinity for sulfur and nitrogen rich biomolecules throughout the cytoplasm. There are multiple sites within the cell that are targeted by cisplatin, therefore the interactions with biomolecules are complicated. The most consistent intracellular target for cisplatin is the nitrogen rich DNA bases.

The ultimate target for cisplatin is DNA. Speciation of cisplatin in the cytoplasm prepares it for covalent binding to the N7 of purine DNA bases (Reedijk, 1999). The N7 is a highly nucleophilic site that is known to attract a number of genotoxic xenobiotics. Less attention has

been focused on cisplatin's binding to phosphate groups within the DNA. Even though such interactions would be weak, they may contribute to DNA conformational changes. Cisplatin is able to access the major groove of the DNA and bend the helix towards the major groove which increase the minor groove of the DNA molecules (Jamieson and Lippard, 1999). The guanine residues are targeted particularly by cisplatin because of their nucleophilic nature. The structural and chemical alterations of DNA induced by cisplatin are heterogeneous.

Cisplatin DNA adducts induce a variety of structural and chemical alterations to DNA that lead to various consequences. The three most profound consequences are the inhibition of replication, inhibition of transcription and induction of apoptosis. Inhibition of replication may facilitate the death of dividing cells but may not explain the death of non-dividing cells, such as hair cells and neurons. Transcription inhibition (by stalling of RNA polymerase and/or molecular hijacking) is sufficient to initiate cell death among dividing and non-dividing cells. Molecular hijacking also may facilitate the shielding of cisplatin DNA adducts from DNA repair factors which increases the tenure of platinum on the DNA helix leading to cell death. The major cell death process induced by cisplatin is apoptosis.

Human temporal bone studies reveal that cisplatin treatment induces variable damage to the cochlea. A characteristic finding is that basal outer hair cells (OHCs) and neurons are more severely damaged than apical OHCs and neurons. This pattern of damage is consistent with the high frequency sensorineural hearing loss revealed by patients after cisplatin treatment (Strauss et al., 1983). Cisplatin also induces OHC damage in experimental animals. The vestibular epithelium is less vulnerable than the cochlear epithelia and histomorphological studies may reveal normal vestibular epithelia (Schweitzer et al., 1986). There is great variability in the degree and incidence of cell damage, but OHC damage at the base is very consistent. These

morphological findings in animal models support the observations of human temporal bone studies (Hinojosa, Riggs, Strauss & Metz, 1995).

Different cell types exhibit varying degrees of vulnerability to cisplatin. For instance, terminally differentiated cells (e.g., hair cells and neurons) are more vulnerable than non-terminally differented cells (e.g., cells of stria vascularis). Additionaly, hair cells at the base of the cochlea are more vulnerable than those at the apex. A possible bassis for these cell-type vulnerabilities is DNA repair capacity. In tumor biology it is known that cells that are vulnerable to cisplatin are deficient in DNA repair while cells that are invulnerable are proficient. The same DNA repair molecules that initiate the repair of cisplatin DNA adducts in tumor cells may be expressed in the inner ear. Cell-type vulnerability in the cochlea may be due to differences in the expression of these DNA repair molecules. In order to assess this possibility a chronic, as opposed to the usual acute cisplatin dosing regimen would be needed. Chronic treatment allows for data collection at multiple time points during treatment before cochlear degeneration. The lack of degeneration of cochlear cells allows for the study of gene and protein expression as a function of cisplatin treatment. In this dissertation, a chronic cisplatin treatment regimen was used, where animals were treated with two cycles of cisplatin. Each cycle consist of four days of treatment (1 mg/kg, i.p., twice daily) separated by 10 days of rest. Standard methods such as real-time reverse-transcription quantitative polymerase chain reaction was used to study gene expression and immunohistochemistry was used to reveal protein distribution among cochlear cell types.

The sections below are divided into chapters. Chapter I provides an integrative view of cisplatin ototoxicity by arguing that the two dominant hypotheses regarding cisplatin ototoxicity are consistent with the molecular toxicology of cisplatin. This chapter further reveals that

cisplatin directly and indirectly induce DNA damage. It is well known in tumor biology that cisplatin treatment results in DNA damage that leads to cell death. This DNA damage induced cell death is mediated by the tumor's ability to express DNA repair molecules. Therefore, chapter I concludes with three specific aims to assess gene and protein expression of DNA repair molecules that are only activated in the presence of damaged DNA. Chapter II describes the methods used to assess gene and protein expression of DNA repair molecules in the cochlea after cisplatin treatment. These methods are standard techniques to assess gene and protein expression in the cochlea. Chapter III presents the results of gene and protein expression after cisplatin treatment. Chapter IV provides a discussion of the results. The results suggest that cisplatin potentiates the molecular DNA repair pathways of nucleotide excision repair in the cochlea. A glossary is provided at the end as an appendix.

II. CHAPTER I

Cisplatin is one of the most ototoxic compounds in routine clinical use today (Arslan, Orzan, & Santarelli, 1999; Kalinec, Webster, Lim & Kalinec, 2003). Both human and animal studies have characterized the ototoxic effect of cisplatin chemotherapy (Kohn et al., 1988; Strauss et al., 1983). Human studies typically involve human volunteers and/or human post-mortem temporal bones. Animal studies are predominantly clustered into two types, those that focus on morphological alterations and those that focus on functional alterations as a consequence of cisplatin treatment. The sections below provide a review of both human and animal studies on cisplatin ototoxicity.

A. HUMAN STUDIES ON CISPLATIN OTOTOXICITY

Table I summarizes human studies related to csiplatin ototoxicity. Studies on cisplatin ototoxicity were conducted on patients with advanced malignancies who are fighting for their lives and hearing assessments are often not a priority. Nephrotoxicity and ototoxicity are the two dose limiting variables to cisplatin chemotherapy, however severe ototoxicity may occur with minimal nephrotoxicity (Pollera et al., 1988). The degree of hearing loss varies widely among cisplatin treated patients (Naggy et al., 1999). The antineoplastic effect of cisplatin is dose dependent but as the treatment dose increases the degree of hearing loss also increases. There is no clear association between the degree of hearing loss and the route of administration (Pollera et al, 1988). The first phase I clinical trials of cisplatin revealed that it induces severe permanent bilateral sensorineural hearing loss (Krakoff & Lippmann, 1974). Hearing loss is predominantly in the high frequencies and may progress to low frequencies with increasing dosage. Speech discrimination is impaired particularly in background noise. Additionally, the relationship between dose and tinnitus is poorly understood. The incidence of tinnitus may range from 2-36% (Schweitzer, 1993b). Cisplatin treatment does not lead to significant functional and morphological vestibular alterations and clinical reports of vestibular dysfunction are rare (Black, Gianna-Poulin & Pesznecker, 2001; Myers, Blakley & Schwan, 1993; Nakayama, Riggs & Matz, 1996; Sergi, Ferraresi, Troiani, Paludetti & Fetoni, 2003; Schweitzer, Rarey, Dolan, Abrams & Sheridan, 1986). Human temporal bone studies reveal that cisplatin treatment induces variable damage to cochlear structures (Cheng et al., 2001; Strauss et al., 1983; Tange, 1987).

Table 1. Summary of Human Studies on Cisplatin Ototoxicity.

Reference	Malignancy	Treatment	Findings/Comments
Talley et al. (1974)	various cancers life expectancy 6 weeks	≥ 15 mg/m2 of cisplatin for 5 days	HFHL & tinnitus first phase I clinical trial in the U.S.
Wiltshaw et al. (1974)	ovarian tumors	cisplatin	NHS & tinnitus first phase I clinical trial in the U.K.
Krakoff et al. (1974)	various tumor types	cisplatin (>4.0 mg/kg)	bilateral HFHL that increase with dosage
Ellerby et al. (1974)	various incurable cancers	cisplatin combined with 5-FU	HFHL phase I clinical trial using combination therapy
Wallace et al. (1974)	advanced genitourinary cancer	cisplatin & adriamycin	HFHL & tinnitus phase I clinical trial using combination therapy
Hayes et al. (1977)	various cancers	cisplatin (3-5 mg/kg)	HFHL with 90% incidence older patients (54-70) had greater loss than younger patients (19-39)
Aguilar-Markulis et al. (1981)	genitourinary cancer	cisplatin (1 mg/kg, i.v.)	HFHL increased with treatment
McHaney et al. (1983)	various cancers	cisplatin (90 mg/m2, i.v.)	88% incidence of HFHL

Table 1 (continued)

Strauss et al. (1983)	various cancer	cisplatin (130 mg/m2)	sbHFHL with pSDS deg of basal OHC, IHC & SG
Fausti et al. (1984)	genitourinary tumors & head & neck cancer	cisplatin	HFHL developed before LFHL
Forastiere et al. (1987)	squamous cell carcinoma of the head & neck	cisplatin (200 mg/m2)	HFHL
Tang (1987)	ovarian carcinoma	cisplatin (300 mg/m2) bleomycin	HFHL deg of basal OHC disordered cochlear vasculature
van der Hulst et al. (1988)	various cancers	cisplatin (20 mg/m2 or 50 mg/m2) carboplatin (350 mg/m2)	HFHL
Kopelman et al. (1988)	various cancers	cisplatin (150 to 225 mg/m2)	progressive HFHL with 100% incidence, SIN deficit & tinnitus
Pollera et al. (1988)	ovarian cancer	cisplatin (200 mg/m2)	HFHL 48 hrs post treatment with an incidence of 75% and minimal nephrotoxicity
Schell et al. (1989)	various tumors	cisplatin	HFHL, normal renal function
Myers et al. (1991)	various cancers	cisplatin (100 mg/m2)	HFHL
Waters et al. (1991)	ovarian cancer	cisplatin (100 or 50 mg/m2)	progressive HFHL & tinnitus

Table 1 (continued)

Hinojosa et al. (1995)	Various cancer	cisplatin (100-250 mg/m2)	OHC btadg
Park (1996)	various tumors	cisplatin (<150 or >650 mg/m2)	HFHL
Hoistad et al. (1998)	various tumors	cisplatin	loss of SG, IHC, OHC & atrophy of Stv
Yardley et al. (1998)	various cancers	cisplatin or carboplatin	abnTEOAE & ePTA after cisplatin treatment
Burris et al. (1998)	tumors of the head, neck trunk, tongue, oral pharynx and esophagus	cisplatin/epinephrine-injectable gel (0.56-380 mg)	NHS
Fausti et al. (1999)	various cancers	cisplatin	94% detection rate of HFHL
Nagy et al. (1999)	esophagus, lung or head & neck cancer	cisplatin (20 mg/m2/4 day, i.v.) radiation therapy	HL
Lauretis et al. (1999)	various cancers	cisplatin (70 mg/m2) or carboplatin (250 mg/m2)	abnHFABR only after cisplatin treatment
Cevette et al. (2000)	various cancers	cisplatin (75 mg/m2)	fDPOAE with NHS & HFHL with pDPOAE
Cheng et al. (2001)		cisplatin	HFHL, damaged; STv, IHC, OHC, SG at the cochlear base
Stavroulaki et al. (2001)	various cancers	cisplatin (50 mg/m2)	HFHL & aDPOAE
Strumberg et al. (2002)	testicular cancers	cisplatin	70% HFHL & 27% nTEOAE 47% aTEOAE

Table 1 (continued)

HFHL	High Frequency Hearing Loss	abnTEOAE	Abnormal Transient Evoked Otoacoustic Emissions
NHS	Normal Hearing Sensitivity	ePTA	Elevated Pure Tone Average
LFHL	Low Frequency Hearing Loss	HL	Hearing Loss
SIN	Speech In Noise	abnHFABR	Abnormal High Frequency Auditory Brainstem Response
fDPAOE	Fluctuating Distortion Product Otoacoustic Emissions	pDPAOE	Present Distortion Product Otoacoustic Emissions
aDPOAE	Absent Distortion Product Otoacoustic Emissions	nTEOAE	Normal Transient Evoked Otoacoustic Emissions
aTEOAE	Absent Transient Evoked Otoacoustic Emissions	sbHFHL	Severe Bilateral High Frequency Hearing Loss
pSDS	Poor Speech Discrimination Score	deg	Degeneration
OHC	Outer hair cell	IHC	Inner Hear Cell
SG	Spiral Ganglion	Stv	Stria Vascularis
btadg	Base To Apex Damage Gradient		

B. ANIMAL STUDIES ON CISPLATIN OTOTOXICITY

Table 2 provides a summary of animal studies related to cisplatin ototoxicity. Cisplatin exerts its ototoxic effects mainly on the cochlea. Anatomical preparations reveal that the basal OHCs are the most vulnerable structures in the cochlea while the OHC at the apex are among the least vulnerable. The vestibular epithelium is less vulnerable than the cochlear epithelia and histomorthological studies may reveal normal vestibular structures (Schweitzer et al., 1986). There is great variability in the degree and incidence of cell damage, but OHC loss at the base is very consistent (Cardinaal, de Groot, Huizing, Veldman & Smoorenburg, 2000b; Sergi, Ferraresi, Troiani, Paludetti & Fetoni, 2003). Physiological studies demonstrate that cisplatin inhibits all mechanical and electrophysiological cochlear responses in a dose dependent manner (O'Leary, Klis, de Groot, Hamers & Smoorenburg, 2001). Treatment typically results in high frequency hearing loss. Vestibular function is less affected than cochlear function (Sergi, Ferraresi, Troiani, Paludetti & Fetoni, 2003). Cisplatin cochlear alterations may induce hyperactivity in the cochlear nucleus which may contribute to tinnitus (Rachel, Kaltenbach & Janisse, 2002). Apoptosis is the end result of cisplatin ototoxicity, however, necrosis also may occur with high dosages (Devarajan et al., 2002).

Table 2. Summary of Animal Studies on Cisplatin Ototoxicity.

References	Animal	Treatment	Findings/Comments
Estrem et al. (1981)	guinea pig (180-360 g)	cisplatin bleomycin	deg of OHC
Nakai et al. (1982b)	guinea pig (400-500 g)	cisplatin (2 mg/kg, i.m.)	mOHC
Kohn et al. (1988)	guinea pig (250-350 g)	cisplatin (4 mg/kg/ 5 days s.c.)	deg of: MC & IC damaged: ER & OHC
Saito et al. (1994)	guinea pig (300-400 g)	cisplatin (15 mg/kg or 21 mg/kg, i.m.)	cisplatin was detected in the kidney but not the inner ear using X-ray microanalysis & ion microscopy
Laurell et al. (1995)	Long-Evans's rat (150-275 g)	cisplatin (8 mg/kg, i.p.)	reduced K concentration in the cochlea
Saito et al. (1996)	guinea pig	cisplatin or cisplatin metabolites	cisplatin had no effect on isolated OHC but cisplatin metabolites induced significant damage
Stengs et al. (1997)	guinea pig (250-350 g)	cisplatin (1.5 mg/kg/ 8 days, i.p.)	reduced: CM, SP & CAP
Ford et al. (1997)	Wistar rats (250-350 g)	cisplatin (6 µL of 0.54 mg/ml, r.w. or 16 mg/kg i.p.)	72 hrs post treatment result in mOHC & mIHC. 5 fold up-regulation of adenosine receptors after

Table 2 (continued)

			24 hrs post treatment.
Stengs et al. (1998a)	guinea pig (250-350 g)	cisplatin (0.7-2.0 mg/kg/ 8 days, i.p.)	reduced: CM, SP & CAP
Janning et al. (1998)	chinchillas (450-700 g)	cisplatin (25 µl of 0.25 mg/ 1.0 ml saline, r.w.)	damaged HC
Meech et al. (1998)	Wistar rats (250-400 g)	cisplatin (16 mg/kg)	strial edema
Sie et al. (1999)	gerbils	cisplatin (1 mg/kg/ 4 days, i.p.)	reduced DPOAE
Tampakopoulou et al. (1999)	gerbils	cisplatin (5 mg/kg)	increased ABR & DPOAE thresholds
Cardinaal et al. (2000a)	guinea pig (250-350 g)	cisplatin (0.7-1.5 mg/kg/ 8 days, i.p.)	atrophy of stria vacuolation of RM
Cardinaal et al. (2000b)	guinea pig (250-350 g)	cisplatin (1.5 mg/kg/ 8 dats, i.p.)	damaged OC
Tsukasaki et al. (2000)	chinchillas (500-700 g)	cisplatin (5µl (1 mg/ml) in saline), r.w.	reduced: EP & CM
Ekborn et al. (2000)	guinea pig	cisplatin (8 mg/kg, i.v.)	ABR threshold shift
Alam et al. (2000)	gerbils	cisplatin (4 mg/kg/5 days, i.p.)	deDPOAE & EP poTUNEL in OC
O'Leary et al. (2001)	guinea pig	cisplatin (3, 30 or 300 µg/ml in saline)	reduced: CAP, CM & EP

Table 2 (continued)

Klis et al. (2002)	guinea pig	cisplatin (1.5 or 2.0 mg/ kg, i.p.)	reduced CAP
Rachel et al. (2002)	hamster (45-55 days old)	cisplatin (15, 11.25 & 7.5 mg/kg/5 days, i.p.)	increases spontaneous activity in DCN
Hatzopoulos et al. (2002)	Sprague Dawley rat (210 ± 30 g)	cisplatin (16 mg/kg, i.p.)	elevated ABR & TEOAE thresholds
Sluyter et al. (2003)	guinea pig (250-350 g)	cisplatin (1.5 mg/kg/ 5-18 days, i.p.)	shrinkage of Stv that did not correlate with redunction of EP
Sergi et al. (2003)	guinea pig (250-300 g)	cisplatin (2.5 mg/kg/ 6 days, i.p.)	impaired VVOR & slight loss of HC of crista ampullaris & macula. Massive OHC loss & CAP threshold shift
Wang et al. (2003)	guinea pig (250-300 g)	cisplatin (2 mg/kg/5 days, r.w.)	damaged OC
Ekborn et al. (2003)	guinea pig	cisplatin (4.24 mg/kg, i.v.) or cisplatin MHC (4 or 8 mg/kg, i.v.)	MHC = moderate ABR threshold shift cisplatin = no ABR threshold shift

OHC	Outer Hair Cell	ER	Endoplasmic Reticulum
mOHC	Missing Outer Hair Cell	K	Potassium
deg	degeneration	CM	Cochlear Microphonic
MC	Marginal Cell	SP	Summating Potential
IC	Intermediate Cell	CAP	Compound Action Potential
r.w.	round window	s.c.	subcutaneous

Table 2 (continued)

i.p.	intraperitoneal	mIHC	Missing Inner Hair Cell
ABR	Auditory Brainstem Response	EP	Endolymphatic Potential
RM	Reissner's Membrane	OC	Organ of Corti
DPOAE	Distortion Product Otoacoustic Emissions	MHC	Mono-Hydrated Complex
DCN	Dorsal Cochlear Nucleus		
deDPOAE	Decreased Distortion Product Otoacoustic Emissions		
poTUNEL	Positive Terminal Deoxynucleotidyl Transferase dUTP-biotin Nick End Labeling		
Stv	Stria Vascularis		
VVOR	Vertical Vestibular Ocular Reflex		
HC	Hair Cell		

C. CISPLATIN OTOTOXICITY AN INTEGRATIVE VIEW

This section provides an integrated overview of the two dominant mechanisms that are believed to underlie cisplatin ototoxicity by revealing that each compliments the other. A review of the pharmacokinetics of cisplatin reveals that after systemic treatment the cisplatin molecule exhibits high binding affinity for sulfur, is labile and redox-active within the cell. The high affinity for sulfur suggests that the cisplatin molecule will interact with many biomolecules containing cysteine and methionine residues. Cisplatin's first biomolecular interactions at the level of the cell, occur on the plasma membrane. Ionic homeostasis can be disrupted by binding of cisplatin to ion channels in the plasma membrane. Altering ionic homeostasis is one of the two dominant mechanisms of cisplatin ototoxicity. The lability and redox-activity of cisplatin promotes ROS generation. ROS production is the other dominant mechanism that underlie cisplatin ototoxicity. Alterations to ionic homeostasis and ROS production each result in DNA damage and cisplatin also may directly damage DNA. From the initial systemic treatment, cisplatin's interactions in the blood stream to its associations with plasma membrane and cytoplasmic proteins prepare it for binding to DNA.

1. Chemical Biology of Cisplatin

Cisplatin (*cis*-Diamminedichloroplatinum-II) was first synthesized in 1845 and known for decades as Peyrone's chloride. Since the discovery of its biological activity in the 1960's, it has become one of the most important inorganic compounds in human medicine today. The biological activity of cisplatin was first demonstrated during experiments to evaluate the effects of electricity on the growth of prokaryotic cells (Rosenberg, van Camp & Krigas, 1965). The investigators transferred alternating current through *Escherichia coli* positioned between two platinum electrodes in a solution of ammonium chloride. The results showed that cell division was altered even without applying alternating current. The researchers fortuitously discovered that the combination of the platinum electrodes with ammonium chloride inhibited cell division but not cell growth and induced filamentous growths up to 300 times the length of a normal cell. This new discovery propelled investigations into the antitumor capacity of various platinum coordination complexes (Rosenberg, van Camp, Trosko and Mansour, 1969). Cisplatin was shown to be the most successful anticancer platinum complex in pre-clinical experiments by inhibiting sarcoma 180, leukemia L1210, Dunning ascetic leukemia, Walker 256 carcinosarcoma and methylbenzanthracene-induced mammary carcinoma (Kociba, Sleight & Rosenberg, 1970; Rosenberg, van Camp, Trosko& Mansour, 1969; Welch, 1971).

The core of the cisplatin molecule and the site of reactivity is the metal ion platinum(II) which facilitates a *cis*-geometry between diammine [$(NH_3)_2$] and dichloride (Cl_2) ligands (see Figure 1). The basic coordination chemistry of platinum(II) may predict some biological

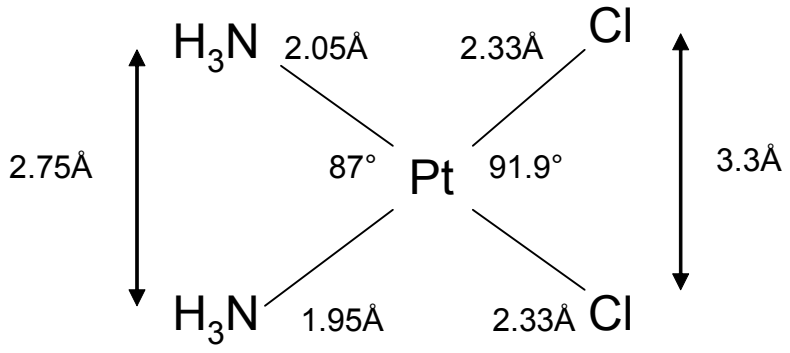


Figure 1: Chemical structure of cisplatin [*cis*-{$(H_3N)_2Pt^{II}Cl_2$}].

reactions of cisplatin. Platinum(II) is a soft (class B) Lewis acid metal and its ligand binding activities are best described by the Hard-Soft-Acid-Base (HSAB) principle. The HSAB principle states that, soft acids (SA) prefer soft bases (SB) and hard acids (HA) prefer hard bases (HB). In equation form the HSAB principle predicts that the following reaction will move to the right.

$$HA[SB] + SA[HB] \quad \rightarrow \quad HA[HB] + [SB]SA \qquad (1)$$

The distinction between hard acids/bases and soft acids/bases is related to parameters such as σ_p (softness parameter), ionic radius, ionic charge and Pauling electronegativity (Newman, McCloskey & Tatara, 1998; Wulfsberg, 2000). The degree of the interaction between metals and

biomolecules (lipids, proteins, enzymes, DNA, RNA) is dependent on the hardness/softness of the metal (Delnomdedieu & Allis, 1993).

The HSAB principle has been used to predict intracellular ligand binding for various metals. Practitioners in the fields of ecotoxicology and environmental safety often assess the level of metal pollution in a particular environment by sampling the soil and/or water for metal levels. They also may study metal uptake and distribution in tissues and cells of invertebrate organisms in the environment (Soto & Marigomez, 1997; Vandenbulcke, Grelle, Fabre & Descamps, 1998; van Straalen, 1993). Decades of such work has revealed that certain metals bind stably and preferentially to certain intracellular ligands. These observations have lead to the development of a biologically relevant metal classification system based on HSAB principles (Duffus, 2002; Hopkin, 1989; Nieboer & Richardson, 1980; Wolterbreek & Verburg, 2001). For instance, metals that preferentially bind oxygen (O) on biomolecules are considered class A metals or hard acids by HSAB principle. Metals that preferentially bind sulfur (S) and nitrogen (N) on biomolecules are considered class B metals or soft acids by HSAB principle. Those that preferentially bind O, S, and N are considered borderline. Platinum(II) belongs to the class B (soft acids) metals that bind preferentially with S and N (Deubel, 2004). This ligand binding activity of platinum(II) carries over to intracellular cisplatin molecules after systemic treatment as revealed by the following reaction in tissue (Guthrie & Balaban, 2004);

$$\text{Pt-ligand} + S^{2-} \rightarrow \text{PtS} + \text{apo-ligand} \qquad (2)$$

where, platinum (Pt) bound to an intracellular ligand (e.g., protein) is titrated by S (a soft base) to form a redox-active (catalytic) platinum-sulfide (PtS) product. The redox-activity of PtS can be

demonstrated intracellularly by autometallographic development in a light insensitive physical developer (see Figure 2 & see discussion below). These results suggest that after systemic treatment intracellular cisplatin is labile, binds preferentially to sulfur bearing biomolecules and redox-active.

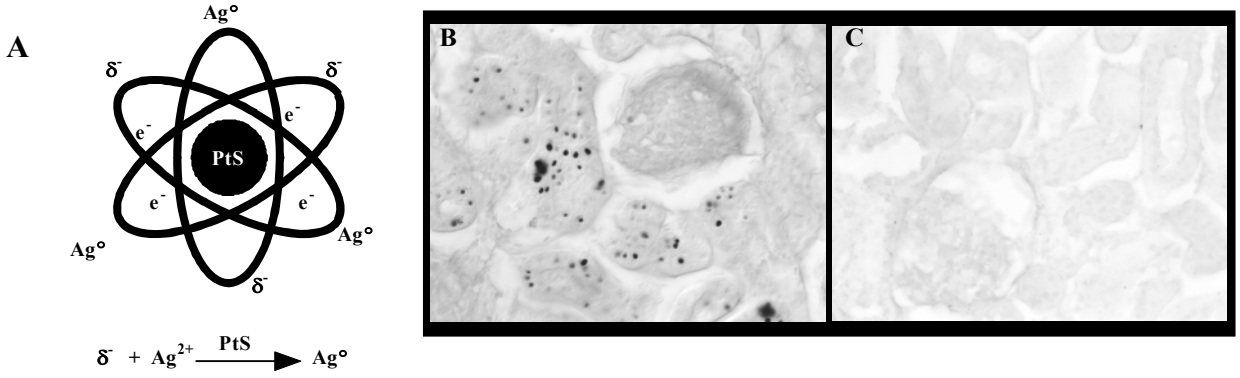


Figure 2: Platinum-sulfide autometallography (PtS^{AMG}). **A**. Schematic of PtS^{AMG}, where platinum bound to sulfur (PtS) creates a redox-active complex that enhances electron (e^-) transfer between a nucleophile (δ^-) and an electrophile (Ag^{2+}). This redox-activity reduces Ag^{2+} to Ag° which chelates PtS and is revealed as black silver spheres under the electron or light microscope. **B**. Photomicrograph of kidney tissue from a Sprague-Dawley rat treated with 13 mg/kg, i.p. of cisplatin. Black silver spheres are present at an intermediate depth within the renal cortex of the kidney. Note the prominent black silver spheres deposited in the nuclei of the proximal tubule cells and lack of deposits within the renal corpuscle. This pattern of deposition was restricted to the middle third of the cortex. The nuclear localization is consistent with the known formation of cisplatin-DNA adducts. The localization of binding to proximal tubule cells in the middle third of the cortex suggest a selective affinity of these cells for cisplatin, which is consistent with the selective damage to proximal tubule cells after cisplatin treatment. **C.** The same region of the kidney developed by autometallography from a control Sprague-Dawley rat.

2. Intracellular Cisplatin is Labile

Reaction (2) only can occur for low-affinity (loosely) bound metals in tissue (Zdolsek, Roberg & Brunk, 1993), which suggests that cisplatin bound to some biomolecules is still labile. Biochemical experiments have shown that cisplatin exhibits low-affinity binding with thioether sulphur (e.g., methionine) which can be disturbed by competing S bearing molecules (Esposito & Najjar, 2002; Lempers & Reedijk, 1990). These findings are particularly important because labile Lewis acid metals promote the generation of ROS (Zdolsek, Roberg & Brunk, 1993). Cisplatin treatment is known to generate the superoxide anion radical ($O_2^{\cdot -}$) in the ear and kidney (Davis, Nick & Agarwal, 2001; Dehne, Lautermann, Petrat, Rauen & de Groot, 2001). In order for dioxygen (O_2) to be metabolized to $O_2^{\cdot -}$, it would need to acquire an electron (e^-). This acquisition known as an electron reduction may be achieved by input energy (e.g., sunlight) or a chemical reducer (Welch, Davis, van Eden & Aust, 2002). It is often thought that in cells, biomolecules may serve as chemical reducers for O_2. However, ground state O_2 is a triplet molecule and its reaction with biomolecules (most of which have a singlet ground state) is physiologically insignificant (Miller, Buetter & Aust, 1990). For instance, although the reaction between O_2 and a biomolecule may be thermodynamically favored, there is a kinetic restriction of O_2 that inhibits its metabolism to $O_2^{\cdot -}$ in the absence of a catalyst (Miller, Buetter & Aust, 1990). Class B Lewis Acid metals are effective catalysts for the metabolism of O_2 to $O_2^{\cdot -}$. Soft Lewis acid metals such as platinum(II) may exist in several spin states and will lift the spin restriction on O_2 which then promotes radical formation. For instance, both O_2 and a biomolecule may coordinate to the *d*-orbital of a soft Lewis acid metal (Valentine, 1973). Here, the metal (M^{n+}) acts as a redox-active bridge between O_2 and the biomolecule (L) which

facilitates the transfer of an e^- to generate the $O_2^{\cdot -}$ but may gain an e^- to generate O_2 (Buetter, 1988; Khan & Martell, 1967).

$$O_2 + \text{L- } M^{n+} \overset{e^-}{\longleftrightarrow} \text{L- } M^{n+1} + O_2^{\cdot -} \qquad (3)$$

The generation of $O_2^{\cdot -}$ facilitates the well known metal mediated Haber-Weiss reaction (see reactions 4-6) which produces the potent hydroxyl radical ($HO^{\cdot}$) that damages biomolecules, such as proteins and DNA via autoxidation (see reaction 7). Cisplatin treatment is known to generate the $HO^{\cdot}$ in ear and kidney tissues (Baliga, Zhang, Baliga, Ueda & Shah, 1998; Clerici, Hensley, DiMartino & Butterfield, 1996).

$$\text{L-}M^{n+1} + O_2^{\cdot -} \longrightarrow O_2 + \text{L-}M^{n+} \qquad (4)$$

$$\text{L-}M^{n+} + H_2O_2 \longrightarrow \text{L-}M^{n+1} + HO^- + HO^{\cdot} \qquad (5)$$

combined

$$O_2^{\cdot -} + H_2O_2 \xrightarrow{\text{L-}M^{n+1}/\text{ L-}M^{n+}} O_2 + HO^- + HO^{\cdot} \qquad (6)$$

in vivo consequence

$$HO^{\cdot} + \text{protein/DNA} \longrightarrow \text{damaged-protein/DNA} \qquad (7)$$

In summary, after systemic treatment intracellular cisplatin is labile. The core of the cisplatin molecule and the site of reactivity is the metal ion platinum(II). Platinum(II) is a class B Lewis acid metal. Such metals are highly destructive to organic polymers and easily generates ROS in biological mileu. Systemic cisplatin treatment results in the generation of lethal ROS

such as $O_2^{\cdot -}$ and $HO^{\cdot}$ in the kidney and ear. These ROS are particularly toxic to DNA and other organic polymers.

3. Cisplatin Preferentially Binds to Sulfur Bearing Biomolecules Initially

Similar to all class B (soft) Lewis acid metals, cisplatin binds preferentially and covalently to available S bearing biomolecules. This is particularly important because many proteins use S and disulfide bonds (-S-S-) to maintain their conformation/function (Ivanov et al., 1998). Cisplatin's first encounter with biomolecules in general and S-bearing biomolecules in particular occurs in the blood stream.

Cisplatin is usually administered intravenously at doses of 20 mg/m^2 per day for 5 days or 100 mg/m^2 once every four weeks. In the blood, cisplatin may exist as protein bound (~90%) or unbound (~10%) (Schweitzer, 1993a). Rapid protein binding occurs soon after administration and the high chloride concentration in serum maintains the remaining cisplatin in the unbound state (Nitiss, 2002; Siddik, Newell, Boxall & Harrap, 1987). After intravenous administration in humans, the elimination kinetics are biphasic with half-lives ($t_{½}\alpha$) of 23-50 minutes and 24-73 hours ($t_{½}\beta$) for cisplatin bound to plasma proteins (Gullo et al., 1980). Unbound cisplatin has a significantly shorter half-life of 21.7-23.6 minutes. Different studies may reveal different times which reflect differences in methods and experimental design. The significance of protein bound vs. unbound cisplatin in terms of antineoplasm and/or toxicity is unresolved. It was previously thought that only unbound cisplatin had antineoplastic activities (Patton et al., 1978). Later research demonstrated that administration of even preformed protein-bound cisplatin was

therapeutically active and cytotoxic (Holding et al., 1992; Hoshino et al., 1995; Vreeburg, Stell, Holding & Lindup, 1992). Protein bound cisplatin results in significantly less urinary secretion and more tissue absorption which may facilitate better therapeutic outcomes as well as increased side effects (Gullo et al., 1980).

When cisplatin enters the blood it reacts readily with S and N rich domains on plasma proteins as predicted from its Lewis acid metal classification (see section C.1.). Albumin is considered the major substrate for cisplatin binding in the blood and is the most studied plasma protein. Human serum albumin (66 kDa) exhibits 17 disulfide bridges (-S-S-) and one free thiol (S) at Cys34 which has high affinity for class B (soft) Lewis acid metals (Esposito & Najjar, 2002; Ivanov et al., 1998). Cisplatin's interaction with albumin is complex and may yield various products such as bifunctional binding to S and N, monofunctional binding to one S and a monofunctional binding with S that expels one amine (NH_3) from cisplatin (Ivanov et al., 1998). Additionally, a *trans* configuration may be achieved where cisplatin cross-links two albumin molecules (Ivanov et al., 1998).

Unbound-unspeciated cisplatin is rapidly speciated in the cytoplasm (see Figure 3). Outside the cell the relatively high chloride concentration (~100 mM) is believed to maintain cisplatin in its native form [*cis*-$\{(H_3N)_2Pt^{II}Cl_2\}$] (Kartalou & Essigmann, 2001). Substitution reactions also may occur outside the cell, where the chloride on cisplatin is replaced by endogenous chloride ions, but the structure remains unchanged. Inside the cell, the relatively low chloride concentration (~4 mM) is believed to facilitate the replacement of one chloride then a second chloride for endogenous anions, which results in [*cis*-$\{(H_3N)_2Pt^{II}Cl(R)\}^{+}$] and [*cis*-$\{(H_3N)_2Pt^{II}(R)_2\}^{2+}$] species, where R may be various types of small ions or molecules (Lippard, 1982). These charged platinum species are potent electrophiles that are ready to react with

proteins, nucleic acids, lipids, enzymes and various organelles in the cell. The toxicity of cisplatin is based predominantly on these charged species, particularly [*cis*-$\{(H_3N)_2Pt^{II}Cl(H_2O)\}^+$], where a chloride ion is replaced by a water molecule. This species is called a monohydrate complex. Administration of the monohydrate complex, results in more hearing loss and systemic toxicity than the unspeciated form of cisplatin (Ekborn et al., 2003).

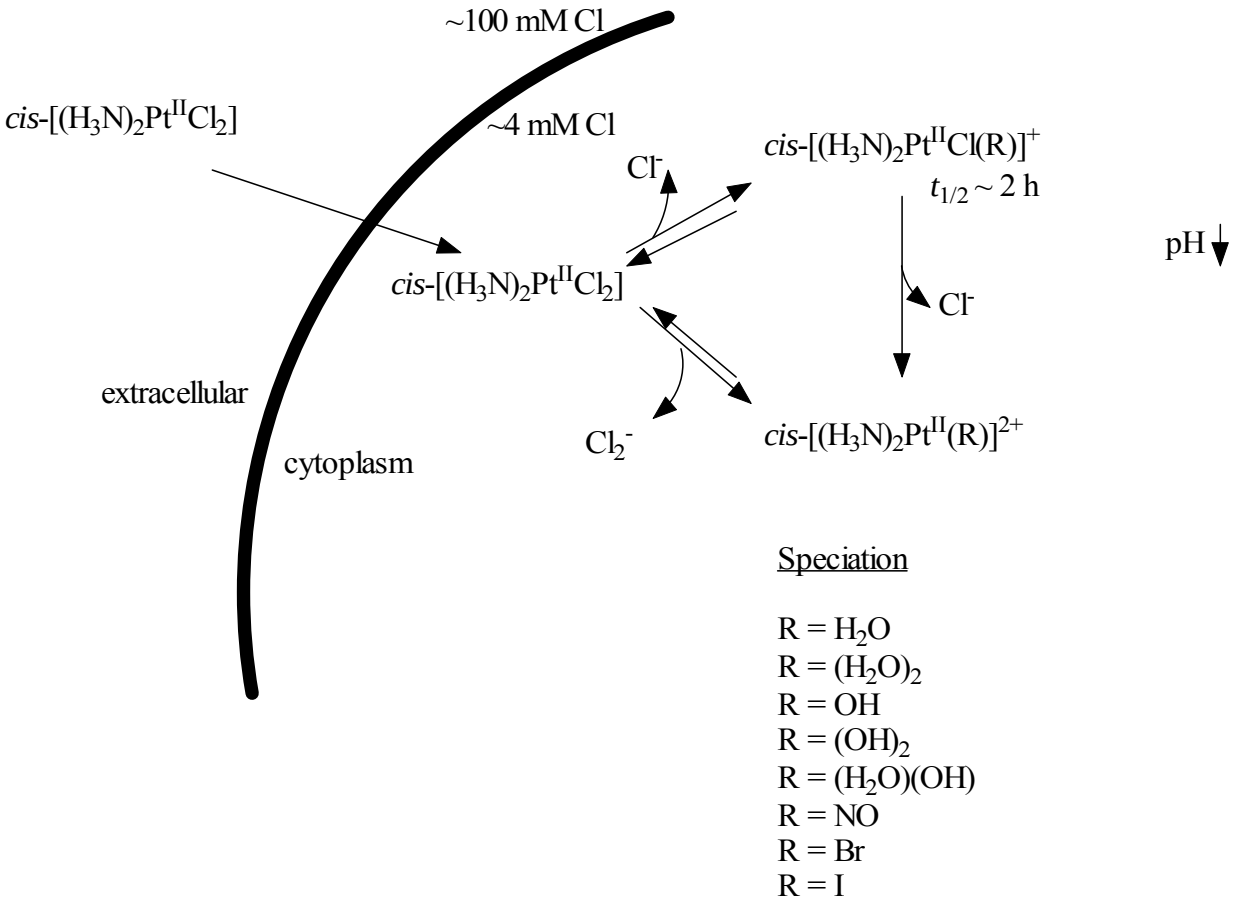
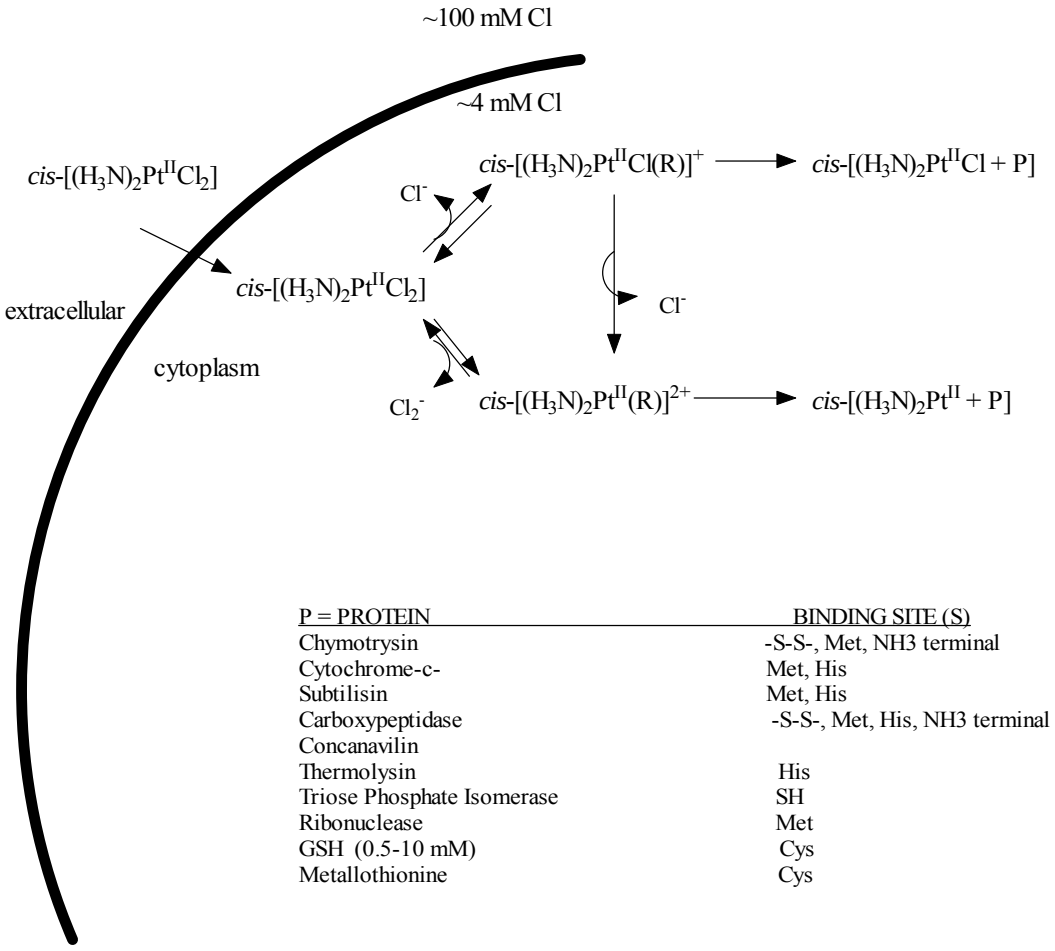


Figure 3: Speciation of cisplatin [*cis*-$\{(H_3N)_2Pt^{II}Cl_2\}$] in the cytoplasm.

Speciation is dynamic, where Rs are weakly coordinated to platinum and may replace each other leading to deprotonation and a lowered pH (acidic) in the cytoplasm. Many cisplatin sensitive cancer cells have a low intracellular pH than resistant cells and high cochlear pH levels protect against cisplatin ototoxicity (Murakami et al., 2001; Tanaka, Whitworth & Rybak, 2004). The charged platinum species are short lived because they readily react with various substrates, particularly proteins in the cytoplasm. They are specifically attracted to S and N donor proteins and peptides such as those with accessible disulfide bridges, methionines, cysteines and histidines (Williams, 1974). Figure 4 illustrates speciation of the cisplatin molecule in preparation for protein binding. The aquated platinum species react more rapidly with thioether sulphur (e.g., methionine) than with thiols (e.g., cysteine) because ligand exchange is slower than direct coordination, but the latter is irreversible (Esposito & Najjar, 2002; Wang, Lu & Li, 1996). Platinum(II) bound to the thioether sulphur on methionine easily can be reversed with S bearing nucleophiles (Lempers & Reedijk, 1990). Interestingly, an ammonia ligand *trans* to the coordinated S may be activated and expelled (Wang, Lu & Li, 1996). This *trans* effect also has been shown when cisplatin coordinates with the methionine of HSA (Ivanov et al., 1998). Speciation does not need to occur for cisplatin to bind to S sites on proteins (Dedon & Borch, 1987). Many proteins and enzymes use disulfide bridges in protein folding in the endoplasmic reticulum. Protein folding or conformation determines protein function. The charged or uncharged platinum species may disrupt protein and enzyme conformation by targeting S and N sites which then result in altered function. For instance, cisplatin will remove S from biomolecules at high concentration and/or prolong incubation time (Ivanov et al., 1998).



P = PROTEIN	BINDING SITE (S)
Chymotrysin	-S-S-, Met, NH3 terminal
Cytochrome-c-	Met, His
Subtilisin	Met, His
Carboxypeptidase	-S-S-, Met, His, NH3 terminal
Concanavilin	
Thermolysin	His
Triose Phosphate Isomerase	SH
Ribonuclease	Met
GSH (0.5-10 mM)	Cys
Metallothionine	Cys

Figure 4: Example of cisplatin's binding to proteins in the cytoplasm.

In summary, cisplatin binds preferentially to sulfur bearing biomolecules initially. Sulfur bearing biomolecules are ubiquitous throughout the cell, therefore a large variety of biomolecules will be tartgeted. The affinity of platinum(II) (the core of the cisplatin molecule) for sulfur is predictable based on HSAB chemistry. Although the cisplatin molecule exhibits

high affinity for sulfur, its binding to sulfur bearing biomolecules is temporary/labile (see reaction 2 and discussion above). This is further supported by the fact that cisplatin binds numerous sulfur bearing biomolecules in the blood and on the plasma membrane but ultimately binds nitrogen rich subunits of DNA in the nucleus. The class B Lewis acid classification of platinum(II) compliments HSAB chemistry by predicting high affinity binding for both sulfur and nitrogen. Recent experiments applying density functional theory and continuum dielectric calculations have revealed that the cisplatin molecule exhibits a kinetic preference for nitrogen over sulfur in biologically relevant conditions (Deubel, 2004). Intracellular cisplatin may bind initially to sulfur bearing biomolecules but it eventually migrates to nitrogen rich biomolecules such as DNA (van Boom, Chen, Maarten & Reedijk, 1999).

4. Cisplatin Bound to Sulfur is Redox-active

Cisplatin binds rapidly to thioether sulfur but slowly to thiols such as cysteine (Esposito & Najjar, 2002). This is because direct coordination to the thioether sulfur is faster than ligand exchange with the thiol. Ligand exchange with the thiol results in PtS covalent bonds (Esposito & Najjar, 2002). The PtS complex may be redox-active which may easily promote oxidative cross-linking of biomolecules in tissue. Results from autometallography are consistent with the redox-activity of the PtS complex (see Figure 2; Guthrie & Balaban, 2004).

Autometallography is the most powerful cytochemical process for characterizing the topographical distribution of low-affinity bound redox-active metals (Garner, Roberg, Qian, Eaton & Truscott, 2000; Zdolsek, Roberg & Brunk, 1993). It has been used to locate and map

the movement of trace amounts of endogenous and exogenous redox-active metals such as; Fe^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+}, Ni^{2+}, Hg^{2+}, Cd^{2+}, Pb^{2+}, As^{2+}, Bi^{2+}, Tl^{2+}, Au^{2+}, and Ag^{2+} (Danscher, 1981; Danscher, 1991). The autometallographic process involves intracellular precipitation of insoluble metallic crystallites from the electrovalent bonding of the target metal to a soft Lewis Base such as S (Danscher, Stoltenberg, Kemp & Pamphlett, 2000; Zdolsek, Roberg & Brunk, 1993). The resulting crystallite is redox-active which may be demonstrated by a physical developer. The physical developer contains a nucleophile such as ascorbate and an electrophile such as Ag^{2+}. Ag^{2+} is reduced on the surface of the metal-sulfide crystallite by the nucleophile forming a metallic Ag° shell around the metal-sulfide crystallite (Csaki, Kaplanek, Moller & Fritzsche, 2003; Jackson & Halas, 2001; James, 1939). Therefore, the physical-development of the metal-sulfide only occurs if the metal-sulfide complex is redox-active (James, 1939). Cisplatin-autometallography has revealed that platinum bound to S yields a redox-active complex in tissue (see Figure 2; Guthrie & Balaban, 2004). This means that cisplatin bound to S or other ligands may actively promote cross-linking between endogenous/exogenous nucleophilic and electrophilic molecules. This effect is similar to what occurs when Fe(III) or Fe(III)-EDTA complex interact with endogenous nucleophiles (e.g., ascorbate, GSH, H_2O_2 or reduced NADH) to induce the generation of $HO^{\cdot}$ and cross-linking of biomolecules (Dizdaroglu, Olinski, Doroshow & Akman, 1993; Murata, Imada, Inoue & Kawanishi, 1998). Cisplatin induced cross-linking of biomolecules *in vitro* is well known and results in loss of molecular function (Ivanov et al., 1998; Pizzo, Swaim, Roche & Gonias, 1988).

In summary, cisplatin bound to sulfur is redox-active. The *d*-orbital of metals such as platinum(II) allows them to serve as a redox-bride between biomolecules (Valentine, 1973). Reaction (2) coupled with autometallography reveals that cisplatin bound to sulfur is redox-

active. This redox-activity leads to oxidative cross-linking of biomolecules. This is particularly important because cisplatin simultaneously binds DNA and sulfur bearing biomolecules (Kartalou & Essigman, 2001).

5. Summary and Implications for Ototoxic Mechanisms

Cisplatin binds preferentially to sulfur and nitrogen rich biomolecules. Most biomolecules exhibit sulfur and nitrogen which suggest that cisplatin will be highly promiscuous in biomolecular binding. The plasma membrane is composed of many sulfur and nitrogen rich biomolecules and serves as the first site of assaul on the cell. Numerous ion channels on the plasma membrane harboring sulfur and nitrogen moieties are direct targets for cisplatin. Cisplatin's interactions with ion channels may be temporary but it elicits signaling cascades that may threaten the life of the cell. Ultimately, cisplatin crosses the plasma membrane to enter the cytoplasm. The redox-capacity of the platinum(II) metal readily induces ROS in the cytoplasm of the cell. Reaction (2) coupled with autometallography reveals that after systemic treatment, the cisplatin molecule is redox-active and low-affinity bound in the cell. The low-affinity binding of cisplatin may serve as an intermediate to DNA binding. For instance, although cisplatin may initially bind biomolecules in the cytoplasm, it eventually migrates to the nucleus to bind nitrogen rich DNA purines (van Boom, Chen, Teuben & Reedijk, 1999). It is likely that the molecular toxicobiology of cisplatin include, interruption of ionic homeostasis due to disruption of plasma membrane ion channels, promotion of ROS due to redox-activity from cross-linking biomolecules in the cytoplasm and DNA damage due to high-affinity for N-rich

purine DNA bases. These mechanisms are probably not independent and each results ultimately in damaged DNA (see Figure 5 for an integrative model of cisplatin ototoxicity).

In hearing research there are two dominant mechanisms of cisplatin induced ototoxicity (Campbell, Kalkanis & Glatz, 2000). The first mechanism is based on the ion homeostasis hypothesis. This hypothesis suggests that cisplatin disturbes plasma membrane ion channels that interferes with cochlear ionic homeostasis leading to apoptosis (Mcalpine & Johnstone, 1990). Figures 6 and 7 reveal molecular mechanisms by which cisplatin may alter ionic homeostasis. The second mechanism of cisplatin induced ototoxicity is based on the ROS hypothesis. This hypothesis suggests that cisplatin induces ROS that oxidizes biomolecules and promote apoptosis (Clerici, DiMartino and Prasad, 1995). Figure 8 reveals the *in vivo* molecular pathway that underlies cisplatin induced ROS and promotion of apoptosis in the cochlea. There are many impirical evidence in the hearing research literature to support these two hypotheses. In the tumor biology literature the primary mechanism by which cisplatin induces cytotoxicity and antineoplasm is based on the formation of cisplatin DNA damage. Recent research has revealed that cisplatin precipitates cisplatin DNA damage among various cell types in the inner ear (van Ruijven, de Groot, Hendriksen & Smoorenburg, 2005). This suggests, that DNA damage may be a third mechanism that underlie cisplatin induced ototoxicity. This is further supported by the fact that irregular ionic homeostasis and ROS production may each induce DNA damage (see the sections below & Figure 5).

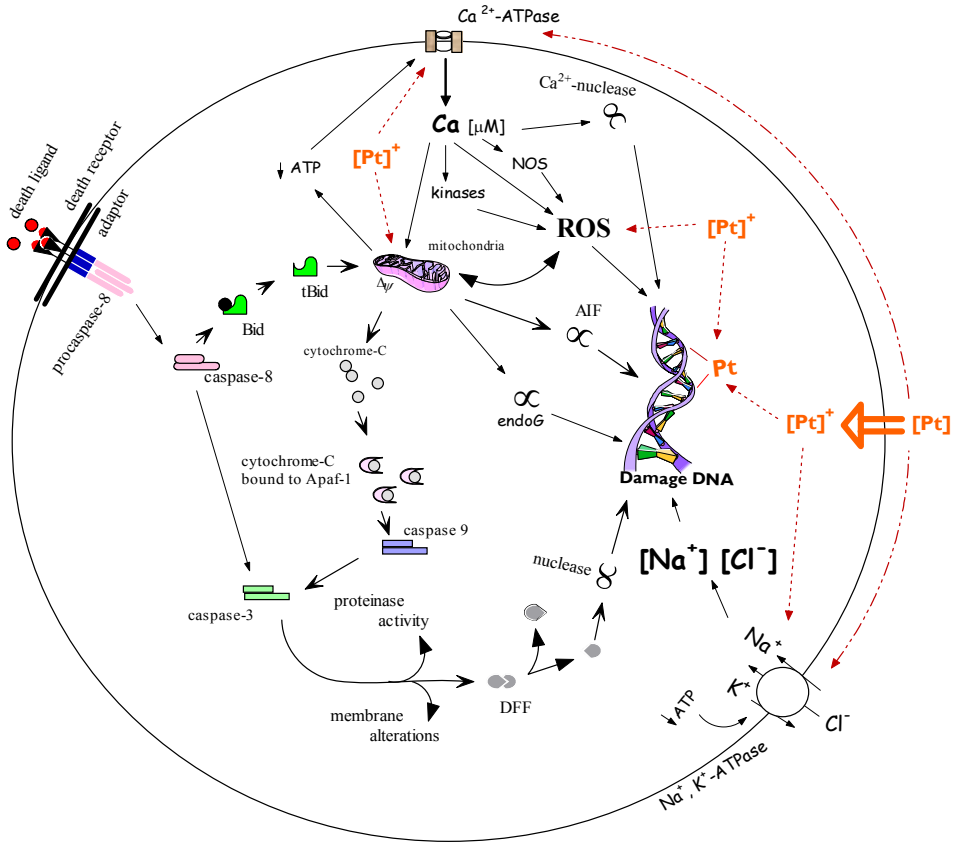


Figure 5: An integrative view of the molecular mechanisms that underlie cisplatin ototoxicity. Before entering the cell cisplatin may interact directly with the plasma membrane and ion channels (see text for details). Such, interactions disrupts ionic homeostasis. Cisplatin easily crosses the plasma membrane both by passive diffusion and active transport (see text for details). Once inside the cell cisplatin targets a wide variety of biomolecules and intracellular organelles (see text for details). Mitochondria is a consistent intracellular target. Cisplatin disrupts the mitochondria membrane potential, initiates the mitochondria apoptosis pathways and reduce the level of ATP available for ion pumps. ATP-dependent ion pumps such as Ca^{2+}-ATPase and Na^{+}, K^{+}-ATPase are specifically disrupted. Depletion of ATP for the Ca^{2+}-ATPase results in excessive influx of calcium (Ca) which intreases intracellular levels to dangerous micromolar levels. High levels of Ca initiates various negative signaling cascades that lead to DNA damage (see text for details). Depletion of ATP for the Na^{+}, K^{+}-ATPase results in excessive influx of sodium (Na^{+}) and chloride (Cl^{-}) and efflux of potassium (K^{+}) (see text for details). Excess Na^{+} and Cl^{-} promotes DNA damage in the form of strand breaks. Intracellular cisplatin is redox-active and easily promotes ROS (see text for details). ROS targets and damage DNA. Cisplatin also targets DNA directly (see text for details). Abbreviations; Pt, cisplatin; AIF, apoptosis inducing factor; endoG, endonuclease G; APAF-1, apoptosis activating factor; DFF, DNA fragmentation factor; tBid, truncated BH3 interacting domain death agonist.

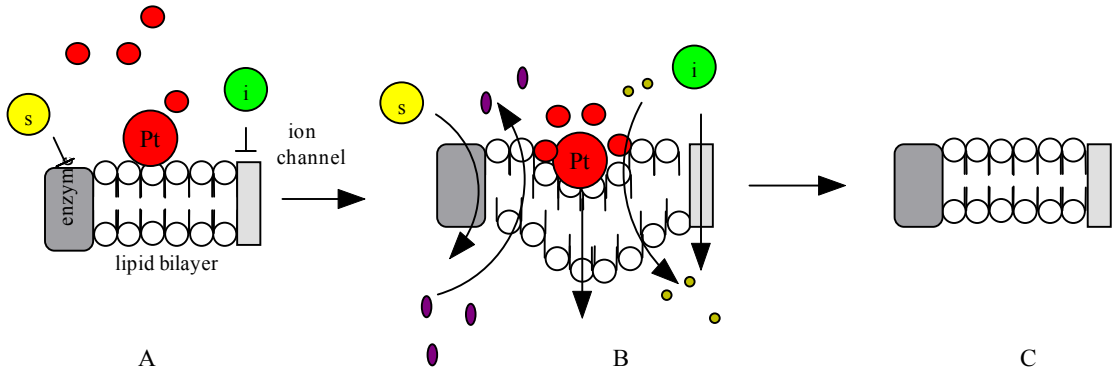
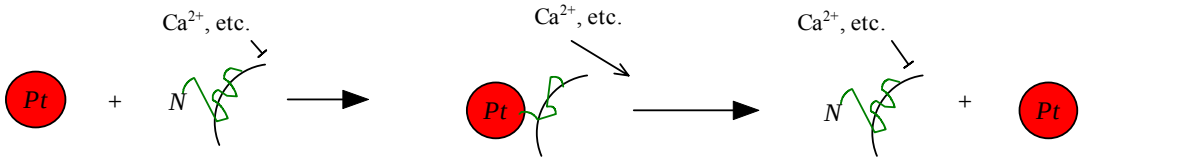


Figure 6: Cisplatin may alter ionic homeostasis by interacting with the plasma membrane. **A.** Enzymes and ion channels regulate passage across the lipid bilayer of substrates (s) and ions (i) respectively. **B.** Cisplatin binds to phospholipids via charge-charge interactions and may facilitate the binding of other cisplatin molecules. The accumulation of cisplatin molecules on the lipid bilayer results in altered fluidity, permeability (efflux/influx of small molecules) and disruption of enzyme and ion channel activities. **C.** These alterations may be temporary but could propagate via signal transduction pathways throughout the cell.



cis-[$(H_3N)_2Pt^{II}M$] and plasma membrane protein

cis-[$(H_3N)_2Pt^{II}N$] induced conformational change

cis-[$(H_3N)_2Pt^{II}M$] released from plasma membrane protein

M	N
M = Cl_2	N = SH
M = Cl (H_2O)	N = -S-S-
M = $(H_2O)_2$	N = COOH
M = plasma protein	N = PO_4
	N = OH
	N = NH_3

Figure 7: Cisplatin may alter ionic homeostasis by interacting with plasma membrane protein(s). Cisplatin [*cis*-$(H_3N)_2Pt^{II}M$] binds to plasma membrane proteins at particular sites (N) and induce conformational changes. This change in conformation results in the loss of biological function for the affected protein(s). For instance, binding to calcium (Ca^{2+}) plasma membrane proteins may lead to excess influx of Ca^{2+}.

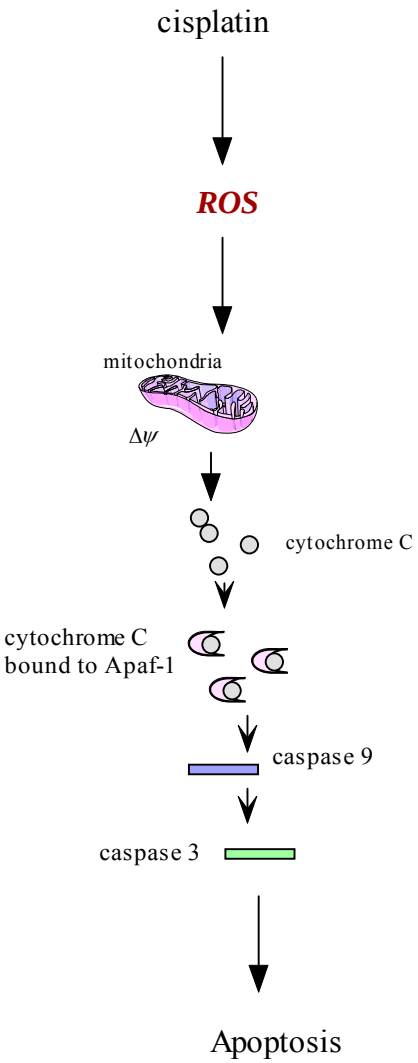


Figure 8: Molecular pathway that underlies cisplatin induced ROS and promotion of apoptosis. Cisplatin cross-links biomolecules and generate ROS. ROS attacks mitochondria and alters the mitochondrial membrane potential ($\Delta\psi$) followed by the release of cytochrome-c. Cytochrome-c binds to apoptotic activating factor-1 (Apaf-1) and activates caspases-9 which activates caspase 3 which then fascilitates cell death (apoptosis).

6. Cisplatin Alters Ionic homeostasis in the Cochlea

McAlpine and Johnstone (1990) reported experiments aimed at elucidating the mechanism of cisplatin ototoxicity. Forty seven pigmented guinea pigs (250-400g) were treated with cisplatin. Cisplatin was administered subcutaneously or iontophoresed into scala media. Electrophysiological results showed that reduction in OHC receptor current (mechanoelectrical transduction) was followed by decreasing N_1 (CAP) thresholds within 20 hours after cisplatin (6 mg/kg, s.c.) treatment. These authors theorized that cisplatin blocked the number of channels available for transduction which lead to hearing loss. Cisplatin was iontophoresed into scala media at the 3 kHz region and N_1 threshold decreased basally (towards the higher frequencies) at a rate consistent with endolymphatic flow. These findings further compelled the authors to theorize that there is no intrinsic difference in vulnerability between high and low frequency regions of the cochlea. They explained that the reason cisplatin induces high frequency hearing loss is because the drug is better able to perfuse the blood-labyrinth barrier at the base than at the apex of the cochlea, however this was not measured directly.

Saito, Moataz and Dulon (1991) studied cisplatin-induced ion channel blockage by isolating OHCs from pigmented guinea pig (200-300g) cochleae. Isolated OHCs treated with cisplatin (1 mM) showed no change in viability out to six hours following removal from the cochlea. Additionally, cisplatin was believed to block calcium ion channels as determined by microspectrofluorometry of the dual emission calcium probe indo-1. These authors argued that cisplatin binds to the plasma membrane of the OHCs and blocks calcium channels. This suggests that cisplatin's interaction with the plasma membrane may alter calcium homeostasis.

Interestingly, similar results had been shown for aminoglycosides in previous studies (Dulon, Zajic, Aran & Schacht, 1989), which suggest similar mechanisms between the drugs.

Kimitsuki, Nakagawa, Hisashi, Komune and Komiyama (1993) evaluated the effects of cisplatin on the mechanoelectrical transduction of the cochlear hair cells from hatched chicks (2-7 days old) by using a whole-cell patch-electrode voltage clamp technique. The results showed that cisplatin treatment modulated mechanoelectrical transduction channels in a dose dependent manner with voltage dependent and independent components. These results support a channel blockade role in cisplatin ototoxicity. Saito and Aran (1994) treated pigmented guinea pigs (300-400g) acutely (7.5 mg/kg/2 days) or chronically (1.5 mg/kg/10 days) with cisplatin. CAP (round window electrode placement) was used to assess ototoxicity. Significant CAP threshold shifts were observed after the second dose of acute treatment. During chronic treatment, threshold shifts were observed only at eight or nine days of treatment. The authors argued that the difference in threshold shift between acute and chronic treatment may be attributed to a blockade in OHC ion channels.

Ernst and Zenner (1995) studied the effects of cisplatin on isolated OHCs from guinea pig cochleae. The whole-cell patch-clamp technique was used to measure the effects of cisplatin. The results showed that cisplatin induced hyperpolarization and cellular elongation. From these results the authors concluded that cisplatin alter ion channels of the plasma membrane. Komune et al. (1995) treated fifty-eight albino guinea pigs (360-480) with cisplatin (12.5 mg/kg, i.m.). The results revealed that cisplatin reduced the EP from 80 mV to 32 mV. Additionally, there was an increase in potassium, sodium and chlorine in the endolymph. In the perilymph only sodium increased while potassium and chlorine stayed the same. These authors reasoned that cisplatin interferes with the normal homeostasis of ion transport in the cochlea. Peters,

Mommersteeg and Heijmen (1999) exposed the electroreceptor organ of the freshwater catfish (120-280 g, n = 8) to cisplatin (330 μM) for 1 hour. Spike trains from the electroreceptor organ primary afferents were studied for ototoxic effects. Cisplatin treatment caused a drop in spontaneous activity, reduced sensitivity and disfiguration of the frequency spectrum. However, these effects were reversed 22 days after treatment. From these results the authors reasoned that ion channels were altered by cisplatin.

In summary, both *in vitro* and *in vivo* studies reveal that cisplatin alters ionic homeostasis. Such observations have generated the hypothesis that the mechanism of cisplatin ototoxicity is disturbance of plasma membrane ion channels that result in altered cochlear ionic homeostasis. Ionic homeotasis may not be the only mechanism of ototoxicity since cisplatin generates ROS and directly bind to DNA in the cochlea.

7. Ionic Mechanisms Can Induce DNA Damage

Altered homeostasis of ions such as $[Ca^{2+}]$, $[Na^{+}]$ and $[K^{+}]$ may induce DNA damage and cell death (see Figure 5). High intracellular levels of ($[Ca^{2+}]_i$) my damage DNA by directly activating $[Ca^{2+}]$-dependent nucleases that fragments DNA (Yakovlev et al., 2000). The altered homeostasis of $[Ca^{2+}]_i$ may promote nitric oxide synthase (NOS), kinases and enery failure of mitochondria, all of which produce ROS induced DNA damage (Fonnum & Lock, 2004). Recent experiments have shown that T-type calcium channel blockers partially reduce DNA fragmentation in the organ of Corti after cisplatin intoxication (So et al, 2005). Blocking $[Ca^{2+}]$ channels only provided partial protection from DNA damage in the cochlea which suggests that

cisplatin may alter cochlear ionic homeostasis by more covert mecahnsims. For instance, cisplatin may reduce intracellular levels of ATP that limits the energy supply available for ion pumps that are vital for maintaining ionic homeostasis (Dursun et al., 2006; Pai & Sodhi, 1992). This line of thinking is supported by recent studies that reveal that cisplatin targets mitochondria and alter activity of both Ca^{+}-ATPase and Na^{+}, K^{+}-ATPase in the cochlea (Cheng, Liu, Hsu & Lin-Shiau, 2005; Devarajan et al., 2002). Reducing the availability of ATP would affect the Ca^{2+}-ATPase by inhibiting the efflux of $[Ca^{2+}]_i$, thereby increasing $[Ca^{2+}]_i$ from nM to lethal μM levels. Additionally, energy depletion by reducing ATP would affect the Na^{+}, K^{+}-ATPase by significantly increasing intracellular level of $[Na^{+}]_i$ followed by concomitant influx of $[Cl^{-}]$. Therefore, excess $[Na^{+}]$ and $[Cl^{-}]$ would accumulate in the cell. Excess $[Na^{+}]$ and $[Cl^{-}]$ induces DNA strand breaks which are chemically and structurally different than cisplatin DNA adducts (Kultz & Chakravarty, 2001). DNA strand breaks have been found in the cochlea after cisplatin treatment (Watanabe et al., 2002). Another effect of reduced ATP on Na^{+}, K^{+}-ATPase is excessive efflux of $[K^{+}]_i$. This is important because $[K^{+}]_i$ prevents apoptosis DNA fragmentation by DNA fragmentation fractors (DFF) (Hughes, Bortner, Purdy & Cidlowski, 1997). Cisplatin is known to induce apoptotic DNA fragmentation in the cochlea (Alam et al., 2000).

8. Cisplatin Mediates ROS Production in the Ear

Class B Lewis acid metals such as cadmium and mercury are known to easily generate ROS by crossing the plasma membrane and accumulate in the cytoplasm. The passage of cisplatin across the plasma membrane is currently a controversial issue because different research groups have

provided evidence to support at least three different mechanisms of influx. For instance, cisplatin is believed to cross the plasma membrane by passive diffusion via lipid solubility, passive diffusion via ion channels and receptor/carrier-mediated transport (Gately & Howell, 1993). Class B metals such as cadmium and mercury are known to passively diffuse across the plasma membrane. Cisplatin also may diffuse passively across the plasma membrane and induce cooperative uptake, where the initial binding to phospholipids facilitates the entry of other cisplatin molecules (Wang, Lu & Li, 1996). Few experimental observations have supported the proposal of passive diffusion of cisplatin across the plasma membrane (Gately & Howell, 1993). Conditions that increase plasma membrane permeability such as hyperthermia, radiation and chemical treatment have supported the notion of passive diffusion (Ohtsubo et al., 1997; Yang, Douple & Wang, 1995). Systematic work on the uptake and distribution of cisplatin using sensitive procedures such as inductively coupled plasma mass spectrometry have concluded that cisplatin enters the cell via passive diffusion (Ghezzi, Aceto, Cassino, Gabano & Osella, 2004).

A few studies have supported the proposal of receptor/carrier-mediated transport of cisplatin across the plasma membrane by showing that cisplatin's accumulation can be manipulated (stimulated or inhibited) by pharmacological means and by altering signal transduction pathways (Canada, 1993; Gately & Howell, 1993). Pharmacological agents that do not alter plasma membrane permeability may inhibit cisplatin uptake which supports the notion of receptor/carrier transport (Andrews, Velury, Mann & Howell, 1988; Desoize & Madoulet, 2002; Gately & Howell, 1993; Kartalou & Essigmann, 2001).

There are multiple routes for the passage of cisplatin across the plasma membrane depending on its modification in the blood and extracellular mileu. Most researchers who disagree on the exact route of influx generally agree that 50% of cisplatin will passively diffuse

across the cell membrane while the remaining 50% is actively transported (Guminski, Harnett & de Fazio, 2002; Kelland, 1993). Regardless of the specific mechanism of influx, cisplatin efficiently crosses the membrane by weak sequential associations and disassociations between biomolecules (Wang, Lu & Li, 1996). Cisplatin's interaction with the plasma membrane is highly complex and there are many routes of entry into the cell (see Figure 9). Iron and copper are transported to various cell types by proteins such as transferin and ceruloplasmin, respectively. Platinum(II) is a more reactive metal than iron and copper therefore molecular mimicry may facilitate passage across the membrane. Once across the membrane, intracellular levels are determined by mechanisms of efflux (see Figure 9).

Cisplatin efflux may be biphasic with an initial rapid phase then a slower phase (Mann, Andrews & Howell, 1990). The efflux of cisplatin may have some relevance to the cochlea. The ability of the stria vascularis to recover from cisplatin intoxication may be due to specialized drug resistance pumps on the plasma membrane. For instance, P-glycoprotein is an energy-dependent efflux pump, also known as the multidrug resistance pump 1 (MRP1). MRP1 has been localized in the stria vascularis and vestibular epithelium but not the organ of Corti (Saito et al., 2001). Experiments on knockout mice suggest that the MRP1 may influence the accumulation of various drugs in the inner ear and may mediate ototoxicity (Zhang et al., 2000). Another member of the MRP1 family of drug resistance pumps is the so called cisplatin efflux pump or canalicular multispecific organic anion transporter (cMOAT), which is now known as MRP2. MRP2 typically functions to transport glucuronide-conguated bile acids into the bile, however it may export GSH-drug conjugates. Experiment on the MRP2 suggested that a cisplatin-resistant phenotype can be achieved through controlling the level of cisplatin in the

cytoplasm (Taniguchi et al., 1996). Such control is vital because intracellular cisplatin is redox-active which means that it will easily promote ROS (Guthrie & Balaban, 2004).

Research supporting a ROS mechanism of cisplatin ototoxicity has been ongoing since the 1990's. For instance, Clerici, DiMartino and Prasad (1995) studied the direct anatomical alterations of ROS on isolated cochlear OHCs. OHCs were superfused with artificial perilymph containing the following ROS; $O_2^{\cdot -}$, $OH^{\cdot}$ or the ROS inducer H_2O_2. The results showed that ROS directly induced OHC bleb formation and diminished cell length. From these results the authors concluded that ROS induced OHC changes are consistent with ototoxic hair cell damage. Clerici and Yang (1996) studied the direct effects of ROS on cochlear physiology. Guinea pigs (250-350 g) were treated with ROS infused through a hole in the cochlea. Analysis of CAP and CM (round window electrode placement) served as functional measurements of the cochlea. ROS treatment lead to significant threshold shifts of the CAP in the high frequencies. There was no statistically significant difference between CM of the ROS treated animals and control animals. Clerici, Hensley, DiMatino and Butterfield (1996) directly demonstrated the generation of ROS as a result of cisplatin ototoxicity. Guinea pig cochlear explants were studied by using electron paramagnetic resonance spectroscopy. The generation of the hydroxyl radical was particularly abundant after cisplatin exposure. Cisplatin was believed to catalyze hydrogen peroxide to the hydroxyl radical. These direct studies engendered the hypothesis of cisplatin ROS induced ototoxicity. Since these direct experiments, subsequent work has relied on indirect methods to support an ROS mechanism of cisplatin ototoxicty (Kamimura, Whitworth & Rybak, 1999; Korver, Rybak, Whitworth & Campbell, 2002; Teranishi, Nakashima & Wakayashi, 2001; Rybak & Kelly, 2003; Wimmer et al., 2004).

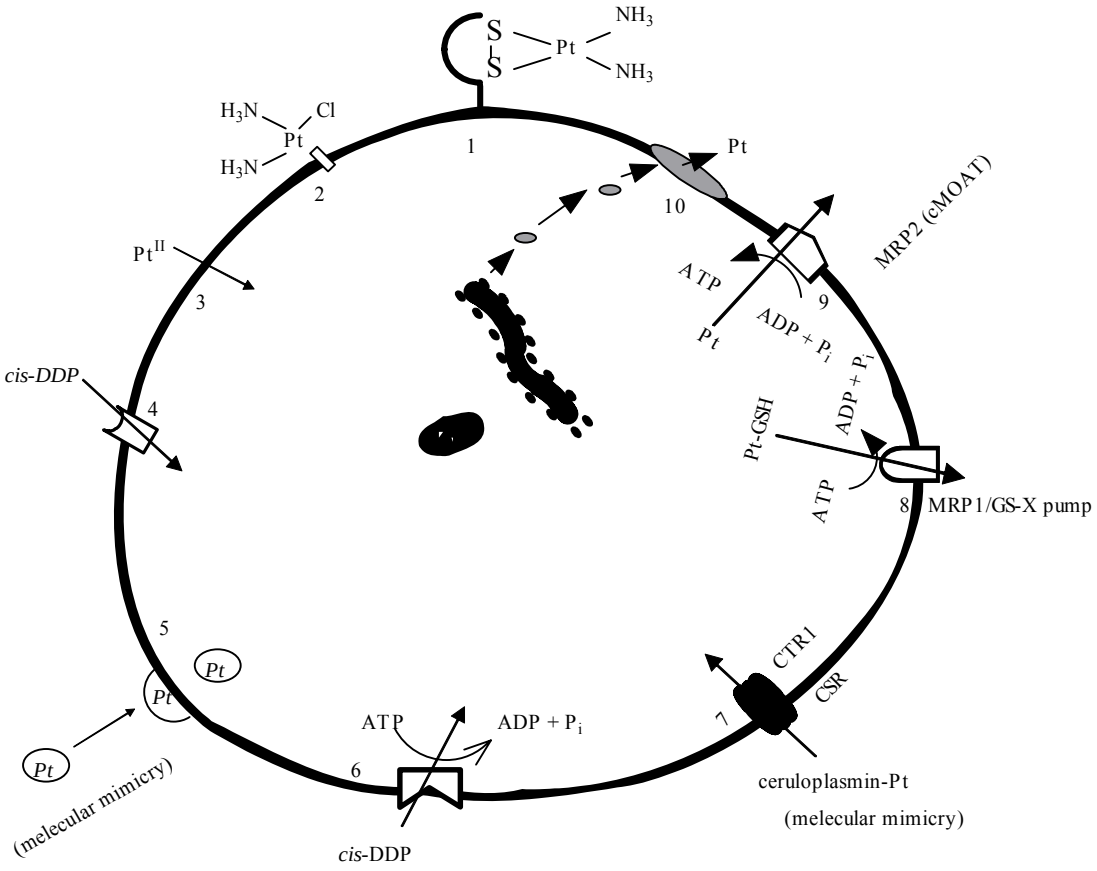


Figure 9: Complex interaction of cisplatin with the cell membrane and routes of influx and efflux. 1. membrane protein with disulfide bridge attracts cisplatin. 2. cisplatin bound to phospholipids. 3. cisplatin may enter the cell via passive diffusion. 4. cisplatin may enter the cell via passive diffusion through channel proteins. 5. cisplatin may enter the cell by transferin & transferring receptor or facilitated diffusion by carrier proteins (energy independent transport). 6. cisplatin may enter the cell via energy dependent transport by carrier proteins. 7. cerulopasmin bound cisplatin, copper transporter 1 (CTR1) & cell surface reductase (CSR) facilitates the transport of cisplatin into the cell. 8. multidrug resistance protein 1 (MRP1) or glutathione S-conjugated export pump (member ABC superfamily) facilitates the efflux of cisplatin. 9. multidrug resistance protein 2 (MRP2) aka canalicular multispecific organic anion transporter (cMOAT) facilitates the efflux of cisplatin. 10. *trans*-Golgi export system facilitates the efflux of cisplatin. Cisplatin abbreviations = Pt^{II}, Pt, *cis*-DDP.

9. Cisplatin Induces DNA Damage

The hearing research literature has provided data to suggest that cisplatin targets ion channels that alters ionic homeostasis or cross-link biomolecules to promote ROS. The cancer literature has provided data to suggest that cisplatin directly binds to DNA and this binding underlies cytotoxicity and antineoplasm. The early studies on prokaryote cells that discovered the biological activity of cisplatin also revealed cisplatin-induced filamentous growths (elongation of bacteria without division due to defective replication from DNA damage). Filamentous growths are characteristic of DNA damaging agents (Adler & Hardigree, 1965; Rosenkranz, Garro, Levy & Carr, 1966; Witkin, 1967). The majority of cisplatin is found bound to DNA (Harder, 1974). Cisplatin is highly attracted to the N-rich purines (guanine & adenine) of DNA (Reedijk, 1999). In fact, the basses of the anticancer action of cisplatin, is its ability to precipitate DNA adducts (Jamieson & Lippard, 1999). The adducts formed by cisplatin are monofunctional and bifunctional (Eastman, 1986; Fichtinger-Schepman, van Oosterom, Lohman & Berends, 1987; Kelland, 2000). Monoaquated or monospeciated cisplatin forms the monofunctional adducts (see Figures 3 and 10). Monofunctional adducts typically bind to the N7 of a single DNA base, particularly a guanine residue while coordinated to H_2O, $HO^{\cdot}$ or a protein. These adducts account for ~2% of the adducts formed. Formation of monofunctional adducts have a $t_{1/2}$ ~0.1 h and they may convert to a bifunctional adduct at a $t_{1/2}$ ~2.1 h (Bancroft, Lepre & Lippard, 1990).

Bifunctional adducts bind to the N7 position of purines to form interstrand and intrastrand cross-links (Jamieson & Lippard, 1999; Kartalou & Essigmann, 2001; Kelland, 2000). The interstrand cross-link is *cis*-[$(H_3N)_2$Pt{d(pGpC)/d(pGpC}] and is often denoted as inter-d(pGpC)/d(pGpC) or *cis*-G/G (see Figure 10). Here cisplatin binds to guanine residues on

opposite DNA strands. The *cis*-G/G accounts for ~2% of bifunctional adducts (Jamieson & Lippard, 1999; Kartalou & Essigmann, 2001). Among the intrastrand cross-links the most abundant is the *cis*-[$(H_3N)_2$Pt{d(pGpG)}] often denoted as 1,2-d(GpG) or *cis*-GG (see Figure 10). Here cisplatin binds to two adjacent guanine (GG) residues. The *cis*-GG are the major adducts and comprise ~60-65% of the adducts formed (Jamieson & Lippard, 1999; Kartalou & Essigmann, 2001). The second most abundant intrastrand adduct is the *cis*-[$(H_3N)_2$Pt{d(pGpA)}] often denoted as 1,2-d(ApG) or *cis*-AG (see Figure 10). Here cisplatin binds to an adenine-guanine (AG) sequence. The *cis*-AG comprise ~20-25% of the adducts formed (Jamieson & Lippard, 1999; Kartalou & Essigmann, 2001). The third most abundant intrastrand adduct is the *cis*-[$(H_3N)_2$Pt{d(GpXpG)}] often denoted as 1,3-d(GpXpG) or *cis*-GXG, where X may be any nucleotide (see Figure 10). Here cisplatin binds to two guanines separated by X. The *cis*-GXG comprise ~5-10% of the adducts formed (Jamieson & Lippard, 1999; Kartalou & Essigmann, 2001).

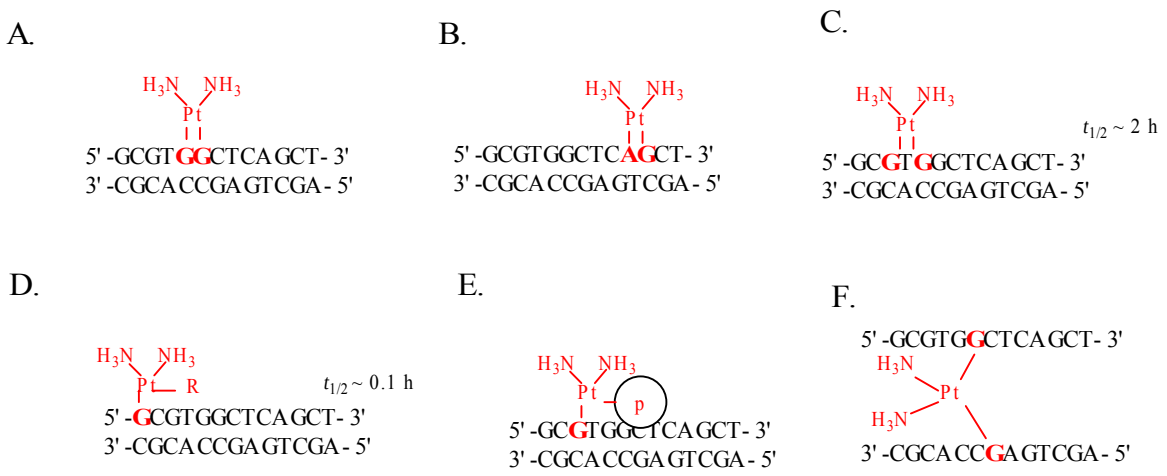


Figure 10: Structure of cisplatin DNA adducts. **A.** *cis*-GG. **B.** *cis*-AG. **C.** *cis*-GXG. **D.** monofunctional adduct, where R = H_2O, $HO^{\cdot}$, etc. **E.** monofunctional adduct, where P = a protein. **F.** *cis*-G/G. See text for details.

All cisplatin DNA adducts may induce structural alterations to the DNA helix. For instance they may significantly bend, unwind and shorten the DNA helix (Bellon, Coleman & Lippard, 1991; Cohen, Bauer, Barton & Lippard, 1979). They bind predominantly in the major groove and may bend the DNA helix by ~20-80º and unwind the helix by ~13-80° (Jamieson & Lippard, 1999; Kartalou & Essigmann, 2001). A single adduct may change the angular orientation of the DNA helix significantly towards the major groove. The *cis*-G/G adduct may bend the helix by ~20-47° and unwind it by ~70-87°. The *cis*-GG adduct may bend the helix by ~32-78° and unwind it by ~13-25°. The *cis*-AG adduct may bend the helix by ~25-55° and unwind it by ~13°. The *cis*-GXG adduct may bend the helix by 20-35 ° and unwind it by 19-23°. Each adduct induces a unique alteration to the classic Watson-Crick DNA structure. These

alterations to the DNA structure are recognized by a variety of different proteins. Many of these proteins are involved in vital cellular functions such as transcription.

Cisplatin DNA adducts accumulate in tumor cell as well as cells from a variety of tissues such as pancreas, liver, kidney, muscle, brain, testis, duodenum, spleen and heart (Terneggen et al., 1987). DNA adducts also have been localized among various cell types in the inner ear (Thomas, Lautermann, Liedert, Seiler & Thomale, 2006; van Ruijven, de Groot, Hendriksen,& Smoorenburg, 2005). In addition to platinated DNA adducts, cisplatin may induce single and double strand breaks in DNA. The mechanisms of such strand breaks are less known but the redox-activity of platinum(II) may induce ROS that could easily induce strand breaks.

In summary, DNA is one target for cisplatin. Speciation of cisplatin in the cytoplasm prepares it for covalent binding to the N7 of purine DNA bases. The N7 is a highly nucleophilic site that is known to attract a number of genotoxic xenobiotics. Less attention has been focused on cisplatin's binding to phosphates on the DNA even though such interactions would be weak. Cisplatin is able to access the major groove of the DNA and bend the helix towards the major groove thus increasing the minor groove. The guanine residues are particularly targeted by cisplatin because of their high nucleophilic nature. The structural and chemical alterations of DNA induced by cisplatin are heterogeneous which suggest that the molecular pathological consequences will be complex.

10. Molecular Pathology of Cisplatin Induced DNA Damage

Cisplatin DNA adducts may affect replication, inhibit transcription and induce apoptosis. Replication is the process by which new DNA is synthesized by polymerases during the S-phase of the cell cycle. Early research on the biological activity of cisplatin suggested that cisplatin DNA adducts inhibited replication which by itself could account for the antineoplastic activity. Cisplatin DNA adducts inhibit a variety of DNA polymerases. For instance, *cis*-GG, *cis*-AG and *cis*-GXG adducts inhibit DNA polymerase I and III, Taq polymerase and bacteriophage T4 and T7 DNA polymerases (Comess, Burstyn, Essigmann & Lippard, 1992). Even a single cisplatin DNA adduct may inhibit DNA polymerases. However, the death of cancer cells may not correlate with inhibition of replication (Chu, 1994; Sorenson & Eastman, 1988). For instance, Chinese hamster ovary cell lines that were deficient in DNA repair died at cisplatin concentrations that failed to disrupt replication (Sorenson & Eastman, 1988). Additionally, cell lines that were proficient at DNA repair survived extreme doses of cisplatin that disrupted replication (Chu, 1994). However, the inhibition of replication does not account for cytotoxicity of non-replicating (non-dividing) cells, such as neurons and hair cells.

Transcription is the process of generating messenger ribonucleic acid (mRNA) leading to protein synthesis. Protein synthesis is essential for cell survival and occurs throughout the cell cycle (Bielas & Heddle, 2004). Tumor cells treated with cisplatin may progress through the S-phase leading to G2 cell-cycle arrest and apoptosis (Allday, Inman, Crawford & Farrell, 1995; Chu, 1994; Kelland 1993). Here transcription products needed to enter M-phase of the cell-cycle are inhibited by cisplatin DNA adducts. For instance, RNA polymerase is stalled by cisplatin DNA adducts during transcription (Corda, Anin, Leng & Job, 1992; Tornaletti, Patrick, Turchi &

Hanawalt, 2003). Additionally, proteins essential for transcription (e.g., hUBF & TBP), are lured to cisplatin DNA adducts which makes them unavailable to participate in transcription (Jordan & Carmo-Fonseca, 2000; Kartalou & Essigmann, 2001). Proteins essential for transcription may have as much as a 175 fold increase in binding affinity for a single cisplatin DNA adduct compared to their normal binding sites (Cohen, Jamieson & Lippard, 2000). The ability of cisplatin DNA adducts to sequester important transcription proteins is called transcription hijacking (Kartalou & Essigmann, 2001; Zhai, Beckmann, Jantzen & Essigmann, 1998). Transcription hijacking can trigger apoptosis (Siddik, 2003). Therefore, a combination of both replication inhibition and transcription hijacking are implicated in cisplatin anticancer activity. For non-cancerous cells and non-dividing cells, transcription hijacking may be a primary event that leads to apoptosis.

The chemical and physical alterations to the DNA duplex by platinated adducts are recognized by a variety of proteins involved in vital cellular processes. The titration of these proteins away from their natural binding sites inhibits cell function. For instance, proteins involved in transcription of vital genes may be titrated away from the transcription process by cisplatin DNA adducts (Treiber, Zhai, Jantzen & Essigmann, 1994). As discussed above, this process is called transcription hijacking and may directly induce apoptosis. Proteins unrelated to transcription also may be hijacked by cisplatin DNA adducts and platinum-protein adducts may hijack other proteins. Therefore, a more general term, "molecular hijacking" is appropriate (see Figure 11). One of the most well studied groups of proteins involved in molecular hijacking is the high-mobility-group (HMG) proteins. HMG proteins are a family of small, nonhistone chromatin-associated proteins involved in gene regulation and maintenance of chromatin structure. These proteins may have a three fold greater binding affinity for cisplatin DNA

adducts than their natural binding sites (Treiber, Zhai, Jantzen & Essigmann, 1994). Multiple HMG domains function in an additive way to increase the binding affinity to cisplatin DNA adducts (Zhai, Beckmann, Jantzen & Essigmann, 1998). The binding of HMG proteins to cisplatin DNA adducts may shield the adducts from DNA repair factors (Patrick & Turchi, 1998). Such repair shielding increases the tenure of platinum on DNA and induces apoptotic cell death (see Figure 11). This is particularly important because cisplatin treatment significantly increase protein levels of HMG1 among spiral ganglion neurons in the cochlea (Li, Liu & Frenz, 2006). Other proteins beyond HMG proteins also may participate in protein hijacking and repair shielding (Yaneva, Leuba, van Holde, & Zlatanova, 1997).

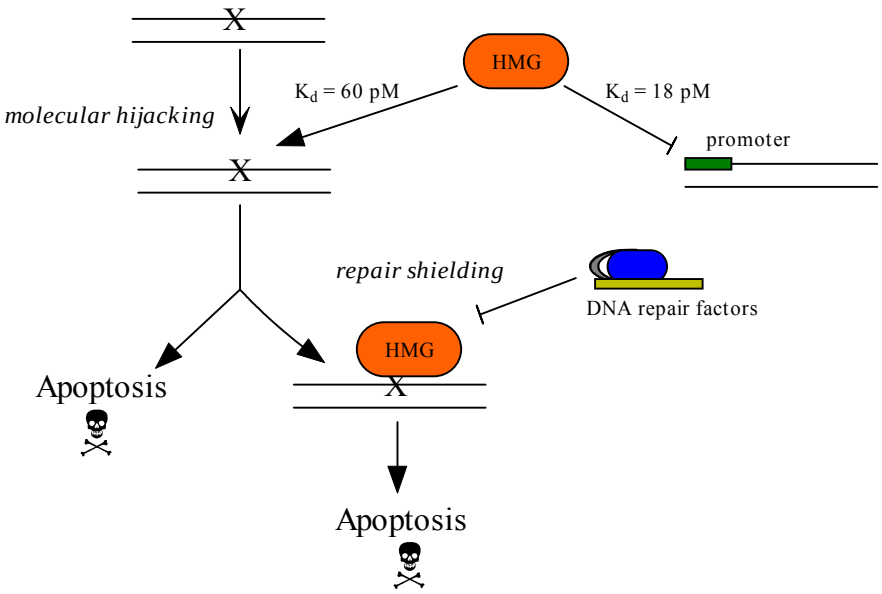


Figure 11: Molecular hijacking and repair shielding. High mobility group (HMG) protein(s) exhibit higher binding affinity for cisplatin DNA adduct (X) than the promoter binding sites which disrupts transcription. Additionally, the binding of HMG protein to cisplatin DNA adduct may shield the adduct from DNA repair factors.

Cisplatin's interaction with DNA often results in apoptosis. Apoptosis can be determined by morphological and biochemical changes. Morphological changes include, cell shrinking with membrane preservation, chromatin condensation, membrane blebbing and release of apoptotic bodies (Choi, 1996). The biochemical changes are many but DNA strand breaks often are used as a diagnostic estimate of apoptosis. The balance between proapoptic (e.g., Bax, Bak, Bad, Bcl-X) and antiapoptotic (e.g., Bcl-2, Bcl-XL, Mcl-1, Bcl-w and A1) proteins determines the progression of apoptosis. Cisplatin increases the level of Bax and Bak while decreasing the level of Bcl-2 in tumor cells (Henkels & Turchi, 1999; Jones, Turner, Mcllwrath, Brown & Dive, 1998).

Nuclear DNA (nDNA) is consistently targeted by cisplatin but mitochondria DNA (mtDNA) may accumulate up to 50-fold more cisplatin DNA adducts than nDNA (Giurgiovich et al., 1997). Cisplatin induces the release of cytochrome-c from mitochondria and subsequent activation of cysteine-aspartate-specific proteases (caspases) among tumor cells which promotes apoptosis (Kojima et al., 1998; Seki et al., 2000). The death receptor (extrinsic) tumor necrosis factor (TNF) apoptotic pathway which also regulates caspases is involved in cisplatin antitumor activity (Fulda, Los, Friesen & Debatin, 1998). These results suggest that multiple apoptotic pathways may be involved in the antitumor and cytotoxicity of cisplatin (see Figure 5).

In summary, cisplatin DNA adducts induces a variety of structural and chemical alterations to DNA that leads to various molecular pathological consequences. The three most profound are inhibition of replication and transcription and induction of apoptosis. Inhibition of replication may facilitate the death of dividing cells but may not explain the death of non-dividing cells, such as hair cells and neurons. Transcription inhibition by stalling of RNA polymerase and/or molecular hijacking is enough to initiate cell death regardless of cell cycle status. Molecular hijacking also may facilitate the shielding of cisplatin DNA adducts from DNA repair factors which increases the tenure of platinum on the DNA helix leading to cell death. The major cell death process induced by cisplatin is apoptosis. Various apoptotic pathways are involved but the mitochondria mediated apoptotic pathway may be particularly important because the majority of genomic platinum is bound to mtDNA (see Figure 5). Mitochondriae are unable to remove cisplatin from there genome because they lacks specialized DNA repair pathways that repair cisplatin induced DNA damage (Salazar & Houten, 1997; Olivero, Cheng, Lopez-Larraza, Semino-Mora & Poirier, 1997).

11. Repair of Cisplatin Induced DNA Damage

Early studies that demonstrated that DNA is a target for cisplatin also revealed that sensitivity to cisplatin depends on the tumor's proficiency at DNA repair (Jamieson & Lippard, 1999). Upon reacting with DNA, cisplatin forms adducts and cross links that globally distort the DNA. These adducts and cross-links, represent covalent bonds between cisplatin and the DNA bases, particularly the purines (guanine & adenine) (Redon, Bombard, Elizondo-Riojas & Chottard, 2003). There is a positive correlation between the accumulation of cisplatin DNA adducts and cell death (Fraval & Roberts, 1979). The removal of these adducts from the DNA helix is accomplished by nucleotide excision repair (NER) pathways (see figure 12). For instance, cisplatin is exceptionally effective at killing testicular cancer cells and may induce a 95% cure rate (Kartalou & Essigmann, 2001). Testicular cancer cells are deficient in NER (Koberle, Masters, Hartley & Wood, 1999). Ovarian cancer and small cell lung cancers may initially respond to cisplatin treatment but later develop resistance (Ferry, Hamilton & Johnson, 2000; Giaccone, 2000). This resistance is correlated with increased expression of NER protein-enzymes (Dabholkar et al., 1994; Selvakumaran, Pisarcik, Bao, Yeung & Hamilton, 2003).

It is accepted that NER is the primary mechanism by which cisplatin resistant tumor cells survive cisplatin DNA damage (Wu, Fan, Xu & Zhou, 2003). For instance, cells that were once insensitive to cisplatin may become sensitive by suppressing the NER protein, xeroderma pigmentosum A (XPA) (Wu, Fan, Xu & Zhou, 2003). XPA (32 kDa) is a zinc-finger DNA binding protein with high affinity for cisplatin DNA adducts. It also commits a cell to NER and coordinates repair activities (Boonstra et al., 2001; de Laat, Jaspers & Hoeijmakes, 1999). Beyond NER, XPA has no other known cellular function (de Laat, Jaspers & Hoeijmakes, 1999;

Thoma & Vasquez, 2003). In fact inhibition of XPA renders tumor cells unable to initiate NER (Koberle, Masters, Hartley & Wood, 1999; Rosenberg, Taher, Kuemmerle, Farnsworth & Valerie, 2001).

NER mechanisms can be partitioned into two pathways which differ by the way they identify lesions (Thoma & Vasquez, 2003; de Laat, Jaspers & Hoeijmakes, 1999). One pathway identifies DNA damage on non-transcribing genes and is called global genomic NER (GG-NER) (Costa, Chigancas, Galhardo, Carvalho & Menck, 2003; de Laat, Jaspers & Hoeijmakes, 1999). Recognition of lesions in this pathway is accomplished by the NER protein, xeroderma pigmentosum C (XPC) (Boonstra et al., 2001; Chen, Xu, Yang & Wang, 2003). XPC (125 kDa) is a DNA binding protein that is charged with the task of scanning the entire genome for lesions (de Laat, Jaspers & Hoeijmakes, 1999). In fact, absence of XPC results in failure of GG-NER (Costa, Chigancas, Galhardo, Carvalho & Menck, 2003; de Laat, Jaspers & Hoeijmakes, 1999).

The other pathway responds to DNA damage on transcribed genes and is called transcription coupled NER (TC-NER) (de Laat, Jaspers & Hoeijmakes, 1999). Recognition of lesions in this pathway is accomplished by RNA polymerase during transcription. Here, RNA polymerase is stalled at the site of lesion, which allows for quick identification and titration of NER enzymes (Tornaletti, Patrick, Turchi & Hanawalt, 2003). XPA is essential to both pathways (de Laat, Jaspers & Hoeijmakes, 1999), for instance, suppression of XPA results in defective GG-NER and TC-NER (Boonstra et al., 2001; Rosenberg, Taher, Kuemmerle, Farnsworth & Valerie, 2001). Both GG-NER and TC-NER are needed in order to defend the genome from toxic insults. Consequently, cells that lack even one of these NER pathways would be highly susceptible to cisplatin. The rate-limiting step for NER activity is lesion identification/verification (Thoma & Vasquez, 2003). In TC-NER this step is accomplished by

transcription factors as well as XPA while GG-NER relies on XPC and XPA, both of which have no other known cellular function (Costa, Chigancas, Galhardo, Carvalho & Menck, 2003). Resent research has demonstrated that XPC first identifies the DNA damage then recruits XPA for verification and subsequent commitment of NER (Thoma & Vasquez, 2003). XPC is a marker for only GG-NER activity while XPA is a marker for NER activity in general (de Laat, Jaspers & Hoeijmakers, 1999). Gene expression for these proteins tends to increase among cisplatin resistant cells resulting in cell survival (Fuertes, Castilla, Alonso & Perez, 2003).

In summary, cisplatin induced DNA damage is primarily repaired by the molecular pathways of NER. In fact, tumor cells that are resistant to cisplatin are proficient at NER while tumor cells that are susceptible to cisplatin are less proficient at NER. Over 30 different proteins are involved in the NER process (see Figure 12). The rate-limiting steps of NER are mediated by XPC and XPA. Unlike other NER proteins, XPC and XPA have no other known celluar function. Gene and protein expression of XPC is often used as a measure of GG-NER activity. Gene and protein expression of XPA is often used as a measure of overall NER (both GG-NER & TC-NER) activity. Both XPC and XPA recognize a large variety of damaged DNA caused by various endogenous and exogenous factors but they exhibit particular affinity for cisplatin DNA adducts. The role of XPC and XPA in cisplatin ototxicity has never been explored but may provide new information about the ear's response to csiplatin intoxication.

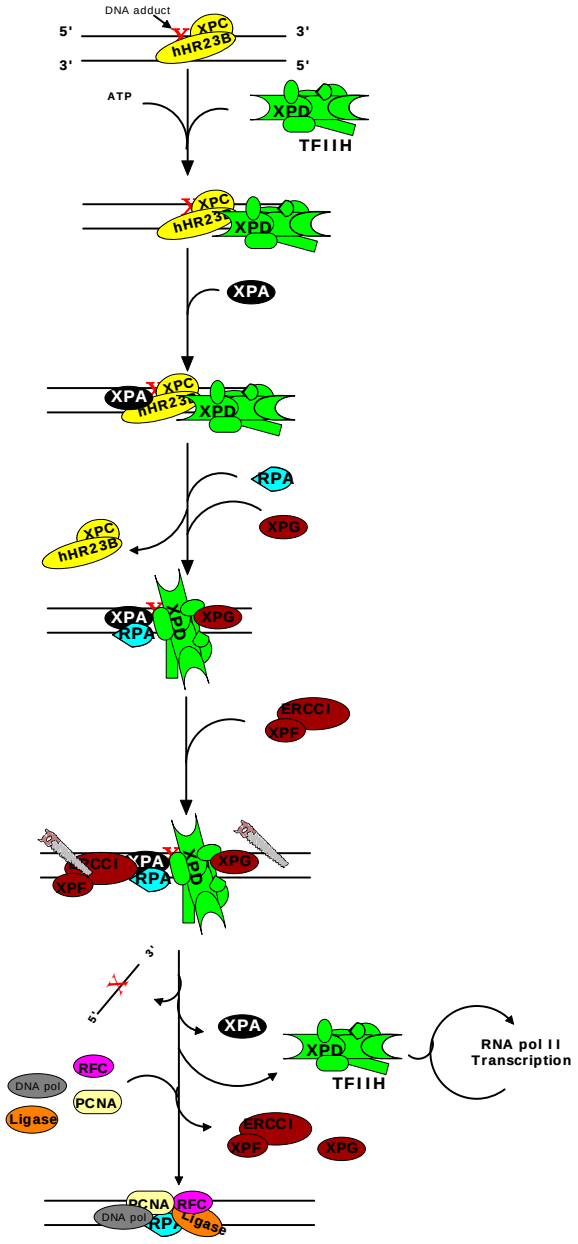


Figure 12: Molecular pathway of global-genomic nucleotide excision repair. See text for details.

D. RELEVANCE OF NER TO CISPLATIN OTOTOXICITY

NER was first demonstrated among prokaryotic cells and believed to be a DNA protection mechanism against short wave ultra violet (UV) components of sunlight (Pettijohn & Hanawalt, 1964). Subsequent experiments revealed that NER pathways exist also among eukaryotic cells and actively repair DNA damage due to UV exposure (Cleaver, 1968). UV exposure leads to bulky DNA adducts such as cyclobutane pyrimidine dimers (CPD) and (6-4) photoproducts that alters the structure of the DNA helix (de Laat, Jaspers & Hoeijmakers, 1999). These structural alterations are similar to cisplatin induced DNA alterations (see section on cisplatin induced DNA damage). The discovery that the NER proteins XPC and XPA exhibit similar affinity for bulky UV DNA adducts and cisplatin DNA adducts suggested that NER substrates extend beyond UV DNA damage (Asahina et al., 1994; Trego & Turchi, 2006). Cisplatin DNA adducts stimulate gene expression of XPC and XPA among tumor cells (Weaver et al., 2005). Although the inner ear is embedded in the temporal bone shielded from UV exposure, cisplatin enters the inner ear and form DNA adducts among various cell types (Schweitzer et al., 1984; van Ruijven, de Groot, Hendriksen & Smoorenburg, 2005). These findings motivated the hypothesis that gene expression for XPC and XPA will increase in the inner ear as a function of cisplatin treatment (see aim 1).

Within the inner ear the cochlear neurosensory epithelium is more vulnerable to cisplatin cytotoxicity than the vestibular epithelium (Black, Gianna-Poulin & Pesznecker, 2001; Myers, Blakley & Schwan, 1993; Nakayama, Riggs & Matz, 1996; Sergi, Ferraresi, Troiani, Paludetti & Fetoni, 2003; Schweitzer, Rarey, Dolan, Abrams & Sheridan, 1986). This is particularly interesting, since the level of cisplatin is greater in the vestibular epithelium than the cochlear

neurosensory epithelia (Schweitzer et al., 1986). The invulnerability of the vestibular epithelium to cisplatin intoxication suggests that it may have a greater capacity to defend its genome from cisplatin DNA adducts. Injury to the mammalian vestibular epithelium is known to induce mitosis and complete phenotype convertion among hair cells and supporting cells (Berggren, Liu, Frenz & van de Water, 2003; Shou, Zheng & Gao, 2003). Such changes do not occur among hair cells and supporting cells of the mammalian cochlea. In fact, cells from the adult mammalian vestibular sensory epithelium have shown pluripotency both *in vivo* and *in vitro* and can give rise to a variety of cell types representative of ectodermal, mesodermal and endodermal lineages (Li, Liu & Heller, 2003). These findings suggest that hair cells and supporting cells of the vestibular and cochlear epithelium are biologically different types of cells in terms of NER capacity.

Nonsensory epithelium cells of the cochlea such as the stria vascularis, inner spiral sulcus cells (ISSC) and outer spiral sulcus cells (OSSC) are able to initiate spontaneous mitosis (Conlee, Gerity & Bennett, 1994; Dunaway, Mhaskar, Armour, Whitworth & Rybak, 2003; Lang, Schulte & Schmiedt, 2003; Zine & Ribaupierre, 1998). They may become more mitotic as a result of direct damage to themselves or damage to the hair cells (Lang, Schulte & Schmiedt, 2003; Sluyter, Klis, de Groot & Smoorenburg, 2003; Vago, Humbert & Lenoir, 1998; Zine & Ribaupierre, 1998). For instance, it is known that they will divide and change their phenotype as a result of noise damage or exposure to aminoglycosides and loop diuretics (Lang, Schulte & Schmiedt, 2003; Vago, Humbert & Lenoir, 1998; Zine & Ribaupierre, 1998). The functional consequence may be alterations in cochlear microstructure that might be revealed in OAE fine-structure. Cisplatin ototoxicity research largely has neglected non-sensory epithelium cells possibly because they tend to be invulnerable to cisplatin compared to hair cells and neurons. It

is conceivable that cisplatin intoxication of the cochlea may stimulate these cells to divide but cisplatin DNA adducts may arrest the cell-cycle during which NER enzymes are up regulated for DNA repair that then prevents apoptosis among these cells. This line of thinking is consistent with the accepted notion that cell cycle arrest is needed in order to mobilize NER activity, which increases cell survival among cancer cells (Siddik, 2003).

Ovarian cancer and small cell lung cancer are susceptible initially to cisplatin but later develop resistance which correlates with increased NER activity (Selvakumaran, Pisarcik, Bao, Yeung & Hamilton, 2003). Similarly, the stria vascularis is susceptible to cisplatin (Klis et al., 2002; Sluyter, Klis, de Groot & Smoorenberg, 2003) but later recovers functionally and morphologically (Klis et al., 2002; Sluyter, Klis, de Groot & Smoorenberg, 2003). This recovery phenomenon may represent cell-cycle arrest leading to up regulation of NER protein-enzymes.

Cells of the stria vascularis are believed to progress through the cell cycle via spontaneous mitosis and damage induced mitosis (Conlee, Gerity & Bennett, 1994; Dunaway, Mhaskar, Armour, Whitworth & Rybak, 2003). It is known that cisplatin attacks the stria vascularis before the hair cells (Tsukasaki, Whitworth & Rybak, 2000). Yet, the stria vascularis survives while hair cells and neurons die (van Ruijven, de Groot & Smoorenburg, 2004). Additionally, systemic administration of radioactive platinum showed a two to three fold uptake in the stria vascularis compared to the organ of Corti (Schweitzer et al., 1984; Schweitzer et al., 1986). This suggests that the stria vascularis is a direct target for cisplatin. The effect of cisplatin on the ISSC and OSSC has not been investigated, however there has been some work with aminoglycosides. *In vivo* and *in vitro* studies have shown that aminoglycoside induced hair cell damage initiates mitosis among ISSC and OSSC (Vago, Humbert & Lenoir, 1998; Zine & Ribaupierre, 1998). It is possible that cisplatin DNA adducts may inhibit mitosis among these

cells until they are removed (repaired) by NER, which may explain their invulnerability to cisplatin.

Supporting cells of the organ of Corti are unable to divide but they can change their phenotype (Smoorenburgh, de Groot, Hamers & Klis, 1999). For instance, damaged hair cells are often replaced by supporting cells, which change their phenotype and become "hair cell like", although in mammals they never fully become hair cells (Cardinaal, de Groot, Huizing, Veldman & Smoorenberg, 2000; Lenoir & Vago, 1997; Smoorenburgh, de Groot, Hamers & Klis, 1999; Zine & Ribaupierre, 1998). Cisplatin treatment has been shown to induce morphological alterations to supporting cells (e.g., Deiter & Hensen) before hair cells and neurons (Ramirez-Camacho, Garcia-Berocal, Bujan, Martin-Marero & Trinidad, 2004; Schweitzer, 1993a; Schweitzer, 1993b). The supporting cells ultimately recover but hair cells and neurons die (Dehne, Lauterman, Petrat, Rauen & de Groot, 2001; Estrem, Babin, Ryu & Moore, 1981) which suggests that the supporting cells are initially targeted by cisplatin but have a higher capacity for DNA repair. Recovery may be due to up regulation of NER (both GG-NER & TC-NER) which then results in survival.

The most vulnerable cells are hair cells and neurons while the least vulnerable cells are nonsensory epithelium cells. One basic biological difference between the vulnerable and invulnerable cells is that the vulnerable cells (hair cells and neurons) cannot divide or change their phenotype while the invulnerable cells are proficient at both cell division and phenotype conversion. This basic biological difference may perpetuate the difference in vulnerability between different inner ear cell types.

It is known in DNA repair biology that terminal differentiation limits NER capacity (Nouspikel & Hanawalt, 2002; Nouspikel & Hanawalt, 2000). Terminally differentiated cells

are cells that lack the ability to divide (post-mitotic) and change their phenotype (Nouspikel & Hanawalt, 2002). Neurons and hair cells are examples of such cells (Devarajan et al., 2002). Since these cells cannot divide, they are free from the chore of replicating their genome. They could dispense with the energy consuming task of searching for and then repairing DNA damage across their entire genome and instead focus on repairing transcribing genes that are essential for their survival. Research on DNA repair has demonstrated that terminally differentiated cells lack the capacity to repair global genomic DNA damage but are able to effectively repair transcribed genes (Houspikel & Hanawalt, 2002; Houspikel & Hanawalt, 2000). More specifically, the GG-NER response is suppressed in terminally differentiated cells while TC-NER activity remains active (Bielas & Heddle, 2004; Nouspikel & Hanawalt, 2002: Nouspikel & Hnawalt, 2000). This means that DNA damage induced by genotoxins such as cisplatin may be allowed to accumulate on non-transcribing genes, since GG-NER is inherently suppressed (Furuta et al, 2002; Tornaletti, Patrick, Turchi & Hanawalt, 2003).

In the cochlea, neurons and hair cells are terminally differentiated. It is known that both cochlear neurosensory and nonsensory epithelium cells precipitate cisplatin DNA adduct, yet the former dies while the later survives. One possible explanation is that cellular vulnerability to cisplatin intoxication based on cell type is due to differential expression of the rate-limiting GG-NER protein, XPC (see aim 2).

Cisplatin treatment leads to the progression of hearing loss from high to low frequencies as a function of increasing dosage (Forastiere, Takasugi, Baker, Wolf & Kudla-Hatch, 1987; Van der Hulst, Dreschler & Urbanus, 1988). Post-mortem temporal bones harvested from patients treated with cisplatin as well as animal experiments have shown a base-to-apex gradient of OHC damage along the cochlear spiral (Hinojosa, Riggs, Strauss & Matz, 1995; Teranishi, Nakashima

& Wakabayashi, 2001). Here, the basal OHCs are severely damaged while the apical OHCs are normal. Cisplatin treatment of isolated OHCs from the base and apex of the cochlea and cochlear explants treated with aminoglycosides reveal similar OHC damage gradient as *in vivo* temporal bone studies (Gabaizadeh et al., 1997; Richardson & Russell, 1991; Saito, Yamada, Manabe, Yamamoto & Saito, 1996; Zine & de Ribaupierre, 1998). The basis of this damage gradient is unknown but it has generated the hypothesis that there is an intrinsic difference between basal and apical OHCs (Schacht, 1993; Zajic & Schacht, 1987).

Glutathione (L-γ-glutamyl-L-cysteinyl-glycine) levels may influence the difference in vulnerability between basal and apical OHCs. Glutathione is known for its cytoprotective activities in detoxifying (inactivate and/or efflux) various electrophilic xenobiotics as well as serving as a potent antioxidant. For instance, aminoglycoside ototoxicity may be supressed by glutathione conjugation and subsequent detoxification as well as free radical scavenging (Garetz, Rhee & Schacht, 1994; Lautermann, Crann, McLaren & Schacht, 1997; Lautermann, McLaren & Schacht, 1995). Cisplatin binds spontaneously with glutathione in solution to form cisplatin-glutathione-conjugates (Bernareggi et al., 1995; Ishikawa & Ali-Osman, 1993). These conjugates also are formed intracellularly and may be extruded from the cell (Ishikawa & Ali-Osman, 1993; Mistry, Loh, Kelland & Harrap, 1993). Similar to aminoglycosides, glutathione may suppress cisplatin cytotoxicity by detoxification and free radical scavenging. For instance, there is a positive correlation between the level of glutathione and the resistance of tumor cells to cisplatin (Kato et al., 2000). Cytosolic glutathione may inhibit apoptosis caused by cisplatin-induced ROS. For instance, during mitochondria mediated apoptosis initiated by ROS, oxidized cytochrome-c may be released into the cytoplasm to promote cell death (see Figures 5 and 8). A

high level of glutathione in the cytoplasm maintains cytochrome-c in a reduced (inactive) state which suppresses mitochondria mediated apoptosis (Jefferies et al., 2003).

In addition to detoxification, free radical scavenging and inactivation of cytochrome-c, glutathione also plays a role in DNA synthesis/repair. Glutathione accounts for the majority of non-protein sulfhydryls (SH groups) in a cell (Lai, Ozols, Young & Hamilton, 1989). Sulfhydryls are required for the normal operation of DNA polymerase in DNA synthesis/repair (Hubscher, Maga, & Spadari, 2002; Lai, Ozols, Young & Hamilton, 1989; Matsukage, Bohn & Wilson, 1975). Previous research has revealed that lowering the level of sulfhydryls suppresses DNA polymerase which disables DNA repair (Costellot, Miller, Lehtomaki & Pardee, 1979; Kane & Linn, 1981; Matsukage, Bohn, Wilson, 1975; Shaper, Grafstrom & Grossman, 1982). Cells with low levels of glutathione may have a more restricted supply of sulfhydryls available for DNA repair compared to cells with higher levels of glutathione (Jamieson & Lippard, 1999; Kelland, 1993).

There is a more direct consequence of glutathione level on DNA repair. For instance, glutathione is used in the synthesis of deoxyribonucleotide triphosphate (dNTP), which is directly involved in DNA repair (Holmgren, 1979; Luthman, Eriksson, Holmgren & Thelander, 1979). Lower levels of glutathione may yield lower amounts of sulfhydryls and dNTP available for DNA repair. This is particularly important because glutathione expression is significantly lower among basal OHCs compared to apical OHCs (Sha, Taylor, Forge & Schacht, 2001). Since, the basal OHCs are selectively vulnerable to cisplatin, it is reasonable to suggest that the lower level of glutathione in the basal OHCs may result in less availability of sulfhydryls and dNTP, which then leads to lower amounts of DNA repair activity. Decreasing the level of glutathione in cisplatin-resistant ovarian cancer cells resulted in the suppression of DNA repair

which then rendered the cells highly vulnerable to cisplatin (Lai, Ozols, Young & Hamilton, 1989). Similar to cancer cells, the basal OHCs may not express XPA and XPC proteins which could suggests that they are unable to initiate NER pathways while the apical OHCs may express XPA after cisplatin treatment which would suggest that they can initiate at least one NER pathway (see aim 3). This would be one possible explanation for the base-to-apex vulnerability among OHCs as a function of cisplatin treatment.

1. Specific Aims

Aim 1: To test the hypothesis that cisplatin treatment potentiates expression of the lesion identification (*xpc*) and verification (*xpa*) genes of global genomic-nucleotide excision repair (GG-NER) in the cochlea.

Aim 2: To test the hypothesis that terminally differentiated cochlear cells will not express the hallmark GG-NER protein, xeroderma pigmentosum C (XPC), following cisplatin treatment.

Aim 3: To test the hypothesis that basal OHCs will not express XPC and xeroderma pigmentosum A (XPA) proteins while apical OHCs will express XPA following cisplatin treatment.

III. CHAPTER II

A. SUBJECTS

Research on NER activity is typically conducted on tumor cell lines, cell-free systems, bacteria and yeast. A recent study on hepatocarcinogenesis successfully evaluated the expression of the *xpc* and *xpa* genes after treatment with tamoxifen (a DNA damaging agent) among female rats (Kasahara et al., 2003). The rat animal model also is used frequently in cisplatin ototoxic research and has the following advantage: wide biochemical characterization, resistance to middle ear infection and the ability to survive chronic and acute ototoxic and anesthetic treatment (Hatzopoulos et al., 2002; Laurell et al., 2002; Martin, et al., 1999). The rat animal model was used in this study. The Fischer344 rat is a widely used albino rat strain, particularly in toxicology and cancer research (Popelar, Groh, Mazelova & Syka, 2003). Previous research revealed that female Fischer344 rats are excellent models for chronic (multiple injections) cisplatin treatment studies (Li et al., 2001; Li et al., 2002; Minami, Sha & Schacht, 2004). A factorial design (2 x 3 x 2) using Fischer344 rats as subjects was implemented in this dissertation (see the next section for a detailed decription of the experimental design). There were two treatment groups (cisplatin & saline vehichle), three survival times and two analysis methods (polymerase-chain reaction and immunohistochemistry) with five animals per group. Five

animals per group typically provide enough tissue in order to detect significant differences in gene expression between experimental and control groups (Balaban, Zhou & Li, 2003; Stankovic & Corfas, 2003). A power analysis (Bausell & Li, 2002) indicated that five rats per group yields a 77% chance (i.e., power = 0.77) of detecting an effect size of 2.0 between the experimental and control groups using an independent samples t-test (two-tailed alpha = 0.05).

All Fischer344 rats (140-160 g) were purchased from Charles River Laboratories, Malvern, PA, USA. The animals were housed in a room (23 ± 2° C) on a 12-h light/dark cycle. All experimental protocols were performed according to National Institute of Health (NIH) guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pittsburgh, Pittsburgh, PA. The IACUC approval process certifies that all protocols are in compliance with the United States Department of Agriculture and NIH guidelines for ethical treatment of animals and that all attempts were made to minimize both animal use and suffering.

B. EXPERIMENTAL DESIGN

Animal studies on cisplatin ototoxicity typically involve acute (single injection) treatment. For instance, in rat studies, a single dose of 16 mg/kg, i.p. is typically administered, which effectively results in hair cell and neural degeneration. Such treatment overdose does not allow for the study of gene and protein expression among vulnerable cochlear cells (e.g., hair cells & neurons) since they easily degenerate. Recent work on cisplatin ototoxicity in the Fischer344 rat

established a treatment regimen that does not overdose the animals and allows for tissue harvesting after cisplatin treatment (Minami, Sha & Schacht, 2004). This treatment regimen prevents adverse effects such as morbidity, excess weight loss and nephrotoxicity. For instance, Fischer344 rats are treated with two cycles of cisplatin, each cycle consists of four days of treatment (1 mg/kg, i.p., twice daily) separated by 10 days of rest. In order to study gene and protein expression of NER factors in the cochlea, this chronic (see Table 3) cisplatin treatment regimen (Minami, Sha & Schacht, 2004) was implimented. Briefly, Fischer344 rats received cisplatin (2 x 1 mg/kg, i.p.) and saline (10 ml, s.c.) injections on days 1-4 of the study (treatment cycle 1). On day five, 10 of these rats received anesthesia for auditory screening followed by pentobarbital overdose and euthanasia. Five of these rats were perfused transcardially with phosphate buffered saline (PBS) followed by periodate-lysine-paraformaldehyde (PLP) for immunohistochemistry; tissue from the other five rats was harvested and frozen for polymerase chain reaction (PCR). The remaining 20 animals received saline (10 ml, s.c.) treatment daily for hydration on days 5-14. On days 15-18 the rats received cisplatin (2 x 1 mg/kg, i.p. daily) and saline (10 ml, s.c., daily) injections (treatment cycle 2). On day 19, 10 of these rats were anesthetized for auditory screening followed by pentobarbital overdose and euthanasia. Five of these rats were perfused transcardially with PBS followed by PLP for immunohistochemistry; tissue from the other five rats was harvested and frozen for PCR. The remaining 10 animals received saline (10 ml, s.c.) treatment daily for hydration on days 19-22. On day 22 the remaining rats (N =10) were anesthetized for auditory screening followed by pentobarbital overdose and euthanasia. Five of these rats were perfused transcardially with PBS followed by PLP for immunohistochemistry; tissue from the other five rats was harvested and frozen for

PCR. A control group received the same treatment as the experimental group except this group received saline vehicle instead of cisplatin.

The aims of this study were concerned with gene and protein expression of cochlear cells (e.g., hair cells and neurons). Cisplatin easily compromises cochlear integrity by damaging cochlear cells. In order to assess gene and protein expression, the cochlear cells must be functioning. Previous experiments using the same cisplatin treatment regimen used in this dissertation (see table 3) revealed that damage to cochlear cells only occur when the mean difference in auditory brainstem response thresholds between cisplatin and saline treated rats exceeded 40 dB at 16 kHz or higher frequencies (Minami, Sha & Schacht, 2004). In this dissertation two physiological measures were used to monitor cochlear integrity after cisplatin treatment to verify appropriate cell function. The auditory brainstem response (ABR) and otoacoustic emission (OAE) served as physiological screening measures of cochlear integrity among experimental and control animals. The OAE measurements are sensitive to changes in cochlear mechanics. This is particularly important because normal cochlear mechanics are dependent on adequate OHC function and cisplatin damage OHCs. The ABR measurements are sensitive to changes in cochlear mechanics and neural synchrony. This is particularly important because cisplatin preferentially kills OHCs and neurons. The combined use of both OAE and ABR allows for monitoring of cochlear and neural alterations as a function of cisplatin treatment.

The ABR was recorded under general anesthesia (ketamine 75 mg/kg, i.p. & xylazine 8 mg/kg, i.p.) (Minami, Sha & Schacht, 2004). The rats were placed on a heating pad maintained at 38° ± 5° C. All recordings were performed in a sound isolation chamber. Six subdermal needle electrodes were used for two channel recordings. Two electrodes (positive) were placed on the vertex, two were placed below the right and left mastoids (negatives) and one electrode

(ground) was placed in the dorsum close to the tail. The stimuli were synthesized digitally and presented through an insert earphone (Etymotic Research ER-3A). The transducer was coupled acoustically to the ear with a pediatric probe tip. The intensity of the acoustic stimuli was expressed in decibel sound pressure level (dB SPL). The animals were presented with a stimulus intensity series that was initially presented at 90 dB SPL and progressively lowered in 10 dB steps. The stimulus frequencies were 4, 8 and 16 kHz tone pips (1 ms rise-fall time, 10 ms plateau). Each ABR recording represented the average of individually amplified and filtered responses. The cubic $2f_1$-f_2 distortion product otoacoustic emission (DPOAE) was recorded using two primaries, f_1 and f_2 (ratio f_2/f_1 = 1.22). The primary tone levels (L) for f_1 and f_2 were L_1/L_2 = 60/50 dB SPL. DP-grams (the function of DPOAE level on increasing stimulus frequency) were recorded with a resolution of four points per octave regarding f_2. Both ABR and DPOAE were recorded using Intelligent Hearing System's Smart EP/DPOAE technology.

Table 3: Cisplatin Treatment Regimen

Group **n = 60**	**Days 1-4** **(cycle 1)** **n = 60**	**Day 5** **n = 60 - 20**	**Days 5-14** **n = 40**	**Day 15-18** **(cycle 2)** **n = 40**	**Day 19** **n = 40 - 20**	**Day 19-21** **n = 20**	**Day 22** **n = 20 - 20**
Experimental N = 30	Cisplatin treatment N = 30	ABR & OAE (N =10) euthanasia for: 1. IHC, N =5 2. PCR, N =5	Rest period for the 20 remaining rats	Cisplatin treatment N = 20	ABR & OAE (N =10) euthanasia for: 1. IHC, N =5 2. PCR, N =5	Rest period for the 10 remaining rats	ABR & OAE (N =10) euthanasia for: 1. IHC, N =5 2. PCR, N =5
Control N = 30	Saline treatment N = 30	ABR & OAE (N =10) euthanasia for: 1. IHC, N =5 2. PCR, N =5	Rest period for the 20 remaining rats	Saline treatment N = 20	ABR & OAE (N =10) euthanasia for: 1. IHC, N =5 2. PCR, N =5	Rest period for the 10 remaining rats	ABR & OAE (N =10) euthanasia for: 1. IHC, N =5 2. PCR, N =5

IHC = immunohistochemistry
PCR = polymerase chain reaction
ABR = auditory brainstem response
OAE = otoacosutic emissions
n = total animals in both groups
N = total animals within a group

C. PROTOCOL FOR AIM 1

In aim one, the hypothesis was that cisplatin treatment potentiates expression of the lesion identification (*xpc*) and verification (*xpa*) genes of GG-NER in the cochlea. Real-time reverse-transcription quantitative polymerase chain reaction (rt-RT-qPCR) was used to quantify changes in expression of the *xpc* and *xpa* genes after cisplatin treatment.

Two month old Fischer344 female rats (140-160 g) were sacrificed by decapitation following pentobarbital anesthesia (100 mg/kg, i.p.). Cochlear tissues were rapidly dissected under a stereomicroscope in ice-cold PBS (pH 7.4), frozen with dry ice and stored in TRIzol™

reagent (Gibco, Gaithersburg, MD, USA) at -80˚C for later processing. Tissues were then thawed and homogenized in TRIzol™ reagent (1 ml per 50-100 mg of tissue). Chloroform was then added and the mixture was centrifuged in order to separate the RNA phase from the DNA phase. The RNA phase was used for RNA precipitation using isopropyl alcohol. The RNA samples were rinsed with 75% ethanol and solubilized with RNase-free water. The RNA was then digested with DNase I (Ambion, Austin, TX, USA) to remove DNA contamination.

The DNA-free RNA was converted to recombinant DNA (cDNA) through RT. The RT reaction included 10 µl of 10 x PCR Taq Gold Buffer II (Applied Biosystems Inc., Foster City, CA), 30 µl of 25 mM $MgCl_2$, 4 µl of 25 mM of each dNTP, 5 µl of 100 µM of random primer (GIBCO), 2 µl of RNasin (40 units; Applied Biosystems Inc.), 1.25 µl of SuperScript II (250 units; GIBCO) and 200 ng of DNA-free total RNA. The reaction mixture was incubated at 25^{o} C for 10 min, 48^{o} C for 30 min, and 95^{o} C for 5 min in a 9600 thermocycler (Applied Biosystems Inc.).

SYBR Green PCR chemistry (Applied Biosystems Inc.) was used for real-time PCR amplification. The PCR reaction included 5 µL of 10X SYBR PCR Buffer, 6 µL of 25 mM $MgCl_2$, 4 µL of each dNTPs (blended with 2.5 mM dATP, dGTP and dCTP, and 5 mM dUTP), 2.5 µL of each gene-specific primer (5 µM), 0.5 µL of AmpErase UNG (0.5 unit), 0.25 µl of AmpliTaq Gold (1.25 units) and 5 µL of cDNA in a final volume of 50 µL. The conditions for the TaqMan PCR were as follows: 50^{o} C for 2 min, 95^{o} C for 12 min, and 40 cycles at 95^{o} C for 15 sec, and 60^{o} C for 1 min in an ABI PRISM 7700 Sequence Detection system (Applied Biosystems Inc.). Gene-specific primers for the target molecules are shown in Table 4.

Table 4: Gene-specific primers for PCR of xpc, xpa and 18S rRNA genes

xpc (accession no. XM_232194)
Forward primer 5'-CAGCCTTTGCCCACCTCCA-3'
Reverse primer 5'-TCCACGACAATACCCAAGGAC-3'
xpa (accession no. XM_216403)
Forward primer 5'-AAGAAGAACCCTCGCCATTCA-3'
Reverse primer 5'-TTCAAGAGCCCGCTTTACAAC-3'
Rat 18S rRNA (accession no. X01117)
Forward primer 5'-AAGCCATGCATGTCTAAGTACGCA-3'
Reverse primer 5'-AAGTAGGAGAGGAGCGAGCGACCA-3'

xpc/a: xeroderma pigmentosum c/a

1. Data Analysis for Aim 1

The 7700 Sequence Detection Software (Applied Biosystems Inc.) was used for instrument control, automated data collection, and data analysis. For each assay, logarithmic amplification plots were constructed based on cDNA copy number of a target gene normalized to the ROX internal passive reference (log ΔRn) versus cycle number. The cycle number at which the signal crossed the mid-linear portion of the log ΔRn-cycle function was defined as the cycle threshold (Ct) (Schmittgen et al., 2000). Because the input cDNA copy number and Ct are inversely related, a sample that contains more copies of the template will have a data line that crosses the Ct at an earlier cycle compared to one containing fewer copies of the template. The level of gene expression was calculated using $2^{-\Delta Ct}$ (Livak & Schmittgen, 2001). The ΔCt represents the Ct

of the target gene normalized to the rat endogenous 18S rRNA ($\Delta Ct = Ct_{Target} - Ct_{18S\ rRNA}$). The $2^{-\Delta Ct}$ calculation satisfies assumptions for the use of parametric statistics such as t-tests and ANOVA (Livak & Schmittgen, 2001).

D. PROTOCOL AND DATA ANALYSIS FOR AIMS 2 AND 3

In aim two, the hypothesis was that terminally differentiated cochlear cells will not express the hallmark GG-NER protein, XPC, following cisplatin treatment. Immunohistochemical staining of para-mid-modiolar cochlear sections was used to demonstrate post-translational protein expression of XPC among various cochlear cells as a function of the cisplatin treatment regiment. In aim three, the hypothesis was that basal OHCs will not express XPC and XPA proteins while apical OHCs will express XPA following cisplatin treatment. Immunohistochemical staining of para-mid-modiolar cochlear sections was used to demonstrate post-translational protein expression of XPC and XPA among basal and apical cells as a function of the cisplatin treatment regiment. Immunohistochemistry reveals the location of a target molecule in cells/tissues. Data analysis is based on microscopic evaluation of cells/tissues embedded on glass slides. Dr. Balaban and the author evaluated the slides and agreed on the presence or absence of positive staining. Spiral ganglion cells with or without positive staining were counted by the author. The proportion of cells with nuclear or cytoplasmic staining were determined from the total number of cells at each cochlear turn.

Animals were sacrificed with pentobarbital anesthesia (100 mg/kg, i.p.) and perfused transcardially with phosphate-buffered saline (PBS; 0.9% NaCl in 50 mM phosphate buffer, pH 7.3), followed by periodate-lysine-paraformaldehyde (PLP) fixative (McLean and Nakane, 1974). The heads were removed, skinned and then post-fixed in 4% paraformaldehyde for at least 24 hrs at 22˚ C. Decalcification occurred in 10% formic acid then neutralized overnight in 5% sodium sulfite by standard methods prior to trimming and paraffin embedding. Paraffin embedded sections were cut with a microtome at 8 μm and mounted on subbed slides. The sections were then stored at 22˚ C prior to immunohistochemistry.

For immunohistochemistry, the sections were de-paraffinized and treated for 10 minutes with 30% H_2O_2 in dH_2O and then rinsed thoroughly with dH_2O. They were then heated for 20 minutes at 90-98˚ C in a low pH (0.80-3.06) sodium citrate-citric acid buffer and rinsed thoroughly with PBS. Afterwards, the sections were pre-treated with a blocking solution of 10% Triton X-100 and 2% bovine serum albumin (BSA; Sigma, St. Louis, MO, USA) in PBS for 1 hour. The primary antibodies were diluted in the blocking solution at a 1:200 concentration. The primary antibodies were anti-XPC and anti-XPA (Santa Cruz Biotechnology, Inc.). For negative controls the primary antibody was omitted. After 24 hour incubation at 22˚ C with the primary anti-bodies the sections were rinsed with PBS. A biotinylated secondary antibody (Vector Laboratories, Temecula, CA, USA) was diluted 1:500 in PBS +2% BSA for one hour at 22˚ C. The sections were then rinsed in PBS, incubated with Vectastain ABC reagent (Vector Laboratories) for one hour, rinsed again with PBS and then treated with a solution of Trizma pre-set crystals (1.58 g; Sigma). After this the sections were washed in PBS and then stained for 10 minutes with 3.3'-diaminobenzidine tetrahydrochloride (DAB staining).

IV. CHAPTER III

Previous research implementing the exact cisplatin treatment regimen as described in this dissertation revealed that cochlear cell loss only occured when the mean ABR threshold shift (threshold after cisplatin treatment for the experimental group minus threshold after saline treatment for the control group) exceeded 40 dB (Minami, Sha & Schacht, 2004). The mean ABR threshold difference between groups from the current work did not exceed 14 dB (see Table 5). The mean OAE amplitude difference between groups (amplitude after saline treatment for the control group minus amplitude after cisplatin treatment for the experimental group) were at worst only 6 dB (see Table 6). Microscopy of the cochlea supported the ABR and OAE results by showing that hair cells and neurons did not degenerate after cisplatin treatment (see Figures 17 & 18). Therefore, the combined results verified that cochlear hair cells and neurons did not degenerate. This is important because cochlear hair cells and neurons are needed to measure gene and protein expression after cisplatin treatment.

Table 5: mean ABR difference between groups

Day	4 kHz	8 kHz	16 kHz
5	14(6)	14(5)	13(4)
19	3(5)	8(4)	9(4)
22	9(5)	11(4)	12(5)

Numbers in parenthesis represent standard error

Table 6: mean OAE difference between groups

Day	1kHz	2kHz	3kHz	4 kHz	5kHz	6kHz	7kHz	8 kHz
5	5(4)	2(4)	2(3)	0(3)	2(2)	3(2)	2(3)	6(3)
19	3(5)	-3(4)	0(3)	1(2)	5(4)	0(2)	0(3)	-4(3)
22	-5(3)	-5(2)	-4(2)	-4(2)	-7(2)	-5(2)	-4(2)	-7(3)

Numbers in parenthesis represent standard error

A. RESULTS FOR AIM 1

In aim one, the hypothesis was that cisplatin treatment potentiates expression of the lesion identification (*xpc*) and verification (*xpa*) genes of GG-NER in the cochlea. Real-time reverse-transcription quantitative polymerase chain reaction (rt-RT-qPCR) was used to quantify changes in expression of the *xpc* and *xpa* genes after cisplatin and saline (control) treatment. Figure 13, shows cumulative gene expression level for *xpc* after cisplatin and saline treatment. The level of expression of *xpc* is greater after cisplatin treatment compared to expression after saline treatment. An independent samples t-test comparing mean *xpc* gene expression after cisplatin and saline treatment found a significant difference between the means of the two treatment groups ($t(34) = -6.648$, $p < .001$). Figure 14, shows the level of expression of *xpc* after the first (day 5) and second (day 19) treatment cycles and fours days after the last treatment cycle (day 22). The level of *xpc* mRNA expression was greater after cisplatin treatment on all days compared to saline treatment. An independent samples t-test comparing mean *xpc* mRNA expression for day 5 ($t(10) = -3.164$, $p < .05$), day 19 ($t(10) = -3.817$, $p < .01$) and day 22 ($t(10) = -5.622$, $p < .001$) found a significant difference between the means of the cisplatin and saline treated groups on each of the three days. Further analysis using a one-way repeated measures ANOVA revealed that xpc gene expression did not significantly change across days during saline ($F(2,10) = 1.763$, $p > .05$) or cisplatin ($F(2,10) = .503$, $p > .05$) treatment. Although, the difference in mRNA expression between cisplatin and saline treatment is statistically significant this difference is less that 2-fold and represents a small change in gene expression.

Figure 15 shows cumulative gene expression level for *xpa* after cisplatin and saline treatment. The level of expression of *xpa* is greater after cisplatin treatment compared to

expression after saline treatment. An independent samples t-test comparing mean *xpa* gene expression after cisplatin and saline treatment found a significant difference between the means of the two treatment groups ($t(34) = -3.926$, $p < .001$). Figure 16 shows the level of expression of *xpa* after the first (day 5) and second (day 19) treatment cycles and four days after the last treatment cycle (day 22). The level of *xpa* gene expression was greater after cisplatin treatment on days 5 and 19 compared to gene expression after saline treatment. An independent samples t-test comparing mean *xpa* gene expression for day 5 ($t(10) = -2.706$, $p < .05$) and day 19 ($t(10) = -6.899$, $p < .001$) found a significant difference between the means of the cisplatin and saline treated groups on each day. There was no difference in *xpa* gene expression between cisplatin and saline treated groups four days after the last treatment cycle (day 22). An independent samples t-test was calculated comparing the mean *xpa* gene expression between cisplatin and saline treated groups. No significant difference was found ($t(10) = .186$, $p > .05$) between the means on day 22. Further analysis using a one-way repeated measures ANOVA revealed that *xpa* gene expression significantly changed across days during saline ($F(2,10) = 56.390$, $p < .001$) treatment while there was no significant differences during cisplatin ($F(2,10) = 2.478$, $p > .05$) treatment. Post-hoc analysis using the protected t-test revealed that during saline treatment *xpa* gene expression significantly changed between days 5 and 19, and days 19 and 22. There was no significant difference between days 5 and 22. Although, the difference in mRNA expression between cisplatin and saline treatment on days 5 and 19 is statistically significant this difference is less that 2-fold and represents a small change in gene expression.

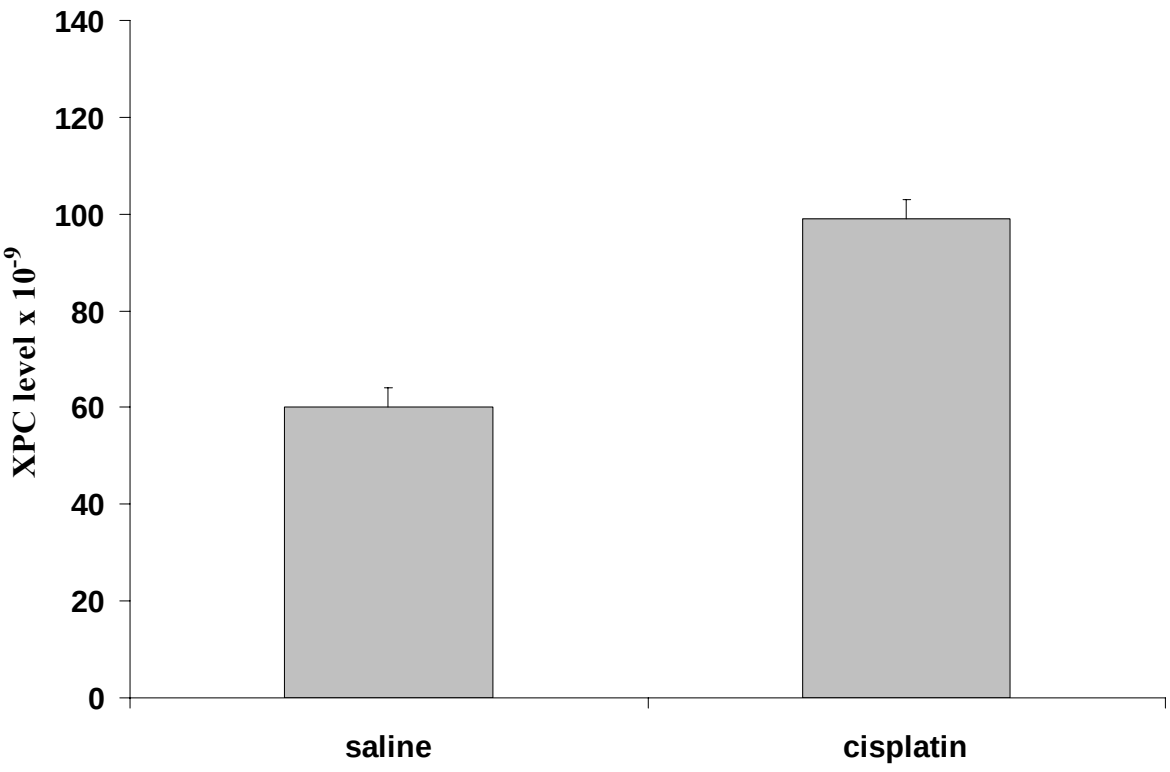


Figure 13: Cumulative XPC gene expression level for saline and cisplatin treated groups. Each bar represents mean ± S.E.

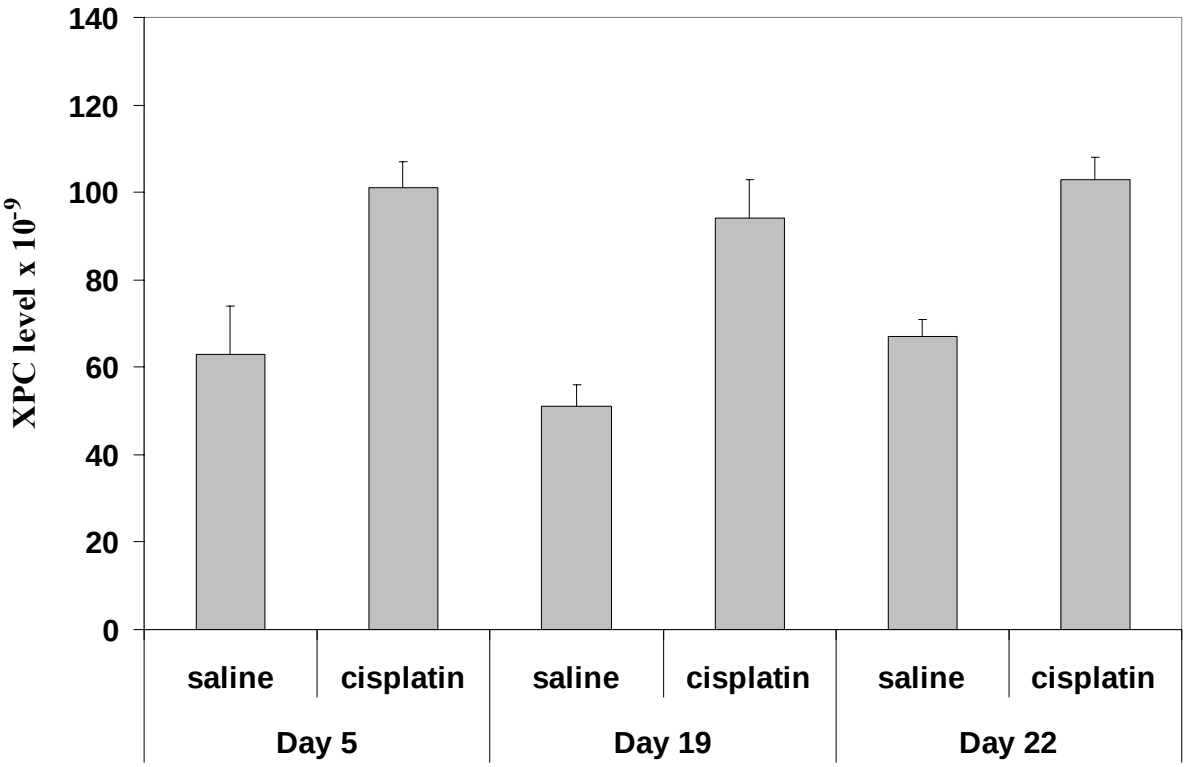


Figure 14: XPC gene expression level for saline and cisplatin treated groups. Each bar represents mean ± S.E.

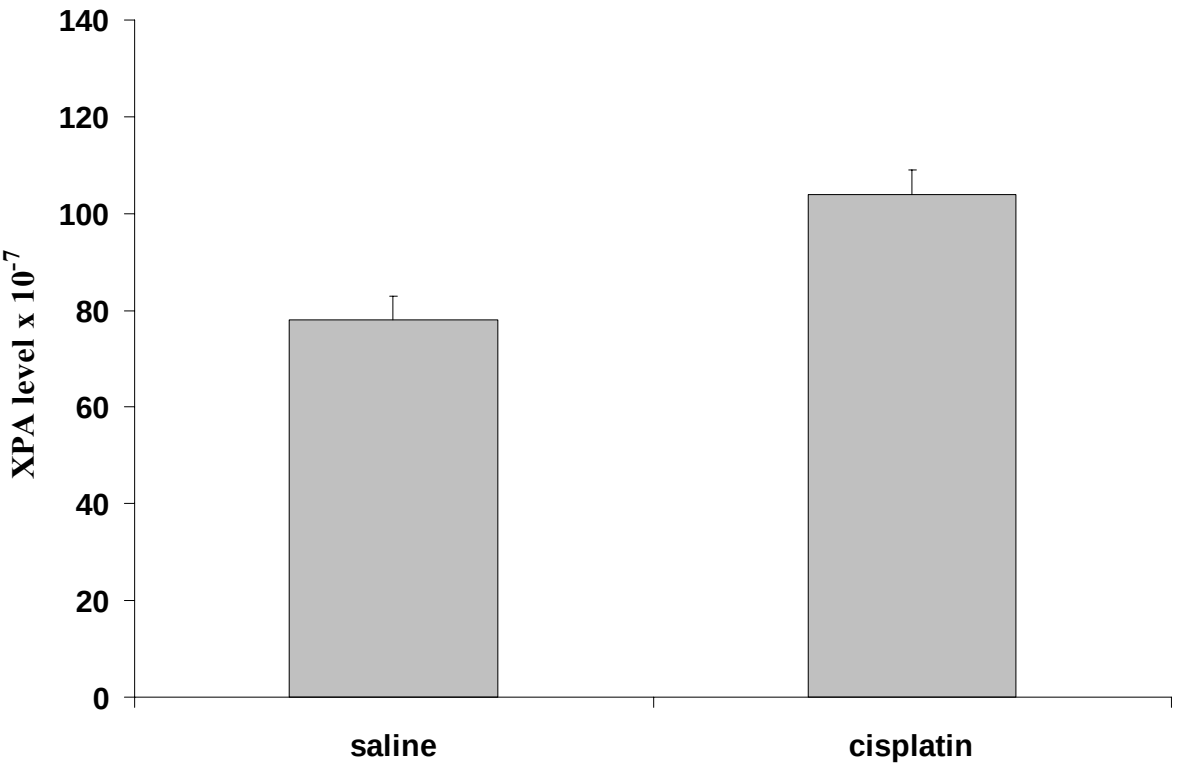


Figure 15: Cumulative XPA gene expression level for saline and cisplatin treated groups. Each bar represents mean ± S.E.

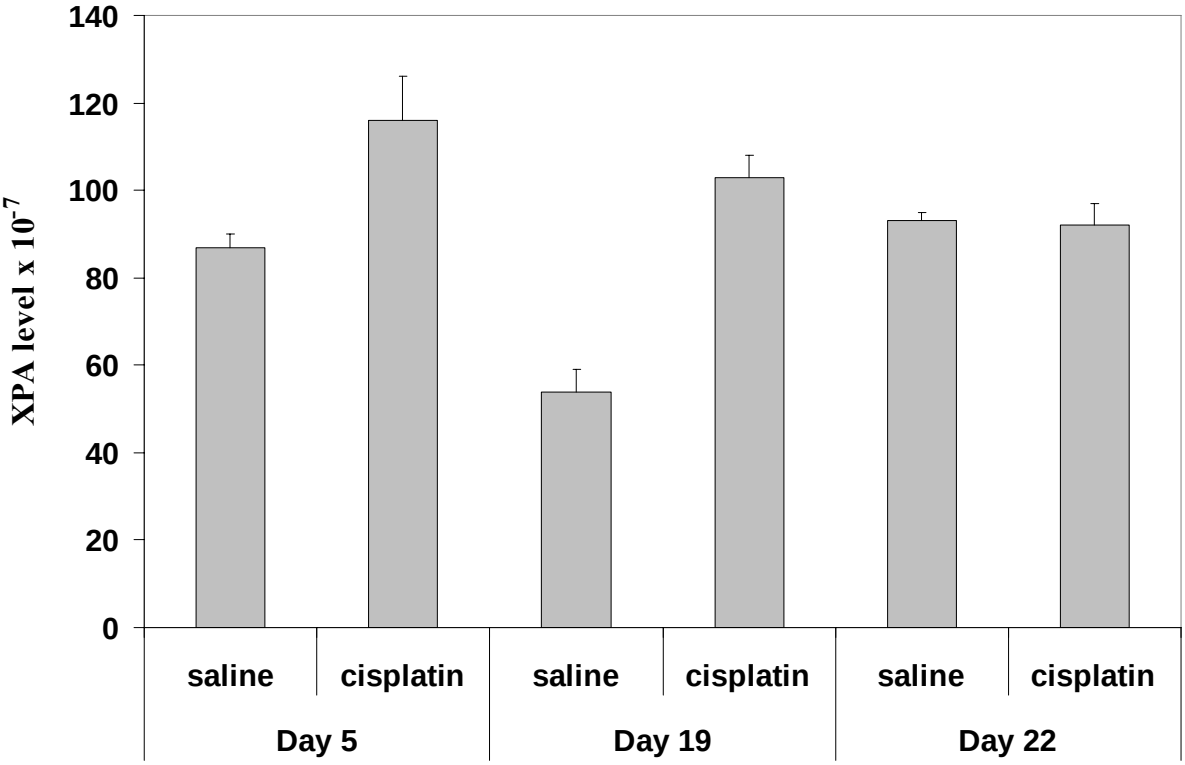


Figure 16: XPA gene expression level for saline and cisplatin treated groups. Each bar represents mean ± S.E.

B. RESULTS FOR AIM 2

In aim two, the hypothesis was that terminally differentiated cochlear cells will not express the hallmark GG-NER protein, xeroderma pigmentosum C (XPC), following cisplatin treatment. Immunohistochemistry was used to assess XPC protein expression among terminally differentiated cochlear cells following cisplatin treatment. In the cochlea terminally differentiated cells are neurons and hair cells. Figure 17 shows XPC protein expression in cochlear neurons (spiral ganglion cells) following cisplatin treatment. The XPC protein is shown localized in the nucleus of spiral ganglion cells after cisplatin treatment. After saline treatment the XPC protein was localized in the cytoplasm of spiral ganglion cells. Figure 18 shows XPC protein expression in cochlear hair cells following cisplatin treatment. The XPC protein is consistently localized in the nucleus of hair cells regardless of saline or cisplatin treatment.

C. RESULTS FROM AIM 3

In aim three, the hypothesis was that basal OHCs will not express XPC and xeroderma pigmentosum A (XPA) proteins while apical OHCs will express XPA following cisplatin treatment. Immunohistochemistry was used to assess XPC and XPA protein expression among hair cells from both the basal and apical coils of the cochlea. Figure 18, reveals that both XPC and XPA were expressed in the nucleus among OHCs at the basal and apical coils of the cochlea.

Further analysis revealed that there was a difference in XPC and XPA protein expression between basal and apical spiral ganglion cells. Figure 19, plots the proportion of spiral ganglion cells expressing XPC in their nucleus or cytoplasm after either cisplatin or saline treatment. A series of 3 (days; 5, 19 & 22) x 2 (treatment; saline vs. cisplatin) between subjects factorial ANOVAs was calculated comparing the proportion of immunoreactive spiral ganglion cells at the apex, middle and basal turns of the cochlea. Table 7 summarizes the ANOVA analysis. Nuclear immunoreactivity at the apex of the cochlea revealed significant main effects for days and treatment and an interaction effect. Post-hoc analysis revealed significant differences between the proportion of immunoreactive cells on day 5 compared to day 19 (Bonferroni/Dunn-test: $p < .05$) and day 22 (Bonferroni/Dunn-test: $p < .05$). Cytoplasmic immunoreactivity at the apex of the cochlea revealed a significant interaction effect and no main effects. Nuclear immunoreactivity at the middle of the cochlea revealed significant main effects for days and treatment and an interaction effect. Post-hoc analysis revealed significant differences between the proportion of immunoreactive cells on day 5 compared to day 19 (Bonferroni/Dunn-test: $p < .05$) but not day 22 (Bonferroni/Dunn-test: $p > .05$). Cytoplasmic immunoreactivity at the middle of the cochlea revealed no significant interaction or main effects. Nuclear

immunoreactivity at the base of the cochlea only revealed a significant main effect for treatment. Cisplatin treatment resulted in significantly greater immunoreactive cells in the nucleus than saline treatment. Cytoplasmic immunoreactivity at the base of the cochlea revealed a significant interaction effect and no main effects. These results suggest that a nuclear pattern of immunoreactivity is characteristic of cisplatin intoxication.

Table 7: Results of Six two-way ANOVAs for XPC.

		***F*-values**					
		Apex		Middle		Base	
Source	*df*	*Nuclear*	*Cytoplasmic*	*Nuclear*	*Cytoplasmic*	*Nuclear*	*Cytoplasmic*
Days (D)	2	9.610[a]	3.047	9.760[a]	0.986	0.941	2.815
Treatment (T)	1	10.521[a]	1.093	13.459[a]	2.170	5.990[b]	0.080
D x T	2	9.675[a]	3.696[b]	5.170[b]	1.750	0.717	3.744[b]
MS_{Error}	24	0.018	0.046	0.006	0.059	0.025	0.026

a = $p < .01$
b = $p < .05$

Figure 20 plots the proportion of spiral ganglion cells expressing XPA in their nucleus or cytoplasm after either cisplatin or saline treatment. A series of 3 (days; 5, 19 & 22) x 2 (treatment; saline vs. cisplatin) between subjects factorial ANOVAs was calculated comparing the proportion of immunoreactive spiral ganglion cells at the apex, middle and basal turns of the cochlea. Table 8 summarizes the ANOVA analyses. Nuclear immunoreactivity at the apex of the cochlea revealed only a significant main effect for treatment where cisplatin treatment resulted in greater nuclear immunoreactivity. Cytoplasmic immunoreactivity at the apex of the cochlea revealed only a significant main effect for treatment where saline treatment resulted in greater cytoplasmic immunoreactivity. Nuclear immunoreactivity at the middle of the cochlea revealed only a significant main effect for treatment where cisplatin treatment resulted in greater nuclear immunoreactivity. Cytoplasmic immunoreactivity at the middle of the cochlea revealed only a significant main effect for treatment where saline treatment resulted in greater cytoplasmic immunoreactivity. Nuclear immunoreactivity at the base of the cochlea revealed only a significant main effect for treatment where cisplatin treatment resulted in greater nuclear immunoreactivity. Cytoplasmic immunoreactivity at the base of the cochlea revealed a significant main effect for treatment and an interaction effect. The total number of immunoreactive cells is approximately equal for the saline or cisplatin treated groups. For instance, after saline treatment about 50% of spiral ganglion cells showed cytoplasmic immunoreactivity and about 50% showed nuclear immunoreactivity after cisplatin treatment. This suggests that protein translocation is occurring among a particular population of spiral ganglion cells. Among this population, under normal conditions the proteins are in the cytoplasm where they are synthesized and primed but after cisplatin treatment they translocate to the nucleus where they are needed.

Table 8: Results of Six two-way ANOVAs for XPA.

		***F*-values**					
		Apex		Middle		Base	
Source	*df*	*Nuclear*	*Cytoplasmic*	*Nuclear*	*Cytoplasmic*	*Nuclear*	*Cytoplasmic*
Days (D)	2	2.186	1.357	2.648	0.740	2.178	0.645
Treatment (T)	1	6.538[b]	8.069[a]	8.740[a]	9.459[a]	6.244[b]	10.609[a]
D x T	2	3.256	1.430	3.119	2.632	3.113	3.595[b]
MS_{Error}	24	0.040	0.056	0.036	0.027	0.040	0.018

a = $p < .01$
b = $p < .05$

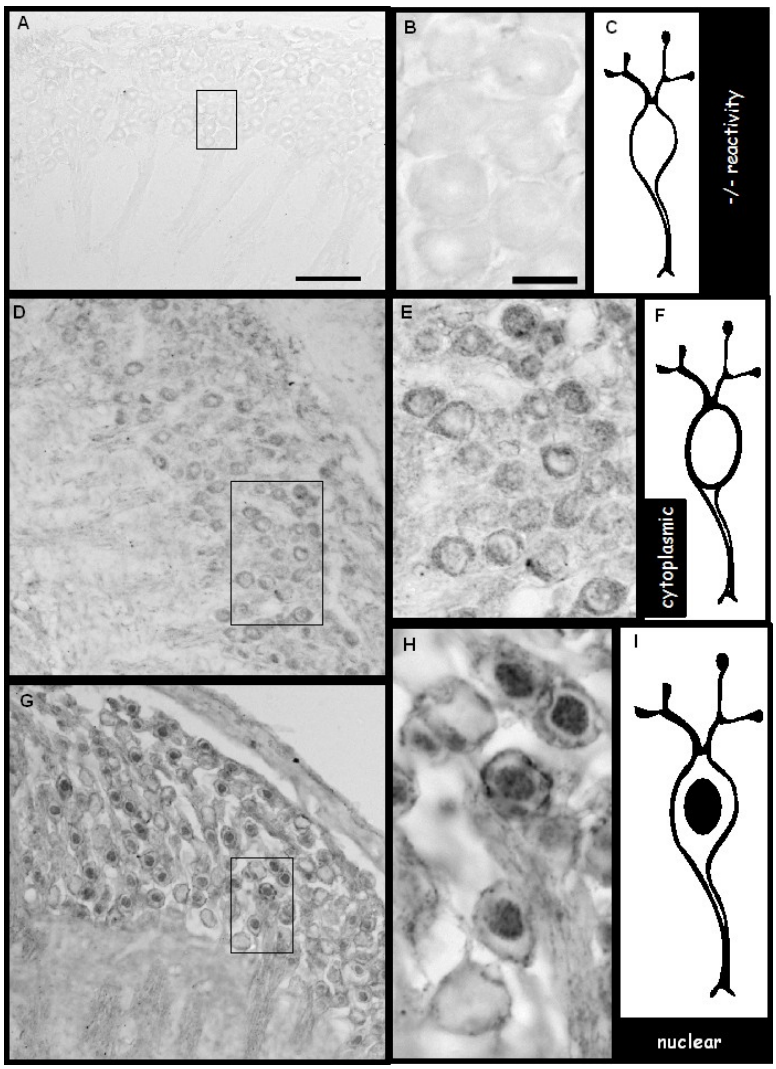


Figure 17: Intracellular distribution of the XPC protein in cochlear neurons (spiral ganglion cells). **A**: Immunoreactivity is absent when anti-XPC antibody is omited (negative control) during immunohistochemistry (Bar = 50 μm). **B**: High magnification (100x oil immersion objective) of a selected region of A (Bar = 10 μm). **C**, Schematic description of cellular appearance with negative immunoreactivity. **D**: XPC is present in spiral ganglion neurons under normal conditions (control). **E**: High magnification of a selected region of D reveals XPC-immunoreactivity in the cytoplasm of normal spiral ganglion neurons. **F**: Schematic description of cellular appearance with cytoplasmic immunoreactivity. **G**: XPC is present in spiral ganglion neurons after cisplatin treatment (experimental condition). **H**: High magnification of a selected region of G reveals prominent XPC-immunoreactivity in the nucleus of spiral ganglion cells. **I**: Schematic description of cellular appearance with nuclear immunoreactivity.

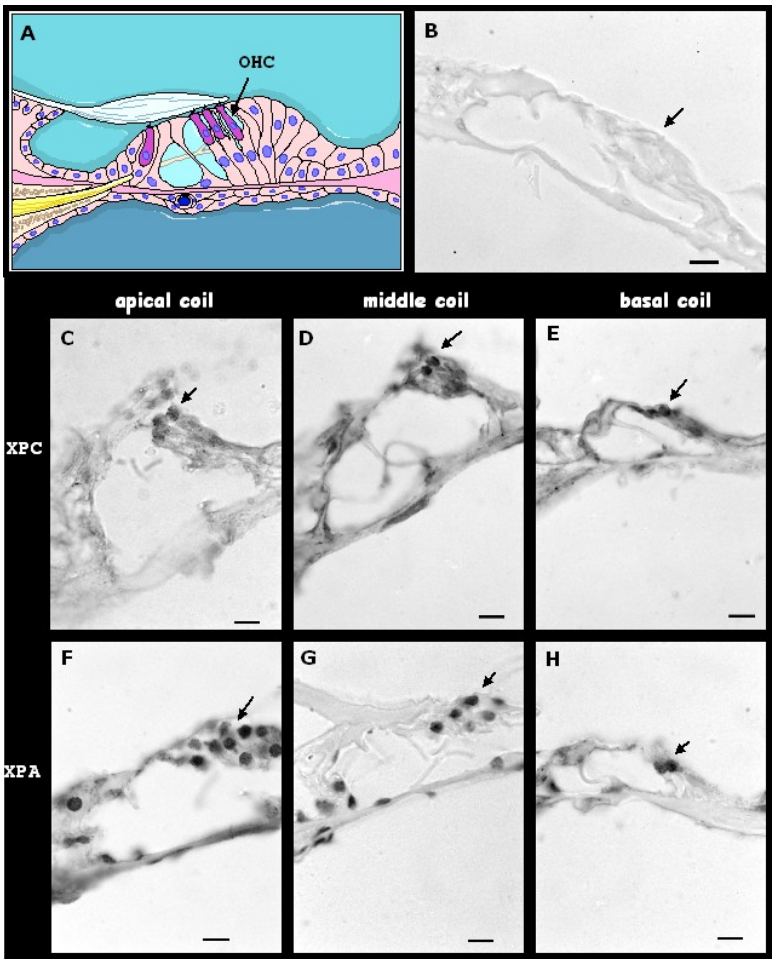


Figure 18: Representative distribution of XPC and XPA proteins among cells of the organ of Corti after cisplatin treatment cycles (days 5 and 19) and four days after the last treatment cycle (day 22). Both proteins were expressed among supporting cells and hair cells. Outer hair cells (OHC, see arrows) showed prominent immunoreactivity. **A**: Schematic representation of the organ of Corti showing hair cells. **B**: Immunoreactivity is absent when anti-XPC or anti-XPA antibody is omited (negative control) during immunohistochemistry. **C-E**: XPC is present among OHCs at all turns of the cochlea. **F-H**: XPA is present among OHCs at all turns of the cochlea. Bars = 10 μm.

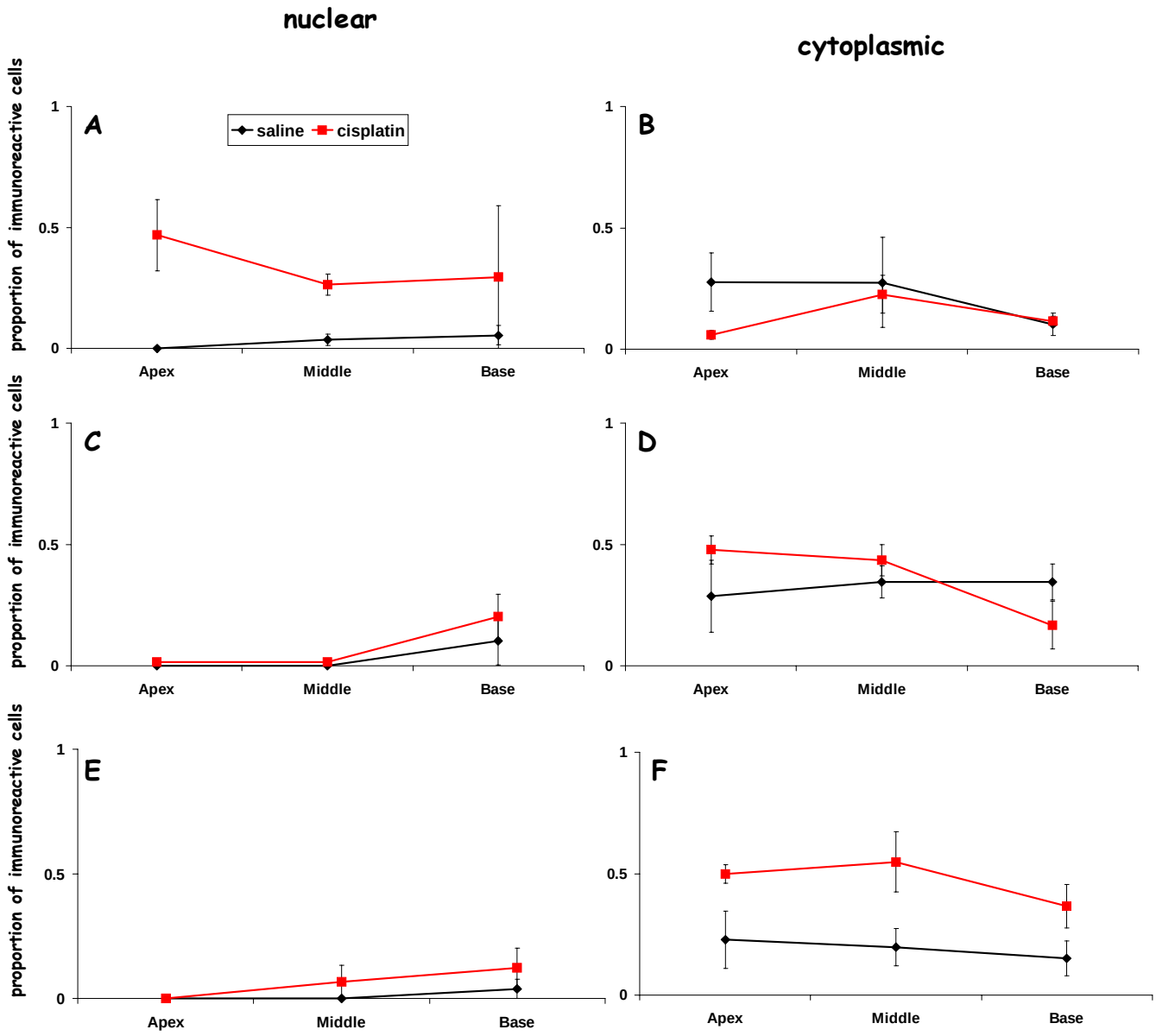


Figure 19: Proportion of nuclear or cytoplasmic XPC immunoreactive spiral ganglion cells at apical, middle and basal turns of the cochlea. **A-B**, immunoreactive cells on day 5. **C-D**, immunoreactive cells on day 19. **E-F**, immunoreactive cells on day 22.

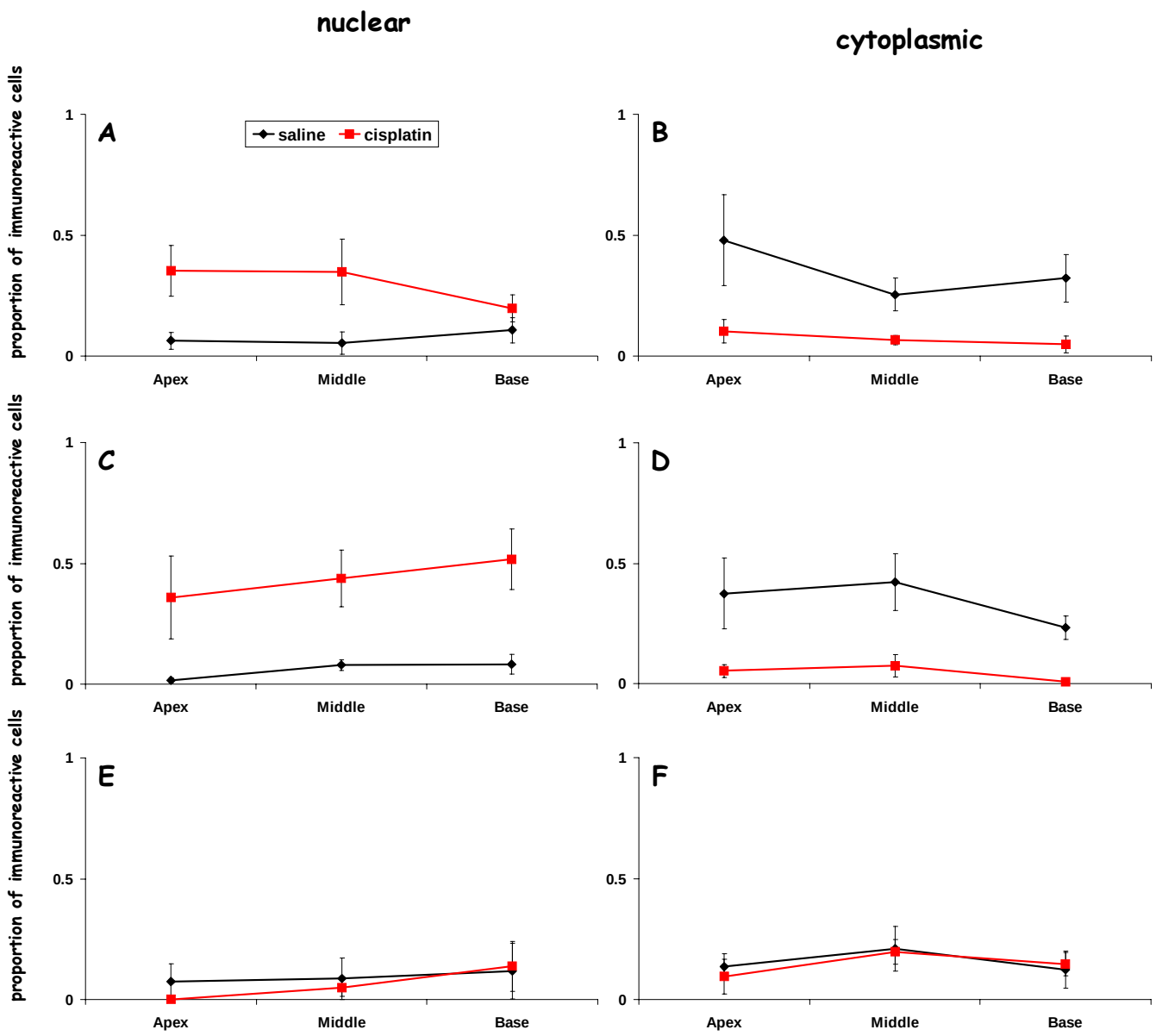


Figure 20: Proportion of nuclear or cytoplasmic XPA immunoreactive spiral ganglion cells at apical, middle and basal turns of the cochlea. **A-B**, immunoreactive cells on day 5. **C-D**, immunoreactive cells on day 19. **E-F**, immunoreactive cells on day 22.

V. CHAPTER IV

Aim 1 of this dissertation tested the hypothesis that cisplatin treatment would potentiate expression of the *xpc* and *xpa* genes. The results revealed that cisplatin treatment lead to a statistically significant increase in gene expression. Aim 2 of this dissertation tested the hypothesis that terminally differentiated cells (e.g., neurons and hair cells) would not express XPC. The results showed for the first time that neurons and hair cells are capable of expressing XPC. Aim 3 of this dissertation tested the hypothesis that basal OHCs will not express XPC and XPA while apical OHCs would express XPA following cisplatin treatment. The results revealed that XPC and XPA were expressed among hair cells from all cochlear turns. An integrated view of these and related findings are discussed below.

The GG-NER pathway is the major defense strategy by which cisplatin resistant tumors cells identify and repair cisplatin induced DNA damage. The rate-limiting steps in this pathway are controlled by the XPC and XPA proteins. Therefore, the production of these proteins is important to defend the genome from cisplatin DNA damage. Protein production first begins with transcription. Transcription is the process of producing mRNA molecules. The mRNAs are short polymers that carry the genetic code represented by the gene being transcribed. The mRNA from the *xpc* and *xpa* genes were quantified among cisplatin and saline treated groups. The level of mRNA was statistically significant between groups but may not be biologically important because the difference was less than two fold. This suggests that the activity of the protein may be more informative than the mRNA level. The second step in protein production is

translation. Translation occurs at ribosomes on rough endoplasmic reticulum (and free ribosomes) which are located in the cytoplasm. Translation is the process of converting the genetic code carried by the mRNA molecules into assembled polypeptides.

XPC and XPA are termed nuclear enzymes because they only function in the nucleus by binding damaged DNA. In cellular terms, this activation requires that the enzyme translocate from the cytoplasm to the nucleus. Figures 17, 19 & 20 reveal that under normal conditions XPC and XPA are expressed predominantly in the cytoplasm of approximately 50% of spiral ganglion cells. This suggests that XPC and XPA are being translated but are not yet needed for any substantial amount of DNA repair. Therefore, accumulation of XPC and XPA in the cytoplasm of a proportion of spiral ganglion cells implies that these cells produce more of the enzymes than are needed to meet the immediate need for DNA damage. Here, cytoplasmic expression of these enzymes in ganglion cells may represent a functional reserve for response to fluxes in DNA damage. Under normal conditions OHCs express XPC and XPA in their nucleus which suggests that all of the translated enzyme is needed to bind to basal levels of damaged DNA. Hence, it is suggested that hair cells lack a functional reserve capacity for GG-NER. This framework is further supported by the observation that cisplatin treatment results in translocation of XPA and XPC from the cytoplasm to the nucleus among spiral ganglion cells. This implies that GG-NER capacity may be calibrated to basal demand in hair cells, but capable of dealing with surges in demand in ganglion cells.

Figures 19 and 20 revealed that XPC and XPA respond (translocate) to cisplatin treatment. The response of XPC and XPA to DNA damage is expected given their roles in GG-NER (Thoma & Vasquez, 2003). XPC binds to sites of cisplatin damage across the genome (Riedl, Hanaoka & Egly, 2003). Once XPC localizes DNA damage it is replaced by XPA at the sight of

damage (de Laat, Jaspers & Hoeijmakes, 1999). XPA verifies the damage and remains at the site until the damage is repaired (de Laat, Jaspers & Hoeijmakes, 1999; Riedl, Hanaoka & Egly, 2003). Figures 19 and 20 reveal that initial treatment with cisplatin (day 5) resulted in a significant increase in the proportion of spiral ganglion cells with XPC translocation to the nucleus (relative to control). This pattern is consistent with mobilization of a GG-NER response to cisplatin DNA damage in the nucleus. XPA also showed significant nuclear translocation on day 5 which further supports a GG-NER response to cisplatin DNA damage in the nucleus. On day 19 XPC revealed significant cytoplasmic reactivity which suggests an increase in translation but no nuclear translocation while XPA remained significantly expressed in the nucleus. This suggests that XPC was no longer needed for lesion localization but remained primed in the cytoplasm. XPA would be expected to remain significantly expressed in the nucleus because it determines whether a particular DNA damage is worth repairing and it oversees the repair process. This is further supported by the fact that no XPA nuclear translocation was observed after the animals recovered from cisplatin treatment (day 22). Additionally, on day 22 XPC remained in the cytoplasm (e.g., no nuclear translocation), which suggest that repair was completed before day 22.

Figures 19 and 20 further revealed that all turns of the cochlea responded similarly to cisplatin treatment; however the degree and latency of response differed between turns. For instance, on day 5 all turns showed XPC and XPA nuclear translocation; however the base revealed the lowest degree of nuclear translocation compared to the other turns and unlike the other turns, was not significantly different from the control. The XPC protein exhibited greater variability in nuclear translocation at the base than the XPA protein. This may be because XPC DNA binding is labile while XPA DNA binding is stable. For instance, XPC binds each site of

DNA damage but it is replaced by XPA which remains at the site of damage until repair is complete. This suggests that the base may initially exhibit greater levels of DNA damage than other cochlear turns and is manifested as labile XPC DNA binding coupled with stable XPA DNA binding. On day 19 all turns showed nuclear translocation of XPA which was significantly different from the control; however the base revealed the highest degree of nuclear translocation compared to the other turns, which suggest a latent response by the base. No significant nuclear translocation was observed for XPC regardless of cochlear turn, which would be expected because XPC is no longer needed on day 19. On day 22 (after recovery) there was no difference in response between cochlear turns for either protein. This uniformed response by all cochlear turns supports the hypothesis that repair was completed before day 22.

In summary, the current work has demonstrated that spiral ganglion cells exhibit a reserved capacity of translated XPC and XPA in their cytoplasm primed for nuclear translocation from cisplatin intoxication. This nuclear translocation is more efficient among apical spiral ganglion cells than basal spiral ganglion cells. Basal spiral ganglion cells translocate XPC and XPA slower and with less magnitude than apical cells. This novel finding is important because basal spiral ganglion cells degenerate before apical cells and typically exhibit greater damage than apical cells. Therefore, the difference in nuclear translocation between basal and apical ganglion cells may underlie their difference in susceptibility to cisplatin ototoxicity.

APPENDIX

GLOSSARY

Acid	a substance that has the ability to react with bases and certain metals to form salts
Adenine	a purine
Adduct	a combination of two or more independently stable compounds by means of van der Waals' forces, coordinate bond or covalent bonds. An example of an adduct would be platinum or cisplatin bound to DNA
Affinity	preference
Amino acid	protein constituent
Antineoplastic	destroying, inhibiting or preventing the growth of tumors
Apoptosis	cell death
ATP	adenosine triphosphate, energy source in a cell
Autometallography	a chemical proceduce used to reveal the distribution of metals in cells/tissues
Base	a substance that reacts with an acid to form a salt
Conformation	structure
Coordination	the binding of a non-metal to a metal
Cysteine	amino acid
Cys	cysteine
DNA	deoxyribonucleic acid
Efflux	refers to the exit of substances from the cell
Electrophilic	electron accepting
Eukaryote	single celled or multicellular organism whose cells contain a distinct membrane bound nucleus
Filament	a threadlike structure, fiber
Free radical or radical	ion or molecule with an unpaired (extra) electron
Genotoxic	toxic to DNA
Glutathione	A polypeptide of glycine, cystine (a sulfur containing amino acid) and glutamic acid that occurs widely in plant and animal tissues and is important in biological oxidation-reduction reactions.
Ground state	the electron configuration of a an atom, ion or molecule
Guanine	purine
hHR23B	human homologue of RAD 23B, a protein that binds XPC
Homeostasis	the tendency of physiological systems to maintain internal stability

Influx	refers to the entry of substances in the cell
In vivo	occurring within a living organism
In vitro	in an artificial environment outside the living organism
Labile	constantly undergoing or likely to undergo change
Lewis acid classification	a classification system that considers acids as an electron pair acceptor
Ligand	a molecule that is attached to another molecule
Ligase	an enzyme (protein) that connects broken ends of DNA
Methionine	one of two amino acids that contain sulfur
Mitosis	cell division
Mitochondria	an organelle in the cytoplasm of nearly all eukaryotic cells that is important for cellular metabolism
Monohydrate	a hydrate that contains one molecule of water
Necrosis	the sum of the morphological changes indicative of cell death and caused by the progressive degenerative action of enzymes. It may affect groups of cells or part of a structure or an organ
Nephrotoxicity	the state of being toxic to the kidney
NER	nucleotide excision repair
Nucleophilic	electron emitting
Ototoxicity	the state of being toxic to the inner ear
PCNA	proliferating cell nuclear antigen, a protein involved in cell replication
pH	the symbol for the logarithm of the reciprocal of hydrogen ion concentration in gram atoms per liter, used to express the acididty or alkalinity of a solution on a scale of 0 to 14, where less than 7 represents acidity, 7 represents neutrality and more than 7 is alkalinity
Pharmacokinetics	the branch of pharmacology that studies the fate of pharmacological substances in the body, as their absorption, distribution, metabolism and elimination
Phenotype	appearance
Polymerase	a molecule that synthesizes DNA or RNA
Prokaryote	a cellular organism that has no nuclear membrane
Purine	DNA bases (adenine and guanine)
Rate-limiting	the rate-controlling step (bottleneck) in a reaction
RFC	replication factor C, a protein involved in replication
RPA	replication protein A, a protein that binds to single-stranded DNA
ROS	reactive oxygen species (an oxygen based free radical)
Redox-active	refers to the ability of an atom, ion or molecule to catalize oxidation or reduction reactions
Replication	DNA synthesis
RNA	ribonucleic acid

Speciation	the exact chemical form or compound in which an element occurs in a sample
Spin state	the direction in which an electron circles an atom or ion
Sulfhydryl	a sulfur atom (S) bonded to a hydrogen (H) atom is a sulfhydryl (SH).
Terminally differentiated cells	cells that do not divide or change their appearance
Termini	the final point; the end
TFIIH	transcription factor two H, a protein complex
Thermodynamic	using or producing heat
Titration	method or process of determining the concentration of a dissolved substance in terms of the smallest amount of a reagent of known concentration required to bring about a given effect
Translocation	to move from one site to the other as in movement from the cytoplasm to the nucleus
Trans	having a pair of identical atoms on opposite sides of two atoms linked by a double bond
Transcription	gene expression
Transition metal	a metallic ion that is able to change electron configuration
Up-regulate	increase
Uptake	entry into the cell by either an active or passive process
Xenobiotic	a substance foreign to living systems
XP	xeroderma pigmentosum, seven proteins labeled from A-G (e.g., XPA to XPG). Mutations in the genes that represent these proteins results in NER failure and increased cancer incidence.

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