Prevention of cisplatin ototoxicity by curcumin loaded nanoparticles

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A thesis submitted to the faculty of graduate studies and research of McGill University in partial fulfilment of the requirements of the degree of

Doctor of Philosophy

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Cisplatin is an effective chemotherapeutic agent which causes oxidative and inflammatory damages to the cochlea, the hearing organ of the auditory system. The degenerative changes of the cochlea as a result of cisplatin chemotherapy lead to permanent hearing loss in patients, especially in younger children.

Curcumin is a phytochemical compound known to exert various biological properties including acting as an effective antioxidant agent. In this study, curcumin was encapsulated by NIPAAM/VP/PEG-A nanoparticles to increase the bioavailability of the drug.

Our hypothesis is that a combination therapy of curcumin loaded nanoparticles and dexamethasone can reduce the cisplatin-induced oxidative and inflammatory stress to the hearing organ. In a first step, characterization of the curcumin nanoparticles showed moderate stability in aqueous matrix, and temperature related slight release pattern of the curcumin from the nanoparticles. In the next phase, the protective effect of curcumin nanoparticles and dexamethasone against cisplatin damage were tested on auditory cells and in a guinea pig model. The in vivo experiments included the auditory brainstem response, antioxidant enzyme assays and morphological assessment of the cochlea.

The results of our experiments showed that a combination treatment of curcumin loaded nanoparticles and dexamethasone reduced cisplatin-induced degenerative changes both in vitro and in vivo.
RÉSUMÉ

Le cisplatin est un agent chimiothérapeutique efficace qui cause des effets néfastes oxydatifs et inflammatoires à la cochlée, l’organe jouant un rôle primordial dans l’audition. Les changements dégénératifs de la cochlée suite au traitement avec le cisplatin entrainent une perte auditive permanente chez les patients, particulièrement chez les jeunes enfants.

Le curcumin est un composant phytochimique qui peut exercer diverses propriétés biologiques, particulièrement comme agent antioxydant efficace. Dans cette étude, le curcumin a été encapsulé par des nanoparticules NIPAAM/VP/PEG-A afin d’augmenter la biodisponibilité du médicament.

Notre hypothèse est qu’un traitement avec du curcumin encapsulé en nanoparticules en combinaison avec la dexaméthasone peut réduire le stress oxydatif et l’inflammation induite par le cisplatin à l’organe auditif. Dans une première étape, la caractérisation des nanoparticules de curcumine a montré une stabilité dans la matrice aqueuse, ainsi qu’une légère libération du curcumin des nanoparticules. Dans la phase suivante, l’effet protecteur des nanoparticules de curcumin combiné avec de la dexaméthasone contre les effets néfastes du cisplatin ont été testés sur des cellules auditives et un modèle de cochon d’inde. Les expériences in vivo comprenaient des réponses auditives cérébrales, des tests d’enzymes anti-oxydantes et des évaluations morphologiques de la cochlée.

Les résultats in vitro et in vivo ont affirmé qu’un traitement de curcumin encapsulé en nanoparticules en combinaison avec la dexaméthasone réduit les changements dégénératifs induits par le cisplatin.
ACKNOWLEDGEMENTS

This thesis would not exist without the help and support of many people to whom I am extremely grateful. First, I would like to thank my supervisor, Dr. Sam Daniel for giving me the opportunity to learn and practice the basic science research under his guidance, and enormous support.

I would also like to thank the members of my advisory committee, Dr. Robert Funnell, Dr. Mario Chevrette, Dr. Melissa Vollrath, Dr. Jacques Lapointe, and Dr. Tony Leroux for their constructive criticisms and insightful suggestions throughout our supervisory committee meetings.

I consider myself very lucky to be supported and guided by our great collaborator, Dr. Janusz Rak and his amazing group. I realize that much of my research would not have been possible without the great advice and technical support from Brian Meehan.

I would also like to thank the chemists Ranjan Roy and Andrew Golsztajn from the Department of Chemical Engineering, McGill University. I would not have been able to complete my first project without the great guidance of Ranjan Roy in my chemical experiments and the help of Aref Taghizadeh in writing my first research article.

Finally, I would like to extend my gratitude to all my friends and lab colleagues for their great help and support. I would like to acknowledge in particular Dan Citra, Sofia Waissbluth, Emilia Peleva, Victoria Akinpelu and Cathy Lemieux.

This thesis is dedicated to all patients suffering from cancer
&
to my family, especially my lovely mother
who offered me love and support throughout the course of my work
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CONTRIBUTION OF AUTHORS

The work of this thesis was performed under the supervision of Dr. Sam Daniel with collaborative supervision by Ranjan Roy for the chemical experiments in chapter II, and Dr. Janusz Rak and Brian Meehan for cell culture experiments in chapter III.

The manuscripts for these chapters were written by myself and revised by co-authors especially Ranjan Roy and Aref Taghizadeh, as well as Meenakshi Malhotra for chapter II; and Emilia Peleva and Sofia Waissbluth for chapter III.

Chapter II is a preprint of an article whose final and definitive form has been published in Journal of Biomaterials Science © Journal of Biomaterials Science Copyright Taylor & Francis; Journal of Biomaterials Science is available online at: http://www.tandfonline.com/doi/full/10.1080/09205063.2012.700111

In Chapter II, AFM and FTIR experiments were completed by Georges Makhoul, and the design of the zeta potential experiments was carried out by Meenakshi Malhotra.

In Chapter III, all samples were collected by the candidates. Scanning electron microscopy was performed at the McGill Electron Microscopy Research Centre with the assistance of Victoria Akinpelu. Light microscopy slides were prepared with the assistance of Dr. He and Victoria Akinpelu at the Montreal Children’s Research Institute. Sample collection and ABR experiments were completed with the assistance of Sofia Waissbluth and Victoria Akinpelu.
<table>
<thead>
<tr>
<th>ABR</th>
<th>Auditory brainstem response</th>
</tr>
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<tbody>
<tr>
<td>AFM</td>
<td>Atomic Force Microscope</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulfate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ATR</td>
<td>Attenuated Total Reflection</td>
</tr>
<tr>
<td>CAT</td>
<td>Catalase</td>
</tr>
<tr>
<td>COX-2</td>
<td>Cyclooxygenase 2</td>
</tr>
<tr>
<td>DAD</td>
<td>Diode array detector</td>
</tr>
<tr>
<td>dB</td>
<td>Decibels</td>
</tr>
<tr>
<td>dH2O</td>
<td>Deionized water</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic laser light scattering</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DMPC</td>
<td>Dimyristoyl phosphatidylcholine</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FAS</td>
<td>Ferrous ammonium persulfate</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FLD</td>
<td>Fluorescence detector</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infrared spectroscopy</td>
</tr>
<tr>
<td>HEI-OC1</td>
<td>House Ear Institute-Organ of Corti 1</td>
</tr>
<tr>
<td>HER2</td>
<td>Human Epidermal Growth Factor Receptor 2</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-pressure liquid chromatography</td>
</tr>
<tr>
<td>IC50</td>
<td>Inhibitory concentration 50%</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1β</td>
</tr>
<tr>
<td>kHz</td>
<td>Kilohertz</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>LCST</td>
<td>Low critical solution temperature</td>
</tr>
<tr>
<td>LD50</td>
<td>Lethal dose 50%</td>
</tr>
<tr>
<td>LM</td>
<td>Light microscopy</td>
</tr>
<tr>
<td>MBA</td>
<td>N,N'‑Methylene bis-acrylamide</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>MTS</td>
<td>3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium</td>
</tr>
<tr>
<td>NCUR</td>
<td>Nanocurcumin</td>
</tr>
<tr>
<td>NF-κβ</td>
<td>Nuclear factor-kappa β</td>
</tr>
<tr>
<td>NIPAAM</td>
<td>N isopropylacrylamide</td>
</tr>
<tr>
<td>NTA</td>
<td>Nanoparticle tracking analysis</td>
</tr>
<tr>
<td>OHCs</td>
<td>Outer hair cells</td>
</tr>
<tr>
<td>PEG-A</td>
<td>Polyethylene glycol monoacrylate</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SGCs</td>
<td>Spiral ganglion cells</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SV</td>
<td>Stria vascularis</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TM</td>
<td>Tympanic membrane</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>uPA</td>
<td>Urokinase Plasminogen Activator</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VP</td>
<td>N-vinyl-2-pyrrolidone</td>
</tr>
<tr>
<td>VPOL</td>
<td>Void polymer</td>
</tr>
</tbody>
</table>
CHAPTER 1: INTRODUCTION

1.1. Anatomy of the auditory system

The ears are sensory organs involved in sound detection and in the maintenance of equilibrium. The ear can be divided into 3 regions, namely the external ear, the middle ear and the inner ear.

The external ear consists of the pinna and external auditory canal which collect and direct the sound waves to the middle ear. The tympanic membrane (TM) separates the external ear from the middle ear cavity. The middle ear is an air filled space between the tympanic membrane and the internal ear and contains 3 ossicles (malleus, incus, stapes). These ossicles transfer the vibration of the tympanic membrane to the oval window membrane of the inner ear. The eustachian tube connects the middle ear to the nasopharynx with the function of equalizing the air pressure in the middle ear. The bony labyrinth of the inner ear holds the vestibule, semicircular canals and the cochlea, which are the sensory organs responsible for balance and hearing.

The cochlea is a spiral shaped tube that is coiled two and half turns around the central pillar and is divided into three spaces: scala vestibuli, scala media, and scala tympani. The scalae are separated by Reissner’s membrane and the basilar membrane. The scala vestibuli and tympani are filled with perilymph and the scala media is filled with endolymph. The oval window membrane is connected to the plate of the stapes. This ossicle transfers the vibrations of the tympanic membrane to the perilymph. The round window vibrates with opposite phase to vibrations of the oval window to set the back and forth movement of the cochlear fluid.

The organ of Corti, the sensory receptor element of the cochlea, sits on the basilar membrane and contains the hair cells and the cochlear nerve fibres. One row of inner hair cells and 3 to 4 rows of outer hair cells (OHCs) are located in the human organ of Corti. The spiral ganglion cells are neurons found in the conical shaped central axis in the cochlea. The dendrites of these neuronal cells stretch to the inner and outer hair cells and their axons form a bundle in the eighth cranial nerve.
The stria vascularis is a layer of stratified epithelium on the lateral wall of the scala media of the cochlea. This layer is penetrated by capillaries and is known to be responsible for producing the ion gradients found in the endolymph.

1.2. The physiology of hearing

Hearing is a process that transforms the sound waves into the nerve impulses to be processed and interpreted as sound by the auditory cortex. The process begins with the collection of sound waves by the pinna and consequent direction of the waves by the external auditory canal towards the tympanic membrane. The vibrations of the tympanic membrane are transmitted through the middle ear ossicles to the oval window and perilymph. Displacement of the perilymph in the scala vestibuli transfers to the scala tympani through the helicotrema, a passage between the scala tympani and scala vestibuli. The movements of the perilymph cause a reflection movement on the round window, with opposite phase to the oval window. Since the area of the central portion of the TM is about
13 times larger than the area of the stapes footplate, the pressure of the movements increase from the TM to the oval window. The larger diameter of the tympanic membrane as compared to the oval window, and the lever action of the middle ear ossicles boost the pressure at the tympanic membrane almost to 200 fold when the wave reaches the inner ear.\(^1\)

![Cochlea cross section](http://en.wikipedia.org/wiki/File:Cochlea-crosssection.png)

**Fig. 2.** Cochlea cross section: Wikipedia: http://en.wikipedia.org/wiki/File:Cochlea-crosssection.png

This mechanism shows how the inner ear overcomes the acoustic impedance, the resistance to the passage of sound between air and fluid. Back and forth movements of the perilymph cause endolymph vibration, and subsequently up and down movements of the basilar membrane. The analysis of sound frequencies is initiated by the basilar membrane vibrations. It is known that the fibers of the basilar membrane become progressively wider and more supple from the base of the cochlea towards the apex. This causes a progressive flexibility of the basilar membrane from the basal turn to the apex of the cochlea. The
variations in the flexibility of each area enables the basilar membrane to vibrate to a particular sound frequency. As a result, the high frequency sound waves (1500-20000 Hz) cause the vibrations at the base of the cochlea, whereas medium frequency waves (600-1500 Hz) stimulate the middle turn, and low frequency waves (200-600 Hz) affect the apex of the basilar membrane.\(^2\) The vibration of the basilar membrane leads to the movement of the outer hair cells toward and away from the tectorial membrane.

![Fig.3. Sound waves in cochlea. Modified from the original picture from McGraw-Hill Companies, Inc.](image)

As a result of these movements, the deflection of the stereocilia toward the tallest and shortest one causes depolarization and hyperpolarization of OHCs.\(^1\) Passive influx of potassium through transduction channels causes depolarization of the OHCs.\(^3\) Depolarization then causes Ca\(^{++}\) influx as a result of the opening of the voltage gated calcium channels. Finally, the ionic imbalance leads to the neurotransmitter (glutamate) being released from the basal region of the hair cells onto the auditory nerve endings, which initiate the transmission of nerve impulses.
1.3. Hearing loss

Hearing loss is diminished sensitivity to sound in one or both ears. It can be divided into three categories: conductive, sensorineural and mixed hearing loss. Conductive hearing loss results from a problem in conduction of sound by the external ear canal, the middle ear ossicles or the tympanic membrane. Sensorineural hearing loss occurs because of the malfunction of the inner ear sensory organ (cochlea), the vestibulocochlear nerve or the central sound processing centre of the brain.

Hearing and the severity of hearing loss are measured in decibels (dB). Hearing threshold is the lowest sound pressure that the human ear can detect at a given frequency. The severity of hearing loss is categorized into mild, moderate, moderately severe, severe, and profound. Mild hearing loss represents the hearing threshold ranging from 26 to 40dB, whereas moderate and moderately severe represent hearing thresholds of 41-55dB and 56-70dB respectively. Severe hearing loss represents hearing threshold of 71-90dB, whereas hearing threshold over 90dB is considered profound.4

1.4. Ototoxicity

Ototoxicity refers to the degeneration and functional impairment of the cochleovestibular system including the cochlea, the vestibular organs and the inner ear neurons. These degenerative changes can be related to drug or noise exposure.

Over 130 drugs have been identified to be potentially ototoxic. These drugs mainly belong to the families of aminoglycosides, platinum based chemotherapeutic agents and loop diuretics.5

1.5. Cisplatin

Cisplatin (cis-diamminedichloro-platinum (II)) is one of the most effective chemotherapeutic agents in the treatment of head and neck, ovarian, bladder, testicular, esophageal and lung cancers.6 The anticancer effect of cisplatin was discovered in 1965 by Barnett Rosenberg. The structure of cisplatin consists of a platinum atom, two chlorine atoms and two amine groups. When cisplatin penetrates the cells, the chlorine ligands are replaced by water molecules to form positively charged monoaquated platinum species.
Cisplatin can then react with one of the nucleobases of DNA, usually guanine, to form intrastrand DNA adducts.\textsuperscript{7}

Although cisplatin is one of the most commonly used antineoplastic agents in the treatment of solid tumors, its dose limiting side effects of ototoxicity and neurotoxicity remain a major challenge.\textsuperscript{8}

\textbf{1.6. Cisplatin Ototoxicity}

Ototoxicity is the major dose limiting side effect of cisplatin chemotherapy. Previous studies have found that 60\% to 80\% of children treated with cisplatin show elevations of hearing thresholds.\textsuperscript{9,10} Cisplatin treatment initially causes hearing loss in the high frequencies (12-16 kHz) and progresses to the lower frequencies with increases in the cumulative dose.\textsuperscript{11} The systemic use of cisplatin leads to non-reversible degenerative changes in the organ of Corti, the stria vascularis and the spiral ganglion neurons of the cochlea.\textsuperscript{12} Since the auditory hair cells of adult mammals do not regenerate, it is known that the cytotoxicity of cisplatin in the inner ear is irreversible and cumulative.\textsuperscript{13}

\textbf{1.7. Mechanisms of cisplatin cytotoxicity}

During the last decade, numerous studies investigating the mechanisms of cisplatin cytotoxicity have suggested DNA damage, reactive oxygen species (ROS) formation and caspase activation, increased release and synthesis of proinflammatory cytokines, and mitochondrial dysfunction as possible mechanisms.\textsuperscript{14-18}

Although cross linkage of DNA and subsequent oxidative stress have been proposed as major mechanisms of cellular damage, DNA synthesis arrest as a main proposed mechanism of cisplatin cytotoxicity has been challenged by other studies indicating that less than 1\% of cisplatin in the cell binds to DNA; moreover, there is a poor correlation between the level of DNA platination and sensitivity of these cells to cisplatin cytotoxicity.\textsuperscript{19} Oxidative stress due to cisplatin has been identified as increased ROS production and decreased activity of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-peroxidase) and glutathione reductase (GSH-reductase), four major antioxidant enzymes in the cochlea.\textsuperscript{7,20} Caspases are cysteine proteases that play important roles in necrosis and programmed cell death.\textsuperscript{21} Caspase activation including caspase-9 (initiator)
and caspase-3 (effector) is one of the identified pathways leading to programmed cell death following systemic administration of cisplatin in the guinea pig animal model. Cisplatin exposure also increases the release of inflammatory cytokines interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α) from organ of Corti cells. Mitochondriae are cellular organelles functioning as cellular power plants by supplying adenosine triphosphate (ATP) as the molecular unit of intracellular energy transfer. Translation of mitochondrial DNA leads to synthesis of 13 proteins participating in the electron transport chain. Mitochondrial DNA damage is another proposed mechanism of cisplatin cytotoxicity which might interfere with supplying cellular energy.

As with many pathological processes, the damage caused by cisplatin can trigger many molecular events which might be secondary to some unknown key lesions. Hence, the mechanism of cisplatin ototoxicity remains uncertain. During the last decade, numerous antioxidants and anti-inflammatory agents have been employed to prevent cisplatin's ototoxicity. Yet there is no antioxidant, anti-inflammatory agent or combination of these drugs with proven clinical effectiveness.

1.8. Curcumin

Curcuminoids are yellow pigments present in the turmeric spice, extracted from rhizomes of the Curcuma Longa plant from the ginger (Zingiberaceae) family. Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1-6-heptadine-3,5-dione; C21H20O6], the most bioactive compound of the curcuminoid group, makes up around 2-5% of the turmeric powder. The other two isoforms are demethoxycurcumin and bisdemethoxycurcumin. Commercial curcumin powder contains 77% curcumin, 17% demethoxycurcumin, and 3% bisdemethoxycurcumin. Curcumin is soluble in ethanol, ketone, chloroform, and insoluble in water. The molecular mass of curcumin is 368.38 gram per mole. Curcumin is more stable in acidic media and undergoes hydrolytic degradation at alkaline pHs (pH>7).

1.9. Curcumin biological properties

Turmeric has been used as a traditional remedy in the treatment of a variety of diseases such as hepatic disorders, rheumatoid arthritis, diabetic wounds, anorexia, cough
and sinusitis. In vitro and in vivo animal studies have shown curcumin unique anticarcinogen, antioxidant, and anti-inflammatory properties.

Molecular targets of curcumin are divided into 2 categories. The first targets are directly involved with curcumin, and their function is modulated by the direct activity of curcumin. These targets include Glutathione, albumin, DNA polymerase, lipoxygenase (LOX), cyclooxygenase (COX)-2, phosphorylase-3 kinase, tubulin, N-aminopeptidase, xanthine oxidase, focal adhesion kinase (FAK), human a1-acid glycoprotein, thioredoxin reductase (TrxR), topoisomerase II, pp60 src tyrosine kinase, and P glycoprotein.

The targets suggested being indirectly up or down-regulated by curcumin include transcription factors (i.e. NF-kB, p53), enzymes (i.e. glutathione S-transferase), protein kinases (PKA, PKC, Src, FAK, CSN-associated kinase), growth factors (EGFR, HER2), inflammatory mediators (i.e. TNF, IL-1, IL-6, COX-2), antiapoptotic proteins (Bcl-2, Bcl-xL, XIAP, IAP, cFLIP), chemokines and their receptors (CXCL1, CXCL2, CXCR4), cell cycle regulatory proteins (Cyclin D1, Cyclin E, c-myc, p21), and invasion and angiogenesis biomarkers (MMP-9, VEGF, uPA).

Curcumin has been recognized as a unique antioxidant due to the fact that it possesses a variety of antioxidant functional groups, including the B-diketo group, phenyl rings and carbon carbon double bonds.

The extensive research on the antioxidant activity of curcumin suggests that it works as an effective reactive oxygen species scavenger and also that it increases the activity of antioxidant enzymes including catalase, glutathione reductase and glutathione peroxidase.

1.10. Curcumin bioavailability

Despite the considerable benefits of curcumin as an effective and safe antioxidant, anti-inflammatory, and anti-neoplastic compound, it has not been embraced by the cancer community or other clinical researchers due to its low bioavailability through oral and intraperitoneal administration. Low bioavailability of curcumin is due to hydrolytic degradation, instability of curcumin in alkaline pH, and hepatic and intestinal metabolism.
1.11. Curcumin loaded nanoparticles

During the last decade, various drug delivery strategies have been used to enhance the delivery of curcumin to the target organs. These drug delivery techniques load curcumin into polymeric nanoparticles, liposomes, phospholipid complexes, polymeric micelles, and nanogels.\textsuperscript{34}

Polymeric curcumin loaded NIPAAM/VP/PEG-A nanoparticles, utilize micellar aggregates of cross linked N-isopropylacrimide (NIPAAM) with N-vinyl-2-pyrrolidone (VP) and poly ethyleneglycol monoacrylate (PEG-A).

Transmission electron microscopy (TEM) and dynamic laser light scattering (DLS) studies of curcumin nanoparticles confirm an average particle size of 45 to 1000 nm.\textsuperscript{34} Nanocurcumin demonstrates the ability to enhance antioxidant levels, reduce production of pro-inflammatory cytokines and growth inhibition of various cancer cell lines including glioblastoma, medulloblastoma and pancreatic cancer.\textsuperscript{35-37}

This study investigates the synthesis and characterization of curcumin loaded NIPAAM/VP/PEG-A nanoparticles. The combination of curcumin nanoparticles and dexamethasone was used as a prophylactic strategy against degenerative changes in the inner ear of cisplatin treated guinea pigs.

1.12. Hypothesis:

Our central hypothesis is that the systemic administration of nanoencapsulated curcumin and dexamethasone can protect the inner ear against cisplatin-induced cytotoxicity.

1.12.2. Objectives:

- Modify the synthesis method of curcumin loaded NIPAAM/VP/PEG-A nanoparticles in order to produce large volume of nanocurcumin for \textit{in vivo} studies
- Investigate the drug release under various pH and temperatures
- Investigate the hydrodynamic size of the curcumin nanoparticles
- Assess the chemopreventive effect of nanocurcumin on cancer cells
- Investigate the morphological changes of the cochlear tissue in guinea pigs receiving cisplatin, nanocurcumin and dexamethasone
• Investigate the hearing threshold shifts of the guinea pigs receiving cisplatin, nanocurcumin and dexamethasone
• Investigate the antioxidant activity in cochlear tissue following the administration of cisplatin, nanocurcumin and dexamethasone
1.13. References:


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33. Pan MH, Huang TM, Lin JK. Biotransformation of curcumin through reduction and glucuronidation in mice. Drug Metabolism and Disposition 1999;27:486-494


CHAPTER 2:

Curcumin loaded NIPAAAM/VP/PEG-A nanoparticles: physicochemical and chemopreventive properties
2.1. Preface

Although a number of potent phytochemical extracts have been identified as active compounds in the treatment of various diseases, curcumin represents one of the most investigated natural compounds with over 5900 published articles according to a recent PubMed search. These studies mostly described the in vitro anti-neoplastic, antioxidant, and anti-inflammatory properties of curcumin.

The limited efficacy of curcumin in clinical studies is attributed to its poor water solubility and low bioavailability. To overcome this problem, various drug delivery systems such as liposomes and nanoparticles have been shown to enhance the delivery of the drug to the target organs. The key feature in these studies is to prepare a stable water soluble compound which can easily penetrate the cell membrane.

In this chapter, we attempted to synthesize and characterize curcumin loaded NIPAM/VP/PEG-A nanoparticles. The main focus of these experiments was on drug release pattern, hydrodynamic size, and stability of the nanoparticles in aqueous media. Bioactivity of the curcumin nanoparticles and toxicity of the non-loaded polymer was tested on 2 different types of cancer cell lines.

2.2. Abstract

This study aims at modifying the synthesis method of preparing N isopropylacrylamide (NIPAAm)/N-vinyl-2-pyrrolidone (VP)/Polyethylene glycol monoacrylate (PEG-A) polymeric nanoparticles encapsulating curcumin as a model drug. The optimal concentration of nanoparticle reagents was determined using Fourier Transform Infrared Spectroscopy (FTIR). Curcumin nanoparticle mean hydrodynamic size was found to be 104 nm with zeta potential of 3 ± 13mV. The release kinetic study of curcumin nanoparticles indicates that a maximum release of curcumin at 24 h positively correlates with increase in temperature; however, change in pH did not produce any substantial drug release. In vitro cell viability assay performed on cancer cells exposed to various concentrations of model compound displayed the inhibitory concentration 50% (IC50) ranging between 100 and 200 μg/mL for human prostate cancer cells (PC3 cells) and 50 and 200 μg/mL for epidermoid carcinoma (A431 cell line). The Hoechst staining and phase contrast micrographs for 48 h exposure of curcumin nanoparticles at a
concentration of 400 μg/mL resulted in almost 92% of cells death in both cell lines. This study concludes that the physiochemical characteristics of NIPAAM/VP/PEG-A polymer with key features of water solubility, sustained drug release, and small particle size make these nanoparticles a prominent drug delivery device.

2.3. Introduction

Curcumin is a natural dietary phytochemical compound with a wide spectrum of biological properties including anti-neoplastic, anti-oxidant, and anti-inflammatory activities.\textsuperscript{1-5} Curcumin is a potent inhibitor of the transcription factor nuclear factor-kappa β (NF-κβ)\textsuperscript{6-9} and downregulates the production of many cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1β,\textsuperscript{9} and interferon gamma.\textsuperscript{8-10} It also inhibits lipid peroxidation by scavenging reactive oxygen/nitrogen species and increasing the activity of anti-oxidant enzymes.\textsuperscript{11-12}

Several studies have shown the safety of the compound even at doses as high as 8 g/day.\textsuperscript{13-14} Despite the promising potential for the treatment of various malignancies, the clinical efficiency of curcumin is limited by its low bioavailability.\textsuperscript{15-16} Regardless of the administration route, curcumin levels are low in serum and tissue due to its poor water solubility and rapid hepatic and intestinal metabolism/elimination.\textsuperscript{14-18} Acid and base hydrolysis, oxidation,\textsuperscript{19} poor intestinal permeability,\textsuperscript{20} glucuronidation and sulfation by liver, kidney, and intestinal mucosa are the main proposed mechanisms of curcumin low bioavailability.\textsuperscript{21} Researchers have undertaken various drug delivery strategies to improve water solubility and bioavailability of curcumin.

The main strategies include synthesis of different dosing formulations of liposomes, phospholipid complexes, and polymeric nanoparticles.\textsuperscript{22-27} It has been previously reported that poly(lactide-co-glycolide),\textsuperscript{28-30} N-isopropylacrylamide (NIPAAM),\textsuperscript{31-36} butylcyanoacrylate,\textsuperscript{37} chitosan,\textsuperscript{22} and dimyristoyl phosphatidylcholine (DMPC) based liposomes increase bioavailability and biological half-life of curcumin.\textsuperscript{24} Polymeric nanoparticles have been increasingly used in the field of drug delivery due to their physicochemical and biological characteristics. These characteristics include nanoscale size, sustained drug release kinetics, water solubility, biocompatibility, pH and ionic strength sensitivity, thermosensitivity, and targeted drug delivery.\textsuperscript{38} A promising
approach to enhance the delivery of water-insoluble curcumin is to employ its encapsulation in a water soluble, thermosensitive polymer called NIPAAM, which undergoes a structural rearrangement in aqueous solution at temperatures above the engineered low critical solution temperature (LCST). The ability to undergo physical changes under easily controlled conditions classifies it as a ‘smart material’ offering extensive applications in the field of drug delivery and tissue engineering. The principal advantages of NIPAAM, when used in combination with polyethylene glycol monoacrylate (PEG-A) and N-vinyl-2-pyrrolidone (VP) as the main components of the nanoparticle formulation, render a hydrophilic nature and hydrophobic functionality to the nanoparticles, which can be adjusted by altering temperature and pH. The mechanism by which NIPAAM polymer leads to drug release involves instability of the hydrogen bonds between the water molecule and amide group of the NIPAAM chains, which happens above the LCST of the polymer leading to contraction of polymeric chains. This contraction causes physical remodeling of the polymer with increased release of the entrapped drug. Moreover, transition from soluble to insoluble form in response to increased temperature, i.e., slightly above 37 °C makes NIPAAM a potential vehicle for targeted drug delivery applications.

The present study investigates the characteristics of the NIPAAM/VP/PEG-A polymer to enhance delivery of the curcumin. The polymerization kinetics and synthesis of this type of reaction have been described by Medeiros et al. and Bisht et al., respectively. Nanoparticles produced using this synthesis acquire a hydrophilic external surface and a hydrophobic core making it an ideal carrier for hydrophobic drugs/substances such as curcumin. In this article, the synthesis method has been modified to enhance the production and delivery of curcumin nanoparticles. The modified method has been explained in detail to facilitate the synthesis of nanocurcumin (NCUR). Furthermore, this study validates the loading efficiency and examines the nanoparticle response to the alterations of pH and temperature. As described by Yan et al., the curcumin should load into the cationic center of the nanopolymer due to the strong van der Waals forces between the hydroxyl group of the curcuma and the amine structure in the interior of the nanoparticles.
Considering the swelling ability of the poly NIPAAM, two different methods have been used to compare the particle size in aqueous media at room temperature and as freeze-dried samples. Finally, the bioactivity of the proposed nanoparticle formulation has been evaluated through viability assays on cancer cell lines (A431 and PC3) using MTS assay and Hoechst 33342 staining.

2.4. Materials and methods

2.4.1 Materials

NIPAAM, 99%, and VP, 99% were purchased from Acros Organics, Canada. The polyethylene glycol 200 monoacrylate (PEG-A) was purchased from Monomer Polymer & Dajac Labs, USA. N,N’-Methylene bis-acrylamide (MBA), 99%, tetramethylethylene diamine (TEMED) 99% and curcumin, 94% were purchased from Sigma-Aldrich, Canada. Ferrous ammonium persulfate (FAS), 99%, 0.5% w/v was purchased from J.T. Baker, USA, and ammonium persulfate (APS) obtained from ACP, Canada. The MTS \(3-(4,5\text{-dimethiazol}-2\text{-yl})-5\text{-}(3\text{-carboxymethoxyphenyl})-2\text{-}(4\text{-sulfophenyl})\text{-2H-tetrazolium}\) assay kit was purchased from Promega, USA.

2.4.2. Fourier transform infrared spectroscopy analyses (FTIR)

A Thermo Model 6700 equipped with a Diamond Attenuated Total Reflection (ATR) and a diffuse reflectance accessory. The samples were analyzed without any pre-treatment for the ATR and mixed in a 1:15 ratio with Potassium Bromide (KBr) powder for diffuse reflectance. The FTIR detection range was 500–4000 cm\(^{-1}\).

2.4.3. Purification of the reagents/APS concentration optimization

The recrystallized NIPAAM was prepared by adding 95% N-hexane to a flask containing 2 g of amorphous NIPAAM. The contents of the flask were heated until the NIPAAM was completely dissolved and then cooled down for complete precipitation of NIPAAM crystals. Subsequently, vacuum filtration was performed to isolate and dry the purified NIPAAM. The PEG-A solution (1% w/v) was prepared and washed with N-hexane three times. The molecular structures of recrystallized/amorphous NIPAAM and washed/unwashed PEG-A solutions were compared using FTIR spectroscopy.
In a typical experimental protocol, three concentrations of APS (0.2, 0.4, 0.8 g/mL) were used for polymer synthesis. The molecular structures of the samples were compared using FTIR spectroscopy.

2.4.4. Preparation of polymeric NIPAAM/VP/PEG-A nanoparticles

Three different combinations of polymeric nanoparticles were prepared by adding 4500 mg of NIPAAM, 250 μL of VP, and 25mL of PEG-A (1% w/v) in 450mL of deionized water (dH2O). Subsequently, 1500 μL of MBA at a concentration of 20 mg/mL was added to the solution to cross link the polymer chains. Oxygen was removed from the solution by bubbling N2 for 30 min. The polymerization reaction was initiated by adding 1mL of FAS, 1.5mL of APS (0.4 g/mL), and 960 μL TEMED. The polymerization was performed under N2 atmosphere and at 30 °C for 24 h. To remove residual monomers and catalysts, the polymer solution was subsequently dialyzed for 48 h using a Spectra/Por, MWCO: 12–14 kDa dialysis membrane at room temperature. The dialyzed polymer solution was then freeze dried. To verify the success of the dialysis procedure, residual catalysts and monomers in the dH2O were traced using an Evolution UV–Visible 600 spectrophotometer at 190–560 nm.

2.4.5. Percentage loading efficiency of curcumin in NIPAAM/VP/PEG-A nanoparticles

Curcumin was dissolved in chloroform at a concentration of 10 mg/mL and was slowly added to the polymeric (NIPAAM/VP/PEG-A) solution (10 mg/mL), under constant vortex to obtain 10% (w/w) curcumin in the nano polymer and form NCUR nanoparticle formulations. The solution was sonicated for 30 min. Subsequently, nitrogen gas was passed through the NCUR solution for 30 min and the sample was freeze dried. The spectra of the lyophilized samples were compared using FTIR.

The concentration of nanoencapsulated curcumin was determined by calculating the curcumin mass loaded into the polymer. Since chloroform contained both polymer and free curcumin, a standard addition method was performed. Standard samples of curcumin were added to the chloroform and the absorbance of each sample was recorded by UV–vis detector at 450 nm. Using the data from the original sample and from vials spiked with curcumin, a linear equation was plotted. The original curcumin concentration was obtained
from the absolute value of the x-intercept. The percentage loading efficiency of curcumin into the nanoparticles was quantified by subtracting the amount of remaining curcumin in the chloroform from the total curcumin used for loading.

2.4.6. Effect of pH and temperature on release of curcumin from NIPAAM/VP/PEG-A nanoparticles

To evaluate the drug release due to change in pH conditions, the NCUR (0.4 mg/mL) in volumes of 2mL were centrifuged at 1000 rpm for 10 min, prepared in triplicate at a pH ranging from 1.5 to 9.5 and extracted with 2mL of chloroform. The samples were subsequently evaporated under nitrogen atmosphere at 40 °C and reconstituted in 1mL high-pressure liquid chromatography (HPLC) mobile phase. pH measurements were performed with Orion pH/ISE meter Model 710A.

For temperature-dependent drug release studies, the same samples were prepared in triplicate (sample volume of 1 mL) and kept at 25, 37, and 45 °C for 12, 24, and 48 h. These solutions were later centrifuged at 1000 rpm for 10 min and the pellets obtained were reconstituted in a 1mL HPLC mobile phase. The samples were injected into the HPLC for quantification of curcumin release from the NCUR nanoparticles.

2.4.7. High-pressure liquid chromatography (HPLC)

Agilent 1200-Reverse Phase HPLC equipped: quaternary pump set to 1.0 mL/min; diode array detector (DAD) (254 and 425 nm); fluorescence detector (FLD) (excitation: 423 nm; emission: 500 nm); column Zorbax Eclipse C8 (4.6_150mm, 3.5 μm), Guard column 4.0_3.0mm (Agilent, USA), column compartment (30 °C); Auto-sampler with 20 μL injection volume. Mobile Phase: 40% phosphate buffer 0.1mM adjusted to pH 4.0 and 60% acetonitrile. The external calibration curve was prepared using curcumin in a range of 1500–3000 ng/mL. All standards were prepared in the mobile phase. Correlation coefficients were 0.9995 for DAD and 0.9998 for the FLD.
2.4.8. Particle size and zeta potential of NIPAAM/VP/PEG-A nanoparticles

A Solver NT-MDT Atomic Force Microscope (AFM) in semi-contact mode was employed to calculate the particle size of the freeze-dried NCUR nanoparticles. A 0.4 mg/mL of NCUR sample was immobilized on quartz micro dish and freeze dried. The immobilized sample was then analyzed on a temperature controlled stage. A background correction was applied to remove the quartz micro dish from the image. To measure the hydrodynamic diameter of NCUR and void polymer, nanoparticle tracking analysis (NTA) was performed using a digital microscope NanoSight NS500 system (NanoSight, UK). A solution of NCUR nanoparticles (4 μg/mL) was injected into the chamber. The size and number of the particles were measured at room temperature for 30 s with a 913 camera shutter, and 14 frames per second. The data were captured and analyzed with NTA 2.2 software.

Surface charge of the NIPAAM/VP/PEG-A nanoparticles, void nanoparticles, and curcumin alone was analyzed using zeta potential electrophoresis (Zeta Plus, Brookhaven Instruments, USA). The nanoparticles analyzed were formed at a drug concentration of 0.4 mg/mL and at pH 4.

2.4.9. Cells and culture conditions

A431 and PC3 cells were generously provided by Dr. Janusz Rak, McGill University, Montreal. These cells were maintained as monolayer cultures in high glucose Dulbecco’s Modified Eagle Medium (DMEM; Life Technologies, Canada) supplemented with 10% fetal bovine serum (FBS; Wisent, Canada) at 37 °C and 5% CO2.
2.4.10. Cell viability test: MTS assay and Hoechst staining

Cells were seeded in 96-well plates at a density of 4000 cells/well in 200 μL DMEM and left to attach for 24 h at 37 °C. Subsequently, cells were exposed to different concentrations of NCUR and void (without curcumin) nanoparticles ranging from 25 to 200 μg/mL, and 250 to 2000 μg/mL, respectively. The MTS solution (25 μL) Promega, USA, was added to each well after 48 h of drug exposure and the plates were incubated for 90 min at 37 °C. The metabolic activities of the cells were recorded as relative colorimetric changes, measured at 492 nm.

To count the viable cells in each well, PC3 and A431 cells treated with concentrations of NCUR and void nanoparticles from 50 to 400 μg/mL, and 500 to 4000 μg/mL, respectively, were fixed for 10 min in 4% (w/v) paraformaldehyde, permeabilized for 5 min with Triton™ x-100 (0.1% v/v) and stained for 10 min with fluorescent dye Hoechst 33342 (0.1 μg/mL). The cells were washed with Phosphate buffered saline and examined with a Leica DMIRB fluorescence microscope at an excitation wavelength of 350 nm. Cells were counted at magnification of _200 in three visual fields which represented the whole well.

2.5. Results

2.5.1. Synthesis modification and physicochemical characterization of NIPAAM/VP/PEG-A nanoparticles (FTIR, release kinetics, AFM, NTA, and zeta potential)

The FTIR spectra of the amorphous NIPAAM and PEG-A were identical to purified reagents described by Bisht et al.31 (Supplementary Fig. 6). The data were collected from 450 to 4000 cm⁻¹ using the ATR. The FTIR results of polymer synthesis at three concentrations of APS (0.2, 0.4 and 0.8 g/mL) showed incomplete reaction at concentration of 0.2 g/mL. The spectra of the polymers using APS concentrations of 0.4 and 0.8 g/mL resulted in complete reaction (Supplementary Fig. 7). The optimal concentration of APS (0.4 g/mL) was used for NCUR synthesis for all the experiments explained in this article. Figures 1(A) and (B) are the spectra of the freeze-dried nanopolymer with and without curcumin loaded.
respectively. Figure 1(C) is a spectra of the curcumin. The data set was generated using the diffuse reflectance accessory. The loading efficacy of NCUR varied from 78 to 81%.

The release of curcumin from NIPAAM/VP/PEG-A nanoparticles as an effect of change in pH (Fig. 2(A)) and temperature (Fig. 2(B)) was analyzed by HPLC method. The results indicate that a greater release of curcumin was detected when the curcumin loaded nanoparticles were exposed to an acidic environment of pH 4.0. Decreased curcumin release was observed at pH 7.5 and 9.5, although greater variability was noted at pH 7.5. A positive effect of increase in temperature on release of curcumin from nanoparticles was observed as represented in Fig. 2(B). Results indicate, for all the temperatures tested, 25, 37, and 45 °C, the maximum release of curcumin was obtained at 24 hours represented in Fig. 2(B).

![Graph A](image)

![Graph B](image)

**Fig. 2.** In vitro release of curcumin based on pH and temperature changes measured by HPLC. NCUR nanoparticles (0.4 mg/mL) were exposed to changes in pH, varying from 1.5 to 9.5. Greater amounts of curcumin are released at pH 4.0 with a secondary peak at pH 8.5 (A). The same sample was also exposed to varying temperatures (25, 37 and 45 °C).
Greater curcumin release is observed at higher temperatures, reaching peak levels at 24 hrs for all temperatures tested (B).

Using the AFM features, the majority of the nanoparticles had a size distribution between 20 and 60 nm (Fig. 3(A)). The mean particle size of NCUR nanoparticles was found to be 38 nm and the 3σ distribution was between 23 and 53 nm. The mean hydrodynamic diameter by NTA was determined to be 104 ± 39 nm. The number of the particles at NCUR’s concentration of 4 μg/mL was 7.93_108. The mean hydrodynamic diameter of void nanoparticles as determined by NTA was found to be 173 ± 63 nm (Fig. 3(B)).

Surface charge analysis using zeta sizer of NCUR nanoparticles indicated an average surface charge of 3 ± 13mV. The average surface charge of void nanoparticles and curcumin was determined to be 29 ± 11mV and _35 ± 5mV as indicated in Fig. 3(C).
**Fig. 3.** Sample size distribution: The 2-D AFM Semi-Contact Mode image analysis of the immobilized particles is summarized in this histogram. The particle size distribution is skewed; however, the mean value was determined to be 38 nm (A). NCUR nanoparticles size distribution by nanoparticle tracking analysis (4 µg/mL) in dH₂O: Size distribution shows a mean peak at 104 nm; SD: 39 nm (B). Zeta potential analysis of void nanoparticles, curcumin and NCUR nanoparticles: NCUR sample (0.4 mg/mL) was centrifuged at 1000 rpm at ambient temperature and pH 4; the nanoparticle surface charge was measured using a Zeta Potential Analyzer (C).

**2.5.2. NCUR nanoparticle inhibits the growth of prostate cancer and epidermoid carcinoma cells**

MTS assay was conducted to determine the anticancer activity of NCUR nanoparticle formulation on cancer cells. A431 and PC3 cells were exposed to various concentrations of NCUR nanoparticles (2,550,100, and 200 µg/mL). As shown in Fig. 4, the lowest cellular metabolic activity was observed at concentration of 200 µg/mL, whereas NCUR concentration of 25 µg/mL demonstrated no effect on either of the cell lines.

**Fig. 4.** Cell viability (MTS) assay following the 48 hr exposure of cells to various concentrations of NCUR nanoparticles showing the IC50 was found to range between of 100-200 µg/mL, and 50-200 µg/mL for PC3 and A431, respectively.
Results of the cell count experiment following Hoechst staining at lowest concentration of NCUR nanoparticles showed 46.5 and 16% of cell death for the A431 and PC3 cell lines, respectively, whereas the highest concentration resulted in almost 92% of cell death for both cell lines (Table 1). Void nanoparticle in ranges up to 10-fold higher than experimental conditions caused no statistically significant effect on both cell lines (Supplementary Fig. 8).

<table>
<thead>
<tr>
<th>NCUR nanoparticles concentration (µg/mL)</th>
<th>CTL</th>
<th>50</th>
<th>100</th>
<th>200</th>
<th>400</th>
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<tr>
<td>A431</td>
<td>591.0(10.1)</td>
<td>322*(12.2)</td>
<td>134.7*(8.3)</td>
<td>89.3*(11.4)</td>
<td>46*(12.5)</td>
</tr>
<tr>
<td>PC3</td>
<td>474.7(9.5)</td>
<td>399.3*(12.2)</td>
<td>407.7*(12.7)</td>
<td>74*(11.8)</td>
<td>32*(4.6)</td>
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</table>

<table>
<thead>
<tr>
<th>Void nanoparticles concentration (µg/mL)</th>
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<th>500</th>
<th>1000</th>
<th>2000</th>
<th>4000</th>
</tr>
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<tbody>
<tr>
<td>A431</td>
<td>474.7(9.5)</td>
<td>578.0(17.8)</td>
<td>590.0(16.4)</td>
<td>585.3(8.1)</td>
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<tr>
<td>PC3</td>
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<td>484.0(12.5)</td>
<td>469.3(17.0)</td>
<td>485.7(18.2)</td>
</tr>
</tbody>
</table>

Table 1. Cell viability (3 wells) after treatment with NCUR and void nanoparticles as determined by Hoechst 33342 staining. Data are expressed as mean (standard deviation). (*) p<0.01.

Phase contrast and Hoechst DNA micrographs of PC3 cells following 48 hour exposure to NCUR nanoparticles show that higher concentration of NCUR nanoparticles is associated with increased cell death (Fig. 5).
Fig. 5. Phase contrast micrographs of PC3 cells following 48 hour exposure to (NCUR) and void nanoparticles demonstrates greater cell loss and increased number of floating cells at higher concentrations of NCUR compared to the control. B. Hoechst staining shows that higher concentration of NCUR is associated with increased cell death. Bar equals 300 microns.
Fig. 6. FTIR spectroscopy of: NIPAAM before and after recrystallization (A); PEG-A before and after wash (B).

Fig. 7. FTIR: Incomplete synthesis of NCUR using the lowest (0.2 g/mL) APS experimental concentration (A); FTIR of NCUR demonstrates complete polymerization using 0.4 g/mL and 0.8 g/mL APS.

Fig. 8. Cell viability (MTS) assay following the exposure of PC3 and A431 cells to various concentrations of VPOL (x10 concentration of NCUR) no cytotoxicity was observed on either cell line.
2.6. Discussion

In order to develop an efficient and reliable carrier for delivering curcumin, a hydrophobic substance, this experiment modified the synthesis of NIPAAM/VP/PEG-A polymeric nanoparticles as previously described.\textsuperscript{31} Bisht et al. have demonstrated that this nanoparticle passes through cellular membranes and has a therapeutic efficacy comparable to free curcumin against pancreatic cancer lines.\textsuperscript{31} The synthetic method described herein provides a rapid procedure that can be adapted by any research laboratory. Chemical reactions of NIPAAM and PEG-A can take place at a relatively low temperature by means of catalysts, without the need for special reactors.

The FTIR analysis of the monomers (NIPAAM and PEG-A) before and after washing as described earlier by Bisht et al. proved unnecessary, as the purity remained the same (Supplementary Fig. 6).

The FTIR spectra of NIPAAM polymer synthesized with APS at a concentration 0.2 g/mL showed that this concentration does not initiate the reaction of the alkene. In this figure, a strong stretch can be seen in the region of 1640–1680 cm\textsuperscript{-1}, corresponding to the alkenes in the unreacted monomers. Furthermore, there was absence of amine stretch between 3300 and 3500 cm\textsuperscript{-1} (Supplementary Fig. 7). In the case of NCUR nanoparticles prepared at APS concentration of 0.4 g/mL there is an absence of the alkene stretch at 1640–1680 cm\textsuperscript{-1}. In addition, there was the presence of the amine stretch at 3500–3300 cm\textsuperscript{-1} and the alkyl stretch at 610–700 cm\textsuperscript{-1} (Fig. 1(A)). The complete absorption of energy at the spectra of 600–1500 cm\textsuperscript{-1} and 2600–3800 cm\textsuperscript{-1} indicated the presence of a cross link between two oxygen atoms, one potentially coming from curcumin and the other one from the polymer (Fig. 1(A)). A clear difference was observed in the above-mentioned ranges when the NCUR nanoparticle was compared to the void polymer which suggests the penetration of curcumin into the polymer spheres (Fig. 1(B)).

As shown in Fig. 2(B), the thermo sensitivity of the polymer facilitates in vitro maximum release of curcumin at 37 and 45 °C. However, the release is reduced by 50\% at 25 °C. Hence, a sustained release of curcumin in the blood or in tissues is possible. The sustained release pattern of the drug from nanoparticles into the target tissues should increase the level of free curcumin and prevent rapid intestinal and hepatic metabolism.\textsuperscript{18}
The curcumin release pattern in the pH study supports the theory of internal entrapment versus attachment to the outer surfaces of the polymer spheres (Fig. 3(A)). In the latter case, greater curcumin release would be detected in response to changes in pH. The minimal downward trend in curcumin levels with increasing pH may be explained by the instability of curcumin in alkaline solutions. When the pH is adjusted to neutral or alkaline conditions, the removed proton from the phenolic group leads to the degradation of this structure.\textsuperscript{49,50}

Gradual decrease in curcumin levels after 24 h at the various temperatures could be the result of hydrolytic degradation over time (Fig. 3(B)).\textsuperscript{49} The results of the NCUR nanoparticles exposure to the various pH conditions indicate that the drug release rate varies between 0.9 and 2%.

The drug release pattern following exposure of the NCUR nanoparticles to the temperatures ranging between 25 and 42 °C demonstrates that there is no significant curcumin release at ambient temperature. This study observed 0.4–1% curcumin release from nanoparticles at 37 °C (pH 4) between 12 and 24 h. The drug release rate from NIPAAM/VP/PEG-A nanoparticles was lower compared to chitosan grafted poly NIPAAM as described by Rejinold.\textsuperscript{35,36} This suggests that using the NIPAAM/VP/PEG-A nanoparticle formulation may allow greater quantities of curcumin to reach the target site in encapsulated form.\textsuperscript{51}

The size of the drug-carrier particle is an important factor for drug delivery, as particles smaller than 400 nm can cross vascular endothelia and reach the target site.\textsuperscript{52} The size of nano-encapsulated curcumin in the present study is comparable to the one from other studies.\textsuperscript{14,25,50,53}

The mean particle size by AFM was 38 nm in a freeze-dried sample; however, the results of this study showed that there was an almost 60 nm increase in mean particle size when the lyophilized NCUR nanoparticle was resuspended in an aqueous matrix. The determination of the particle size using two techniques confirmed the mechanism of hydration of the nanopolymer described by Guven and Sen.\textsuperscript{43} The NTA results showed marked shrinkage in hydrodynamic size (69 nm) when the void nanoparticles were loaded with curcumin. Shrinkage of the polymeric spheres seems to be the result of electrostatic attraction of the positively charged polymer with the negatively charged curcumin.
The zeta potential data confirms the model proposed by Yan et al. The observed variability in the zeta potential of NCUR nanoparticles when compared to void nanoparticles and curcumin (Fig. 3(B)) is likely due to entry of the negatively charged curcumin into the positively charged nanopolymer, where it expectedly donates its proton to the amine functional group of the nanopolymer and migrates outside the polymer sphere in its electronegative form. It then likely picks up a proton from the acidified water and migrates back into the nanopolymer. As a result, the zeta potential shifts continuously from positive to negative charge for NCUR nanoparticles. This hypothesis is supported by the curcumin release data at pH 4.3 (Fig. 2(B)).

The MTS assay is based on bioreduction of the MTS salt (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) into formazan. The absorbance of formazan at 490 nm shows mitochondrial activity which is representative of the cellular metabolic activity. In this study, the metabolic activity of A431 and PC3 cell lines is decreased by 49 and 53%; respectively, when the cells were exposed to NCUR nanoparticles at a concentration of 200 μg/mL. The reduction in metabolic activity of the treated cells was significant when compared to controls, which received no treatment. To ensure that the decreasing metabolic activity was due to cell death versus the alteration of mitochondrial activity [54], Hoechst 33342 staining and phase contrast microscopy were conducted in parallel to the MTS assay. As shown in the phase contrast micrographs of PC3 cells, the NCUR nanoparticle concentrations of 200 and 400 μg/mL led to the greatest level of cell loss and cell detachment (Fig. 5). It is well-known that apoptotic cells detach from the culture flask and float in the medium. Hoechst DNA indicates that the numbers of viable cell in each visual field decreased at NCUR nanoparticle concentrations greater than 100 μg/mL (Table 1).

Combining the results of the MTS assay, phase contrast microscopy, and Hoechst staining provided a more reliable interpretation of cell reactions to the NCUR regimens. The results reveal that decreased metabolic activity, shown by the MTS assay and cell counts, is due to the anti-neoplastic activity of the NCUR nanoparticles that was also investigated by other studies. Based on the overall results, the modified method presented in this article seems to be a rapid and effective alternative to the other drug formulations for delivery of curcumin.
Fig. 9 NCUR synthesis: NIPAAM (4500mg) was dissolved in 450mL dH$_2$O, VP 99% (250 µL), PEG-A (25 mL) at concentration of 112mg/mL added to 500 mL dH$_2$O; MBA (1500 µl) at concentration of 20mg/mL, bubbling nitrogen for 20 minutes, added 1 mL of FAS (5mg/mL) and 1.5 mL APS (0.4g/mL) and 1 mL of TEMED. Polymerization process continued for 24 hours at 37 °C under nitrogen atmosphere, followed by 48 hours of dialysis, loading curcumin, separation and lypholization.

2.7. Conclusions

The present study enhances the synthesis method of the NIPAAM/VP/PEG-A polymer to circumvent the pitfall of curcumin's low bioavailability. The current method adjusted the concentration of APS and investigated the unknown physicochemical characteristics of the curcumin nanoparticles. Moreover, a simple standard addition method was developed to quantify the amount of drug loaded onto polymer spheres. The anti-proliferative effect of NCUR nanoparticle on two cancer cell lines confirmed the biological activity of the proposed nanoparticle.

The main characteristics of the proposed curcumin nanoparticle, including small size, water solubility, thermosensitivity, and sustained release pattern make it a unique choice for future in vivo experiments.

2.8. Acknowledgements

Authors thankfully acknowledge Dr. Janusz Rak and Brian Meehan for help with cell biology. We also thank Aref Taghizadeh, Dan Citra, Emilia Peleva for editorial assistance and Andrew Golsztajn for help with the preparation of the graphics and providing valuable comments.
2.9. References


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CHAPTER 3:

Attenuation of Cisplatin Ototoxicity by Otoprotective Effects of Nanoencapsulated Curcumin and Dexamethasone in a Guinea Pig Model
3.1. Preface

Platinum-based chemotherapy is one of the most effective treatments for various types of malignancies including ovarian, head and neck, testicular, lung, bladder, and colorectal cancers.

Ototoxicity, neurotoxicity, and nephrotoxicity are 3 major dose limiting side effects of Cisplatin chemotherapy. Although the exact mechanism of cisplatin cytotoxicity is still unknown, these side effects are mainly attributed to increased oxidative and inflammatory stress in hearing organ of the ear, peripheral neurons, and kidneys. Therefore, it is hypothesized that reducing the level of the oxidative stress and inflammation might be a reasonable preventive strategy in patients receiving cisplatin chemotherapy.

In the previous chapter, we developed a simplified synthesis method to produce curcumin loaded NIPAAM/VP/PEG-A nanoparticles in large scale for *in vivo* experiments. In this chapter, curcumin loaded nanoparticles were used as antioxidant agent. Dexamethasone which is a well-known steroid was added to the treatment to reduce the inflammatory effect of the cisplatin. The experiments were conducted on hearing hair cells *in vitro* and in a guinea pig model. The functional assessment of the hearing organ was completed by comparing the hearing thresholds of the animals before and after cisplatin treatment. The cochleae of the guinea pigs were used for morphological assessment and antioxidant enzyme assays.

3.2. Abstract

Cisplatin, one of the most effective and widely-used chemotherapeutic agents in the treatment of head and neck malignancies, has severe dose-limiting side effects including ototoxicity. This study evaluates the effectiveness of nanoencapsulated curcumin and dexamethasone in preventing degenerative changes in inner ear cells caused by cisplatin. For this purpose, cultured auditory cells (HEI-OC1) and a guinea pig model were used for *in vitro* and *in vivo* experiments, respectively. First, cell viability assays were conducted to compare the direct toxicity of cisplatin against auditory cells in the presence or absence of pre-treatment with nanoencapsulated curcumin and dexamethasone. Such pre-treatment resulted in significant attenuation of cisplatin toxicity. To recapitulate these effects *in vivo*, guinea pigs received cisplatin alone, or along with dexamethasone, nanoencapsulated
curcumin or the combination of both products. Outcome measures included auditory brainstem response, cochlear morphology under both light and scanning electron microscopy, and antioxidant enzyme assays. In this animal model, cisplatin caused an average hearing loss of 50 dB which was attenuated by nanoencapsulated curcumin and dexamethasone across all of the hearing frequencies. There was also greater preservation of histological structures in this group. Superoxide dismutase and catalase activities were increased in cisplatin-treated animals whereas the nanoencapsulated curcumin with dexamethasone led to a diminution of this effect. Thus, nanoencapsulated curcumin administered in combination with dexamethasone provides a partial but marked protection against cisplatin-induced hearing loss, likely due to reduced toxic damage to auditory cells.

3.3. Introduction

Platinum-based chemotherapy is an effective treatment for a variety of malignancies.\(^1,2\) Platinum compounds, such as cisplatin, exert their cytotoxic effects through the cross-linkage of DNA, the production of reactive oxygen species with subsequent caspase activation, the stimulation of the immune response as well as the inflammatory response through the up-regulation of inflammatory cytokines and mediators.\(^2\) These, however, also lead to dose limiting side effects including ototoxicity, nephrotoxicity and neurotoxicity. Approximately 60 to 80% of the patients treated with cisplatin will develop hearing loss, which is usually permanent, bilateral and progressive.\(^2-4\) Development of hearing loss often leads to therapeutic dose reductions, potentially decreasing the efficacy of the treatment. Despite tremendous research efforts, there is currently no known treatment or preventive measure for cisplatin-induced hearing loss.

Curcumin, a polyphenol isolated from turmeric (\textit{Curcuma longa}), has received increased attention over the last few decades due to its antioxidant, anti-inflammatory and antineoplastic activities.\(^5\) Regardless of the administration route, the bioavailability of curcumin is minimal due to hydrolytic degradation, chemical instability in alkaline pH, and rapid intestinal and hepatic metabolism/elimination processes.\(^6,7\) Recent nanoencapsulation of curcumin has provided a strategy for increasing its solubility and stability in aqueous media, enhancing delivery of the compound to the target organs.\(^8\)
to these properties, it is plausible that nanoencapsulated curcumin (NCUR) could reach many cellular compartments, including inner ear cells, which are the main target of cisplatin ototoxicity.

It is known that cisplatin can elicit an inflammatory response in the inner ear leading to cellular injury.\(^2\) Corticosteroids have been evaluated as a potential otoprotective measure on the principle of their anti-inflammatory activities.\(^9\) Dexamethasone is one of the most potent corticosteroids available and is used to treat a variety of inflammatory conditions of the inner ear, but reports on the protective effect of dexamethasone against cisplatin ototoxicity are controversial.\(^{10-14}\) For example, there is evidence of partial protection against cisplatin-induced morphological changes in the ear, especially in the stria vascularis.\(^{15}\) Dexamethasone is also currently used in the prevention of cisplatin-induced acute emesis in a large proportion of patients receiving this form of chemotherapy.\(^{16}\)

In this study, we present a novel experimental approach to protect the inner ear against the damaging effects of cisplatin by using a combination therapy of NCUR and dexamethasone.

### 3.4. Materials and Methods

#### 3.4.1. Synthesis of polymeric nanoparticles

NCUR nanoparticles were synthesized using a previously described method.\(^{17}\) Briefly, 4320 mg N-isopropylacryamide (Acros Organics), 240 µL N-vinyl-2-pyrrolidone (99%, Acros Organics), 24 mL polyethylenglycol monoacrylate (112 mg/mL, Acros Organics) and 1440 µL N-methylene bis acrylamide (Sigma-Aldrich, 20 mg/mL) were added to 450 mL distilled water in a distilling flask. Subsequently, 960 µL ferrous ammonium sulfate (5 mg/mL, J.T Baker) and 1440 µl ammonium persulfate (0.4 mg/mL, J.T Baker) were added to the solution. Polymerization reaction was initiated by adding 960 µL tetramethylethlenediamine (99%, Sigma-Aldrich) at 30 °C under a nitrogen atmosphere. To load the drug, free curcumin (500 mg) was dissolved in 50 mL of chloroform and was then slowly added to the polymer solution under constant vortex. The NCUR solution was then separated and freeze dried to obtain a dry powder.
3.4.2. Cell culture and viability

HEI-OC1 cells (a generous gift from Dr. Kalinec from the House Research Institute, Los Angeles, CA) were maintained as monolayer cultures in high glucose Dulbecco’s Modified Eagle Medium (Life Technologies, Canada) supplemented with 10% fetal bovine serum (Wisent, Canada) at 33 °C and 10% CO2. The cells were seeded at 3000 cells/well and left to attach for 24 hours. HEI-OC1 cells were exposed to cisplatin for 8 hours to determine the lethal dose 50% (LD50). Cisplatin at an LD 50 concentration of 70µM (8 hours exposure) was selected for subsequent experiments.

Culture cells were also treated with various concentrations of dexamethasone ranging from 50 to 900 nM. The dosage of 200 nM was selected for subsequent experiments for two main reasons. Firstly, considerable decreases in inflammatory mediators have been observed in cultured cells exposed to an inflammatory environment, and dexamethasone at a dosage of 100 nM. The anti-inflammatory effects appear to increase with dosage; however, the changes in mediator release have been less dramatic between 100 to 1000 nM. Secondly, Takahashi et al observed that patients receiving dexamethasone when receiving cisplatin chemotherapy, had a maximum plasma dexamethasone level of 80 ng/mL (≈ 200 nM). Further evidence is provided by Rinehart et al; as the dexamethasone dose chosen by this group was 80 ng/mL (8 ug/dL – 200 nM equivalent), and achieved desired biological effects.

To evaluate the cytotoxic effect of the NCUR, HEI-OC1 cells were exposed to various concentrations of the drug ranging from 5 to 80 µg/mL for 48 hours. The cell viability was determined with the use of the MTS assay which detects metabolic activity. The effect of NCUR on cisplatin-treated cells was examined with the same tested concentrations. The last group of cells received NCUR and dexamethasone (200 nM) 24 hours prior to cisplatin exposure to the end of the experiment. Subsequently, cells were washed and incubated in media containing NCUR and dexamethasone at 33 °C for 24 hours. The MTS solution (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (25µL/well) (Promega) was added and the cells were incubated for 90 minutes. The absorbance at a wavelength of 490 nm was measured using a microplate reader (Biotek, USA).
3.4.3. Animals

The study was approved by the institutional review board of the McGill University Health Centre. Sixty-eight female Hartley guinea pigs (500-600 g) were used in this study. The guinea pigs were randomly divided into seven groups (8-14 animals each; Table 1): cisplatin (group 1), dexamethasone (group 2), NCUR (group 3), cisplatin/void polymer (group 4), cisplatin/dexamethasone (group 5), cisplatin/NCUR (group 6), and cisplatin/NCUR/dexamethasone (group 7). Cisplatin was administered intraperitoneally (IP) over 7 consecutive days for a total dose of 13 mg/kg. Dexamethasone and NCUR (0.5 mg/mL) were administered IP over 9 consecutive days, starting one day prior to the first cisplatin administration and ended one day after the last cisplatin injection, at doses of 10 mg/kg/day and 20 mg/kg/day, respectively. Dexamethasone and/or NCUR administration was started one day prior to the cisplatin administration. The dose of NCUR was selected based on the maximum-tolerated volume of IP injections for guinea pigs. The dexamethasone dosage was selected based on previously tested, non-toxic high doses in a guinea pig animal model.

3.4.4. Hearing assessment

Auditory brainstem response (ABR) tests were performed using the Smart EP Device (Intelligent Hearing Systems, Miami FL). Acoustic stimuli were generated by high frequency transducers and presented separately to each ear through plastic tubes connected to the earphones that were inserted into the external ear canal of the animals. Acoustic signals were emitted at 8, 16, 20 and 25 kHz tone bursts (rise-fall time of 5 millisecond, 44 Blackman envelope) at a rate of 39.1 bursts/seconds after 1600 sweeps. Threshold recording started with administration of a 100 dB SPL signal for the click, 8, and 16 kHz stimuli, and a 85 dB SPL signal for the 20, and 25 kHz tone bursts. Evoked responses were recorded using two subdermal needle electrodes positioned at the pinna of the test ear. The analysis time window for each response was 16 milliseconds. The evoked potentials were filtered with a bandpass filter between 100 and 1500 Hz. The ABR thresholds were determined by an examiner who was blinded to the treatments administered, as the lowest sound wave to which a visible, repeatable wave V could be identified.
Hearing assessments were performed before treatment (baseline measurement) and 24 hours after the last cisplatin injection (post measurement). The hearing thresholds were compared to obtain a threshold change for each of the tested frequencies.

We were unable to perform long term follow up ABR due to the high mortality rate of guinea pigs receiving 13 mg/kg cisplatin. The animals show progressive sickness starting 3 days after the last cisplatin injection, possibly related to nephrotoxicity. We suggest using a mouse model to evaluate the long term effect of NCUR/dexamethasone on cisplatin-induced ototoxicity for the future experiments.

3.4.5. Light microscopy (LM)

Three randomly-selected cochleae from each group were evaluated using light microscopy. Three guinea pigs were used as controls. The cochleae were freed from surrounding soft tissue and bone, and were fixed in 10% formalin for 24 hours. The samples were then decalcified with 10% ethylenediaminetetraacetic acid (EDTA) in phosphate buffered saline (pH 7.4). After decalcification, the samples were taken through graded alcohol dehydration. The tissues were then cleared in xylene and embedded in paraffin. Eight micron thick sections were obtained, mounted, and stained with haematoxylin and eosin. The sections were visualized under a light microscope (Carl Zeiss, Germany) at 40x, 100x, 200x and 400x. Detailed assessment of the stria vascularis (SV), spiral ganglion cells (SGCs) and outer hair cells (OHCs) was completed at 400x.

3.4.6. Scanning electron microscopy (SEM)

SEM was performed on three randomly-selected animals in groups 1 (cisplatin), 5 (cisplatin/dexamethasone), 6 (cisplatin/NCUR), 7 (cisplatin/NCUR/dexamethasone) and controls in order to evaluate the appearance of the outer hair cells (OHCs). The effect of the dexamethasone on cisplatin ototoxicity and related morphological changes of the OHCs were presented in a previous study from our laboratory; cochlear micrographs were prepared using the previously described methods. Using the modified version of the method described by Saito et al., the OHCs were counted at a magnification of 1000x across the basal turn of the cochlea on 3 visual fields. The OHCs of the basal turn were counted in this study since the damage caused by cisplatin is most evident in this area of the
cochlea. OHCs with intact stereocilia were considered as normal, whereas cells corresponding to grades 1 and 2 (i.e. grade 1: OHC with 10% to 50% damage or loss of stereocilia; grade 2: OHC with less than 50% of stereocilia remaining) according to Waissbluth et al. were considered as the abnormal stereocilia group in this study. Also, grade 3 was considered as total absence of stereocilia and rupture of the cuticular plate. The results in each group were presented as percentages and represent an approximation of the extent of damage caused to the outer hair cells, which is not necessarily conclusive of hair cell death.

3.4.7. Determination of antioxidant enzyme activity

The bony shell of the cochlea was removed and the cochlear tissues (wet weight ranging from 5 to 15 mg) of the euthanized animals were extracted and homogenized in 100 µL of 50 mM phosphate buffer (pH 7) containing 1mM EDTA. Samples were centrifuged at 10000 rpm at 4 °C for 15 minutes and the supernatants were removed and kept at – 80 °C. Superoxide dismutase (SOD) assay was performed using the SOD assay kit-WST (Dojindo, USA). SOD activity is calculated based on the formation of formazan dye from tetrazolium salt upon reduction with a superoxide anion. The rate of the reduction with O$_2^-$ is linearly related to the xanthine oxidase activity which is inhibited by SOD. The catalase assay was conducted using the catalase assay kit from Cayman chemical (USA). This assay is based on the reaction of catalase with methanol in the presence of an optimal concentration of H$_2$O$_2$. The formaldehyde production was measured by spectrophotometer according to the protocol provided by the manufacturer.

3.4.8. Statistical analysis

Statistical analysis for the ABR experiments was performed by evaluating of the differences between all groups with the use of the two-way analysis of variance (ANOVA). The Tukey-Kramer multiple comparisons test was subsequently performed for further analysis between two groups. The analysis for the cell viability assay was performed with a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. The Kruskal-Wallis and Dunn's multiple comparison tests were conducted to compare the antioxidant enzyme activities and the SEM cell counts between experimental groups. Data is reported
as mean and standard error of the mean unless otherwise stated. Statistical significance was set at a $P$-value $\leq 0.05$.

3.5. Results

3.5.1. NCUR combined with dexamethasone improves the viability of auditory cells in vitro (MTS assay)

In our search for a product that could reduce cisplatin ototoxicity, we surmised that the effects of this drug may result from direct damage to auditory cells in the inner ear, and could be prevented by agents that attenuate the related oxidative damage, such as dexamethasone and nanocurcumin. Firstly, the cells exposed to varying concentrations of dexamethasone (50 to 900 nM) did not exhibit any significant toxicity ($p<0.5$), further sustaining the dose chosen for the following experiment (200 nM).

We tested the effects of the respective agents on the metabolic activity and survival of HEI-OC1 cells and a statistically significant difference between the means for all groups evaluated ($p<0.001$) was observed. NCUR significantly reduced the viability of HEI-OC1 cells to 92% and 84%, at high concentrations of 60 and 80 µg/mL respectively, at 48 hrs exposure ($p<0.01$ and $p<0.001$ respectively). NCUR at concentrations of 5, 10, 20, and 40 µg/mL showed 11%, 100%, 111%, and 96% viable cells as compared to controls respectively ($P>0.05$). Cisplatin exposure (70µM) caused a 53% decrease in cell viability as compared to controls which was statistically significant ($p<0.001$). Interestingly, addition of NCUR at non-toxic concentrations of 5, 10, 20, and 40 µg/mL did not significantly protect HEI-OC1 cells against cisplatin cytotoxicity (54%, 60%, 63%, 59% viable cells respectively, $P>0.05$).

An effect was observed with the NCUR/dexamethasone combination, in which case the cells exhibited 57%, 58%, 60%, 63% and 57% viability at NCUR concentrations of 5, 10, 20, 40, and 60 µg/mL respectively as compared to controls (Fig. 1). Pre-treatments of the cells with dexamethasone alone or in combination with the void polymer (0.8 mg/mL) had no protective effect against cisplatin cytotoxicity (data not shown).
Fig. 1. Cell viability (MTS) assay of the HEI-OC1 cells pre-treated with NCUR and NCUR/dexamethasone before cisplatin exposure. Cells receiving cisplatin/NCUR/dexamethasone exhibited greater survival as compared to the group receiving cisplatin only (p<0.001). Significant differences were observed for NCUR concentrations <80 µg/mL (p<0.05). No significant differences were observed between the cells receiving cisplatin/NCUR and cells receiving cisplatin only (p>0.05). (Results are given as means and standard error of the mean).

3.5.2. NCUR combined with dexamethasone protects hearing function in vivo (ABR)

In order to test whether the aforementioned changes in the viability of auditory cells translate into hearing gains in vivo, we employed a guinea pig model and the well-established ABR screening method. The results of the ABR test are presented in Table 1. These results indicated partial protection by NCUR and dexamethasone against cisplatin-induced hearing loss. The cisplatin treated group showed average threshold elevations which corresponds to mild-moderately severe hearing loss in humans. The average threshold shifts, below 10 dB, were observed in groups 2 and 3 (dexamethasone or NCUR alone). Groups 4 (cisplatin/polymer), 5 (cisplatin/dexamethasone) and 6 (cisplatin/NCUR), exhibited similar threshold elevations as group 1 (cisplatin only). However, in cisplatin/NCUR/dexamethasone group, a statistically significant reduction in ABR threshold elevations were observed as compared to the cisplatin and the
cisplatin/dexamethasone groups (p<0.01). These results suggest that neither modulator is sufficient to engender an effective protection, while their combination seems to provide a protective effect. This protection was observed across all frequencies tested. However, the greatest protection was observed at 25 kHz where the mean ABR threshold shift was less than 20 dB.

Table 1. Mean ABR threshold shifts in decibels for all groups. Statistically significant differences were observed across all frequencies in group 7 (cisplatin/NCUR/dexamethasone) as compared to group 1 (cisplatin) (p<0.01). ABR results are given as mean ± standard deviation. The calibrated smart-EP device has an upper limit when testing 20 and 25 kHz. We use the sign "≥" when the threshold shifts are above the limit, therefore the actual threshold elevations might be greater than what is reported in this table. Cis: cisplatin, Dex: dexamethasone.

3.5.3. NCUR and dexamethasone pre-treatment preserve structural integrity of the inner ear (LM and SEM evaluation)

To link the functional and structural changes observed upon exposure to cisplatin, NCUR and dexamethasone, the ears of treated animals were analyzed using LM and SEM.
Fig. 2. LM of the basal turn of the cochleae showed preserved morphology of the SV, the organ of Corti, and the SGCs in control (non-treated) animals. Intact marginal cells of the SV (a), inner hair cells (b), OHCs (c), tunnel of Corti (d) and supporting cells (e) are shown in
control group. Gross morphological changes of the SV including extensive vacuolization of
the intermediate layer (f) and destruction of the marginal cells (g), total destruction of the
organ of Corti (h), loss of all OHCs, destruction of tunnel of Corti, and degenerated SGCs (i)
were observed in group 1 (cisplatin). Lower level of vacuolization of the intermediate layer
and less destruction of the marginal cells of the SV (j, k), less structural damage of organ of
Corti (l,m) and less SGCs degeneration (n,o) were observed in group 5 (cisplatin/dexamethasone) and group 6 (cisplatin/NCUR) as compared to group 1 (cisplatin). A decreased level of the damage to the SV (p), the organ of Corti (q), and the
SGCs (r) was observed in group 7 (cisplatin/NCUR/dexamethasone) as compared to groups
1 (cisplatin) and 6 (cisplatin/NCUR). Detailed qualitative assessment of the SV, SGCs and
OHCs was completed at 400x. Bar equals 30 microns. Cis: cisplatin, Dex: dexamethasone.

LM of the cochleae obtained from the control (non-treated) animals showed
preserved morphology of the organ of Corti, the stria vascularis (SV), the spiral ligament,
and the spiral ganglion cells (SGCs). Gross morphological changes were observed in the SV
of groups 1 (cisplatin) and 4 (cisplatin/polymer). These included bulging of the marginal
cells into the scala media and the presence of vacuolization. The groups receiving
dexamethasone or NCUR only (groups 2 and 3) showed a preserved SV. Also, the group
receiving cisplatin/dexamethasone (group 5) exhibited SV morphology which was
comparable to the control group. The cochleae obtained from the animals in groups 6
(cisplatin/NCUR) and 7 (cisplatin/NCUR/dexamethasone) showed an improved
morphology of the SV as compared to cisplatin treated animals (Fig. 2).

OHCs are known to be greatly affected by cisplatin and were analyzed in more
detail. The greatest amount of absent OHCs and visible changes to the architecture of the
organ of Corti were observed in cisplatin treated animals. Less severe damage was
observed in groups 5 (cisplatin/dexamethasone) and 6 (cisplatin/NCUR), while group 7
(cisplatin/NCUR/dexamethasone) demonstrated a decreased level of OHC damage. The
apical turns showed more preserved OHC morphology as compared to basal turns in all
cisplatin treated animals.

The severity of the injury was greater at the bases of all of the cisplatin treated
guinea pigs. Group 7 (cisplatin/NCUR/dexamethasone) showed the presence of the organ
of Corti with variable degrees of distortion but with lower degree of injury as compared to groups 5 (cisplatin/dexamethasone) and 6 (cisplatin/NCUR) (Fig. 2). As for the spiral ganglia, there was degeneration of SGCs as evidenced by cell loss, in groups 1 (cisplatin) and 4 (cisplatin/polymer). The SGCs in the remaining five groups exhibited similar morphology (Fig. 2).

Fig. 3. SEM of the basal turn of the organ of Corti. (A) Non-treated control showing three intact rows of OHCs. (B) Severe damage of all rows of OHCs in cisplatin treated animals. Severe damage to the OHCs was observed in cisplatin/NCUR (C) and cisplatin/dexamethasone (D) treated animals which was comparable to cisplatin only treated animals. (E) SEM of the animals pre-treated with dexamethasone and NCUR showing less damage to the OHCs as compared to the cisplatin treated guinea pigs. Bar equals 100 microns.

To assess the cellular damage under higher resolution, SEM analysis was completed for groups 1 (cisplatin), 6 (cisplatin/NCUR), 7 (cisplatin/NCUR/dexamethasone), 5
(cisplatin/dexamethasone) and for non-treated animals (controls). The SEM findings in group 1 (cisplatin) demonstrated greater damage to the OHCs of the basal turn of the cochlea as compared to groups 5 (cisplatin/dexamethasone), 6 (cisplatin/NCUR) and 7 (cisplatin/NCUR/dexamethasone), in which most of the cells exhibited deformed stereocilia or completely ruptured cuticular plates (Fig. 3).

![Graph](image)

**Fig. 4.** SEM: OHC counts at the basal turn. This figure represents the percentages of the counted cell per group to the controls (control group= 100% normal stereocilia). Significant differences were observed in all three OHC grades. A decreased quantity of absent cells were seen in group 6 (cisplatin/NCUR) as compared to group 1 (cisplatin) (p<0.05). A greater percentage of normal cells and a lower percentage of cells with abnormal stereocilia or absent cells were observed in the group treated with cisplatin/NCUR/dexamethasone (group 7) as compared to the cisplatin treated animals. Total number of 63±4 cells per visual field was counted at a magnification of x1000. (*): p<0.01, (+): Presence of drug, (-): Absence of drug, Cis: cisplatin, Dex: dexamethasone.

Statistically significant differences were observed for all the grades evaluated in all groups (p<0.001). Samples obtained from the control group (non-treated) showed 100% of
OHCs graded as normal. For the cochleae obtained from group 1 (cisplatin), we observed 6.8% of the cells with normal stereocilia and a greater proportion of cells with abnormal stereocilia (62.5%) while 30% of the cells had complete destruction of their cuticular plates. The cisplatin treatment caused a significant change in the OHC counts, as compared to the non-treated group (p<0.001 for all grades). In group 5 (cisplatin/dexamethasone), 55.6% abnormal stereocilia, 19.5% absent cells, and 24.9% showed normal stereocilia. In the samples from group 6 (cisplatin/NCUR), a significant change was observed in the quantity of absent OHCs (14.6%) as compared to group 1 (cisplatin) (p<0.05). In this group OHCs showed 59.5% and 25.5% abnormal and normal stereocilia, respectively. Interestingly, the combination treatment group (group 7) did provide significant protection against cisplatin as evidenced by the changes in the OHC counts with 40.3% of the cells considered as normal, 49% presenting abnormal stereocilia and 11.4% of the cells being absent. These changes were statistically significant for all of the grades (p<0.01) (Fig. 4).

3.5.4. Changes in antioxidant enzyme activities in tissues exposed to cisplatin, NCUR and dexamethasone

There are reasons to believe that the cytotoxic damage of auditory cells exposed to cisplatin may occur due to oxidative damage, and could be prevented by re-balancing the activity of oxidative enzymes involved. As shown in Fig. 5, a cisplatin administration of 13 mg/kg (given in 7 days) resulted in an increased activity of the SOD and catalase enzymes in the cochlear tissue lysates. A significant difference in the means was observed between the control group, the cisplatin treated group, the cisplatin/dexamethasone group, the cisplatin/NCUR group and the cisplatin/NCUR/dexamethasone group for SOD and catalase enzyme activities (p<0.01). Cochlear SOD activity of the cisplatin and the cisplatin/dexamethasone group reached 48% and 45% as compared to non-treated controls (42%), whereas the cisplatin/NCUR and the cisplatin/NCUR/dexamethasone group showed a decreased SOD activity, to 44% and 30% respectively. The cisplatin/NCUR/dexamethasone group exhibited a significant decrease in the activity of SOD as compared to the cisplatin treated group (p<0.05). Catalase activity demonstrated the same trend. For example, cochlear catalase activity in groups 1 (cisplatin) and 5 (cisplatin/dexamethasone) increased to 6.08 and 6.41 µM/min/mg tissue respectively.
Groups 6 (cisplatin/NCUR) and 7 (cisplatin/NCUR/dexamethasone) showed decreased activity of the enzyme with 4.89 and 3.86, respectively, whereas control was at 5.21 μM/min/mg tissue. Indeed, the cisplatin/NCUR/dexamethasone showed a statistically significant decrease in the enzyme activities as compared to the cisplatin treated group for both assays (p<0.05).

Fig. 5. Antioxidant enzyme activity: (A) Catalase and (B) SOD activity. Significant differences were observed in the activity of both enzymes (p<0.05). Statistically significant decreases were detected for group 7 (cisplatin/NCUR/dexamethasone) as compared to the cisplatin treated group (p<0.05). (n=5 guinea pigs per group, results are given as means± standard error of the mean; Cis: cisplatin, Dex: dexamethasone).
3.6. Discussion

During the last decade, there have been many attempts, using *in vivo* and *in vitro* models, to counter the ototoxic side effects of cisplatin chemotherapy. Some studies have aimed at mopping up the generated reactive oxygen species by the use of antioxidants or countering the intense inflammatory response using steroids. These efforts have not led to the development of a robust otoprotective strategy, and yielded negative or conflicting results.\(^{10-13,15}\) The multi-therapeutic properties of curcumin, including its antioxidant, anti-inflammatory and antineoplastic activities, make it a good candidate in this regard. It is reasonable to propose that bioavailable preparations of this agent could attenuate some of the chemotherapy side effects in the ear, especially those related to oxidative stress. The results of the present study demonstrate that NCUR has potential in reducing the deleterious effects of cisplatin on the sensory cells of the cochlea and in hearing preservation.

The results of the cell viability assay suggest that the toxic effects of cisplatin may include intrinsic damage to auditory cells rather than being solely dependent on the inflammatory microenvironment, and so are the counter-effects of NCUR/dexamethasone. Thus, we observed that a non-cancerous auditory cell line (HEI-OC1 cells) receiving exposure to NCUR and dexamethasone in combination with cisplatin had an improved metabolic activity, as compared to cells treated with cisplatin alone, or pre-treated with either NCUR or dexamethasone. It was also reported in our previous study that NCUR (60 \(\mu\)g/mL) lowered the survival rate of two tested cancer cell lines.\(^{17}\) Considering that an ideal antineoplastic agent acts against cancer cells with minimal side effects on normal cells, NCUR appears to be a promising candidate to be used in combination with other chemotherapeutic agents. The potential for a synergistic effect of NCUR with antineoplastic agents (including cisplatin) against cancer cells, the sensitization of resistant cancer cells to chemotherapy along with its antioxidant and anti-inflammatory properties merit further assessment.\(^{26,27}\)

The cell viability assay results in this study are consistent with the ABR results, which demonstrate that a combination therapy of NCUR and dexamethasone significantly improved the hearing thresholds at all of the tested frequencies when animals were given cisplatin. It is well known that systemic cisplatin administration causes greater damage at
the basal turn of the cochlea, with a major impact on high frequency hearing thresholds.\textsuperscript{28} As shown in Table 1, reduced hearing threshold shifts at the 20 and 25 kHz frequencies were observed as compared to the lower frequencies, which is not in keeping with previous reports. The lower level of threshold shifts at 20 and 25 kHz is due to limitation of the smart EP device in recording the hearing thresholds above 85 dB for these frequencies.

The LM and SEM findings also demonstrated that OHCs are more preserved in the group receiving the combination therapy (cisplatin/NCUR/dexamethasone) as compared to the other groups. A greater number of intact OHCs were observed in this group. This may be related to the combined antioxidant properties of NCUR and the effects of dexamethasone, since none of these two drugs had a protective effect against cisplatin ototoxicity when administered separately. The SGCs appeared to have a somewhat similar morphology in all of the groups except for groups 1 (cisplatin) and 4 (cisplatin/polymer) which showed evidence of cellular degeneration. At the same time, it is thought provoking that in our experiments \textit{in vitro} dexamethasone added a level of cytoprotection to NCUR effects in the context of cisplatin toxicity against auditory cells. This suggests that there could be a direct manner, unrelated to interaction with inflammatory cells, in which corticosteroids may act on auditory cells, through gene expression changes or other mechanisms which still need to be identified. Still, the magnitude of the cytoprotective effects of NCUR/dexamethasone was far greater \textit{in vivo} than \textit{in vitro}, which suggests that indirect influences, such as inflammation, play a role.

Previous studies investigating the effect of cisplatin on oxidative stress reported a decreased activity of antioxidant enzymes in the cochlea, kidney, and liver;\textsuperscript{29-31} however, there are also reports of increased activity of antioxidant enzymes such as SOD and catalase in the inner ear following cisplatin administration.\textsuperscript{32,33} Ravi et al. indicated that the increased activity of SOD might be due to the inhibition of glutathione peroxidase, or an increased generation of reactive oxygen species in the cochlea of the cisplatin treated animals.\textsuperscript{32} Zhang et al. stated that an increased activity of SOD and catalase caused by cisplatin is a non-specific response to oxidative stress, which includes depletion of glutathione and lipid peroxidation. This study also demonstrated that higher doses of cisplatin cause greater SOD and catalase activities \textit{in vitro}\.\textsuperscript{33} Our results are in keeping with, and extend these findings.
In our study, the activity of SOD and catalase increased in the cochlear tissue of the cisplatin treated animals (p>0.05). It was also observed that coadministration of cisplatin and dexamethasone did not cause any significant changes in the activity of these antioxidant enzymes as compared to the animals receiving cisplatin only. Interestingly, the animals receiving cisplatin/NCUR/dexamethasone showed lower SOD and catalase activities as compared to non-treated controls and animals receiving cisplatin only. The decreased activity of these antioxidant enzymes in the cochlea can be compensatory to the oxidative stress following cisplatin administration. Since increased activity of both antioxidant enzymes could be related to a non-specific response to oxidative stress, we expect to observe lower level of enzyme activity in extended duration of the cisplatin treatment. Comparison of the antioxidant enzyme activity of the cisplatin treated animals with controls at different time points might create different results; therefore, we compared our experimental groups with cisplatin only treated animals (not the controls) to eliminate any possible effect of cisplatin dose or duration of the treatment on antioxidant enzyme activity.

In conjunction with previous reports, our present study offers a tantalizing possibility that curcumin derivatives, such as NCUR, in conjunction with dexamethasone and other treatments may exert antithetical and advantageous effects in the context of cancer treatment. We suggest that these agents could simultaneously contribute to the antitumor activity while protecting certain normal tissues, such as cochlear epithelium. By extension, similar protective effects could be explored in the case of nephrotoxicity, neurotoxicity and other side effects of cisplatin.

The *in vitro* antineoplastic effect of the NCUR formulation used in this study has been previously reported for various cancer cell lines;\textsuperscript{17,34,35} however, our present project did not directly evaluate the antineoplastic effects of NCUR with concomitant use of cisplatin, perhaps allowing a choice of lower less toxic doses. Further experiments are needed to investigate the anti-tumor activity of NCUR *in vivo*, and the exploration of the related opportunities in the context chemotherapy. Nonetheless, while the exact mechanism for otoprotection and the interactions between NCUR and dexamethasone are currently unknown, our results clearly indicate that combining NCUR with dexamethasone markedly reduced the degree of hearing loss following cisplatin administration.
3.7. Conclusion

NCUR appears to be a promising candidate in the prevention of cisplatin-induced ototoxicity. Due to its antioxidant, anti-inflammatory, and antineoplastic properties, NCUR would potentially allow for a lower dose of cisplatin, thereby reducing the risk of hearing loss. This is the first study to demonstrate the otoprotective effects of NCUR and dexamethasone against platinum-induced ototoxicity. Further research is needed in order to elucidate the effects of this otoprotective strategy in the presence of tumor cells as well as the exact mechanism of otoprotection.
3.8. References

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Curcumin is a phytochemical compound that has been used as a traditional remedy in treatment of various diseases including cancer. The therapeutic effects of curcumin are due to antioxidant, anti-inflammatory and anti-neoplastic properties. Most of the evidence regarding biological properties of curcumin are preclinical due to rapid intestinal and hepatic metabolism/elimination, and chemical instability in alkaline pH.

During the last decades, scientists have tried various drug delivery strategies curcumin to reach the target organs of interest. This study used curcumin loaded NIPAAM/VP/PEG nanoparticles (NCUR), which were initially presented by Bisht et al. The selected curcumin nanoparticle has the advantage of a small size (mean: 110nm) as compared to the other curcumin nanoparticle formulations. The small size of the nanoparticle possibly increases the chance of the penetration of the nanoparticle to the targeted cells.

In this study, we aimed to facilitate and develop the synthesis method of the curcumin loaded nanoparticles in a larger scale for in vivo studies and also to investigate the unknown physico-chemical characteristics of this drug model.

The effect of the formulation variables on the polymerization process, including the PEG-A washing and the NIPAAM recrystallization, was determined by Fourier Transform Infrared spectroscopy. PEG-A washing and NIPAAM recrystallization steps were initially suggested by Bisht to remove any possible inhibitors during the polymer synthesis process. The results of the FTIR study showed that using washed PEG-A and crystallized NIPAAM does not change the polymer synthesis process as compared to the polymer synthesized by using the amorphous products. These results suggested eliminating of PEG-A washing and NIPAAM recrystallization from the polymer synthesis method.
This study also investigated the release kinetics of the drug by exposing the curcumin nanoparticles to various pHs and temperatures. The temperature drug release test was designed based on the idea of thermosensitivity of the NIPAAM polymers which cause remodelling of the polymer structure. The results of the temperature experiments showed that the highest level of drug release occurred at temperatures above 37 °C. These findings suggest a possible greater release of curcumin in the locally heated body organs as a targeted drug delivery strategy. Transition of the NIPAAM polymers from water soluble to insoluble, when the temperature is exceeding 37 °C, can be another factor that leads to a greater concentration of nanoparticles inside the heated target organs.

The results of the pH tests do not show any specific trend of the drug release. These findings support the theory of internal entrapment of the drug versus attaching to the external surface of the polymer spheres.

This study also compared the particle size of the freeze dried nanoparticles versus the nanoparticles dispersed in the aqueous media. Since some laboratory techniques including scanning electron microscopy and atomic force microscopy use freeze dried samples in the process of sample preparation, we hypothesized that these read outs might not give us a real estimation of the particle size when it is dispersed in body fluids. Nanoparticle tracking analysis was used to investigate the hydrodynamic size of the curcumin loaded NIPAAM/VP/PEG nanoparticles in order to be compared to AFM measurements. NTA results showed an average of 66 nm increase in hydrodynamic particle size as compared to freeze dried samples. These results suggest NTA as a proper technique for measuring the size of the thermosensitive nanoparticles in future experiments.

Zeta potential measurements were performed in order to determine the stability of the nanoparticles in aqueous media. Zeta potential refers to the potential difference between the stationary layer of fluid attached to the dispersed particle and the dispersion medium. The significance of zeta potential measurements is related to the degree of repulsion versus attraction between adjacent nanoparticles. When the zeta potential is close to zero, the power of attraction exceeds repulsion which leads to the aggregation of the nanoparticles.

A positive or negative charge of 25 mV is considered as an arbitrary value that separates high charged surfaces from low charged surfaces, therefore the zeta potential
ranging from 0 to ±5 represents rapid coagulation and the values of ±10 to ±30, ±30 to ±40, ±40 to ±60 and over ±61 are representative of the incipient, moderate, good and excellent stability respectively.\textsuperscript{10}

The results of the zeta potential of the non-loaded polymer showed incipient stability, whereas the mean value for loaded polymer showed low stability of the nanoparticles. We concluded that a low positive charge of 3 ± 13mV for NCUR nanoparticles is due to the calculation of the average point between positive and negative values in 6 consecutive runs. We suggested that the positive values referred to NCUR nanoparticles and non-loaded polymers, whereas the negative values represented the free curcumin dispersed in nanocurcumin solution. It is suggested that removing the negative values from zeta potential read outs can give us a better estimation of the real surface electric charges of the nanoparticles.

NCUR nanoparticles have been shown to have antineoplastic effects by decreasing the survival rate of the human prostate cancer cells (PC3) and epidermoid carcinoma cells (A431). A431 cells appeared to be more sensitive to the drug as compared to the PC3 cell line. Since the non-loaded polymer was not toxic to these cell lines, it is concluded that the chemopreventive effect of the NCUR nanoparticles is related to loaded curcumin into the polymer spheres. Further experiments are needed to investigate the effect of NCUR nanoparticles on tumor growth \textit{in vivo}. Bisht \textit{et al} demonstrated that curcumin loaded nanoparticles accumulate more in the pancreas and that it is recommended for the treatment of the pancreatic cancer cell lines.\textsuperscript{11}

Various formulations of curcumin nanoparticles have been shown to reduce the survival rate of many cancer cell lines; hence, there is no screening experiment to compare the sensitivity of the cancer cell lines from all body organs using the same concentration of a specific nanoparticle formulation. Regardless of the variety of the sensitivity of the cellular subtypes of a specific cell line to the antineoplastic drugs, NCUR nanoparticles possibly would play an important role if it could increase the sensitivity of the chemoresistant cell lines. Increasing the sensitivity of the chemoresistant ovarian cancer cells following the exposure of the cells to curcumin nanoparticles was shown by Yallapu \textit{et al}.\textsuperscript{12} Pretreatment of the other chemoresistant cell lines with curcumin nanoparticles prior to the exposure of these cells to the drug of choice might result in a more effective
chemotherapy. It is also necessary to investigate the biosafety, biodegradation and clearance of the polymer from the tissue in different body organs.

4.2. Attenuation of cisplatin ototoxicity by otoprotective effects of nanoencapsulated curcumin and dexamethasone in a guinea pig model

This study was designed to investigate the otoprotective effect of NCUR against the degenerative damages of cisplatin to the auditory sensory organ in a guinea pig model. We hypothesized that NCUR's antioxidant and anti-inflammatory properties make it a unique candidate for the prevention of oxidative and inflammatory damages of cisplatin to the cochlea. This research project demonstrated that a combination of NCUR and dexamethasone attenuated cisplatin-induced ototoxicity in a guinea pig model. This work mostly focuses on functional and morphological changes of the cochlea using ABR, antioxidant enzyme assays, LM and SEM.

As a first step, we attempted to optimize the dose of NCUR to prevent cisplatin ototoxicity in a guinea pig model. The results of our pilot studies testing various concentrations of NCUR ranging from 1 to 25 mg/kg showed no significant changes in hearing thresholds in the cisplatin/NCUR group versus cisplatin-only treated guinea pigs. Interestingly, we observed redness of the feet in cisplatin/NCUR and cisplatin treated animals which seemed to be an inflammatory response to the cisplatin treatment; therefore, dexamethasone was added to the treatment to reduce the inflammatory response.

For the ABR measurements, the frequencies of 8, 16, 20 and 25 kHz were tested and these results were presented as hearing threshold shifts. The ABR experiment investigated the effects of the drugs on hearing threshold at each individual frequency and the tested frequencies were not considered as covariates. Since the calibration of the smart EP device does not allow us to measure the hearing thresholds over 100 dB for high frequencies (20, 25 kHz), the threshold shift measurements at these frequencies cannot be as accurate as 8 and 16 kHz tone bursts. The limitation of the smart EP device in detecting the thresholds over 100 dB for high frequencies explains the lower level of high frequency threshold shifts compared to 8 and 16 kHz. The result of the ABR test showed significant differences of
threshold shifts between cisplatin and cisplatin/NCUR/dexamethasone groups which supported our study hypothesis.

The morphological changes of the cochlea observed by LM and SEM micrographs also correlated with ABR findings by showing the structural preservation of the stria vascularis, organ of Corti and auditory hair cells in the cisplatin/NCUR/dexamethasone group as compared to cisplatin treated animals.

It was also observed that cisplatin elevated the catalase and superoxide dismutase activity in the cisplatin group. It is believed that cisplatin can decrease the activity of the antioxidant enzymes in the cochlea. We believe this elevated activity can be related to an initial host self-defence response to the cisplatin-induced oxidative stress. It is expected that an acute high dose cisplatin treatment causes a drop in the antioxidant enzyme activity when compared to long term low dose treatments. The lower level of antioxidant enzyme activity in the cisplatin/NCUR/dexamethasone group as compared to the cisplatin group seems to be related to the lower level of oxidative stress.

Attenuation of cisplatin ototoxicity by various reported synthetic or phytochemical compounds like thiamine pyrophosphate, D-methionine, N-acetylcysteine, KR-22335, micronized flavonoid fraction, Ginkgo biloba, and resveratrol have been shown in several studies. Although, a proper comparison between these agents and NCUR requires the same experimental set up, we believe that along with antioxidant properties, NCURs' antineoplastic effect makes it a unique compound when compared to the other protective agents evaluated.13-19

Although over 2900 research articles have been published with respect to the chemical and biological properties of curcumin, further experimental work is needed to investigate the detailed mechanisms of action of curcumin loaded nanoparticles as an anti-inflamatory, antioxidant and antineoplastic agent. The antioxidant and anti-inflammatory activity of curcumin nanoparticles can be compared to other known antioxidant and anti-inflammatory agents such as vitamin E and dexamethasone.

Comparison of the biological properties of different formulations of curcumin nanoparticles would be very useful in choosing the right formulation for any specific in vivo or clinical experiment. Comparison of the level of the drug distribution in body organs can help determine the most effective drug delivery strategy. Although it has been previously
described that a specific formulation of curcumin nanoparticles distribute more in the pancreas, further investigation is needed to verify the biodistribution of the other nanoparticle formulations.

Finally, comparison of the safety and biodegradation of the different curcumin nanoparticle formulations in different animal models would play an essential role in choosing the right formulation for future clinical experiments.

4.3. Conclusions

Taken together, the results of this study suggest that systemic treatment of curcumin loaded NIPAAM/VP/PEG nanoparticles and dexamethasone reduce the degenerative changes of the cochlea in a cisplatin treated guinea pig model.
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