Effect of Genipin on Cisplatin-Induced Nephrotoxicity

Eglal Orner Mahgoub

Follow this and additional works at: https://scholarworks.uaeu.ac.ae/all_theses
Part of the Medical Pharmacology Commons, and the Medical Toxicology Commons

Recommended Citation
https://scholarworks.uaeu.ac.ae/all_theses/475
EFFECT OF GENIPIN ON CISPLATIN – INDUCED NEPHROTOXICITY

Eglal Omer Mahgoub

This thesis is submitted in partial fulfillment of the requirements for the degree of Master of Medical Sciences (Pharmacology and Toxicology)

Under the Supervision of Dr. Rajesh Mohanraj

November 2016
Declaration of Original Work

I, Eglal Omer Mahgoub, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled “Effect of Genipin on Cisplatin-Induced Nephrotoxicity”, hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Dr. Rajesh Mohanraj, in the College of Medicine and Health Sciences at UAEU. This work has not previously been presented or published, or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

Student’s Signature:   Eglal Omer   Date: 3/1/2017
Advisory Committee

1) Advisor: Dr. Rajesh Mohanraj
Title: Assistant Professor
Department of Pharmacology
College of Medicine and Health Sciences, UAE University

2) Co-advisor: Dr. Shreesh Ojha
Title: Assistant Professor
Department of Pharmacology
College of Medicine and Health Sciences, UAE University

3) Member: Prof. Samir Attoub
Title: Professor
Department of Pharmacology
College of Medicine and Health Sciences, UAE University

4) Member: Dr. Fakhreya Yousuf
Title: Assistant Professor
Department of Pharmacology
College of Medicine and Health Sciences, UAE University
Approval of the Master Thesis

This Master Thesis is approved by the following Examining Committee Members:

1) Advisor (Committee Chair): Dr. Rajesh Mohanraj
   Title: Assistant professor
   Department of Pharmacology and Therapeutics
   College of Medicine and Health Sciences
   Signature ___________________________ Date 24/11/2016

2) Member: Professor Ernest Adeghate
   Title: Professor and Chair
   Department of Anatomy
   College of Medicine and Health Sciences
   Signature ___________________________ Date 24/11/2016

3) External Examiner: Dr. Gautam Sethi
   Title: Associate Professor
   Department of Pharmacology
   Institution/University: National University of Singapore, Singapore
   Signature ___________________________ Date 24/11/2016
This Master Thesis is accepted by:

Vice Dean of the College of Medicine and Health Sciences: Dr. Suliman Al Hammadi

Signature ___________________________ Date 8.1.2017

Dean of the College of the Graduate Studies: Professor Nagi T. Wakim

Signature ___________________________ Date 8.1.2017

Copy 5 of 6
Abstract

Cisplatin (CSP) is a potent and widely used chemotherapeutic agent. However, clinical efficacy of CSP is compromised due to the elicitation of nephrotoxicity and ototoxicity. In this study, I have investigated the nephroprotective effects of a phytochemical – genipin (GP) isolated from gardenia flower (Gardenia jasminoides), on a murine model of cisplatin-induced nephropathy. CSP-induced renal tissue injury was characterized by elevated levels of serum blood urea nitrogen (BUN), creatinine (Cr) and cystatin-C. In addition, levels of kidney injury molecule-1 (KIM-1) were increased in the renal tissues of CSP administered animals. CSP also induced renal oxidative stress, evidenced by increased NADPH oxidase, and diminished superoxide dismutase (SOD) activities. Furthermore, elevated levels of renal 4-hydroxynonenal (4-HNE) and 3-nitrotyrosine (3-NY) and diminished glutathione (GSH) levels signified profound oxidative stress in CSP administered animals. Renal inflammation was exaggerated in CSP treated animals, which was revealed by elevated levels of proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-1beta (IL1-β). Increased activation of mitogen activated protein kinases (MAPKs) was observed in CSP administered animals. Finally, CSP also induced apoptosis in the renal tissues, revealed by increases in caspase-3/7 and poly (ADP-ribose) polymerase [PARP] activities, DNA fragmentation, and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining in the kidney sections. Interestingly, GP treatment markedly attenuated the cisplatin-induced oxidative/nitrative stress, inflammation, and cell death in the kidney, and improved renal function. Thus, results from this present suggest that GP may represent a promising new protective strategy against cisplatin-induced nephrotoxicity.

Keywords: Cisplatin, genipin, Gardenia jasminoides, nephrotoxicity, oxidative stress, inflammation, apoptosis, phytochemicals.
تأثير النبات الطبي، مسحوق الغاردينيا (Genipin) على تسمم الكليتين الناتج عن السيسبلاتين (Cisplatin)

الملخص

السيسبلاتين (Cisplatin) هو علاج كيميائي قوي ويستخدم على نطاق واسع. ومع ذلك، تسمم الكليتين وتسمم الأذينين تعد من الأثار الجانبية الجسيمة عند استخدام هذا العلاج. في هذه الدراسة الحالية، فهنا باستكشاف الخصائص الوقائية عن مايسمى مسحوق الغاردينيا Gardenia (genipin) وهو مستخلص نباتي طبي مزوزل عن زهرة الغاردينيا (jasminoides) في نموذج الفئران الذي تعاني من اعتلال الكليتين بعد معالجتها بالسيسبلاتين.

وقد تميزت إصابة نسبة الكلى الناجم عن استخدام السيسبلاتين بارتفاع مستويات كل من النيتروجين والبوريا في الدم (BUN)، الكرياتينين (creatinine)، الكرياتينين (cystatin-C) في (KIM-1) و(KIM-1) بالإضافة إلى ذلك، تم زيادة مستويات جزيء إصابة الكلى -1 في (Oxidative stress) الإجهاد التأكسدي الكلوي (NADPH oxidase) من زيادة مؤكسد فسفات ثنائي نكلوتيديئ النائديين والأدينين المختزل (Superoxide dismutase (SOD).

وتقلص نشاط الأنزيم مضاد الأكسدة سوبر أكسيد ديميوتاز (Renal 4-hydroxynonenal (4-HNE) و(GSH) مما يدل على حدوث تقلص مستويات الجلوتاثيون (3-nitrotyrosine (3-NY) أكسدة عميقة في الحيوانات المعالجة بالسيسبلاتين. بالإضافة إلى ذلك فقد تم الكشف عن مستويات من الالتهاب الكليي الموجود في أنسجة الفئران عن طريق وجود مستويات مرتفعة.

علاوة على ذلك، لوحظ مستويات مرتفعة من (Renal 4-hydroxynonenal (4-HNE) و(GSH) مما يدل على حدوث أكسدة عميقة في الحيوانات المعالجة بالسيسبلاتين. بالإضافة إلى ذلك فقد تم الكشف عن مستويات من الالتهاب الكلوي الموجود في أنسجة الفئران عن طريق وجود مستويات مرتفعة.
من السيتوكينات المحفزة للالتهاب مثل عامل نخر الورم ألفا (TNF-α) والتريلوبين (1β).
وقد لوحظ زيادة تفعيل ونشاط MAPKs. وأخيراً موت الخلايا المبرمج، فعمل السيسلياتين poly و Caspases 3/7 و تقسيت الحمض النووي، وأيضاً عن طريق تلوين (ADP-ribose) polymerase (PARP) TUNEL staining.
وتصبغ خلايا الكلى المختلفة باستخدام

ومن المثير للاهتمام، أن العلاج باستخدام هذا المستخلص النباتي الطبي، مسحوق الغاردينيا (Genipin)، يؤدي بشكل ملحوظ إلى تقليل التأكسد الناتج عن السيسلياتين، والالتهابات، وموت الخلايا المبرمج في الكلى، وتحسين وظائف الكلى. وهكذا، نجد أن النتائج تشير إلى أن هذا المستخلص (Genipin) قد يمثل استراتيجية وقائية جيدة واعدة ضد الالتهاب الكلوي الناجم عن السيسلياتين.

مفاهيم البحث الرئيسية: سيسلياتين. مسحوق الغاردينيا. غاردينيا باسمينة. تسمم الكليتين، الأكسدة، الالتهاب، موت الخلايا المبرمج. النياتات الطبية.
Acknowledgements

First, I would like to express my gratitude to my supervisor, Dr. Rajesh Mohanraj for his patience, mentorship, and support.

I also appreciate the sincere advice and feedback from the faculty members from the department of pharmacology and therapeutics. I would also like to convey my sincere appreciation to all the members of my advisory committee, Prof. Samir Attoub, Dr. Shreesh Ojha and Dr. Fakhreya Y Jalal. Furthermore, I extend my special thanks to Prof. Salim Bastaki (Chair, Department of Pharmacology and Therapeutics) and Prof. Abdu Adem (Former Chair, Department of Pharmacology and Therapeutics) for accepting me in this program and for their support and encouragement.

I would also like to extend my gratitude to Dr. Maryam Al Shamsi (Assistant Dean for Scientific Research and Graduate Studies) for the encouragement and support.

Special thanks go to my parents, brother, sister and husband who helped and supported me along the way.
Dedication

I would like to dedicate my thesis to my parents, siblings, husband, and my son
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Title</td>
<td>i</td>
</tr>
<tr>
<td>Declaration of Original Work</td>
<td>ii</td>
</tr>
<tr>
<td>Copyright</td>
<td>iii</td>
</tr>
<tr>
<td>Advisory Committee</td>
<td>iv</td>
</tr>
<tr>
<td>Approval of the Master Thesis</td>
<td>v</td>
</tr>
<tr>
<td>Abstract</td>
<td>i</td>
</tr>
<tr>
<td>Title and Abstract (in Arabic)</td>
<td>viii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>x</td>
</tr>
<tr>
<td>Dedication</td>
<td>xi</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>xii</td>
</tr>
<tr>
<td>List of Figures</td>
<td>xv</td>
</tr>
<tr>
<td>Chapter 1 : Introduction</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Cisplatin (CP) Discovery and Clinical Applications</td>
<td>1</td>
</tr>
<tr>
<td>1.2 Disadvantages of CP Chemotherapy</td>
<td>3</td>
</tr>
<tr>
<td>1.2.1 Risk Factors for CP-Induced Nephrotoxicity</td>
<td>4</td>
</tr>
<tr>
<td>1.2.2 Biomarkers for Detecting CP-Induced Nephrotoxicity</td>
<td>4</td>
</tr>
<tr>
<td>1.3 Why Kidneys are Prime Target for CP Toxicity?</td>
<td>6</td>
</tr>
<tr>
<td>1.3.1 Transporter-mediated Mechanism for CP Accumulation in Kidneys</td>
<td>6</td>
</tr>
<tr>
<td>1.3.2 Metabolic Conversion of CP to Nephrotoxins and Accumulation in</td>
<td>6</td>
</tr>
<tr>
<td>Kidneys</td>
<td></td>
</tr>
<tr>
<td>1.4 Clinical Pharmacology of CP and its Mechanism of Anti - Cancer</td>
<td>7</td>
</tr>
<tr>
<td>Activity</td>
<td></td>
</tr>
<tr>
<td>1.5 Biochemical and Molecular Mechanisms Purported for CP-Induced</td>
<td>8</td>
</tr>
<tr>
<td>Nephrotoxicity</td>
<td></td>
</tr>
<tr>
<td>1.5.1 DNA Damage and Mitochondrial Dysfunction</td>
<td>8</td>
</tr>
<tr>
<td>1.5.2 Cell Transport System Alterations</td>
<td>10</td>
</tr>
<tr>
<td>1.5.3 Oxidative Stress</td>
<td>10</td>
</tr>
<tr>
<td>1.5.4 Inflammatory Response</td>
<td>11</td>
</tr>
<tr>
<td>1.5.5 Apoptosis Pathways Activation</td>
<td>13</td>
</tr>
</tbody>
</table>
1.6 Strategies for Ameliorating CP - Induced Nephrotoxicity .......................... 15

1.6.1 Phytochemicals as Cytoprotective Agents for Mitigating CP – Induced Nephrotoxicity.......................................................................................................................... 16

1.7 Aims and Objectives of the Study ................................................................. 17

Chapter 2 : Materials and Methods ................................................................... 19

2.1 Animals and Drug Treatment ..................................................................... 19

2.2 Determination of Renal Function ............................................................... 19

2.3 Determination of Serum Cystatin C ......................................................... 20

2.4 Determination of Kidney Injury Molecule -1 (KIM-1) in Renal Tissues..... 20

2.5 Histological Evaluation of CP-Induced Renal Injury ................................ 20

2.5.1 Periodic acid-Schiff Staining (PAS) of Paraffin Sections...................... 21

2.6 Determination of NADPH oxidase Activity in Renal Tissues ..................... 22

2.7 Determination of Superoxide dismutase (SOD) Activity in Renal Tissues .. 23

2.8 Determination of GSH content in Renal Tissues....................................... 23

2.9 Determination of 4-hydroxynonenal (4-HNE) in Renal Tissues ............... 24

2.10 Determination of 3-nitrotyrosine (3-NY) in the Renal Tissues................. 25

2.11 Determination of Pro-Inflammatory Cytokines in Renal Tissues ............. 26

2.12 Determination of MAPK Activation ......................................................... 26

2.13 Determination of Caspase 3 Activity ....................................................... 26

2.14 Determination of Poly (ADP-ribose) polymerase (PARP) Activity......... 27

2.15 Determination of DNA Fragmentation .................................................... 27

2.16 Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) Staining in Paraffin Embedded Renal Sections ......................................................... 28

2.17 Statistical Analysis of the Data................................................................. 29

Chapter 3 : Results .......................................................................................... 30

3.1 GP Treatment Attenuates CP-Induced Renal Tissue Injury ....................... 30
3.2 GP Treatment Attenuates CP-Induced Renal Tissue Injury ........................................ 32
3.3 Effect of CP and/or GP on Renal Histology ................................................................. 34
3.4 Effect of GP on CP-induced Oxidative Stress in Renal Tissues .................................. 36
3.5 Effect of GP on CP-induced Nitrative Stress in Renal Tissues .................................... 38
3.6 Effect of GP on CP-induced Renal Inflammation ......................................................... 39
3.7 Effect of GP on CP-induced MAPK Activation ............................................................ 39
3.8 Effect of GP on CP-Induced Apoptotic Cell Death ....................................................... 40
3.9 Effect of GP on CP-induced Apoptotic Cell Death – TUNEL staining ...................... 43
3.10 Effect of GP Treatment after the Established Renal Tissue Injury ......................... 44
3.11 Therapeutic effect of GP on Renal Histology ............................................................. 45

Chapter 4 : Discussion ........................................................................................................ 46
Chapter 5 : Conclusions and Future Directions ............................................................... 50
References .......................................................................................................................... 51
List of Publications ............................................................................................................. 60
List of Figures

Figure 1.1: Cisplatin structure ........................................................................................................ 1
Figure 1.2: Mechanism of CP action ............................................................................................ 8
Figure 1.3: CP-induced DNA damage and cell death ................................................................. 9
Figure 1.4: Structure of genipin and its natural source ............................................................... 17
Figure 2.1: GSH assay principle ................................................................................................ 24
Figure 2.2: Protocol for 3-NY determination ............................................................................. 25
Figure 2.3: DNA fragmentation assay principle ........................................................................ 28
Figure 3.1: Effect of GP on CP-induced Renal Tissue Injury (serum creatinine and BUN) ....... 31
Figure 3.2: Effect of GP on CP-induced Renal Tissue Injury on (Cystatin C and KIM-1) ........ 33
Figure 3.3: Effect of CP and/or GP on Renal Histology .............................................................. 35
Figure 3.4: Effect of GP on CP-induced Oxidative Stress in Renal Tissues ......................... 37
Figure 3.5: Effect of GP on CP-induced Nitrative Stress in Renal Tissues ....................... 38
Figure 3.6: Effect of GP on CP-induced Renal Inflammation .................................................. 39
Figure 3.7: Effect of GP on CP-induced MAPK Activation ...................................................... 40
Figure 3.8: Effect of GP on CP-induced Apoptotic Cell Death ............................................... 42
Figure 3.9: Shows the representative TUNEL staining images in the respective group of animals ........................................................................................................................................... 43
Figure 3.10: GP therapeutic effect against CP-induced renal tissue injury ....................... 44
Figure 3.11: The representative images of PAS stained paraffin embedded kidney sections from the respective group of animals ................................................................. 45
Chapter 1: Introduction

1.1 Cisplatin (CP) Discovery and Clinical Applications

Cisplatin (CP) is a chemotherapeutic agent. International union of pure and applied chemistry (IUPAC) nomenclature refers CP as cis-diamminedichloroplatinum II. Chemically, CP was the first molecule in the class of platinum-containing anti-cancer drugs synthesized. Subsequently, carboplatin and oxaliplatin have been developed. CP was first synthesized by Michel Peyrone in 1845 (Burchenal, Kalaher, Dew, & Lokys, 1979; Galluzzi et al., 2014). Then, American chemist Barnett Rosenberg characterized the anti-proliferative effects of cisplatin, first in *Escherichia coli*, and then in solid and hematopoietic tumor xenografts (Galluzzi, et al., 2014; Rosenberg, Vancamp, & Krigas, 1965). In fact, Rosenberg’s findings laid the foundation for unraveling the molecular mechanisms governing CP anti-cancer effects, clinical efficacy, safety and adverse effects (L. Kelland, 2007; L. R. Kelland et al., 1999; Lebwohl & Canetta, 1998).

![Cisplatin Structure](image)

Figure 1.1: Cisplatin Structure

However, in 1978 it was approved by United States food and drug administration (USFDA) for clinical use to treat testicular and bladder cancers (L. Kelland, 2007). Since then, cisplatin has been licensed worldwide for the treatment of
several solid tumors originating from head and neck, lung, colorectal, cervical, ovarian and bladder (Galanski, 2006; Lebwohl & Canetta, 1998; Prestayko, D’Aoust, Issell, & Crooke, 1979).

CP has been found to be highly efficient for treating testicular germ cell cancers (Feldman, Bosl, Sheinfeld, & Motzer, 2008; Winter & Albers, 2011). However, significant number of patients with lung, prostate and colorectal cancer patients tend to develop resistance to CP therapy (Galluzzi, et al., 2014 present and future; Koberle, Tomicic, Usanova, & Kaina, 2010). Despite being a well-known and powerful anti-proliferative drug, CP usage is accompanied with moderate-to-severe nephrotoxicity, ototoxicity and neurotoxicity (Galluzzi, et al., 2014).

Therefore, efforts have been made towards the development of novel derivatives of CP that would elicit robust therapeutic responses along with minimized side effects (Galluzzi, et al., 2014). Thus, it led to the development of additional platinum derivatives in clinical practice such as carboplatin and oxaliplatin (Mandala, Ferretti, & Barni, 2004; Tattersall, 2002). FDA has approved carboplatin in 1989 for the treatment of ovarian cancer and oxaliplatin in the 5-fluorouracil (5-FU) and folinic acid-containing cocktail regimen for treating colorectal cancer (Mandala, et al., 2004; Tattersall, 2002). Carboplatin antineoplastic actions are similar to cisplatin, reflecting the fact that the active forms of these drugs are identical. However, carboplatin appears to be less nephron and neurotoxic than cisplatin, and this perhaps reflects its reduced biological potency (Harrap, 1985).

However, significant proportion of CP-resistant tumors are resistant not only to carboplatin but also to oxaliplatin as well as to other chemotherapeutic agents (Gore et al., 1989; Hamaguchi et al., 1993; Negoro et al., 2009). These findings had
led to the development of additional platinum derivatives such as picoplatin and satraplatin. However, phase II clinical trials revealed that neither picoplatin nor satraplatin provide consistent advantages over CP, oxaliplatin or carboplatin-based chemotherapy (Choy, 2006; Eckardt et al., 2009; Monneret, 2011; Sternberg et al., 2009).

1.2 Disadvantages of CP Chemotherapy

Clinical studies have revealed that approximately 30-50% of patients treated with CP could develop hearing loss and 14% - 57% affected by neurotoxicity. Furthermore, 70% of patients treated with CP could be affected by nephrotoxicity (Rathinam, Ghosh, Neumann, & Jamesdaniel, 2015 renal, and neuronal cells is associated with nitration and downregulation of LMO4; Rubin et al., 1995; Skinner et al., 1998; Travis et al., 2014). In spite of these disadvantages, CP continues to remain as standard chemotherapy regimen for treatment of cancers arising from head and neck, testicular, cervical, ovaries and bladder. This could be attributed to the findings that other platinum based drugs failed to provide firm clinical efficacy (Coppin et al., 1996; Loehrer, Gonin, Nichols, Weathers, & Einhorn, 1998; Planting et al., 1999; Rose et al., 1999).

Nephrotoxicity is the most serious dose-limiting side effect of CP therapy (Sastry & Kellie, 2005). CP-induced nephrotoxicity can manifest with various types of symptoms such as acute kidney injury (AKI), hypomagnesemia, Fanconi-like syndrome, distal renal tubular acidosis, hypocalcemia, renal salt wasting, renal concentrating defect, hyperuricemia, transient proteinuria and erythropoietin deficiency (Miller, Tadagavadi, Ramesh, & Reeves, 2010). However, the most
serious and life-threatening side effect is AKI, which occurs in 20-30% of patients treated with CP (G. S. Oh et al., 2014).

1.2.1 Risk Factors for CP-Induced Nephrotoxicity

Several risk factors have been identified that could pre-dispose the patients to develop CP-induced nephrotoxicity. These include, high cumulative doses, increased frequency of treatment, geriatric population, smoking and hypoalbuminemia. While organic cation transporter-2 (OCT2) polymorphisms have been associated with decreased risk for the development of CP-induced nephrotoxicity (Miller, et al., 2010).

1.2.2 Biomarkers for Detecting CP-Induced Nephrotoxicity

Since the kidneys are the major organ affected with CP treatment, it is important to diagnose and treat nephrotoxicity at the earliest to avoid the morbidity and mortality associated with AKI. Therefore, robust and validated biomarkers are vital to aid for prompt diagnosis and management of CP-induced AKI. The most commonly used biomarkers till date are serum creatinine (Cr) and blood urea nitrogen (BUN). Moreover, several enzymes secreted in urine such as N-acetyl-β-d-glucosaminidase (NAG), lactate dehydrogenase (LDH) and aspartate aminotransferase (AST), have been suggested as nephrotoxic biomarkers in different studies (Tonomura, Tsuchiya, Torii, & Uehara, 2010). Unfortunately, the conventional biomarkers such as Cr and BUN are characterized by low detection sensitivity. Therefore, there is an urgent need for development of biomarkers with higher sensitivity to ensure that CP-elicited nephrotoxicity is being diagnosed with higher precision. USFDA and the European Medicines Agency (EMEA) recently published guidelines stating that several other urinary nephrotoxic biomarkers
including total protein, albumin, kidney injury molecule-1 (KIM-1), clusterin (CLU), β2-microglobulin (B2M), cystatin-c (CysC), and trefoil factor 3, has been shown in the regulatory decision-making during the management of CP-induced nephrotoxicity (Tonomura, et al., 2010).

Furthermore, neutrophil gelatinase-associated lipocalin (NGAL) has been recently recognized as a novel candidate for detecting drug-induced nephrotoxicity (Haase, Bellomo, Devarajan, Schlattmann, & Haase-Fielitz, 2009). However, KIM-1 with greater accuracy and sensitivity and shows the rapid increase in urine in a time-dependent manner. Regarding the early elevation of KIM-1, it is known that KIM-1 mRNA is rapidly expressed and translated to protein early after the injury of renal tubule (Bonventre, 2009; Tonomura, et al., 2010). KIM-1 is a type I cell membrane glycoprotein, which is involved in the regulation of the innate and adaptive immune responses. Since, the major target site of CP toxicity is the S3 segment of the proximal tubule, urinary KIM-1 can be considered as a specific biomarker of proximal tubular injury. (Bonventre, 2009; Prozialeck et al., 2009).

In contrast, cystatin C (CysC) is known as functional biomarker because it can freely pass through glomerular capillary wall due to its low-molecular weight and is almost completely reabsorbed by epithelial cells in proximal tubules. Therefore, CysC serum concentration correlates closely to the glomerular filtration rate (GFR). CysC is a secreted, extracellular cystatin protease inhibitor that belongs to the cystatin superfamily. Its measurement in serum and/or plasma has been proposed as an indicator of drug-induced nephrotoxicity that is less affected by factors such as gender, age, muscle mass, and preexisting liver diseases (Herget-Rosenthal, van Wijk, Brocker-Preuss, & Bokenkamp, 2007; Tonomura, et al., 2010).
1.3 Why Kidneys are Prime Target for CP Toxicity?

1.3.1 Transporter-mediated Mechanism for CP Accumulation in Kidneys

Cisplatin enters renal tubular cells by passive diffusion or transporter-mediated facilitated diffusion (Peres & da Cunha, 2013). Two different membrane transporters have been identified that are capable of transporting CP into the cells, chiefly Ctr1 (copper transporter) and OCT2. Ctr1 transporter is highly expressed in adult kidney cells and it localizes to the basolateral membrane of the proximal convoluted tubule (Pabla, Murphy, Liu, & Dong, 2009). Downregulation of Ctr1 transporter expression decreases both CP uptake and cytotoxicity effects, suggesting that Ctr1 transporter could be considered as an important uptake mechanism of CP in cells (Pabla, et al., 2009). OCT2 also plays an important role in transporting CP into cells especially in the proximal tubules of kidneys. Three isoforms of OCT were identified in human subjects. OCT2 is mainly expressed in renal tissues, while OCT1 in liver, and OCT3 in placenta. Downregulation of OCT2 accounts for lowering the risk of CP uptake, accumulation and development of nephrotoxicity (Miller, et al., 2010). Interestingly, CP does not interact with OCT1, which could emphasize the drug's organ and cell-specific toxicity (Peres & da Cunha, 2013).

1.3.2 Metabolic Conversion of CP to Nephrotoxins and Accumulation in Kidneys

Upon entry into the cells, CP undergoes metabolic activation in the kidneys to become a more potent toxin. Formation of glutathione conjugates is the first step in this process, mediated by glutathione-S-transferase. Once glutathione conjugates pass through the kidneys, they are cleaved by gamma glutamyl transpeptidase (GGT)
expressed on the surface of proximal tubule cells into cysteiny1-glycine-conjugates. These are further metabolized to cysteine conjugates by enzymes expressed on the surface of the proximal tubule cells called aminodipeptidases. Cysteine conjugates are finally transported to proximal tubule cells where it is further metabolized to highly reactive thiols (Townsend, Deng, Zhang, Lapus, & Hanigan, 2003). These highly reactive thiols interact with macromolecules, affect their structure, function, and eventually leading to renal cell death. It is pertinent to note that mice deficient in GGT were resistant to the nephrotoxic effects of CP (Peres & da Cunha, 2013).

1.4 Clinical Pharmacology of CP and its Mechanism of Anti-Cancer Activity

Upon systemic infusion, unbound CP has been estimated to have final plasma elimination half-life of 30-45 minutes, with renal elimination accounting for more than 25% of the total elimination. CP is administered either intravenously or short-term infusion in physiological saline or intraperitoneally, binds to serum protein by about 90%, distributes to most tissues and is cleared by the kidney in unchanged form (Royer et al., 2005) (Murry, 1997). In the aqueous environment, chloride atoms of CP are replaced by water molecules, and this result in the formation of positively charged electrophile. The electrophile reacts with nucleophilic sites of the DNA, RNA and protein to form different adducts. This interaction between cisplatin and DNA, results in the formation of either interstrand or intrastrand crosslinks (Figure 1.2), thereby arresting DNA synthesis and replication in rapidly proliferating cancer cells (D. Wang & Lippard, 2005).
1.5 Biochemical and Molecular Mechanisms Purported for CP-Induced Nephrotoxicity

1.5.1 DNA Damage and Mitochondrial Dysfunction

Platinum compounds mediate their cytotoxic effect through their interaction with the DNA. A positively charged metabolite of CP is mostly accumulated within the negatively charged mitochondria. This could explain the sensitivity of the renal proximal tubule to CP, as this particular segment possesses the highest mitochondrial density in kidneys (Miller, et al., 2010; Townsend, et al., 2003). Due to poor DNA repair machinery, mitochondrial DNA is more prone for CP damage rather than nuclear DNA (Figure 1.3) (Olivero, Chang, Lopez-Larraza, Semino-Mora, & Poirier, 1997).
Energy production in the mitochondria are also disrupted by CP. Fatty acids serve as the major source of energy in the proximal tubule cells and are the primary target of CP-induced kidney injury. CP causes fatty acid oxidation inhibition in proximal tubule cells through interfering with PPAR-α mediated expression of genes involved in cellular fatty acid utilization (Li et al., 2004). In addition, CP also inhibits mitochondrial respiration. As a result of this, intracellular ATP generation is decreased, that causes further disruption in the mitochondrial energy metabolism through cascade of biochemical changes culminating in the apoptotic cell death of renal parenchyma (Kruidering, Van de Water, de Heer, Mulder, & Nagelkerke, 1997). Specifically, mitochondrial dysfunction occurs following CP treatment is due to the decline in membrane electrochemical potential, marked decrease in
mitochondrial calcium uptake and depletion of mitochondrial antioxidant defense mechanisms (Kruidering, et al., 1997). In fact, a recent study demonstrated that rodents treated with mitochondria targeted antioxidants protected against CP-induced nephrotoxicity without compromising its anti-cancer activity (Partha Mukhopadhyay et al., 2012).

1.5.2 Cell Transport System Alterations

CP interferes with transporter of water and renal tubular cell nutrients that is mediated by Na\(^+\) pumps in the apical and basolateral face of the renal proximal tubular cells, such as Na\(^+\)/K\(^+\)/ATPase, Na-K-2Cl cotransporter, and type III Na\(^+\)/H\(^+\) exchanger, and water-permeable channels such as aquaporin 1, 2, and 3. CP also inhibits the activity of transporters in the brush border. CP-induced renal injury may compromise the integrity of the cytoskeleton and cell polarity, thus interfering with H\(^+\), K\(^+\), Mg\(^{2+}\), and Ca\(^{2+}\) ions homeostasis and contributing to lower ion reabsorption rates in the proximal and distal tubules and increased excretion of these ions in urine. Additionally, loss of the tubular epithelial cell barrier and/or junctions between cells in CP-induced tubular injury could also force the glomerular filtrate to flow back into blood circulation, producing significant decrease in glomerular filtration rate (GRF) (Lajer et al., 2005; Peres & da Cunha, 2013).

1.5.3 Oxidative Stress

Oxidative stress is involved in CP-induced nephrotoxicity. Several mechanisms have been proposed that are associated with renal injury, which include, reactive oxygen species (ROS) generation, depletion of antioxidant systems, and stimulation of renal accumulation of lipid peroxidation products (Peres & da Cunha, 2013). These mechanisms lead to the activation of oxidative metabolism secondary to
the stimulation of ROS production, including superoxide anions (\(O_2^{-}\)), hydrogen peroxide (\(H_2O_2\)), and hydroxyl radicals (\(^*\)OH). Furthermore, CP also depletes the antioxidant defense mechanisms such as glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). In addition, nitrative stress is also involved in the renal damage through production of high levels of nitric oxide (NO), possibly as a result of induced synthesis of nitric oxide synthase (iNOS), leading to the continuous formation of peroxynitrite (ONOO\(-\)), which contribute to CP-induced renal damage by reacting with \(O_2^{-}\). Therefore, both reactive oxygen and nitrogen species generation following CP treatment leads to significant damage to cell structure and function, including lipid peroxidation, protein nitration, enzyme inactivation, and DNA breaks. Eventually, this phenomenon leads to cellular dysfunction and signaling for the activation of pro-apoptotic pathways, causing cell death and kidney damage (Cetin et al., 2006; Peres & da Cunha, 2013) (Pál Pacher, Joseph S. Beckman, & Lucas Liaudet, 2007).

1.5.4 Inflammatory Response

In addition to direct toxicity that exists following CP administration, inflammation plays a pivotal role in renal injury due to several inflammatory mediators. Different chemokines and cytokines are produced and released to the site of renal tissue injury. CP activates phosphorylation and subsequent translocation of nuclear factor kappa -B (NFkB) transcription factor to the nucleus that consequently promotes the transcription of specific genes encoding inflammatory mediators and causing immune, proliferative, anti-apoptotic, and inflammatory responses (Miller, et al., 2010). This event leads to increased expression of tumor necrosis factor alpha (TNF-\(\alpha\)) in kidney tubular cells. TNF-\(\alpha\) is the prototypical inflammatory cytokine that
plays an important role in the host-defense mechanisms against infectious diseases and other chronic inflammatory diseases. Several studies have addressed the role of TNF-α in the pathogenesis of CP-induced acute renal failure. In fact, treatment of animals with specific TNF-α inhibitors or genetic ablation of TNF-α conferred marked renal protection against CP-induced nephrotoxicity (Ramesh & Reeves, 2002). These observations revealed the important contribution of TNF-α in the pathogenesis of CP-induced renal toxicity. Furthermore, several studies independently have confirmed the above findings (Y. K. Kim et al., 2003; Tsuruya et al., 2003).

TNF-α can stimulate renal injury and tubular cell death directly via TNF receptor type 1 (TNFR1) and indirectly by evoking strong inflammatory response through TNF receptor type 2 (TNFR2). Moreover, TNF-α/TNFR2 signaling contributes to CP nephrotoxicity and may enhance the pro-apoptotic effects arising from the activation of TNFR1. Additionally, TNF-α is responsible for the activation of a large network of proinflammatory cytokines such as interleukins IL-1β, IL-4, IL-6, transforming growth factor-β1 (TGF-β 1) and monocyte chemotactic protein-1 (MCP-1). Furthermore, TNF-α induces the expression of adhesion molecules, such as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), resulting in the invasion of inflammatory cells into the renal parenchyma (Peres & da Cunha, 2013).

Mitogen-activated protein kinases (MAPK) are protein kinases that are involved in directing cellular responses to a diverse range of stimuli, such as mitogens, osmotic stress, heat shock and proinflammatory cytokines. Moreover, MAPK’s regulate cell functions including proliferation, gene expression,
differentiation, mitosis, cell survival, and apoptosis. Different stimulus such as physical, biological or chemical, causes initiation of cellular stress, inflammatory response and apoptotic pathways that eventually induced the production of c-Jun N-terminal kinases (JNKs) and p38 MAPK kinases. In addition, a cascade of extracellular-signal-regulated kinases (ERKs) is induced mostly by cell death and cell survival growth factors. Therefore, different activation patterns of these three main MAPK pathways (ERK, JNK, and p38) have been involved in the pathogenesis of CP-induced nephrotoxicity (dos Santos, Carvalho Rodrigues, Martins, & dos Santos, 2012; Pabla & Dong, 2008). p38 MAPK activation leads to the synthesis of TNF-α in CP-induced renal inflammation. It has been demonstrated that the activation of MAPK p38 in neutrophils causes IκB (an inhibitor of NF-κB) degradation, consequently promoting the activation and migration of NF-κB to the nucleus, which culminates in the generation of pro-inflammatory cytokines including TNF-α. Consequently, TNF-α and some other inflammatory mediators, form a loop feedback which induces the transcription of genes of inflammatory mediators (Peres & da Cunha, 2013; G. Ramesh & W. B. Reeves, 2005).

1.5.5 Apoptosis Pathways Activation

Nephrotoxic dose of CP has been shown to initiate several apoptotic pathways in renal epithelial cell death via extrinsic and intrinsic pathways (Shiraishi et al., 2000). CP recruits extrinsic pathway by activating death receptors such as TNF receptors located in the basolateral membrane and their main ligands including Fas and TNF-α, and the activation of these receptors increases the activity of caspase 8 and eventually resulting in the apoptosis. Intrinsic pathway or mitochondrial and endoplasmic reticulum stress pathway enhances the pro-apoptotic proteins Bax and
Bak to undergo structural modifications and alter the integrity of the mitochondrial membrane to cause the release of apoptogenic factors such as cytochrome C (caspases activator) and apoptosis-inducing factor (AIF), a caspase-independent cell death promoter (Peres & da Cunha, 2013). Both pathways induces the activation of specific proteases called executioner caspases (caspase-3 and caspase-7), resulting in the characteristic morphological changes such as membrane degradation, cell shrinkage, and DNA fragmentation, which are reminiscent of apoptosis (Pabla & Dong, 2008; Peres & da Cunha, 2013; Sancho-Martinez, Prieto-Garcia, Prieto, Lopez-Novoa, & Lopez-Hernandez, 2012).

Caspases are a family of cell death proteases that plays an important role in the execution phase of apoptosis in the renal tubular epithelial cell death both in vivo and in vitro (Jiang, Wang, Huang, Yang, & Dong, 2009). Activation of caspases occurs after CP treatment of renal epithelial cells and inhibition of caspase activity suppress its role in cell death induction. P53 is a tumor suppressor gene that causes cell cycle arrest or apoptosis in response to DNA damage, hypoxia and oncogene activation (Bassett, Wang, Rastinejad, & El-Deiry, 2008). CP administration has been reported to activate the p53 transcription. However, pharmacological inhibition of p53 transcriptional activity or genetic ablation/repression reduces CP-induced caspase activation, apoptosis, ameliorated CP-induced renal apoptosis, and renal tissue injury (Wei et al., 2007). In sum, CP-induced cell injury and cell death involves number of pathways including oxidative stress, pro-inflammatory cytokines generation, and activation of intrinsic and extrinsic apoptotic pathways.
1.6 Strategies for Ameliorating CP - Induced Nephrotoxicity

Vigorous hydration with saline and simultaneous administration of mannitol before, during and after cisplatin administration have been shown to reduce the risk for CP-induced nephrotoxicity (Ali & Al Moundhri, 2006; G. S. Oh, et al., 2014). Amifostine (an organic thiophosphate) also reduces CP-induced nephrotoxicity. Amifostine mechanism of renoprotection is purported through the scavenging of oxygen free radicals, via generation of GSH (Ali & Al Moundhri, 2006; Asna et al., 2005). In addition, erythropoietin has been shown to confer protection against CP-induced nephrotoxicity (Yalcin et al., 2003). In addition to its essential role as hematopoietic agent, erythropoietin inhibits apoptotic cell death, enhances tubular epithelial regeneration and promotes renal functional recovery in hypoxic or ischemic acute renal injury (Yalcin, et al., 2003).

Salicylates are anti-inflammatory agents used in the treatment of various inflammatory ailments. Salicylates confer anti-inflammatory activity by inhibiting cyclooxygenase activity and prostaglandin synthesis. Furthermore, high doses of salicylates are able to stabilize the inhibitor of kappa-B (IκB) enzyme as well as reduce NFκB transcriptional activity, that consequently attenuates TNF-α generation and reduces renal inflammatory response during the CP-induced nephrotoxicity(G. S. Oh, et al., 2014). (Ramesh & Reeves, 2004). However, these approaches have met with limited clinical success, since CP-induced nephrotoxicity could not be abrogated. Therefore, there is an urgent need to develop agents that can confer renoprotection, without compromising the anti-cancer activity of CSP.
1.6.1 Phytochemicals as Cytoprotective Agents for Mitigating CP – Induced Nephrotoxicity

Phytochemicals are bioactive non-nutrient plant compounds that are widely distributed in plant kingdom. Regular consumption of fruits and vegetables were associated with reduced risk for the development of major chronic diseases such as metabolic syndrome, cardiovascular and inflammatory diseases. Presently, more than 5000 phytochemicals were identified. Despite this development, their pharmacology and health benefits are hitherto unknown (R. H. Liu, 2003). In fact, USFDA approved anti-cancer drug paclitaxel and anti-malarial drug artemisinin are phytochemicals derived from *Taxus brevifolia* and *Artimesia annua* (Morejohn & Fosket, 1991) (Tu, 2016). In addition, anti-diabetic drug belonging to the chemical class biguanide was originally isolated from *Galega officinalis* (Witters, 2001). These developments provide indication that phytochemicals have tremendous translational potential for the treatment of various diseases. In this direction, several phytochemicals were evaluated for cytoprotective effects against CP-induced nephrotoxicity (Ojha et al., 2016).

In the present study, we have investigated the nephroprotective actions of genipin (GP) in a murine model of CP-induced nephrotoxicity. GP is a phytochemical extracted from the fruit of *Gardenia jasminoides* (Figure 1.4). This plant belongs to the coffee family and it is native to the subtropical countries of Africa, Asia and Pacific islands (Manickam, Sreedharan, & Elumalai, 2014). In traditional Chinese medical practice, GP formulations is used for the treatment of inflammation associated pain, hypertension, hepatic disorders (Ko & Moon, 2015). Recently, GP chemo-preventive effects were also recognized (Ko & Moon, 2015).
1.7 Aims and Objectives of the Study

GP is a terpinoid molecule and it is widely used in the pharmaceutical industry as a cross-linking agent in the synthesis of various biopolymers, and used as drug delivery agents. Because of natural availability and low cytotoxicity, GP is pursued for the development of novel cross-linking agents. In addition, GP also serves as backbone for the synthesis of various alkaloids during medicinal chemistry research (Tsai, Tseng, Chen, & Tsai, 2002). Previous studies have indicated that GP possesses anti-inflammatory and anti-oxidant properties (Koriyama et al., 2010). Furthermore, GP has been shown to ameliorate hepatic ischemia reperfusion injury, steatosis, autoimmune hepatitis and fibrosis in rodents (J. Kim, Kim, & Lee, 2013) (Ma et al., 2012) (S.-J. Kim, Kim, Lee, Kwak, & Lee, 2010) (Inao et al., 2004). Similarly, GP has been reported to attenuate myocardial tissue injury (Paul et al., 2012) and suppresses neuro-inflammation (Koo et al., 2004). In addition, GP
inhibited hyperglycemia-induced renal tissue injury by diminishing oxidative stress and inflammation (Qiu et al., 2012).

However, there are no reports available on GP nephroprotective activity against CP-induced renal toxicity. Hence, we have undertaken this study to determine and investigate the GP nephroprotective actions, in murine model of CP-induced nephrotoxicity. Specific objectives of this project are outlined below.

1. Investigate the GP nephroprotective effect against CP-induced nephrotoxicity in mice.
2. Investigate the pre-treatment (prophylaxis) and post-treatment (therapeutic) effects of GP against CP-induced nephrotoxicity.
3. Investigate the biochemical mechanisms governing GP nephroprotective actions.
4. In specific, the role of oxidative stress, inflammation and cell death pathways in ameliorating CP-induced nephrotoxicity will be determined.
Chapter 2: Materials and Methods

2.1 Animals and Drug Treatment

All animals experimentation protocols adhered to the National Institutes of Health (Bethesda, MD, USA) guidelines for responsible use of laboratory animals and were approved by institutional animal care and use committee of the United Arab Emirates University. C57BL/6J (6-8 week-old male mice) were purchased from the Jackson laboratory (Bar Harbor, ME, USA). Animals were maintained in temperature-controlled facility, with 12-hr light/dark cycle and provided access to water and food *ad libitum*. Genipin (GP) was obtained from Santa Cruz biotechnology (Dallas, TX, USA). Cisplatin (CP) was purchased from Sigma chemicals (St Louis, MO, USA). GP (1 – 10 mg/kg) was administered via intraperitoneal injections (I.P) either 2hrs before or 12hrs after CP administration. Animals were sacrificed 72hrs after single dose of CP (20 mg/kg; I.P). Animals were anesthetized with tribromomethanol (0.2 ml/10 gm body weight). Blood samples were collected by retro-orbital puncture and later euthanized by cervical dislocation. After ensuring the death of animals, they were dissected and kidneys were removed and snap frozen in liquid nitrogen for biochemical investigations or placed in 10% neutral buffered formalin (Electron microscopy sciences, Hatfield, PA, USA) for histological evaluations. Unless specified, all fine reagents were obtained from Sigma chemicals.

2.2 Determination of Renal Function

Serum levels of blood urea nitrogen (BUN) and creatinine (Cr) was determined using IDEXX VetTest chemistry analyzer (IDEXX Lab, Hoofddorp, The...
Netherlands). Test kits were procured from IDEXX lab and samples were analyzed per the protocol supplied by the manufacturer (Partha Mukhopadhyay et al., 2010).

2.3 Determination of Serum Cystatin C

Levels of cystatin c in serum samples were determined using commercially available ELISA kit (R & D systems, MN, USA). Samples were subjected to ELISA assay as per the protocol supplied with the kit. In brief, 50 μL of assay diluent was added to the 96-well microtiter plate, followed by 50 μL of serum samples or standards, and incubated for 2 hours at room temperature (RT). After washing, 100 μL of cystatin c conjugate solution was added and incubated at RT for 2 hours. Subsequent to washing, wells were probed with 100 μL substrate solution and absorbance was determined at 450 nm using microtiter plate reader [Molecular Devices, Sunnyvale, CA, USA]. Values are expressed as ng/ml.

2.4 Determination of Kidney Injury Molecule -1 (KIM-1) in Renal Tissues

Renal tissues were homogenized with tissue lysis buffer (Thermo Fischer Scientific, Paisley, UK). Protein content in the homogenates was determined with Lowry reagent (Bio Rad, CA, USA). Levels of KIM-1 in the renal tissues were determined with the commercially available kit (R & D systems, MN, USA) and the protocol was identical to that of cystatin C measurement. Values were expressed as pg/mg protein.

2.5 Histological Evaluation of CP-Induced Renal Injury

After fixation of the renal tissues in 10 % neutral buffered formalin for 72 hours, samples were processed as follows:

1. 70 % ethanol for 1 hour
2. 95 % ethanol for 1 hour
3. Absolute ethanol for 1 hour X 3 changes
4. Xylene 1 hour X 2 changes
5. First wax (Paraplast X-tra; Thermo Fischer Scientific, Paisley, UK) at 58°C for 1 hour
6. Second wax (Paraplast X-tra) at 58°C 1 hour
7. Processed samples were embedded in tissue cassettes and allowed to cool down to RT.
8. 5 μm paraffin sections were obtained using microtome (Thermo Fischer Scientific, Paisley, UK). Sections are air dried and placed at hot plate at 58°C for 2 hours. The sections were stored in slide boxes, until staining.

2.5.1 Periodic acid-Schiff Staining (PAS) of Paraffin Sections

Slides were placed in a rack, and deparaffinization was performed using glass chamber as per the protocol provided below:

<table>
<thead>
<tr>
<th>Solution</th>
<th>Incubation time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylene</td>
<td>3 min</td>
</tr>
<tr>
<td>Xylene</td>
<td>3 min</td>
</tr>
<tr>
<td>Xylene : Ethanol (1:1)</td>
<td>3 min</td>
</tr>
<tr>
<td>100% Ethanol</td>
<td>3 min</td>
</tr>
<tr>
<td>100% Ethanol</td>
<td>3 min</td>
</tr>
<tr>
<td>95% Ethanol</td>
<td>3 min</td>
</tr>
<tr>
<td>70% Ethanol</td>
<td>3 min</td>
</tr>
<tr>
<td>50% Ethanol</td>
<td>3 min</td>
</tr>
</tbody>
</table>

After deparaffinization, slides are placed in distilled water, until further processing of the sections. PAS staining of the renal sections was performed using the kit purchased from American Master Tech (Lodi, CA, USA). The sections were immersed in 0.5 %
periodic acid and incubated for 7 min and washed in running tap water for 1 min. Next, sections were covered with Schiff solution and incubated for 15 min, followed by washing in running tap water for 5 min. Sections were counterstained with Mayer’s hematoxylin for 2 min and washed in running water for 1 min. Finally, sections were dehydrated in absolute alcohol for 1 min, and xylene, and permanently mounted with cover slips. Slides were air-dried and observed using light microscope (Carl Zeiss, Germany). Tubular necrosis was characterized by the loss of proximal tubular brush border, blebbing of apical membranes, epithelial detachment from basement membrane or intraluminal hyaline cast formation (Partha Mukhopadhyay, et al., 2010).

2.6 Determination of NADPH oxidase Activity in Renal Tissues

Renal tissues were washed in ice-cold phosphate buffered saline (PBS), homogenized in buffer containing 20 mM KH₂PO₄ (pH 7.0), protease and phosphatase inhibitors cocktail tablet (Calbiochem) NADPH oxidase activity of the renal homogenates was measured using the method described earlier (Abid, Spokes, Shih, & Aird, 2007). In brief, photon emission from the chromogenic substrate lucigenin as a function of acceptance of electron/O₂⁻ generated by the NADPH oxidase complex was measured every 15 seconds for 10 min in a luminometer (Molecular Devices). The composition of NADPH oxidase assay buffer is: 250 mM HEPES (pH 7.4), 120 mM NaCl, 5.9 mM KCl, 1.2 mM MgSO₄, 1.75 mM CaCl₂, 11mM glucose, 0.5 mM EDTA, 100 μM NADH and 5 μM lucigenin. The data were converted to relative light units/min/mg of protein and expressed as % activity.
2.7 Determination of Superoxide dismutase (SOD) Activity in Renal Tissues

SOD activities in renal tissues were determined using the assay kit obtained from Trevigen, Inc. (Gaithersburg, MD, USA). Protocol for determining the enzyme activity was supplied with kit. In this assay, $O_2^\cdot$ generated from the conversion of xanthine to uric acid and $H_2O_2$ by xanthine oxidase (XOD), reduced WST-1 (colorless) to WST-1 formazan (chromogen), which absorbs light at 450 nm. SOD present in the samples would reduce the $O_2^\cdot$ concentration and inhibits the generation of WST-1 formazan (chromogen). Therefore, the extent of reduction in the production of WST-1 formazan is proportional to the SOD activity in the tissues. For instance, higher SOD activity in the samples will result in diminished formation of WST-1 formazan. Values were expressed as units/mg protein.

2.8 Determination of GSH content in Renal Tissues

GSH levels in the tissues were determined using the kit obtained from Trevigen, Inc. (Gaithersburg, MD, USA). This kit uses the enzymatic recycling method for the quantification of GSH. GSH reductase reduces the oxidized glutathione (GSSG) to reduced GSG. As shown in Figure 2.1, the sulfhydryl group of GSH reacts with 5,5’ dithiobis-2-nitrobenzoic acid (DTNB) to generate yellow chromogen -5-thio-2-nitrobenzoic acid (TNB), which absorbs at 405 nm. The rate of TNB production is directly proportional to the concentration of GSH in tissues. GSH content was expressed as $\mu$mol / mg protein.
2.9 Determination of 4-hydroxynonenal (4-HNE) in Renal Tissues

Lipid peroxides are unstable indicators of oxidative stress in cells that decompose to form more complex and reactive compounds like malondialdehyde and 4-HNE. Determining the end products of lipid peroxidation is one of the most widely accepted assays for oxidative damage. 4-HNE in the renal tissues were determined using ELISA kit procured from Cell Biolabs, Inc (San Diego, CA, USA). The assay was accomplished by following the protocol supplied with the kit. First, an HNE conjugate was coated on an ELISA plate. The unknown HNE protein samples or HNE standards are then added to the HNE conjugate reabsorbed ELISA plate. After incubation for 1 hour at RT, an anti-4HNE polyclonal antibody was added, followed by an HRP conjugated secondary antibody, and incubated for 1 hour at RT. After washing of wells, substrate solution was added and absorbance was measured at 450 nm. 4-HNE content in the samples was expressed as nmol/mg protein.

Figure 2.1: GSH assay principle
2.10 Determination of 3-nitrotyrosine (3-NY) in the Renal Tissues

Nitrotyrosine has been considered as footprint of peroxynitrite formation (P. Pacher, J. S. Beckman, & L. Liaudet, 2007). 3-NY content in the renal tissue homogenates was determined with the aid of ELISA kit procured from Hycult Biotech (Frontstraat, Uden, Netherlands). Protocol is depicted below.

![Protocol for 3-NY determination](image)

Figure 2.2: Protocol for 3-NY determination
2.11 Determination of Pro-Inflammatory Cytokines in Renal Tissues

Levels of pro-inflammatory cytokines such as TNFα and IL-1β in renal tissue homogenates were determined using commercially available ELISA kit (R & D systems, MN, USA). In brief, 50 μL of assay diluent was added to the 96-well microtiter plate, followed by 50 μL of serum samples or standards, and incubated for 2 hrs at room temperature (RT). After washing, 100 μL of cystatin c conjugate solution was added and incubated at RT for 2 hours. Subsequent to washing, wells were probed with 100 μL substrate solution and absorbance was determined at 450 nm using microtiter plate reader [Molecular Devices, Sunnyvale, CA, USA]. Values are expressed as pg / mg protein.

2.12 Determination of MAPK Activation

JNK and p38 MAPK activation in the renal tissues was determined with the aid of commercially available kit procured from Cell signaling technology (Danvers, MA, USA). Tissue homogenates 100 μL was added to microtiter wells and incubated for 2hrs at 37°C. Contents of the wells were aspirated and washed four times with wash buffer provided in the kit. Then 100 μL of detection antibody was added to the corresponding wells and incubated for 1hr at 37°C. After repeating the washing step, wells were probed with tetra methylene blue (TMB) substrate solution and absorbance was measured at 450 nm.

2.13 Determination of Caspase 3 Activity

Caspase 3 activity in the renal tissue homogenate was determined using the kit purchased from Promega (Madison, WI, USA). Caspase 3 is a protease that specifically cleaves the aspartate residue of the sequence DEVD (Asp-Glu-Val-Asp).
In this assay, the 100 μL of substrate solution (rhodamine Z-DEVD-R100) is mixed with 100 μL of renal tissue homogenate in a fluorescence microtiter plate and incubated for 1 hour at RT. Substrate is cleaved by caspase 3 in the samples and this produces fluorescent rhodamine. Fluorescence is measured by excitation at 499 nm and emission at 521 nm, using microtiter plate reader at fluorescence reading mode [Molecular Devices, Sunnyvale, CA, USA].

2.14 Determination of Poly (ADP-ribose) polymerase (PARP) Activity

PARP activities in the renal tissues were determined using the reagents obtained from Trevigen, Inc. This assay measures the incorporation of biotinylated poly(ADP-ribose) on to the histone pre-adsorbed in 96-well microtiter strips. In brief, 50 μL/well of PARP buffer was added on to microtiter plate to rehydrate histones for 30 min at RT. Contents of the wells were aspirated and 25 μL of diluted renal tissue homogenate and 25 μL assay buffer is added and incubated at RT for 60 min. This was followed by washing and probing of wells with 50 μL of diluted streptavidin-HRP conjugate and incubated at RT for 60 min. After washing if wells 50 μL of substrate solution was added and incubated in dark for 15 min. The reaction was arrested by adding 50 μL of 0.2M HCl and absorbance was measured at 450 nm.

2.15 Determination of DNA Fragmentation

The assay is based on quantitative sandwich ELISA using mouse monoclonal antibodies against DNA and histones (Sigma Chemicals). This facilitates the specific determination of mono and oligonucleosomes in the total tissue homogenates. 20 μL of tissue homogenates were placed on to streptavidin-coated wells. 80 μL of anti-histone-biotin and anti-DNA-peroxidase was added and incubated at RT for 2 hrs.
Then unbound components were removed by washing and 100 μL of 2,2'-azino-di-(3-ethylbenzthiazoline sulfonyl acid(ABTS) solution was added and the absorbance was measured at 405 nm.

Figure 2.3: DNA fragmentation assay principle

2.16 Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) Staining in Paraffin Embedded Renal Sections

TUNEL staining was performed with the kit obtained from Sigma chemicals. Cleavage of genomic DNA during apoptosis yields double-stranded, low molecular weight DNA fragments as well as single strand breaks (nicks) in high molecular weight DNA. These DNA strand breaks can be identified by labeling free 3’-OH termini with modified nucleotides in an enzymatic reaction. Paraffin sections were dewaxed as per the procedure described in section 2.5.1. Then slides were placed in plastic coplin jar containing 50 ml 0.1 M citrate buffer pH 6.0 (Sigma chemicals) and microwave irradiated (750 W) for 1 min. Slides were immediately cooled by adding 80 ml of distilled water and then transferred to coplin jar containing PBS. Sections were then immersed for 30 min in Tris-HCl (0.1 M, pH 7.5) containing 3% bovine serum albumin (BSA) at RT. After careful washing with PBS, 50 μL of TUNEL
reaction mixture was carefully overlaid on to the sections and incubated at 37°C for 60 min in humidified side staining chamber (Electron microscopy sciences, Hatfield, PA, USA). After washing of slides with PBS, sections were mounted in glycerol medium and observed under fluorescence microscope using 515-565 nm filter [Fluorescein isothiocyanate (FITC) filter; EVOS FL, Thermo Fischer Scientific, Paisley, UK]. Images were acquired at final magnification of X100.

2.17 Statistical Analysis of the Data

Values expressed are mean ± SEM. One-way analysis of variance (ANOVA) followed by Tukey’s post hoc test was performed. Probability values P < 0.05 were considered as significant. Analysis was performed with Graph Pad prism V software (La Jolla, CA, USA).
Chapter 3 : Results

3.1 GP Treatment Attenuates CP-Induced Renal Tissue Injury

Animals were treated as described in the methods section (2.1) and sacrificed after 72 hrs. Serum samples were subjected to the determination of clinical markers for renal tissue injury. Specifically, levels of BUN and creatinine were determined. As shown in the Figure 3.1, CP treatment resulted in significant renal tissue injury, which was characterized by elevated levels of BUN (Figure 3.1A) and creatinine (Figure 3.1B). However, GP elicited dose-dependent nephroprotective actions and ameliorated CP-induced nephrotoxicity. The maximum nephroprotective activity of GP was observed at 10 mg/kg. Therefore, in further experiments this concentration was used in animal experiments.
Figure 3.1: Effect of GP on CP-induced Renal Tissue Injury (serum creatinine and BUN). GP at the indicated doses was administered to animals 2 hrs, prior to CP administration and then sacrificed after 72 hrs. (A) Shows the serum levels of BUN in the respective group of animals. (B) Shows the serum levels of creatine in the respective group of animals. n=6/group; # P < 0.001 vs. Veh/Genipin; * P < 0.01 vs. CP; @ P < 0.001 vs. CP
3.2 GP Treatment Attenuates CP-Induced Renal Tissue Injury

We further evaluated the nephroprotective effect of GP against CP-induced nephrotoxicity by determining the specific biomarkers of acute kidney injury such as cystatin C and KIM-1 in serum and renal tissue samples. Our observations indicated that CP induced significant elevations in the levels of cystatin C (Figure 3.2A) and KIM-1(Figure 3.2B). Upon treatment with GP (10 mg/kg), the levels of cystatin C and KIM-1 were reduced, indicating the amelioration of CP-induced nephrotoxicity (Figure 3.2).
Figure 3.2: Effect of GP on CP-induced Renal Tissue Injury on (Cystatin C and KIM-1). (A) Shows the serum levels of cystatin-C as determined by ELISA (B) Shows the renal tissue levels of KIM-1 as determined by ELISA. N=6/group; # P < 0.001 vs. Veh / GP; * P < 0.001 vs. CP
3.3 Effect of CP and/or GP on Renal Histology

The effects of different treatment modalities on renal histological features were evaluated by PAS staining as described in the methods section. CP administration induced severe and widespread tubular necrosis with dilatation, marked degeneration, intraluminal epithelial anoikis and cast formation. Furthermore, CP treatment is also characterized by infiltration of immune cells, which are characteristic of inflammation. However, treatment of animals with GP significantly improved the histological architecture by attenuating the tubular necrosis and inflammation (Figure 3.3).
Figure 3.3: Effect of CP and/or GP on Renal Histology. Shown are the representative images of PAS stained paraffin embedded kidney sections from the respective group of animals. Asterisk symbol denotes tubular necrosis with cast formation. Arrowhead denotes tubular epithelial anoikis and dashed arrow indicated the inflammation foci. Magnification is X 200
3.4 Effect of GP on CP-induced Oxidative Stress in Renal Tissues

Oxidative stress is one of the key mechanisms by which CP perpetuates renal tissue injury (G.-S. Oh et al., 2016). Therefore, we have investigated the various endogenous markers of oxidative stress and anti-oxidant defense systems in renal tissues. First, we determined the $O_2^\cdot$ generating enzyme (NADPH oxidase), which has been shown to play pivotal role in CP-induced renal damage (Y. Wang et al., 2015). Our observations revealed that CP significantly induced NADPH oxidase activity (Figure 3.4A) and this was mitigated upon GP treatment. SOD a major endogenous defender against oxidant damage was diminished by CP and its activity was restored by GP (Figure 3.4B). Similar trend was observed with the renal content of GSH (Figure 3.4C). $O_2^\cdot$ is powerful oxidant and rapidly reacts with the membrane lipids, and produces lipid peroxides, which affect the structure and function of the lipids (Arany & Safirstein, 2003). In fact, our results suggest that levels of 4-HNE was elevated in the renal tissues of CP treated animals and this was attenuated by GP (Figure 3.4D).
Figure 3.4: Effect of GP on CP-induced Oxidative Stress in Renal Tissues. GP attenuates CP-induced oxidative stress in renal tissues. (A) depicts NADPH oxidase activity; (B) SOD activity; (C) GSH content and (D) 4-HNE in the respective group. N=6/group; # P < 0.001 vs. Veh/GP ; * P < 0.01 vs. CP
3.5 Effect of GP on CP-induced Nitrative stress in Renal Tissues

Reactive nitrogen species (RNS) plays pivotal role in the pathophysiology of several diseases. \( \text{ONOO}^- \) is the formed by coupling of \( \text{O}_2^- \) and NO, when they are simultaneously generated by the phagocytes that invade the renal parenchyma (P. Pacher, et al., 2007). \( \text{ONOO}^- \) being a powerful oxidant rapidly nitrates lipids, proteins and nucleic acids, due to the lack of endogenous defense system, which could detoxify them (P. Pacher, et al., 2007). In fact, our results suggest that CP significantly induced the accumulation of 3-NY in renal tissues and this was ameliorated upon GP treatment (Figure 3.5).

![Figure 3.5: Effect of GP on CP-induced Nitrative Stress in Renal Tissues. GP attenuates CP-Induced nitrative stress in renal tissues. Quantification of 3-nitrotyrosine levels in the renal tissues. N=6/group; # P < 0.001 vs. Veh/GP; * P < 0.01 vs. CP](image-url)
3.6 Effect of GP on CP-induced Renal Inflammation

Since our earlier histological observation indicated that GP inhibited renal inflammation, we further confirmed this by quantification of pro-inflammatory cytokines (TNF-α and IL1β) in the renal tissues by ELISA. As expected, CP provoked significant increase of these pro-inflammatory cytokines and this was diminished when the animals were treated with GP (Figure 3.6).

![Graph showing effect of GP on CP-induced Renal Inflammation](image)

**Figure 3.6:** Effect of GP on CP-induced Renal Inflammation. GP attenuates CP-induced pro-inflammatory cytokine expression in renal tissues. (A) TNF-α and (B) IL1-β in the respective group of samples. N=6/group; # P < 0.001 vs. Veh/GP; * P < 0.01 vs. CP

3.7 Effect of GP on CP-induced MAPK Activation

MAPK plays crucial role in cell proliferation, migration, and apoptosis during growth and development. In addition, they are also implicated in the pathogenesis of several human diseases. ROS are the potent activators of MAPK and since CP induces ROS generation, we evaluated the MAPK activation in renal tissues
There was robust activation of p38 MAPK (Figure 3.7A) and JNK (Figure 3.7B) in renal tissues obtained from CP treated animals, and this was inhibited upon GP treatment (Figure 3.7).

![Figure 3.7: Effect of GP on CP-induced MAPK Activation. GP attenuates CP-induced MAPK activation in renal tissues. (A) depicts the p38 MAPK activation and (B) shows JNK activation in the respective group of animals. n=6/group; # P < 0.001 vs. Veh/GP; * P < 0.001 vs. CP](image)

3.8 Effect of GP on CP-Induced Apoptotic Cell Death

Apoptotic cell death is the major pathway, were renal tissue succumbs to CP-toxicity (Schrier, Wang, Poole, & Mitra, 2004). Therefore, we determined the various molecular determinants of apoptosis in the kidney tissues (Figure 3.8). There were
significant increase in the caspase 3 activity (Figure 3.8A) and PARP activation (Figure 3.8B) in animals that received CP. In addition, DNA fragmentation was increased in CP treated animals. However, this phenotypic change was mitigated upon GP treatment.
Figure 3.8: Effect of GP on CP-induced Apoptotic Cell Death. GP attenuated CP-induced apoptotic cell death in renal tissues. (A) caspase 3 activity, (B) PARP activity and (C) DNA fragmentation in the respective groups. n=6/group; # P < 0.001 vs. Veh/GP; * P < 0.001 vs. CP
3.9 Effect of GP on CP-induced Apoptotic Cell Death – TUNEL staining

We further confirmed the nephroprotective of GP on mitigating CP-induced apoptotic cell death by TUNEL staining in the paraffin embedded kidney sections. In fact, there was marked apoptosis characterized by increased TUNEL positive cells in animals that were administered CP. Interestingly, GP elicited marked nephroprotective action by mitigating the apoptosis, which was evidenced by reduced number of TUNEL positive cells.

![TUNEL staining images](image)

Figure 3.9: Shows the representative TUNEL staining images in the respective group of animals. White arrowhead indicates the TUNEL positive cells. Magnification is X100
3.10 Effect of GP Treatment after the Established Renal Tissue Injury

In this section, we have investigated the therapeutic effect of GP on mitigating/reversing the established renal tissue injury imparted by CP. To our surprise, GP significantly attenuated renal tissue injury, which was evidenced by diminished levels of serum BUN and creatinine levels (Figure 3.10).

![Figure 3-10: GP exhibits therapeutic effect against CP-induced renal tissue injury. (A) Serum BUN levels and (B) serum creatinine levels in respective group of animals. n=6/group; # P < 0.001 vs. Veh/GP; * P < 0.001 vs. CP.](image-url)
3.11 Therapeutic effect of GP on Renal Histology

The therapeutic effect of GP on abrogation of CP-induced nephrotoxicity was confirmed by PAS staining of respective paraffin embedded kidney sections. In fact, GP inhibited CP-induced tubular necrosis, degeneration, and renal inflammation (Figure 3.11).

Figure 3.11: The representative images of PAS stained paraffin embedded kidney sections from the respective group of animals. Asterisk symbol denotes tubular necrosis with cast formation. Arrowhead denotes tubular epithelial anoikis and dashed arrow indicated the inflammation foci. Magnification is X 200
Chapter 4: Discussion

Cisplatin (CP) is a potent chemotherapeutic agent that induces AKI by recruiting oxidative stress, inflammation, and cell death pathways (Yolanda I. Chirino & Pedraza-Chaverri, 2009). Previous studies have reported that GP elicited anti-oxidant and anti-inflammatory activity in various pre-clinical studies (S. X. Yu et al., 2015). In this study, we have investigated the potential nephroprotective actions of GP against CP-induced nephrotoxicity. Animals administered with CP revealed marked tubular necrosis, degeneration, and inflammation. Treatment with GP significantly ameliorated renal tissue injury. Various mediators such as ROS, pro-inflammatory cytokines and adhesion molecules, can activate leukocytes. In fact, we have observed reduced renal inflammation, which corroborated with diminished leukocyte storming to the site of tissue injury (Faubel et al., 2007) (Donnahoo et al., 1999).

GSH is the pivotal endogenous antioxidant molecule that maintains the redox homeostasis in the cells and tissues (Atessahin, Yilmaz, Karahan, Ceribasi, & Karaoglu, 2005). GSH protects the biomolecules from oxidative tissue damage by scavenging ROS (Atessahin, et al., 2005). Previous studies had reported depletion of GSH by CP via forming direct adducts (Yilmaz et al., 2004). Decreased GSH levels in the renal tissues further perpetuates CP-induced renal damage. Herein, we have observed that CP-induced GSH depletion was inhibited by GP treatment. In fact, our findings are consistent with previous reports that GP could restore the cellular thiol pool. Diminished endogenous antioxidant reserve capacity is often observed in patients receiving cancer chemotherapy. In particular, this scenario is more pronounced in subjects on CP regimen.
ROS can be generated by endogenous or exogenous sources. In particular, NADPH oxidase has been implicated as key source for CP induced O$_2^•$ in the renal tissues (Y. Wang, et al., 2015). It is pertinent to note that NADPH oxidase (phagocyte oxidase) is highly expressed in leukocytes, particularly in macrophages and neutrophils (Y. Wang, et al., 2015). In our present study, we have observed that GP inhibited NADPH oxidase activation by CP, probably via mitigation of leukocyte activation. Levels of 4HNE depict the ROS mediated lipid peroxidation. Previous study has shown that SOD plays pivotal role in limiting ROS-induced tissue damage (B. P. Yu, 1994). GSH participates in regeneration of cellular lipid molecules via reacylation of cell membrane components (B. P. Yu, 1994). Therefore, to investigate the antioxidant effects of GP against CP-induced renal tissue damage, we have determined GSH levels, SOD activity, and 4HNE content. In fact, we observed that GP significantly inhibited oxidative stress by restoring GSH levels, SOD activity and diminishing 4HNE accumulation.

ONO$O^-\,$ is an important mediator implicated in the reactive nitrogen species mediated tissue injury. ONOO$^-\,$ generation is accelerated in cells and tissues, due to the lack of endogenous defense system available to detoxify this noxious and highly reactive oxidant molecule (Speckmann, Steinbrenner, Grune, & Klotz, 2016) (Trujillo, Ferrer-Sueta, & Radi, 2008). The other factor contributing to enhanced generation of ONOO$^-\,$, could occur in scenario wherein, NO and O$_2^•$ are simultaneously generated by activated leukocytes (Pál Pacher, et al., 2007). Therefore, it is pertinent to note that genetic ablation of inducible nitric oxide synthase (iNOS) or treatment with chemical ONOO$^-\,$ decomposition catalysts
prevented CP-induced renal tissue damage (Y. I. Chirino, Hernandez-Pando, & Pedraza-Chaverri, 2004) (Y. I. Chirino et al., 2008). Our findings are also in agreement with these observations, since GP inhibited accumulation of 3NY in CP treated animals.

Previous studies have indicated that production of pro-inflammatory cytokines is mediated by NFκB activation (Barry, Behnke, & Eastman, 1990). In this study, we have observed that GP attenuated pro-inflammatory cytokine expression in the renal tissues. However, the exact role of NFκB in this context needs to be explored further. ROS has been reported to be the key mediator involved the activation of MAPK (Persons, Yazlovitskaya, Cui, & Pelling, 1999) (G. Ramesh & W. B. Reeves, 2005). CP has been shown to stimulate the hydroxyl radical generation (H. Liu & Baliga, 2003). Production of hydroxyl radical by CP in renal tissues, is demonstrated due to the release of iron from the heme group of cytochrome P 450 2E1 (H. Liu & Baliga, 2003). Furthermore, previous studies have reported that genetic ablation of TNF-α resulted in decreased ROS generation, MAPK activation and cell death pathways activated by CP in renal tissues (Zhang, Ramesh, Norbury, & Reeves, 2007). In fact, our findings accede with these observations, because GP attenuated leukocyte activation, ROS generation and MAPK activation.

CP targets neoplastic and naïve cells resulting in DNA damage. To counteract the DNA damage, DNA repair enzymes are pressed in to service. PARP is a ubiquitous nuclear enzyme that actively participates in DNA repair mechanism (Bai, Nagy, Fodor, Liaudet, & Pacher, 2015). However during the enhanced tissue injury, hyper activation of PARP results in depletion of cellular ATP levels, that activates extrinsic pathway of apoptosis (Pacher & Szabo, 2008). Interestingly, either
pharmacological inhibition of PARP or genetic ablation, protected the mice from CP-induced renal tissue damage (P. Mukhopadhyay et al., 2011). In our present study, we have also observed that GP inhibited extrinsic pathway of cell death via inhibition of PARP, caspase3 activation and DNA fragmentation. In summary, findings emanating from the present study indicated that inhibition of oxidative stress, inflammation, and apoptosis pathways by GP could represent as novel strategy to counteract CP-induced AKI.
Chapter 5 : Conclusions and Future Directions

1. To our knowledge, this is the first study to report the nephroprotective activity of GP against CP-induced renal tissue injury. Specifically GP attenuated CP-induced renal toxicity via mitigation of oxidative stress, inflammation, and cell death pathways.

2. GP exhibited both prophylactic and therapeutic nephroprotective effects against CP-induced renal tissue damage.

3. Further studies are warranted to completely understand the molecular mechanisms governing GP nephroprotective activity.

4. To establish the GP renoprotective actions, further studies are warranted, in rodents harboring tumors that are sensitive to CSP. This is to ascertain that GP beneficial effects do not compromise/interfere with the CP anti-tumor activity.
References


List of Publications