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# THE ROLE OF HAEM OXYGENASE-1 MODULATION BY ISOTHIOCYANATES IN DRUG-INDUCED RENAL FAILURE

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*To my parents, brothers and sisters for being always besides me*

*To my wife for her love and outstanding support*

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# SUMMARY

The effect of two isothiocyanates (ITCs), S-(N-benzylthiocarbamoyl)-L-cysteine (BTCC) and S- (N-3- phenylpropylthiocarbamoyl)-L-cysteine (PTCC), in modulating the haem oxygenase-1 (HO-1) pathway was investigated in order to identify a possible strategy for protecting renal cells and tissue against drug-induced toxicity. HO-1 is a versatile inducible enzyme that provides cytoprotection by generating the signalling molecule carbon monoxide (CO) and the antioxidant couple biliverdin (BV) and bilirubin (BR). When cultured renal tubular epithelial (LLC-PK1) cells were exposed to BTCC and PTCC, an increase in HO-1 expression and activity was observed in a time- and concentration-dependent manner. This effect required activation of MAPKs, PKC and PI-3K signal transduction pathways. Interestingly, both BTCC and PTCC caused apoptosis in LLC-PK1 cells when used at concentrations above 20 μM but rendered these cells resistant to apoptotic cell death when low concentrations (5 and 10 μM) were used. In fact, pre-incubation of LLC-PK1 cells with 5 or 10 μM BTCC or PTCC conferred major resistance to apoptosis mediated by cisplatin (CP), an effective anti-neoplastic agent known to cause nephrotoxicity by damaging proximal tubular cells. The anti-apoptotic effect mediated by ITCs against CP was abolished by tin protoporphyrin IX (SnPPIX), an inhibitor of HO activity. Administration of BTCC or PTCC (25 mg/kg i.p.) to CP-treated rats markedly attenuated the resulting renal dysfunction as indicated by a significant decrease in both serum urea and creatinine levels and by a marked reduction in proximal tubular cell necrosis. Once again, administration of SnPPIX to CP-treated rats abolished the reno-protective effect of ITCs. CP-treated cells were

also incubated with increasing concentrations of BV, BR or CO releasing molecule-3 (CO-RM-3), a newly discovered CO carrier. Among the three by-products of HO, we found that only CORM-3 decreased CP-mediated apoptosis in a concentration-dependent manner. Moreover, administration of CORM-3 to CP-treated rats *in vivo* normalized renal function, improved the histological profile and completely alleviated CP-induced tubular cell necrosis indicating that the HO-1-mediated protective effect against CP-renal dysfunction is mediated by CO.

## PUBLICATIONS ORIGINATING FROM THIS THESIS

1. **Tayem Y, Johnson TR, Mann BE, Green CJ, Motterlini R**. Protection against cisplatin-induced nephrotoxicity by a carbon monoxide-releasing molecule. *Am J Physiol Renal Physiol* 290: F789-94, 2005.

2. **Sandouka A, Balogun E, Foresti R, Mann BE, Johnson TR, Tayem Y, Green CJ, Fuller B, Motterlini R**. Carbon monoxide-releasing molecules (CO-RMs) modulate respiration in isolated mitochondria. *Cell Mol Biol* 51: 425-32, 2005.

## EDITORIALS FOCUSING ON THE DATA OF THIS THESIS.

**Hill-Kapturczak N, Agarwal A**. Carbon monoxide: from silent killer to potential remedy. *Am J Physiol Renal Physiol* 290:F787-8, 2005.

# PRESENTATIONS OF THE DATA ORIGINATING FROM THIS THESIS.

1. **Motterlini R, Sandouka A, Tayem Y, Fuller B, Mann BE, Foresti R, Green CJ.** Beneficial effects of carbon monoxide-releasing molecules (CO-RMs) on stress-induced renal injury. The winter meeting of the Pathological Society of Great Britain and Ireland, January 2006, Cambridge, UK.

2. **Motterlini R, Sandouka A, Tayem Y, Fuller B, Mann BE, Foresti R, Green CJ.** Therapeutic actions of carbon monoxide-releasing molecules (CO-RMs) in renal injury. The fourth international congress on haem oxygenase, October 2005, Boston, USA.

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| | |
|---|---|
| Act D | Actinomycin D |
| ADH | Anti-diuretic hormone |
| ADP | Adenosine diphosphate |
| AIF | Apoptosis-inducing factor |
| AIN | Acute interstitial nephritis |
| AKI | Acute kidney injury |
| ALA | Aminolevulinic acid |
| Apaf-1 | Apoptosis activating factor-1 |
| ARE | Anti-oxidant response element |
| ATN | Acute tubular necrosis |
| ATP | Adenosine triphosphate |
| AP-1 | Activator protein-1 |
| BIR | Baculovirus inhibitor of apoptosis repeats |
| BMK-1 | Big MAPK-1 |
| BR | Bilirubin |
| BVIX-α-FeIII | Ferribiliverdin IX- α |
| COX | Cyclooxygenase |
| CHX | Cycloheximide |
| Cyt C | Cytochrome c |
| Diablo | Direct inhibitor of apoptosis protein (IAP)-binding protein with low pI |
| DPX | Depex polystyrene |
| BV | Biliverdin |
| cGMP | Cyclic guanosine monophosphate |
| CNC | Cap "n" collar |
| CO | Carbon monoxide |
| CT | Collecting tubule |
| CP | Cisplatin |
| DCT | Distal convoluted tubule |
| DD | Death domain |
| DED | Death effector domain |
| DEFT | Death effector domain containing testicular molecule |
| DR | Death receptor |
| EpER | Electrophile response element |
| EPO | Erythropoietin |
| ERK | Extracellular-regulated kinase |

| | |
|---|---|
| FADD | Fas-associated death domain |
| $Fe^{+2}$ | Iron |
| FLASH | FLICE-associated huge protein |
| FLICE | FADD-like IL-1β converting enzyme |
| FLIP | Flip-like inhibitory protein |
| GC | Guanylate cyclase |
| GSH | Glutathione |
| GSHPx | Glutathione peroxidase |
| GST | Glutathione-S-transferase |
| HO | Haem oxygenase |
| $H_2O_2$ | Hydrogen peroxide |
| IAP | Inhibitor of apoptosis |
| ICAM | Intercellular adhesion molecule |
| IL | Interleukin |
| I/R | Ischaemia/reperfusion |
| Keap1 | Kelch like E associated protein1 |
| MAPK | Mitogen-activated protein kinase |
| MRC | Membrane receptor complex |
| MRP-1 | Multi-drug resistance protein-1 |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NO | Nitric oxide |
| NQO | NADPH quinone oxidoreductase |
| $O_2^{\cdot-}$ | Superoxide |
| $OH^{\cdot}$ | Hydroxyl radical |
| PCT | Proximal convoluted tubule |
| PEITC | Phenyl ethyl isothiocyanate |
| $PGI_2$ | Prostaglandin $I_2$ |
| PTCC | S- (N-3-phenylpropylthiocarbamoyl)-L-cysteine |
| PIK | Phophatidyl inositol kinase |
| PKC | Protein kinase C |
| $QH^{\cdot}$ | Ubisemiquinone radical |
| QR | Quinone reductase |
| UCP | Uncoupling protein |
| ROI | Reactive oxygen intermediates |
| SMAC | Second mitochondrial-derived activator of caspase |
| SOD | Superoxide dismutase |
| SODD | Silencer of death domain |
| TNF | Tumour necrosis factor |

# 1 INTRODUCTION

## 1.1 Urinary system

The major function of the urinary system is to maintain fluid and ion homeostasis by sustaining a critical balance between intake and expenditure of water and electrolytes.

## 1.2 Anatomy of the kidney

The kidney is a retroperitoneal organ that lies on the ventral surface of the quadratus lumborum muscle and lateral to the psoas muscle and vertebral column (79). The kidney is covered directly by a fibrous capsule (renal or true capsule) that can be stripped readily from the surface and by the peritoneal fascia of Gerota (known as the false capsule) (79). Furthermore, the kidney is surrounded by pararenal fat which is most prominent in the posterolateral region (79). No structural differences have been demonstrated between mammalian kidneys (177).

### *1.2.1 Renal internal structure*

The kidney is composed of two structurally distinct parts which are the outside cortex and the inside medulla (Fig 2-1) (79). The cortex is located directly below the renal capsule and extends between the renal pyramids as the renal columns (columns of Bertin) (79). The inner medulla, however, is composed of renal pyramids (known also as pyramids of Malpighi) whose tips terminate at renal papillae that merge to form the renal pelvis and end up forming the ureter (79).

The collecting system of the kidney is composed of 5-11 cup-shaped structures called minor calyces that surround each renal papilla, 2-3 major calyces formed by the fusion of minor calyces and the renal pelvis which is the main urine collection chamber and is continuous with the ureter at the ureteropelvic junction (79). The walls of the calyces, pelvis and ureters are lined with smooth muscle cells that can contract to force urine towards the bladder by peristalsis.

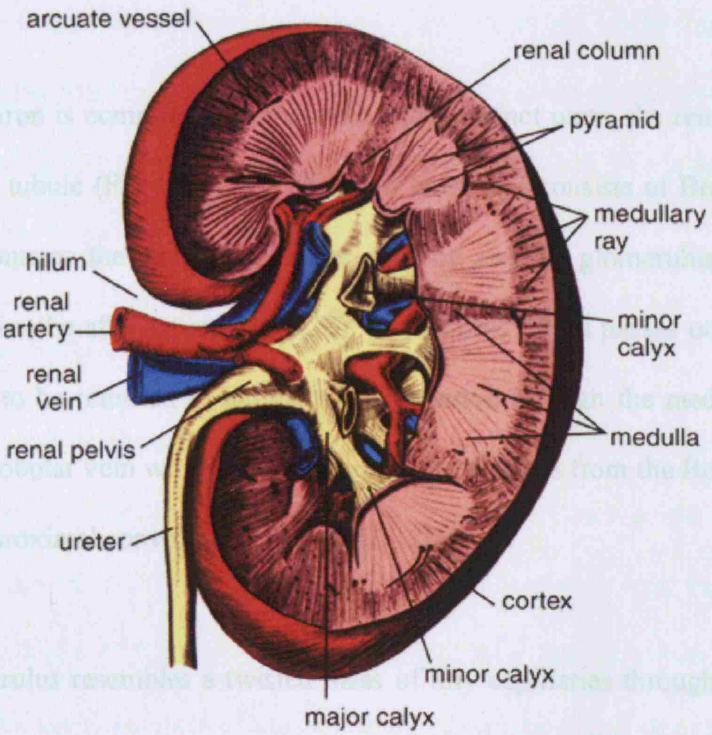



**Figure 1-1 Schematic diagram of human kidney anatomy adapted from the Department of Anatomy and Cell Biology, Indiana University, USA. Web page: Phttp://anatomy.iupui.edu/courses/histo_D502/D502f04/lecture.f04/urinaryf04/urinaryf04.html.**

### *1.2.2 The nephron*

The nephron is the functional unit of the kidney in which all the filtration processes take place. Roughly, one million nephrons exist in a normal human kidney of which two groups have been characterized; the superficial cortical and the juxtamedullary nephrons (38). The superficial nephrons are concentrated in the cortex and typically have a short loop of Henle (LOH) while the juxtamedullary nephrons lie deep in the cortical side of the cortico-medullary region and have a long (LOH).

The nephron is composed of two structurally distinct parts; the renal corpuscle and the renal tubule (Fig 1-2) (38). The renal corpuscle consists of Bowman's capsule which contains the main filter of the nephron and the glomerulus which receives blood from the afferent arteriole (38). The filtered blood passes out to the efferent arteriole to be returned to the general circulation through the medullary plexus to the interlobular vein while the resulting filtrate diffuses from the Bowman's capsule into the proximal convoluted tubule (PCT) (38).

The glomerulus resembles a twisted mass of tiny capillaries through which blood is filtered. The glomerular capillary membranes are permeable to water and soluble wastes which pass to the Bowman's space (the area separating Bowman's capsule and glomerulus) and then excreted out of the Bowman's capsule as urine (38).

The walls of the nephron are made up of a single layer of epithelial cells. In Bowman's capsule, there are specialized epithelial cells called podocytes which

surround the glomerular capillaries and contribute to the filtration barrier (38). The cells lining the convoluted tubules carry a brush border of microvilli on their luminal surface and contain a large number of intracellular mitochondria. These features are particularly pronounced in the proximal convoluted tubule (PCT) and provide a large surface area and a rich ATP supply to aid in the active transport that takes place in this part of the nephron (38). In contrast, in the descending part of the LOH the epithelium is flat with few organelles consistent with a low level of active transport and great permeability to fluids and electrolytes in this part of the nephron. However, the cells of the thick part of the LOH are more like those in the tubules. These cells are involved in active ion transport but have low permeability to water manifested by tight junctions between adjacent cells.

### *1.2.3 Renal tubule*

The renal tubule is composed of three continuous but functionally distinct parts. The tubule starts from Bowman's capsule as the PCT then extends as the LOH and finally ends with the DCT (Fig 8-2). Urine then leaves nephrons and flows through the collecting tubules (CTs) and collecting ducts (CDs). The CDs then fuse when they approach the renal pelvis to form larger papillary ducts that deliver urine into the minor calyces via papillae of the pyramids (38).

PCT is the longest and most active part of the renal tubule in which 75% of the reabsorbtion processes take place (38). Most of the PCT length is located in the cortex but a small part of it (*vasa recta*) lies in the medulla and continues as the LOH (38).

PCT contains S1, S2 and S3 cells which are active in the renal reabsorption and secretion mechanisms (259).

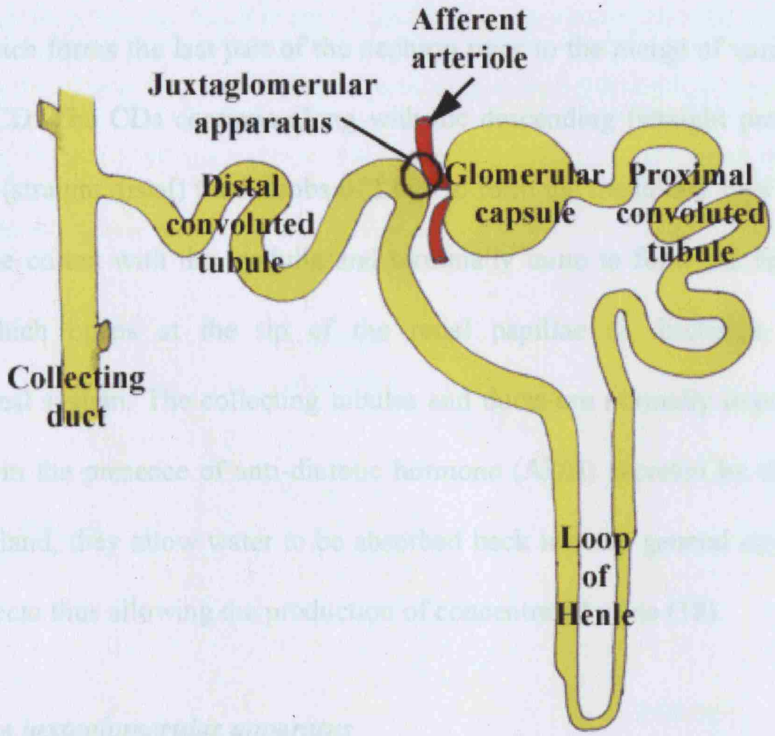



**Figure 1-2 An illustration of the renal nephron adapted from the Western Kentucky University, USA . Web page: http://bioweb.wku.edu/courses/Biol131/stokes/131f96chap26.html.**

LOH is a U-shaped tubule, extending through the medulla from the end of the PCT to the beginning of the DCT (38). It begins with the descending limb starting as a thick-walled segment called the proximal straight tubule, followed by a thin-walled part called the thin or attenuated tubule. This is followed by the ascending limb, which

sometimes includes the distal end of the attenuated tubule and always ends with a long thick-walled segment called the distal straight tubule.

DCT is a continuum of the LOH when it enters the medulla (38). DCT continues as the CT which forms the last part of the nephron prior to the merge of various CTs to form the CD. The CDs continue along with the descending (straight proximal) and ascending (straight distal) thick limbs of LOH to form the medullary rays (79) which connect the cortex with the medulla and terminally unite to form the large duct of Bellini which opens at the tip of the renal papillae to discharge urine into pelvicalyceal system. The collecting tubules and ducts are normally impermeable to water but in the presence of anti-diuretic hormone (ADH) secreted by the posterior pituitary gland, they allow water to be absorbed back into the general circulation via the *vasa recta* thus allowing the production of concentrated urine (38).

### *1.2.4 The juxtaglomerular apparatus*

The juxtaglomerular apparatus (JGA) is a small endocrine organ associated with individual nephrons within the kidney and comprised of an area of specialized epithelial cells in the thick ascending limb of the LOH called the *macula densa* and specialized vascular cells known as the juxtaglomerular (JG) cells which secrete renin (Fig 1-3) (38). The JG cells are found in the afferent arterioles of the glomerulus and act as an intra-renal pressure sensor. The macula densa senses fluid flow rate in the DCT and secretes a locally active vasopressor which acts on the adjacent afferent arteriole to decrease glomerular filtration rate (GFR). Renin secretion by the JGA

depends on several factors including fall in $Na^+$ concentration, prostaglandins, sympathetic nerve activity, circulating catecholamines and a fall in blood pressure. Renin secretion is decreased by a rise in $Cl^-$ concentration, angiotensin II, ADH and increased blood pressure. Another set of cells known as mesangial cells are positioned adjacent to the glomerular capillary basement membrane and control the surface area of capillary filtration by contraction. Moreover, they act as phagocytes that remove macromolecules which escape from capillaries.

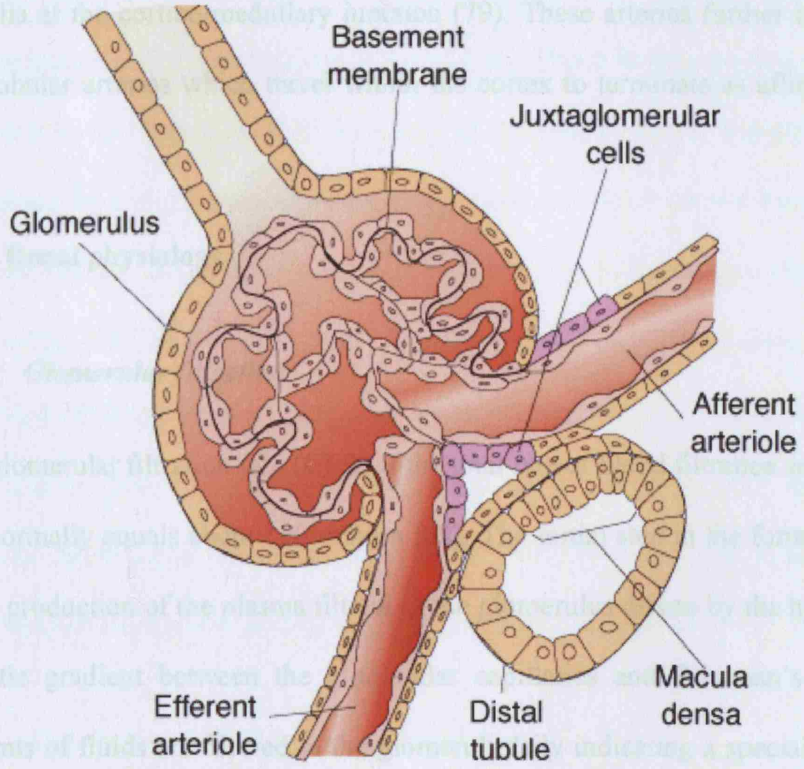



**Figure 1-3 An illustration of the juxtaglomerular apparatus adapted from Perth's Pathophysiology, Concepts of Altered Health State, seventh edition, Lippincott Williams & Wilkins, 2005.**

### *1.2.5 Renal vasculature*

The kidney receives 25% of cardiac output and major blood supply to the kidney is provided by the renal artery which branches into 5 segmental arteries (79). Collectively, there are 4 anterior segmental arteries supplying the anterior segments of the kidney and 1 posterior segmental artery which supplies the posterior parts (79). The segmental arteries further branch inside the renal columns forming the interlobar arteries which branch into arcuate arteries which travel between the cortex and medulla at the cortico-medullary junction (79). These arteries further branch to give interlobular arteries which travel within the cortex to terminate as afferent arterioles (79).

## 1.3 Renal physiology

### *1.3.1 Glomerular filtration*

The glomerular filtration rate (GFR) is the total rate of blood filtration in both kidneys and normally equals about 120 ml/min (62). The initial step in the formation of urine is the production of the plasma filtrate in the glomerulus driven by the hydrostatic and osmotic gradient between the glomerular capillaries and Bowman's space. Huge amounts of fluids are filtered in the glomeruli daily indicating a specialized structure favouring filtration. The kidneys are adapted to a high level of filtration due to the large glomerular surface area and low resistance to the movement of water across the wall due to the fenestrated structure of the glomerular endothelium (38). This barrier is highly permeable to water and electrolytes but is impermeable to large molecules

such as plasma proteins. Therefore, the fluid entering Bowman's space has the same composition as plasma but is poor in proteins.

### *1.3.2 Forces controlling glomerular filtration*

Hydrostatic pressure within the glomerular capillaries is very high (50 mmHg) and is largely caused by the high outflow resistance offered by the efferent arteriole resulting in an increase in the upstream pressure (38). Moreover, there is very little difference in pressure between the afferent and efferent arterioles as a result of the low resistance in the glomerular capillaries (38). These factors create a net difference in pressure reaching 40 mmHg favouring glomerular filtration along the glomerular capillaries. In addition, there is an osmotic pressure which results from the fact that proteins cannot cross the glomerular wall. At the afferent arteriolar end of the glomerulus this equals the normal plasma colloid osmotic pressure (15 mmHg) creating a net filtration (hydrostatic-osmotic) gradient of about 15 mmHg at this point. It should be borne in mind, however, that along the glomerulus the hydrostatic pressure might equal the osmotic pressure but never exceeds it so there is no absorption of fluid at any point along the glomerular capillaries.

### *1.3.3 Regulation of GFR and renal blood flow*

The physiological control of GFR is complex and not fully understood. However, it has been shown that GFR is increased by changes in the glomerular capillary hydrostatic pressure, glomerular capillary flow rate and glomerular capillary surface

area (38). Moreover, renal blood flow, neural stimulation and renin-angiotensin-aldosterone system play a role in controlling GFR (62).

Renal blood flow is effectively maintained at 1.2 L/min by the mechanism of autoregulation (62). In this process, when blood pressure falls afferent arteriolar resistance drops to maintain renal blood flow. However, at very low blood pressure such as in septic shock autoregulation fails to maintain renal blood flow and neural stimuli manifested by reflex stimulation of sympathetic nerves reduce GFR and hydrostatic pressure by constricting the afferent arteriole causing an increase in renal injury. In addition, GFR is regulated by renin release with the concomitant production of angiotensin II and aldosterone as discussed below.

### *1.3.4 Tubular reabsorption*

Tubular reabsorption is an extremely efficient process because out of 125 ml of plasma filtered by the glomeruli 124 ml is reabsorbed through the renal tubules. Tubular reabsorption is a transepithelial process carried out in the PCT, LOH, DCT and CD (38). Water, electrolytes, glucose and amino acids are reabsorbed either passively along their electrochemical gradient, by simple diffusion, facilitated diffusion or osmosis (38). Moreover, they might be actively reabsorbed by cotransporters which often couple the free energy released by the energetically favourable movement of $Na^+$ along its electrochemical gradient to the transport of substances such as amino acids and glucose against their electrochemical gradient. Collectively, the entire length of the renal tubule is involved in reabsorption but the

cells of different regions of the renal tubule are adapted to perform specific transport functions, and consequently, the absorptive capacities of the different regions of the renal tubule differ (62).

The proximal tubule is the major site of reabsorption (62). Normally, all the glucose, lactate and amino acids from the filtrate are reabsorbed in the PCT, together with 65% of the $Na^+$ by symport with other solutes or by a $Na^+$-$H^+$ antiporter. Because $Na^+$ movement is coupled with water transport, a similar percentage of water moves into the tubule. In addition, 90% of the $HCO_3^-$, 50% of the $Cl^-$, and 55% of the $K^+$ are reabsorbed in the PCT. Similarly, the majority of the active transport-dependent reabsorption of electrolytes such as calcium, phosphate and magnesium occurs in the PCT (62).

In the LOH, water reabsorption is no longer coupled to solute reabsorption. Water can leave the descending limb but not the ascending limb and these permeability differences play an essential role in the kidney's ability to produce dilute and concentrated urine. A further 25% of the $Na^+$, 10% of the water, 35% of the $Cl^-$, and 30% of the $K^+$ are reabsorbed in this part of the tubule. While $K^+$ is reabsorbed in the ascending limb and secreted from the descending limb, $Na^+$ is reabsorbed into the lumen via a $Na^+$-$K^+$-$2Cl^-$ cotransporter, $Na^+$-$H^+$ antiporter and via a paracellular route (62).

When the filtrate reaches the DCT it contains only 10% of the original filtered NaCl, and 25% of the water. $Na^+$-$Cl^-$ cotransporters reabsorb most of the $Na^+$ and $Cl^-$ and the

remaining $Na^+$ uptake is hormonally controlled by aldosterone (62). This hormone causes the principal cells of the collecting ducts to open or produce more $Na^+$ channels and more $Na^+$-$K^+$ transporters. As a result, virtually no $Na^+$ leaves the body in urine, and as a secondary effect, more $K^+$ is removed. In contrast to aldosterone, atrial natriuretic peptide (ANP), a hormone released by atrial myocytes under conditions of raised blood pressure, inhibits $Na^+$ reabsorption in the collecting ducts whereas ADH released from the posterior pituitary increases water permeability in the CD (62).

### *1.3.5 Tubular secretion*

Secretion is an essential mechanism for removing unwanted particulates from the plasma. Substances such as $H^+$, $K^+$, creatinine, $NH_4^+$ and certain organic acids move from the blood of the peritubular capillaries through the tubule cells or from the tubule cells themselves into the filtrate (38). With the exception of $K^+$ (which is mainly secreted from the DCT and CD), the proximal tubule is the main site of secretion. Tubular secretion plays a major role in the removal of substances not already in the filtrate such as drugs, removing urea and uric acid, excessive $K^+$ and controlling blood pH. In this context, when blood becomes acidic, the renal tubule cells actively secrete $H^+$ into the filtrate and retain more $HCO_3^-$, and $K^+$. By contrast, if blood becomes alkaline, $Cl^-$ is reabsorbed and more $HCO_3^-$ leaves the body in urine.

## 1.4 Hormones of the kidney

### *1.4.1 Renin-angiotensin system*

The renin-angiotensin system is a hormone system that helps regulate long term blood pressure and blood volume in the body (Fig 1-5) (62). This system is activated when there is loss of blood volume or a drop in blood pressure such as in cases of haemorrhage or dehydration states. In these cases, a fall in JGA perfusion causes the JG cells to release renin which stimulates the production of angiotensin II that in turn increases the release of aldosterone from the adrenal gland.

### *1.4.2 Aldosterone*

Aldosterone is a steroid hormone secreted by the adrenal gland. This hormone plays a major role in regulating the body's electrolyte balance by decreasing the rate of $Na^+$ excretion in the kidney (with accompanying retention of water) while it increases $K^+$ excretion (62). The secretion of aldosterone appears to be regulated by the concentration of $Na^+$ and the blood flow to the kidney which regulates renin release. This hormone in turn causes the conversion of the inactive angiotensinogen in the blood into angiotensin I which is rapidly converted into the active form (angiotensin II) by the angiotensin converting enzyme in the blood and in endothelial cells (231). This peptide, in turn, stimulates the secretion of aldosterone by the adrenal cortex.

### *1.4.3 Anti-diuretic hormone*

Anti-diuretic hormone (ADH) or vasopressin is produced in the hypothalamus but is stored in the posterior pituitary. By acting on the DCT and CD, ADH decreases the amount of water excreted by the kidneys thus allowing the production of concentrated urine (62). ADH secretion by the pituitary is regulated by neural connections from the hypothalamus which are believed to monitor either the volume of blood passing through it or the concentration of water in the blood. Failure of the pituitary to produce ADH results in diabetes insipidus which is characterized by dilute polyuria.

### *1.4.4 Erythropoietin*

Most erythropoietin (EPO) is produced in the renal cortex and to a lesser extent in the liver (mainly in the foetus), brain and uterus. The production of this hormone is stimulated by hypoxia of the interstitial cells of the peritubular capillaries in the kidney (62). Renal tubular epithelial cells secrete EPO because anaemic blood is unable to deliver enough oxygen from the peritubular capillaries to the highly active tubular cells. This hormone subsequently binds to specific receptors on the surface of red cell precursors in the bone marrow stimulating their maturation and eventually improving oxygen supply to the kidney.

### *1.4.5 Calcitriol*

Calcitriol (vitamin D) is a fat-soluble steroid hormone precursor that contributes to the maintenance of normal levels of calcium and phosphorus in the blood in conjunction with parathormone and calcitonin (62). Vitamin $D_2$ is derived from

ergosterol in the diet whereas vitamin $D_3$ is derived from cholesterol via 7-dehydrocholesterol. UV light is responsible for the production of vitamin $D_2$ and $D_3$ in the skin while the active form of the vitamin (calcitriol) is synthesized from either $D_2$ or $D_3$ in the kidney.

### 1.5 Acute kidney injury

Acute kidney injury (AKI) (formerly known as acute renal failure) is defined as a sustained decrease in renal function resulting in retention of nitrogenous (urea and creatinine) and non-nitrogenous waste products that is accompanied by metabolic disturbances such as metabolic acidosis, hyperkalaemia and changes in body fluid balance. AKI ranges from a severe rise in renal dysfunction leading to a rise in serum urea and creatinine that requires dialysis to a minor increase that does not require intervention. Based on serum creatinine concentration, a patient is said to have AKI if he or she has an elevation of serum creatinine and plasma urea above the normal reference values (>120 μmol/l and >8 mmol/l, respectively) and/or urine output below 800 ml/24 h or both (28).

AKI can be broadly classified into three categories: pre-renal, renal and post-renal. In the pre-renal form, renal hypoperfusion as a result of interruption of renal blood flow leads to a reduction in GFR and consequent elevation of serum creatinine and blood urea concentrations. Post-renal AKI, however, occurs as a result of obstruction of the urinary collecting system by either intrinsic or extrinsic masses. The remaining

patients have the renal form in which structures of the nephron, such as the glomeruli, tubules, vessels, or interstitium are affected.

### *1.5.1 Acute pre-renal failure*

Pre-renal azotaemia results from any disease process that is associated with a reduction in renal blood flow (renal hypoperfusion). This can result from true hypovolaemia such as in dehydration or haemorrhage or relative hypovolaemia which is characterized by a reduction in the effective circulating volume such as in low cardiac output heart failure, systemic vasodilatation, or intrarenal vasoconstriction (30). As a result, several neurohumoral vasoconstriction mechanisms are switched on to preserve renal blood flow (19). Specifically, various vasodilator agents such as prostaglandins and nitric oxide (NO) are produced and stimulate dilatation of pre-glomerular arterioles to maximize renal blood flow (72). Drugs that interfere with the autoregulation of renal blood flow and GFR such as non-steroidal anti-inflammatory drugs (NSAIDs) and angiotensin converting enzyme (ACE) inhibitors can provoke acute pre-renal failure (83; 188). Pre-renal azotaemia is typically reversible if the factors causing the renal hypoperfusion are quickly corrected. However, persistent renal hypoperfusion will ultimately lead to ischaemic acute tubular necrosis.

### *1.5.2 Drug-induced renal failure*

The kidney is the main excretory organ in the body and is therefore a frequent site of drug toxicity. Although drug-induced nephrotoxicity can involve one or more of the major structural components of the kidney including interstitium, blood vessels,

glomerulus and tubules, the last two are the most commonly affected resulting in tubulointerstitial nephritis which falls into two different categories. The first is acute interstitial nephritis (AIN) which results from an inflammatory reaction in the interstitium and tubules and is usually caused by allergic reaction to a drug in an idiosyncratic (non-dose-dependent) manner. The second form is acute tubular necrosis (ATN) which occurs due to direct toxicity of the offending drug or its metabolites. In contrast to AIN, this form is dose-dependent and is characterized by tubular injury in the absence of inflammation.

Over the last decade, there has been accumulating evidence for an important role of oxidative stress in the pathogenesis of drug-induced nephrotoxicity as a result of treatment with several therapeutic agents (21). For example, cisplatin (CP), gentamycin and cyclosporine A have been shown to induce iron ($Fe^{2+}$) release which exacerbates oxidative stress by generating excessive reactive oxygen intermediates (ROI) leading to renal tubular cell death by necrosis or apoptosis. The role of ROI and $Fe^{2+}$ in drug-mediated renal injury has been highlighted since $Fe^{2+}$ chelators and radical scavengers have been shown to preserve renal function (21; 160). Therefore, a lot of experimental work has been carried out to examine the potential of anti-oxidants to protect against nephrotoxic renal injury.

The inflammatory response that occurs in AKI plays an important role in renal damage (32; 33). Indeed, endothelial injury is followed by recruitment of inflammatory cells which release various inflammatory mediators and induce the

expression of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and P-selectin which in turn promote endothelial-leukocyte interactions (40). Furthermore, the proximal tubular epithelium can generate a number of mediators that enhance the inflammatory response. These include cytokines such as TNF-α, IL-6, IL-1β (40).

### *1.5.3 Acute interstitial nephritis*

AIN is a milder form of AKI which is rarely oliguric, rarely requires dialysis and often resolves after removal of the precipitating cause. Typically, patients present with signs of a hypersensitivity reaction manifested by fever and skin rash accompanied by renal insufficiency, eosinophiluria, proteinuria and sterile pyuria few weeks after the administration of a drug (65; 145). However, AIN might start earlier if the patient had been previously sensitized to the causual agent. Examples of drugs that cause AIN include several antibiotics and NSAIDs (112).

The pathogenesis of AIN involves hypersensitivity reaction to the causal agent because in most cases eosinophils have been shown to predominate in the interstitial infiltrate in addition to T cells and monocytes (212). It has been thought that certain drugs can act as haptens that induce an immune reaction leading to an inflammatory response. In addition to drug treatment, several bacterial and viral infections have been associated with AIN such as *Staphylococcus aureus*, *Escherichia coli*, salmonellosis, cytomegalovirus, polyomavirus, rubeola, *Toxoplasma gondii*, *Rickettsia rickettsii*, and *Leishmania donovani* (84; 294). Furthermore, AIN

sometimes manifest in conjunction with a variety of immune-mediated diseases such as sarcoidosis, systemic lupus erythematosus (SLE) (178; 214) and Sjögren syndrome (143).

### *1.5.4 Acute tubular necrosis*

Although the terms AKI and acute tubular necrosis (ATN) comprise two different entities, they are commonly used synonymously in the clinical setting. This disorder is largely caused by various nephrotoxic insults such as sepsis, ischaemia/reperfusion (I/R) injury and drug-induced nephrotoxicity and comprises separate histopathological and pathophysiological processes that account for the majority of the causes of AKI (195). ATN is caused by a wide range of pathological conditions but sepsis comprises the major cause especially as a part of multiple organ failure (195). Other causes include the administration of radio-contrast medium (195), major surgery (47) and drugs (212). The renal failure developing in patients with ATN is often severe, usually oliguric and in many cases requires dialysis. Typically the rise in serum urea and creatinine is accompanied by an increase in the fractional excretion of $Na^+$, mild proteinuria and bland urinary sediment.

ATN often has a well-defined sequence of events comprised of three phases. The initiation phase is characterized by an acute severe decrease in GFR with a sudden increase in renal function. The maintenance phase is characterized by a sustained severe reduction in GFR, and this phase continues for a variable length of time but most commonly it lasts for 1-2 weeks. The recovery phase is characterized by an

increase in urine volume (if oliguria was present during the maintenance phase) and a gradual decrease in renal function to its pre-injury level.

The histological features of ATN include vacuolation, effacement and loss of brush border in proximal tubular cells, focal areas of proximal tubular dilatation, appearance of patches of cellular regeneration and sloughing of tubular cells leading to tubular lumen obstruction. This is frequently accompanied by interstitial oedema and mild to moderate leukocyte infiltration. Although the disorder is named necrosis and is associated with severe functional impairment, necrotic cells are rarely a common finding and histological evidence of injury is limited in many cases and is restricted to the outer medullary region (mainly the S3 segment of the PCT and the medullary ascending limb of LOH).

Apoptosis has been reported in the distal and proximal tubule cells in both the ischaemic and nephrotoxic forms of human AKI (49). In addition, peri-tubular capillaries in the outer medulla have been shown to display striking vascular congestion and leukocyte accumulation (33). If the causal agent is discontinued before severe damage takes place, proximal tubular cells can undergo repair, regeneration and proliferation (114).

### *1.5.5 Pathophysiology of acute tubular necrosis*

The pathophysiology of ATN is complex and not fully understood. As a vital body organ, the kidneys receive 25% of cardiac output and most of this blood is directed to

the renal cortex but in established ATN, renal blood flow falls by 30–50%. Several vasoconstrictors have been implicated in the reduction of renal blood flow such as angiotensin II, thromboxane $A_2$, prostaglandin $H_2$, leukotrienes C4 and D4, endothelin 1, adenosine and increased sympathetic-nerve stimulation (254). The mechanisms that mediate these haemodynamic changes in ATN have been related primarily to endothelial injury (176) as a result of the enhanced release of vasoconstrictor endothelin and reduced release of vasodilatory endothelium-derived NO (254).

Tubular cell injury is most prominent in the straight portion of the proximal tubules and in the thick ascending limb of the LOH. The reduction in GFR that occurs from ischemic or nephrotoxic injury is a result not only of reduced filtration but also of casts and debris obstructing the tubule lumen causing back leak of filtrate through the damaged epithelium.

Treatment with nephrotoxic drugs leads to tubule cell death by necrosis or apoptosis which both usually coexist and may represent two extremes of a continuum (136). Necrosis is characterized by loss of membrane integrity, cytoplasmic swelling, nuclear pyknosis, cellular fragmentation and inflammation. Apoptosis is characterized by cytoplasmic and nuclear shrinkage, DNA fragmentation and breakdown of the cell into apoptotic bodies which are rapidly cleared by phagocytosis. Mounting evidence indicates that apoptosis is the major mechanism of early tubule cell death in contemporary clinical AKI (136). Due to its importance as a mode of cell death in AKI, considerable attention has recently been directed towards studying the molecular

pathways involved in renal tubule cell apoptosis. Therefore it has been thought that inhibition of apoptosis might be of therapeutic value for the prevention of renal failure (26). Moreover, AKI induced by drugs and ischaemia/reperfusion (I/R) injury has been found to stimulate the production of ROI which are associated with induction of tubular cell apoptosis by stimulating caspases and/or endonucleases.

## 1.6 Cisplatin-induced renal failure

For 25 years, CP has been one of the few available curative chemothetherapeutic agents used in clinical practice (Fig 1-4). CP shows particular promise for the treatment of several solid tumours particularly head and neck, testicular, ovarian, bladder and small cell lung cancer (101). This alkylating anti-neoplastic agent forms strong electrophilic intermediates that act via nucleophilic reactions to form inter-strand and intra-strand DNA cross-links (314).

Despite being one of the frontline anti-neoplastic agents, the use of CP is limited by its nephrotoxicity which develops even after a single dose in excess of 5 mg/kg (156) while further treatment with CP can cause chronic renal impairment. Ten min following exposure to CP, this agent is concentrated to high levels in different parts of the renal juxtamedullary and cortical areas but the highest concentration is in the S3 segment (150). The abovementioned nephrotoxic effects of CP have encouraged the search of other platinum compounds that could be used with less toxicity whilst maintaining similar anti-cancer activity. Unfortunately this has not been successful

and only a minority of the platinum compounds tested entered the clinical trial phase (97).

### *1.6.1 Pathophysiology of cisplatin-induced nephrotoxicity*

In a similar sense to the pathophysiological changes in response to renal I/R injury, administration of CP progressively reduces renal blood flow prior to any changes in GFR indicating that renal vasoconstriction might be an early and important event in mediating CP nephrotoxic effects (20). Interestingly, both insults produce ATN and similar changes in gene profile (243).

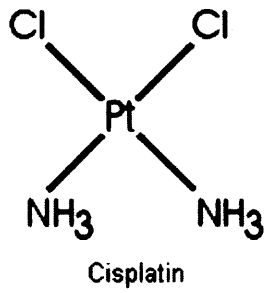



**Figure 1-4 The structure of cisplatin adapted from the Laboratory of Molecular Biology, the Medical Research Council, Cambridge, UK. Web page: http://www2.mrc-lmb.cam.ac.uk/personal/sl/Html/Properties.html.**

CP binds cellular sulfhydryl groups and inactivates thiol-containing enzymes leading to disruption of cellular redox status as a result of several factors including increased intra-cellular ROI generation, suppressing anti-oxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GSH Px) (199), decreasing mitochondrial oxidative phosphorylation and respiratory chain enzyme activity (148)

and increasing lipid peroxidation (81). Since redox mechanisms have been incriminated in CP-mediated cytotoxicity, many anti-oxidants have been tested for their protective effects against CP-mediated toxicity with positive results (76; 225; 303). Moreover, recent evidence has suggested a role for cytochrome (cyt) P450 enzymes in CP-induced nephrotoxicity (23). It has been thought that $Fe^{2+}$ released from cyt P450 enzymes contribute to CP-mediated renal failure because inhibition of these enzymes reduces $H_2O_2$ and $OH^{\cdot}$ generation due to $Fe^{2+}$-mediated oxidative mechanisms and markedly alleviates CP-mediated cytotoxicity (161).

CP-induced apoptosis in renal epithelial cells has been reported by many investigators to play a key part in CP-mediated renal injury (156; 211; 326). However, the pathways that underlie this effect are still under investigation. Park and co-workers reported that CP caused apoptosis in LLC-PK1 cells in a dose and duration-dependent manner. Furthermore, they showed that cell death occurred via activation of the mitochondrial apoptotic pathway (211). In contrast, Tsuruya and colleagues have shown that CP caused apoptosis in rat renal proximal tubule cells (RPTC) through the activation of the receptor-mediated death pathway (287).

Caspase-independent apoptosis has been described also by Cummings and colleagues who reported that at least 50% of cell death induced in renal proximal tubular cells (RPTC) was due to p53 activation and subsequent activation of caspase-3 independently of caspase-8 or caspase-9. As cell death progresses, however, a parallel

and distinct apoptosis-like cell death mechanism ensues which is not inhibited by p53, caspase-8 or caspase-9 inhibitors (70).

Several studies have addressed the role of inflammation in CP-mediated renal injury (223; 224). It has been suggested that oxidative stress resulting from CP exposure leads to the activation of NFkB gene transcription. This factor induces the production of several pro-inflammatory cytokines and chemokines especially TNF-α (223) which in turn recruits and activates other inflammatory mediators. Moreover, CP enhances the expression of the intracellular adhesion molecule-1 (ICAM-1) that increases the adherence of neutrophils to the endothelial cells thus facilitating the inflammatory response. Recently, inhibition of ICAM-1 by specific antibody or in knockout mice has been reported to alleviate CP-mediated renal damage (223).

## 1.7 Haem

### *1.7.1 Functions, biosynthesis and distribution of haem*

Haem is an $Fe^{2+}$–protoporphyrin complex consisting of four substituted pyrrole rings linked by –CH group (Fig 1-5). When the $Fe^{2+}$ atom is in the ferrous state, the complex is called ferroprotoporphyrin or haem, and the molecule is electrically neutral. However, when the $Fe^{2+}$ atom is in the ferric state, the complex is called ferriprotoporphyrin or haemin, and the molecule carries a positive charge.

Haem exists in the blood in free form complexed with haptoglobin or most commonly bound to blood proteins such as haemopexin and albumin. In hepatic cells, haem is taken up by receptor endocytosis and is degraded by the haem oxygenase (HO) enzyme in the hepatic cytosol (165). It is noteworthy that haem exists in free form prior to incorporation into haem proteins and after physiological turn-over of haem-containing proteins. Interestingly, whilst in the free pool, haem can be utilized by another haem-containing protein.

The fraction of free haem under physiological conditions is very small (0.1- 0.2 mM in the liver) but this molecule may accumulate in amounts in excess of that required for haemoprotein synthesis. This occurs under certain conditions such as sickle cell disease, I/R injury and malaria (125). Interestingly, haem function inside the cell is determined by the protein it is incorporated into.

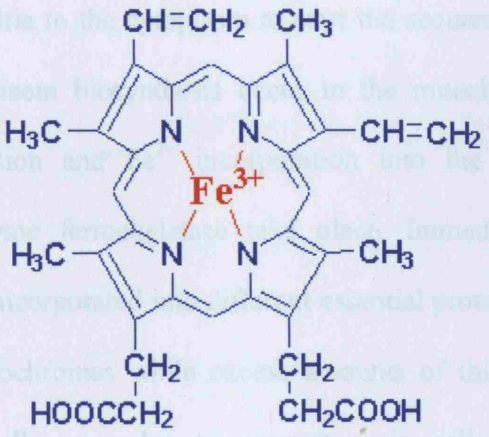



**Figure 1-5 Haem structure adapted from Indiana State University, USA. Web page: http://web.indstate.edu/thcme/mwking/oxidative-phosphorylation.html.**

For instance, it aids in oxygen transport in haemoglobin and it helps to store oxygen in myoglobin while in mitochondria it is incorporated into cytochromes and is involved in ATP synthesis and oxygen transport. Furthermore, haem is indispensable for biologically vital enzyme systems, such as cyclooxygenase (COX) and nitric oxide (NO) synthase (215). Other physiological effects of haem include regulating the expression of various proteins such as globin, haem biosynthetic enzymes, cytochromes, myeloperoxidase (MPO), HO-1 and controlling cellular differentiation and proliferation (215).

Haem synthesis in biological systems from glycine and succinyl Co A passes through different sequential steps starting from amino levulinic acid (ALA) synthase to protoporphyrin 9 (242) (Fig 1-6). ALA synthase is the rate-limiting step of haem synthesis and exists in erythroid and non-erythroid isoforms which are encoded in the liver and other tissues (305). During haem bio-synthesis, this enzyme is translocated from the mitochondria to the cytoplasm to start the sequential steps of the pathway. The last steps of haem biosynthesis occur in the mitochondria where side-chain oxidation, dehydration and $Fe^{2+}$ incorporation into the haem molecule by the mitochondrial enzyme ferrochelatase take place. Immediately after it has been produced, haem is incorporated into different essential proteins such as haemoglobin, myoglobin and cytochromes while excess amounts of this molecule are degraded immediately by cells in order to prevent their cellular toxic effects (276). Interestingly, the accumulation of haem inside the cell is prevented by modulating its synthesis and degradation. Specifically, a negative feedback effect on its synthesis is

facilitated by down regulating ALA synthase whereas it facilitates its degradation by inducing HO-1. By doing this, haem level in the cell is kept under control and the deleterious effects of its accumulation are prevented.

### *1.7.2 Toxic effects of haem*

Porphyrins have several oxidative effects mediated by photochemical reactions. A protoporphyrin in the ground state (P0) is a photosensitizer (89) that absorbs a photon upon exposure to light and is converted into an excited singlet state (P1) which is spontaneously converted into the triplet state (P3). The latter reacts through four different mechanisms:

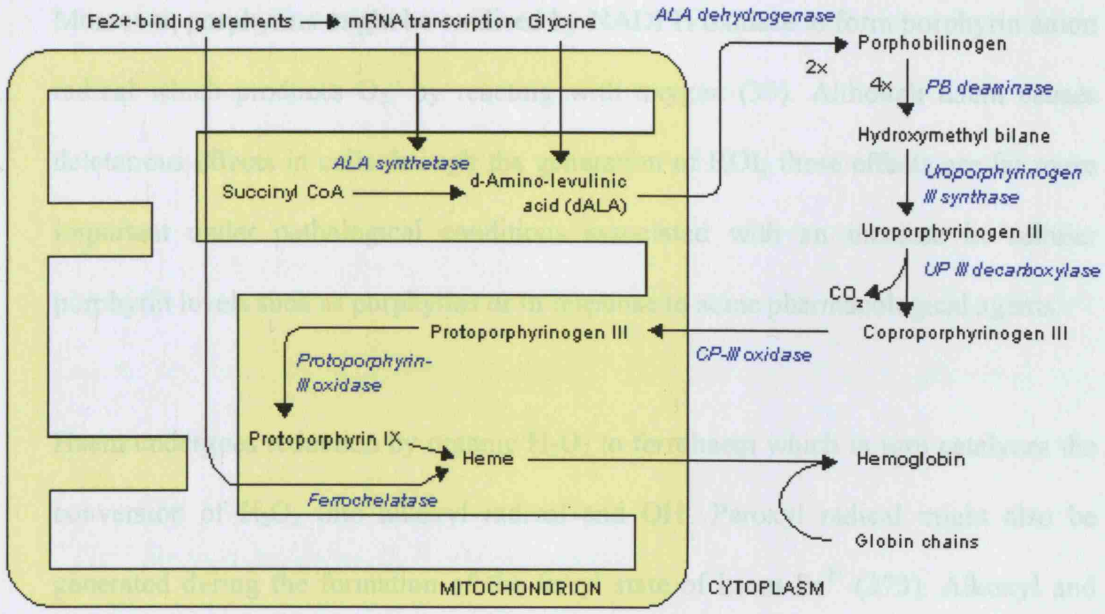



**Figure 1-6 Haem biosynthesis pathway adapted from Wikipedia, the free encyclopedia. Web page:**

**http://en.wikipedia.org/wiki/Heme.**

1. A decay reaction back into the ground state.

2. An anaerobic reaction with a ground state sensitizer.

3. An anaerobic type I reaction with an organic substrate to form radical anionic or cationic states of sensitizers and substrates.

4. Reaction with molecular oxygen (type II) to form singlet molecular oxygen by energy transfer or superoxide radical ($O_2^{\cdot-}$) by electron transfer (288).

So ROI produced during haem bio-synthesis such as $O_2^{\cdot-}$ may target macromolecules such as vital proteins, nucleic acids, carbohydrates and polyunsaturated fatty acids and lead to lipid peroxidation (228). Subsequently, $O_2^{\cdot-}$ and its dismutation $_{product}$ $H_2O_2$ produce the toxic hydroxyl radical ($OH^{\cdot}$) through the Haber-Weiss reaction. Moreover, porphyrins might be oxidized by NADPH oxidase to form porphyrin anion radical which produces $O_2^{\cdot-}$ by reacting with oxygen (35). Although haem causes deleterious effects in cells through the generation of ROI, these effects are far more important under pathological conditions associated with an increase in cellular porphyrin levels such as porphyrias or in response to some pharmacological agents.

Haem undergoes reduction by organic $H_2O_2$ to ferrohaem which in turn catalyzes the conversion of $H_2O_2$ into alkoxyl radical and $OH^{\cdot}$. Peroxyl radical might also be generated during the formation of the ferryl state of haem $Fe^{2+}$ (273). Alkoxyl and peroxyl radical species may further produce alkyl radicals during lipid peroxidation which are converted back into peroxyl radicals via addition of $O_2$ and then another cycle begins. Ultimately, termination of these propagation reactions may occur by

forming cross-links or these radicals may undergo further re-arrangement reactions leading to the formation of various end products such as lipid alcohol, carbohydrates, thiols, epoxides or hydrocarbons (98). In addition to the abovementioned pro-oxidant effects of haem, this molecule has been found to possess pro-inflammatory effects by stimulating the synthesis of ICAM-1, vascular adhesion molecule-1 (VCAM-1) and *E*-selectin with a resultant increase in recruitment of inflammatory cells such as leukocytes and monocytes. This has been related to the ability of haem to stimulate the generation of ROI and the resultant activation of NF-κB (151).

### *1.7.3 Effects of haem on the kidney*

Haem has several effects on the kidney. It increases renal blood flow by decreasing renal vascular resistance as a result of increasing the generation of prostaglandin $I_2$ ($PGI_2$) thus causing an increase in urine volume and $Na^+$ excretion (233). Haem induces a local inflammatory reaction in the kidney leading to renal failure (198) and stimulates oxidative damage to renal epithelial cells by generating ROI and depleting GSH (196). Moreover, haem has been shown to decrease renal tubular epithelial cell growth by inducing apoptosis, provoking cell cycle arrest and enhancing p21 synthesis (315).

### *1.7.4 Haem degradation by haem oxygenase*

For the degradation of haem by HO, the reduced form of nicotinamide-adenine dinucleotide phosphate (NADPH), three molecules of $O_2$ and seven electrons (provided by the NADPH-cyto P450 reductase) are required (Fig 1-7) (Fig1-8) (165).

HO cleaves the haem molecule to form biliverdin IX α (BV IX- α), water, CO and $Fe^{2+}$ (165). CO is formed during the conversion of α–meso-hydroxyhaem into α-verdohaem where the α-methyne carbon of haem is released as CO. During this process, NADPH: cytochrome P450 reductase reduces ferric haem in the HO-haem complex. Subsequently three sequential mono-oxygenation reactions occur in which $Fe^{2+}$ binds molecular oxygen that accepts the second electron from NADPH and the activated oxygen attacks the haem ring. During these cycles, hydroxyhaem, verdohaem and ferribiliverdin IX-α (BVIX-α-FeIII) are produced and the latter is reduced by BV reductase to bilirubin (BR) in a reaction that requires NADPH (165). The released $Fe^{2+}$ is efficiently chelated by apoferritin and stored as ferric iron in ferritin or is transported into the bone marrow by transferrin for recycling and use for haemoglobin synthesis whereas CO is transported as carboxyhaemoglobin to the lung where it is exhaled.

### 1.8 Iron

Recent evidence has suggested an important role for $Fe^{2+}$ in oxidative tissue injury. The relative ease of conversion of $Fe^{2+}$ into the oxidative or reduced state makes it a powerful contributor to the Haber-Weiss reaction with the resultant production of $OH^{\cdot}$ (219).

Ferritin is a 450 KDa protein which is composed of 24 subunits that are composed of either heavy (21 KDa) or light (19 KDa) chains. Ferritin sequesters intracellular $Fe^{2+}$ and maintains it in the ferric state due to its ferroxidase activity (219). Interestingly, it

has been demonstrated that HO induces the *de novo* synthesis of ferritin and it thus enhances $Fe^{2+}$ sequestration and prevents its toxic effects (87).

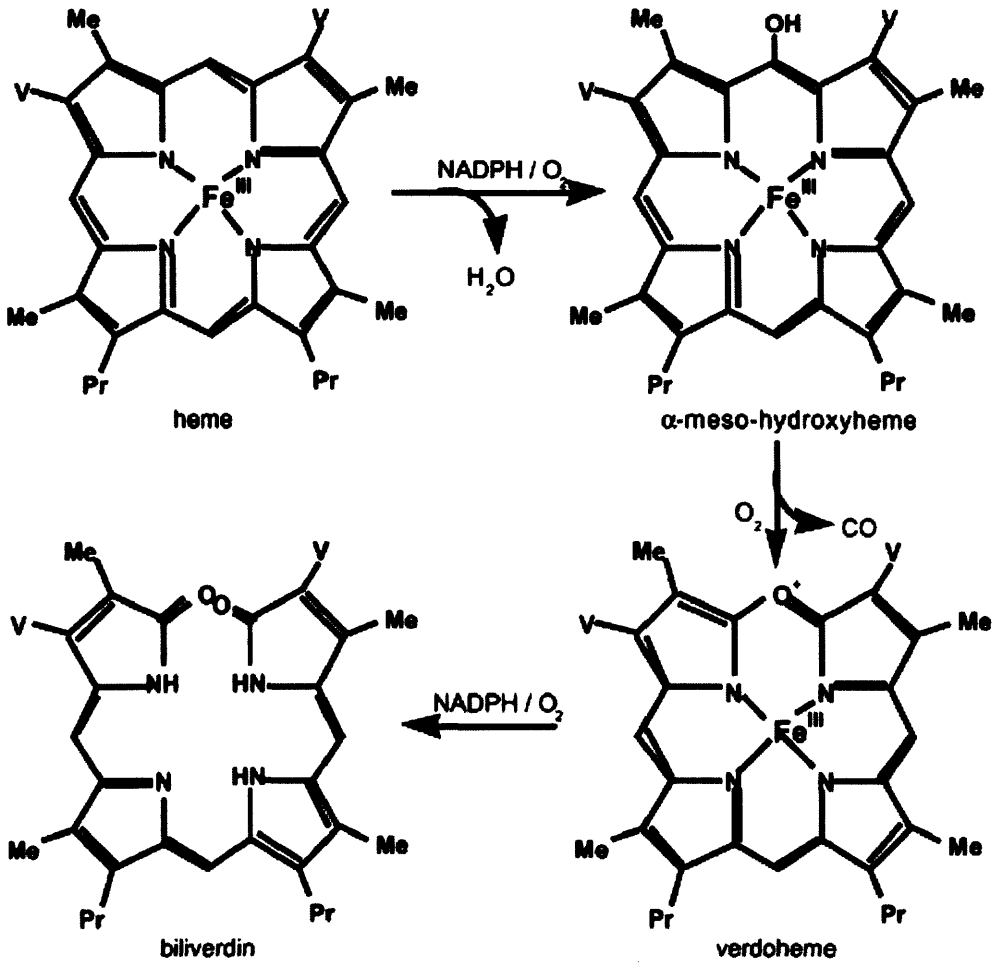



**Figure 1-7 The haem oxygenase pathway adapted from the Science Analysis and Consulting Service. Web page:**
**http://www.sacs.ucsf.edu/home/Ortiz/res-ho.htm.**

Moreover, HO-1 has been found to stimulate the activation of an ATP-dependent $Fe^{2+}$ pump which stimulates its removal from the cell (87). Interestingly, remarkable evidence indicates that $Fe^{2+}$ itself regulates the intracellular expression of ferritin by

inhibiting the $Fe^{2+}$-regulatory protein which binds and inhibits the translation of this protein (248). Although ferritin has been thought to be a cytoprotectant, under stress conditions such as paraquat, adriamycin and $O_2^{\cdot-}$ toxicity it might paradoxically lead to the release of sequestered $Fe^{2+}$ (229).

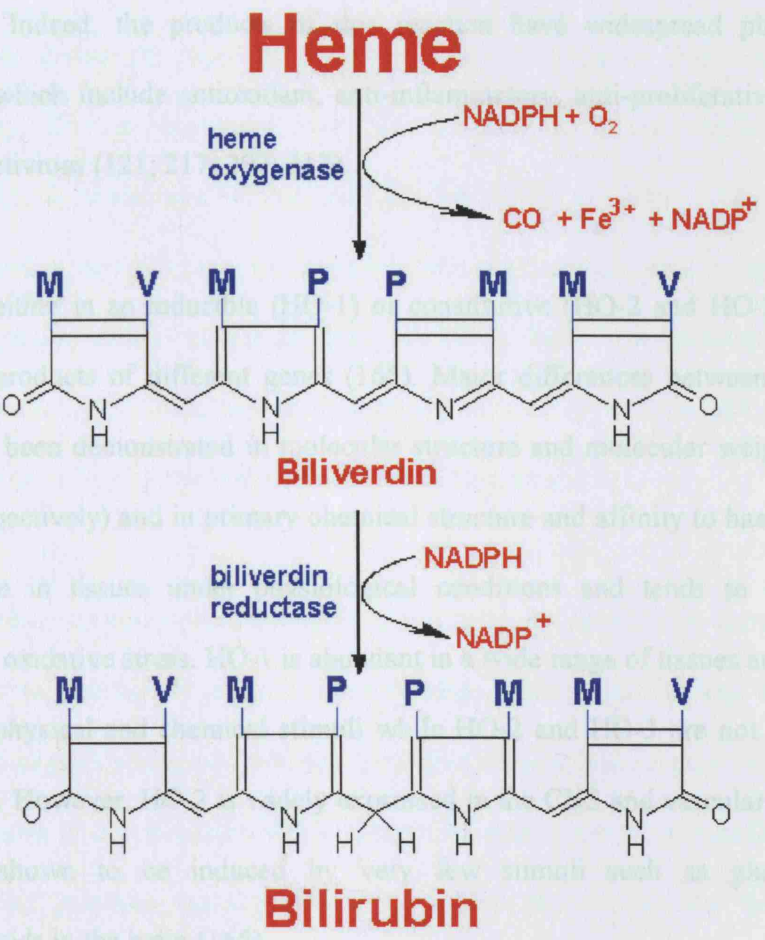



**Figure 1-8 The haem oxygenase pathway adapted from the Medical Biochemistry Page, Indiana State University, 2005. Web page: http://web.indstate.edu/thcme/mwking/heme-porphyrin.html.**

## 1.9 The haem oxygenase enzyme

HO was described for the first time in 1968 by Tenhunen and colleagues as the enzyme which degrades haem into BV and CO (281). This enzyme is ubiquitous in all species including bacteria, fungi, plants and humans (165). Despite the fact that HO undertakes only one metabolic reaction, the biological consequences are far more significant. Indeed, the products of this reaction have widespread physiological influences which include antioxidant, anti-inflammatory, anti-proliferative and anti-apoptotic activities (121; 217; 297; 312).

HO exists either in an inducible (HO-1) or constitutive (HO-2 and HO-3) isoforms which are products of different genes (165). Major differences between HO-1 and HO-2 have been demonstrated in molecular structure and molecular weight (32 and 36 kDa respectively) and in primary chemical structure and affinity to haem. HO-1 is undetectable in tissues under physiological conditions and tends to increase in response to oxidative stress. HO-1 is abundant in a wide range of tissues and responds to various physical and chemical stimuli while HO-2 and HO-3 are not induced by xenobiotics. However, HO-2 is widely expressed in the CNS and vascular tissues and has been shown to be induced by very few stimuli such as glucagon and glucocorticoids in the brain (165).

Besides its basic function in degrading haem, HO-1 possesses potent cytoprotective properties against several oxidative stress stimuli that affect the cell (217). There has been a common consensus that due to its inducible cytoprotective activities, HO-1

induction could be used as a therapeutic strategy to counteract various oxidative and inflammatory conditions (181). In this context, extensive research is being conducted in order to find potent non-toxic HO-1 inducers that could be used clinically to treat several pathological conditions such as chronic inflammatory states and I/R injury.

### 1.10 Bilirubin and biliverdin

BV is formed during the degradation of haem by HO enzymes (Fig 1-7) (Fig 1-8). Subsequently, BV is converted into BR by BV reductase (135) (Fig 1-8). Interestingly, in birds, amphibians and reptiles BV is the final product of haem degradation in direct contrast with mammals where an energy consuming reaction is carried out in the cells to reduce BV into BR (269). Both bile pigments contain an extended system of conjugated double bonds and reactive hydrogen atoms that probably account for their potent anti-oxidant properties (269).

BR is formed in several tissues including blood cells and spleen and circulates in the blood bound to albumin. This anti-oxidant is taken up by liver cells and is detoxified by the enzyme glucoronosyl-transferase through a conjugation reaction to glucoronic acid converting it into a water-soluble compound that is easily excretable in bile or faeces. In the intestine, conjugated BR is further metabolized by the intestinal microbial flora into urobilinogen and urobilin.

Jaundice due to cellular accumulation of BR is an extremely common clinical problem especially in premature babies. Neonatal jaundice occurs in up to 60% of

newborns and is caused by several factors including overproduction due to catabolism of foetal haemoglobin, insufficient activity of UDP-glucoronosyl transferase due to liver immaturity and excess absorption of un-conjugated BR from the gastrointestinal tract. Although BR serves anti-oxidant properties, most newborns with jaundice are treated with phototherapy or exchange transfusion in severe cases to prevent kernicterus and irreversible neurological deficits that develop in severe un-conjugated hyperbilirubinaemia.

### 1.11 Biological functions of bilirubin and biliverdin

BR has been long considered a toxic waste product of haem degradation until its anti-oxidant properties were reported by Stocker and co-workers who showed that this HO reaction product prevents oxidation of polyunsaturated fatty acids in multilamellar liposomes as effectively as α-tocopherol (269). Thereafter, several studies emerged exploring the biological effects of this bile pigment. Collectively, BR has been shown to decrease the incidence of coronary artery disease (115), ameliorate post-ischaemic cardiac dysfunction (63), protect cardiomyocytes against I/R injury (90), decrease mortality in malignant disease (280) and scavenge peroxynitrite, an extremely potent oxidant, formed as a result of the interaction of NO and $O_2^{\cdot-}$ (120). In addition, BR has been shown to exert potent anti-inflammatory effects (110) and to minimize I/R injury in the liver and kidney (2; 134).

Unlike BR, BV is a non-toxic water soluble compound that is quickly converted to BR. Recently, several studies have addressed the cytoprotective effects of BV in

cardiac and small intestine transplantation (194; 313). This bile pigment has been found also to cause a decrease in apoptosis, inducible NO synthase expression, inflammation and an increase in hepatic function, anti-apoptotic genes and animal survival when it was added to the perfusate of an *ex vivo* model of orthotopic liver transplantation (88).

### 1.12 Carbon monoxide

Exogenously produced CO is continuously generated as a result of incomplete combustion of organic materials while endogenous CO is produced as a by-product of haem degradation by HO. In micro-organisms, CO is metabolized by CO oxidase into $CO_2$ via an aerobic oxidation reaction that oxidizes CO at the expense of reducing $O_2$. In anaerobic organisms, CO is metabolized by CO dehydrogenase (CODH) resulting in the production of $CO_2$ (104). CO causes increase in the activity of CODH by interacting with the haem-containing transcriptional factor CooA which enhances the expression of CODH via interacting with the RNA polymerase (232).

The affinity of CO to haemoglobin is significantly higher than that of $O_2$ and its release from haemoglobin is rather slow. As a result, by avidly binding to haemoglobin, CO impairs $O_2$ delivery to tissues resulting in generalized tissue hypoxia. In the mitochondria, CO alters mitochondrial redox status and energy provision in the brain and these effects last even after CO haemoglobin (COHb) has been cleared from the blood (43). Moreover, CO causes a decrease in $CO_2$ dissociation leading to respiratory failure as a result of increasing blood $CO_2$

concentration. Interestingly, CO impairs respiratory centre function and that is why it is called the silent killer as death occurs as a result of impaired tissue oxygenation without prior symptoms.

The clinical manifestations of acute CO poisoning affect several body systems such as the cardiovascular system causing tachycardia and hypertension. Neurological manifestations of CO poisoning include headache, dizziness, paresis, convulsions and coma (59). Moreover, late onset neurological manifestations may occur days or weeks after acute CO poisoning. Other life-threatening effects of CO poisoning include myocardial ischaemia, atrial fibrillation, pneumonia, pulmonary oedema, erythrocytosis, leucocytosis, hyperglycaemia, muscle necrosis, acute renal failure, skin lesions, and changes in the perception of the visual and auditory systems. Delayed neurological sequelae of CO poisoning are common and include mental deterioration, urinary incontinence and gait disturbance which usually occur in middle or older age.

### *1.12.1 Biological activities of CO*

The old perception of CO as the silent killer and the useless by-product of HO-1 has dramatically changed. This odourless, tasteless, and colourless gas has been found to possess fascinating biological functions. Duke and Killick demonstrated for the first time in 1952 that CO decreased pulmonary vascular resistance (80). Over the last decade, CO has attracted special attention as reflected by the growing number of reports in the literature that describe the various biological and physiological

functions of this molecule. Probably most of the interest in CO arises from its positive effects on the cardiovascular system. In addition, CO exerts anti-inflammatory, anti-apoptotic, vasoactive and anti-proliferative effects in several cell types and has been shown to act as a neurotransmitter (42; 250) (179) (246; 293).

The impressive vasoactive properties of CO result from its relaxing effects on the vascular smooth muscle cells in a similar manner to NO (120). Various mechanisms have been described for the vasoregulatory effects of CO. In this context, the rising level of CO in the vascular milieu increases cGMP causing intracellular calcium concentration to fall and blunts the contractile influence of calcium on vascular smooth muscle cells. Moreover, CO has been discovered to modulate $Ca^{+2}$- dependent $K^{+}$ channels content in vascular smooth muscle cells which, under physiological conditions, increase calcium concentration and enhance vasoconstriction (130).

CO has been shown to possess potent anti-inflammatory effects. This important biological influence has been linked to the suppression of inflammatory mediators especially TNF-α released from macrophages during the inflammatory response (250). Furthermore, CO up-regulates anti-inflammatory mediators such as interleukin-10 (IL-10) (205) and has been reported to prevent platelet aggregation and thus down-regulates the pro-inflammatory response that emerges due to thrombus formation (44; 241).

### *1.12.2 Effect of CO on apoptosis*

Exposure to CO has been shown to exert potent anti-apoptotic effects both *in vivo* and *in vitro*. In organ transplantation, CO prevents organ rejection in part by inhibiting the initiation of inflammatory and apoptotic events (262). Furthermore, CO has been shown to induce several NF-kB-dependent anti-apoptotic genes to protect against TNF-α-mediated endothelial cell apoptosis (42). In addition, the anti-apoptotic effects of CO involved inhibition of Fas/FasL expression, and other apoptosis-related factors including caspases (-3,-8,-9), mitochondrial cyt c release, Bcl-2 proteins and poly (ADP ribose) polymerase (PARP) cleavage (319). The anti-apoptotic effect of CO in smooth muscle cells was partially dependent on the activation of soluble guanylate cyclase (sGC) and was associated with suppression of p53 and inhibition of mitochondrial cyt c release (163). However, Zhang *et al* has demonstrated that CO prevents apoptosis in pulmonary artery endothelial cell cultures via activating the p38 pathway (319).

### *1.12.3 Carbon monoxide signal transduction pathways*

GCs which exist in membrane-bound or soluble cytosolic isoforms (sGC) catalyze the formation of cGMP, an intracellular signalling molecule that plays an important role in the regulation of various cellular events such as smooth muscle relaxation, inhibition of platelet aggregation and synaptic transmission (299). This enzyme is a haem-protein and CO, like NO, binds sGC with high affinity but leads to a far lower level of activation of this molecule than NO (270). CO stimulation of cGMP

production in cell and tissue preparations has been demonstrated in processes like inhibition of platelet aggregation and smooth muscle relaxation (120; 246).

Although NO is a more efficient sGC activator than CO, some cells generate this gaseous molecule in much greater quantities than NO which may favour its binding to the haem moiety of sGC (120). Interestingly, it has also been suggested that HO activity may serve, under certain circumstances, to modulate NO synthesis and the subsequent production of the nitrogenous ROI such as peroxynitrite ($ONOO^-$) (265).

In addition to cGMP, several studies have reported the crucial role of the p38 signal transduction pathway in mediating CO biological effects (205). Interestingly, the pathway that is used by CO to exert its effect is cell specific. For instance, CO exerts anti-apoptotic effects in fibroblasts through mechanisms that involve the p38 MAPK pathway (42) while the same effect in pancreatic islet cell line requires the cGMP pathway (107). Not only does CO utilize cGMP and p38 to exert its effects but the ERK pathway has been reported to be activated by CO to mediate its anti-apoptotic effects in human airway smooth muscle cells (263) and to mediate its anti-proliferative effects in T lymphocytes (207).

### *1.12.4 Carbon monoxide releasing molecules (CO-RMs)*

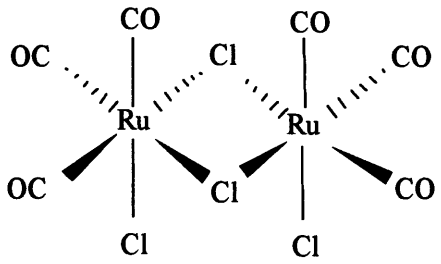
Our group has been working for the past few years on the characterization of transition metal carbonyls as potential carriers of CO in experimental models (180; 184). These compounds contain a heavy metal such as nickel, cobalt, or $Fe^{2+}$

surrounded by carbonyl (CO) groups as a coordinated ligand (180). The discovery of molecules that can carry and liberate CO in tissues and organs would undoubtedly facilitate the use of CO as a therapeutic agent (183). This would have significant advantage over the inhalation method as it avoids the problems related to the systemic effects imposed by CO on oxygen transport and delivery to tissues.

Immense efforts have been directed in our laboratory towards the production of molecules that can carry and deliver CO in biological systems in a controlled fashion that simulates CO release as a result of haem degradation. Transition metal carbonyls have been found to be good candidates for this purpose as they are capable of carrying and liberating CO in physiological buffers (180). A lot of progress has been achieved by our group in the development of these carbon monoxide-releasing molecules (CO-RMs). Initially, two CO-RMs were produced which contain manganese decarbonyl ($[Mn_2(CO2)_{10}]$) and tricarbonyldichlororuthenium(II) dimer ($[Ru(CO_2)_3Cl_2]_2$) termed CORM-1 and CORM-2 (Fig 1-9) respectively. Both CO-RMs are soluble in organic solvents and are pharmacologically active in vessel relaxation, attenuation of coronary vasoconstriction and suppression of acute hypertension (180). Other pharmacological effects of CORM-1 and CORM-2 include cerebral vasodilatation (144), smooth muscle relaxation of internal anal sphincter (226), anti-proliferative effects in smooth muscle cells (266), anti-inflammatory (290) and reno-protective effects (292).

The first water-soluble CO-RM was produced by ligand substitution to enhance CO release (184). Tricarbonylchloro(glycinato)ruthenium(II) (CORM-3) (Fig 1-1), was

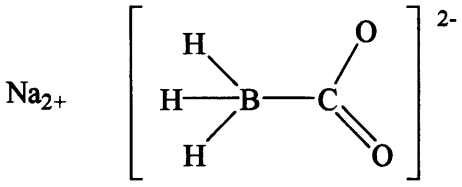
produced and rendered water-soluble by inserting the amino acid glycine onto the metal centre and has been shown to release CO *in vivo*, *ex vivo* and *in vitro* (180). Several studies have been conducted in our laboratory to explore the biological activities of CORM-3 which showed that this compound protects against cardiac I/R injury (64), possesses vasodilatory properties (92) and exerts potent anti-inflammatory effects (250). Subsequently, another water-soluble vasoactive CORM (CORM-A1) was produced (185).




Tricarbonyldichloro ruthenium(II) dimer

$[Ru(CO)_3Cl_2]_2$

**CORM-2**




Sodium boranocarbonate

**CORM-A1**

**Figure 1-9 Chemical structure of CORM-2 and CORM-A1.**

## 1.13 Isothiocyanates (ITCs)

### *1.13.1 Sources, absorption and metabolism of ITCs*

The plant family *Brassicacaeae* which contains more than 350 genera and 300 species is the main source of glucosinolates (86). Cruciferous species of the Brassicacaeae family have been fully investigated and gained special interest recently because all plants which belong to this group contain glucosinolates (Fig 1-11). These biologically active compounds are present in 16 families of dicotyledonous angiosperms and in another 500 species of non-cruciferous species (86).

Many studies have shown that high intake of cruciferous vegetables protects against the development of cancer (68; 129). This favourable effect of cruciferous vegetables has been attributed to their high content of glucosinolates of which more than 120 types have been characterized to date (86). Glucosinolates exist in cruciferous vegetables as N-hydroxysulfates that consist of a sulphur-moiety linked to $\beta$-glucose and a variable chain consisting of alkyl, alkenyl, aromatic or indolyl moiety (86) (Fig 1-10). Glucosinolates have been classified by Fahey *et al* into chemical groups according to their structural similarities. The most common glucosinolates are aliphatic, w-methylthioalkyl, aromatic and heterocyclic compounds (86).

The content of glucosinolates in plants is quite variable and depends on many plant and environmental factors (152). For instance, the glucosinloate distribution differs between roots, seeds and leaves. Furthermore, the age of the plant seems to play a role

in determining the glucosinloate content. For example, old plants may contain about 1-4 μM glucosinloate/g fresh vegetable while seeds or young sprouts of broccoli may contain up to 70-100 μM/g fresh weight.

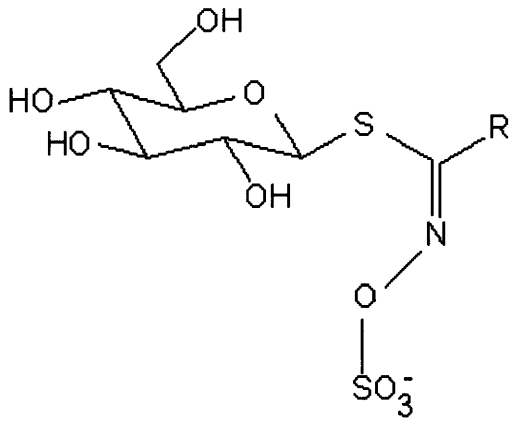



**Figure 1-10 Structure of glucosinolates adapted from the NTNU Cell and Molecular Biology Group, Norway. Web page:**
**http://boneslab.bio.ntnu.no/Paal/glucosin.htm.**

### *1.13.2 ITCs-mediated cytoprotection*

ITCs are naturally occurring compounds that have attracted special interest recently because of their potent chemopreventive properties both *in vivo* and *in vitro* (68; 96; 164; 256). ITCs are abundant in cruciferous vegetables such as broccoli, cabbage, Brussels sprouts, radish and horse radish (86; 324). Glucosinolates in plants are converted into ITCs upon cutting or chewing by the plant enzyme myrosinase (*beta*-thioglucosidase) which is segregated in vacuoles and is physically separated from ITCs in the plant tissues (137). Although myrosinase is inactivated by heating such as

cooking, ITCs can be liberated from glucosinolates by the myrosinase activity of the intestinal microbial flora albeit at a lower level (129).

It has been thought that the various cytoprotective properties of ITCs are dependent on their rapid and efficient absorption into the cell which is facilitated by conjugation to GSH in a reaction catalyzed by the enzyme glutathione-S-transferase (GST) to form dithiocarabamates (DTCs) (46). Surprisingly, many studies have shown that the ITC-GSH conjugates are rapidly exported after absorption into the cell through the multi-drug resistance associated protein-1 (MRP-1) channels (46).

Like many xenobiotics, ITCs are metabolized by the mercapturic acid pathway (321). Once again, in this process ITCs are conjugated with GSH via a reaction mediated by GST and then gradual cleavage of glutamine and glycine occurs resulting in the formation of cysteineylglycine, cysteine and N-acetylcysteine conjugates of ITCs (mercapturates) which are excreted in urine. Recently, it has been postulated in that mercapturates can be used as a bio-marker of the amount of ITC released from glucosinolates (118).

### *1.13.3 ITCs are potent chemprotective compounds*

Although a lot of progress has been achieved in cancer diagnosis and treatment, the incidence and mortality of several human malignancies have not substantially declined. Since it is impossible to eradicate all the carcinogenic factors from the environment, immense efforts have been devoted recently to cancer chemoprevention.

The concept of inhibiting, delaying or reversing the carcinogenic process is called chemoprevention.

The process of transformation of a normal cell into a neoplastic one is a long-term process that appears to proceed through several steps (85). The first stage is called initiation in which DNA damage occurs due to physical, chemical or viral exposure. This leads to several gene and chromosomal abnormalities such as sequence alterations and/or gross structural abnormalities in one or more chromosomes (85). Promotion occurs as a result of chronic exposure of the changed cell to carcinogens such as growth factors, hormones or ultraviolet (UV) irradiation and involves the transformation of an already initiated cell into a group of neoplastic cells. The last stage is called progression in which the pre-neoplastic cells are converted into invasive malignant ones.

Chemopreventive compounds have been classified by Wattenberg *et al* into two categories; blocking and suppressing agents (301). Blocking agents prevent the development of cancer by inhibiting carcinogens from reacting with essential cellular components such as DNA, RNA and vital proteins. Moreover, blocking agents can prevent the conversion of pro-carcinogens into carcinogens. Suppressing agents, however, prevent carcinogenesis by inhibition of malignant transformation of initiated cells at the promotion or progression stages.

Due to their safety, availability and acceptance among the public, a lot of effort has been devoted to search for natural compounds that offer non-toxic and effective chemprotective potential (154). In this context, many plant-derived compounds especially phenolic and sulphur containing ones have been identified with cancer-preventive potential such as ITCs, catechins, resveratrol, curcumin, caffeic acid phenyl ester (CAPE), falvunoids, vitamin C and vitamin E.

### *1.13.4 The mechanisms of ITCs-mediated cytoprotection*

The human body possesses various enzymes that metabolize toxic agents and environmental carcinogens upon their entry into the cell. These harmful compounds are converted into hydrophilic substrates by oxidation, reduction, conjugation or hydrolysis reactions rendering them water-soluble and readily excretetable from the body.

Two enzyme systems have been identified that take over the metabolism of toxic chemicals and carcinogens in the body. Phase I (cyt P450) enzymes are a group of mono-oxygenases that convert pro-carcinogens and foreign molecules into highly reactive intermediates that have the capability to react with vital cellular components such as DNA, RNA and proteins through a two electron oxidation reaction (102; 295). Therefore, phase I enzymes actually enhance the carcinogenesis process and the cyto-damaging effects of oxidative stress through the activation of pro-carcinogens and the production of ROI. Phase II enzymes, on the other hand, are the first line defence against oxidative stress and tumourigenesis (186). Through reduction or

conjugation reactions with endogenous cofactors such as GSH, glucorunoids, or sulphate, these enzymes detoxify pro-carcinogens and toxic ROI such as $H_2O_2$, $O_2^{\cdot-}$ and $OH^{\cdot}$ that are continuously produced from mitochondrial respiration and the biotransformation of xenobiotics. The phase II family includes enzymes such as GST which eliminates ROI and hydrophobic intermediates via conjugating them with GSH, NADPH quinone oxidoreductase (NQO) which catalyzes the reduction and detoxification of quinones, UDP-glucuronosyl transferase (UDP-GT) which catalyzes detoxification of several xenobiotics and drugs by conjugating them with glucoronic acid and HO-1 which catalyzes the detoxification of haem. Thus, it has been thought that a critical balance must be maintained between the activity of phase I and phase II enzymes to minimize the risk of carcinogenesis.

The cytoprotective properties of ITCs have been related to their inherent activation of phase II enzymes and inhibition of phase I enzymes (186) (190). These plant-derived compounds block the activation of pro-carcinogens by down-regulating phase I enzymes while at the same time they induce metabolism and excretion out of the cell by phase II enzymes as harmless conjugates. The degree of induction of phase II enzymes by different ITCs varies depending on the ITC used and the cell type (128; 186).

### *1.13.5 Signal transduction pathways activated in response to ITCs*

MAPKs are largely characterized as a group of evolutionary conserved proline-directed serine/threonine kinases that respond to a plethora of extracellular stimuli

(167) (Fig 1-12). These pathways constitute a group of separate but parallel signal transduction kinases that transmit various extracellular signals and convert them into intracellular responses through serial phosphorylation cascades leading to the modulation of several biologically active genes which affect cell metabolism, proliferation, differentiation and death (167).

To date, three MAPK pathways have been identified, extracellular regulated kinase (ERK), C-Jun-N-terminal kinase (JNK) and p38 (167). Each pathway consists of MAPK kinase kinase (MAPKKK) that phosphorylates and activates MAPK kinase (MAPKK) which in turn phosphorylates and activates MAPK. It has been shown previously that MAPK pathway can regulate the antioxidant response element/electrophile response element (ARE/EpRE) through nuclear regulated factor 2 (Nrf2)-dependent mechanisms (123). A good example of how the MAPK pathways work is the activation of the ERK pathway which is activated usually in response to growth factors (167). Upon receiving an activating signal, Raf1 (a MAPKKK) is activated and in turn activates MEK (a MAPKK) which phosphorylates and activates ERK (1).

The JNK pathway has been identified by its ability to regulate cell proliferation, cell survival, apoptosis, DNA repair and metabolism in response to various stress stimuli (133). JNK-mediated phosphorylation enhances the ability of the transcriptional factor c-Jun to activate gene expression in response to a large number of extracellular

stimuli. The JNK activation leads to induction of AP-1-dependent target genes involved in cell proliferation, cell death, inflammation and DNA repair.

In a similar manner to the JNK pathway, the p38 pathway is activated mainly by stress stimuli such as inflammatory cytokines, endotoxins, UV irradiation and hyperosmolarity (119). The fourth and least studied mammalian MAPK pathway, big MAP kinase 1 (BMK1), also known as ERK5 is activated in response to growth factors and stress. Activation of this signalling pathway has been implicated in cell survival, proliferation and differentiation as well as in pathological processes such as carcinogenesis, cardiac hypertrophy and atherosclerosis (109).

ITCs lead to the activation of MAPKs and the role of these signalling cascades in the modulation of detoxification enzymes by these plant-derived compounds has been observed in many studies. For instance, sulforaphane has been shown to activate the ERK and p38 pathways in mediating its apoptotic effects in human hepatoma (HepG2) cells (82). Furthermore, it has been shown that ITCs stimulate the transcriptional activation of the ARE which exists in the promoter region of several phase II enzyme genes through the activation of various signal transduction pathways such as MAPKs, phosphatidyl inositol 3 kinase (PI-3K) and protein kinase C (PKC) pathways (308).

The PI-3K pathway is a lipid kinase which has been implicated in modulating various cellular events such as cellular growth, differentiation and apoptosis. This pathway

has been shown to regulate ARE/EpRE by enhancing Nrf2 translocation into the nucleus (132).

The PKC pathway has been found to contribute to the ARE/EpRE regulation by direct phosphorylation of Nrf2 and facilitating dissociation from the cytoplasmic actin-binding protein kelch like ECH protein-1 (Keap1) (117). PKC family consists of at least 12 serine-threonine kinases (142). Short-term activation of PKC is often associated with short-term events such as secretion and ion-influx. In contrast, sustained activation is suggested to induce long-term effects such as proliferation, differentiation, apoptosis, migration and tumourigenesis.

### *1.13.6 Mechanisms of ITCs-mediated induction of phase II enzymes*

The intracellular mechanisms that mediate the ITCs-induced up-regulation of phase II enzymes are complex and not fully understood. However, it has been suggested that the cytoplasmic transcriptional factor, Nrf2, plays a crucial role in this process. This factor is a member of the cap "n" collar (CNC) family of basic leucine zipper transcriptional agents which is sequestered under physiological conditions in the cytoplasm by Keap1 (141). However, upon exposure to an oxidative challenge, Nrf2 is released from Keap1 and is translocated into the nucleus where it interacts with the ARE/EpRE which exist in the promoter region of several cytoprotective enzyme genes such as GST, Gamma-glutamyl synthase (gamma-GS), NQO and HO-1 (137). The role of Nrf2 in ITC-mediated regulation of phase II enzymes has been reported in several studies (126; 317).

Nrf2 stabilization is extremely important in increasing its concentration in the cytoplasm since it leads to an increase in its translocation into the nucleus and consequently increases ARE-mediated expression of the detoxification genes. Intracellular protein stabilization depends on the activity of the degradation processes by an ubiquitin-dependent proteasome.

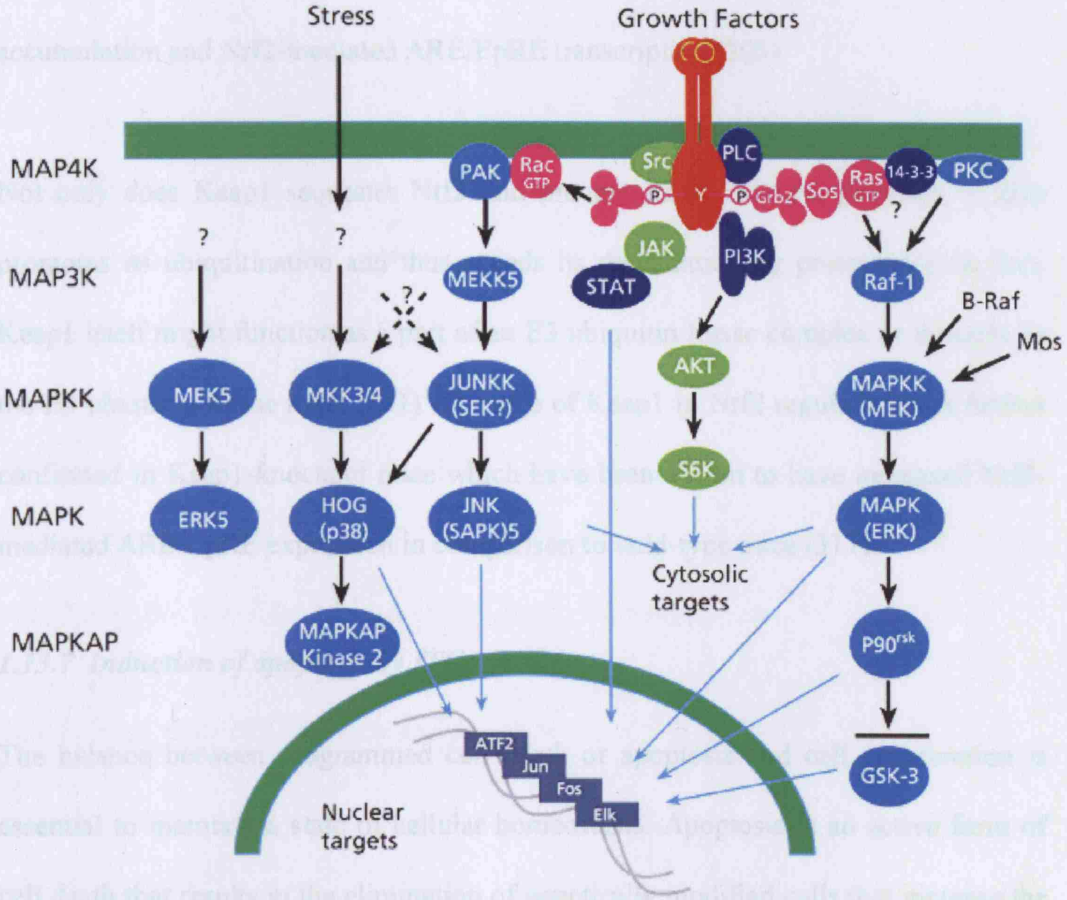



**Figure 1-11 The MAPK signal transduction pathways adapted from SignaGen laboratories. Web page:**

**http://www.signagenlabs.com/index.php?main_page=index&cPath=1_4_79_82.**

In this system, the degradation of a protein is a highly specific process in which, to be recognized by the proteasome, the protein to be degraded is targeted by an E3-ubiquitin ligase which causes changes in certain amino acid sequences or conformational changes in the structure of the protein. More recently, several observations have addressed the importance of Nrf2 phosphorylation in maintaining its stability. In this context, the phosphatase inhibitor okadic acid induced Nrf2 accumulation and Nrf2-mediated ARE/EpRE transcription (203).

Not only does Keap1 sequester Nrf2 and prevent its nuclear translocation, it also promotes its ubiquitination and thus speeds its degradation by proteasome. In fact, Keap1 itself might function as a part of an E3 ubiquitin ligase complex or it might be the E3 ubiquitin ligase itself (141). The role of Keap1 in Nrf2 regulation was further confirmed in Keap1-knockout mice which have been shown to have increased Nrf2-mediated ARE/EpRE expression in comparison to wild-type mice (317).

### *1.13.7 Induction of apoptosis by ITCs*

The balance between programmed cell death or apoptosis and cell proliferation is essential to maintain a state of cellular homeostasis. Apoptosis is an active form of cell death that results in the elimination of genetically modified cells that increase the risk of tumour formation. Induction of apoptosis in deleterious cells has been considered to play a major role in the chemopreventive activity of ITCs (278; 283; 308).

ITCs induce apoptosis in various cell types via mechanisms that involve the activation of the JNK pathway (56). Interestingly, it seems that the caspases that are induced by different ITCs are cell specific. For instance, phenylethyl isothiocyanate (PEITC) induces apoptosis via activation of caspase-3, caspase-8 and caspase-9 in HeLa cells (316) whereas only caspase-8 is required by PEITC to induce apoptosis in HL-60 cells (311).

Although at higher concentrations ITCs have been proved to be cytotoxic by induction of apoptosis in normal cells, at lower concentrations, ITCs protect cells against oxidative and carcinogenic stimuli by the up-regulation of phase II enzymes. Therefore, a model has been suggested in which induction of phase II enzymes or induction of apoptosis by ITCs depends on the concentration used. At lower concentrations, cytoprotective genes are switched on. Nevertheless, by using higher concentrations ITCs lead to cellular damage as manifested by depletion of GSH, JNK stimulation, mitochondrial damage and caspase activation.

### *1.13.8 Mitochondrial structure and function*

Mitochondria are the major source of ATP in eukaryotic cells (Fig 1-12). Each mitochondrion has two membranes, the outer membrane which completely surrounds the organelle and the inner membrane. The latter has infolding called cristae where the mitochondrial respiratory chain (MRC), also known as the electron transport chain, resides. The inner mitochondrial compartment (known as the matrix) contains a large number of enzymes and chemical intermediates involved in energy-yielding

metabolism. The MRC consists of five complexes, namely complexes I, II, III, IV and V. Although more than 80 proteins which comprise these complexes have been identified, only a small group of them is encoded by mitochondrial DNA, which is transcribed and translated within mitochondria. The remaining, however, are encoded by nuclear DNA and transported from the cytosol to the mitochondria.

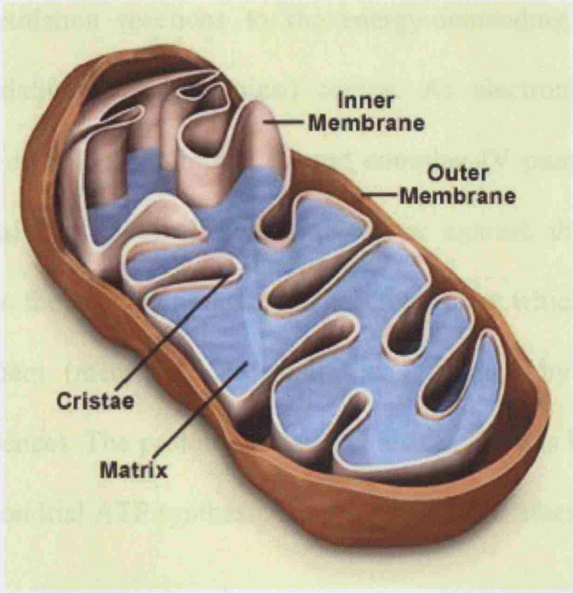



**Figure 1-12 Mitochondrial structure adapted from the ABC Body Building. Web page:**
**http://www.abcbodybuilding.com/magazine03/8weekstobiggertibialis.htm.**

### *1.13.9 Respiratory chain and ATP synthesis*

During mitochondrial oxidative phosphorylation, electrons flow through an electron transport chain from reduced substrates (such as glycerol phosphate, fatty acids, NADH or succinate) (Fig 1-13). Large membrane-bound enzymes, such as glycerol

phosphate dehydrogenase, the dehydrogenases of $\beta$-oxidation, NADH-Q oxidoreductase (complex I) or succinate-Q oxidoreductase (complex II) pass electrons down the gradient of redox potential to the mobile lipid-soluble carrier, ubiquinone (Q). From Q, electrons pass down through Q-cytochrome *c* oxidoreductase (complex III), cyt c (a second, water-soluble, mobile carrier) and cyt c oxidase (complex IV) to the final acceptor, $O_2$. Through a chemiosmotic mechanism, coupling of this series of energy-releasing oxidation reactions to the energy-demanding reactions of ATP synthesis (i.e. oxidative phosphorylation) occurs. As electrons flow down their chemical gradient, complex I, complex III and complex IV pump protons from the mitochondrial matrix to the intermembrane space against their electrochemical gradient resulting in the formation of a protonmotive force, which consists mostly of an electrical gradient (membrane potential), accompanied by a small chemical gradient (pH difference). The protonmotive force drives protons back into the matrix through the mitochondrial ATP synthase, resulting in ATP synthesis.

### *1.13.10 Mitochondrial generation of reactive oxygen intermediates*

The current scientific literature describes oxidative damage in biological systems and the role of various anti-oxidants in physiological states such as ageing and growth as well as in pathological conditions such as atherosclerosis, immunologic, neurodegenerative diseases and cancer (105).

Oxidative stress results when an imbalance between the level of oxidants and reductants in the living cell is disturbed. In this context, an increase in an oxidative

insult or a decrease in the redox buffers or both lead to exacerbation of oxidative stress. ROI are produced endogenously by several enzyme systems especially in the mitochondrial respiratory chain in which electrons flow through multiple enzyme complexes resulting in ATP production (36; 125). During this process ROI are produced but most of them are detoxified under physiological conditions by the cellular anti-oxidant machinery. Other sites of ROI generation in the cell include xanthine oxidase which converts xanthine into uric acid and produces $O_2^{\cdot-}$ as a by-product and NO synthase which produces $ONOO^-$ as a result of the reaction between NO and $O_2^{\cdot-}$ (220; 291). Moreover, during the inflammatory response, neutrophils are activated and eventually burst releasing ROI to aid inactivating foreign materials and bacteria which are introduced into the cell (100).

ROI comprise a group of highly reactive chemically-diverse molecules such as $O_2^{\cdot-}$, $H_2O_2$, $OH^{\cdot}$ and $ONOO^-$. Although most of them originate from $O_2^{\cdot-}$ generated as a by-product of oxidative phosphorylation, they differ in their mechanism of production, cofactors, diffusion range, hydrophobicity, biological targets, detoxification pathways and breakdown products.

The $O_2^{\cdot-}$ damaging effects largely involve disassembly of $Fe^{2+}$–sulphur clusters in proteins such as aconitase (291). However, its conjugate acid, the hydroperoxyl radical ($HO_2^{\cdot}$) induces lipid peroxidation (12). $O_2^{\cdot-}$ is detoxified either spontaneously, by dismutation reaction with $HO_2^{\cdot}$, or is converted to $H_2O_2$ by SOD. Nitrogenous

species include NO· which diffuses easily into mitochondria and may also be produced there (99).

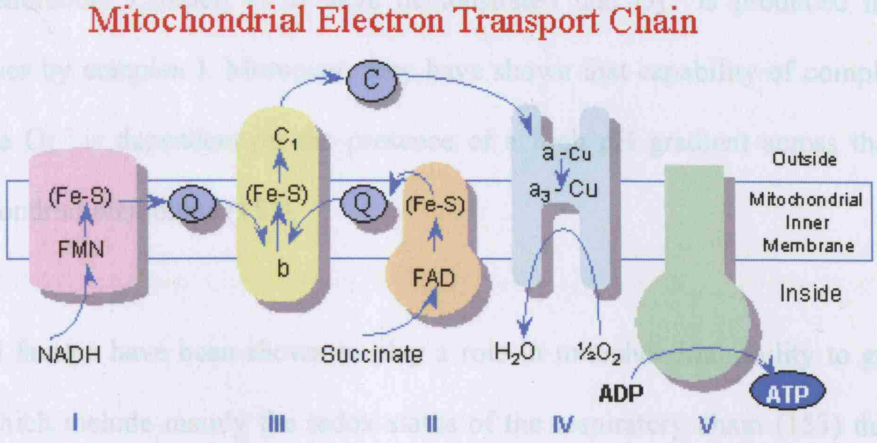



**Figure 1-13 Electron transport chain adapted from the Institute of Neurology, University College London, UK. Web page:**

**http://aix-150.ion.ucl.ac.uk/neurochemistry/nmu/Mitochondrial-Disorders.htm**

$H_2O_2$ produced by $O_2^{\cdot -}$ dismutation is relatively inert, can cross cell membranes easily and is degraded by GPx and peroxiredoxin III. However, propagation reactions can take place in which $H_2O_2$ is also broken down in the presence of $Fe^{2+}$ to form the highly damaging OH·. The cell can control the production of OH· by limiting the concentration of free $Fe^{2+}$. The deleterious effects of $ONOO^-$ and OH· involve vital cellular components such as protein, RNA and DNA.

Mitochondria are indeed the major source of ROI in the living cell (264; 288). Specifically, $O_2^{\cdot -}$ is generated initially and converted to $H_2O_2$ by spontaneous dismutation or by SOD. The major source of mitochondrial $O_2^{\cdot -}$ was until recently

believed to be the ubisemiquinone radical ($QH^{\cdot}$) formed at the complex III $Q_O$ (74; 264). However, complex I has been shown recently to represent a major source of $O_2^{\cdot -}$ generation. Lambert *et al* have demonstrated that $O_2^{\cdot -}$ is produced in large quantities by complex I. Moreover, they have shown that capability of complex I to produce $O_2^{\cdot -}$ is dependent on the presence of a high pH gradient across the inner mitochondrial membrane (153).

Several factors have been shown to play a role in mitochondrial ability to generate ROI which include mainly the redox status of the respiratory chain (153) that is in turn dependent on the *trans*-membrane proton gradient ($\Delta pH$) and the membrane potential ($\Delta\psi_m$) which can inhibit the pumps by exerting a negative feedback effect (267). All of the known regulators of mitochondrial ROI act at one or more of the sites of production of these highly toxic molecules in the respiratory chain. For instance, rotenone affects complex I, antimycin affects complex III (45) and $NO^{\cdot}$ affects complex IV (66). Overall, it seems that most bio-energetic effectors, via their effects on $\Delta pH$ and $\Delta\psi_m$, can modulate mitochondrial ROI generation.

### *1.13.11 Uncoupling proteins*

Uncoupling agents such as dinitrophenol (DNP) are lipid-soluble weak acids that cross the mitochondrial membrane in either the protonated or the unprotonated state and dissipate the protonmotive force and allow substrate oxidation to proceed without providing the driving force for coupled ATP synthesis.

Even in the absence of uncoupling agents, physiological uncoupling is quite common (236). In this context, proton leakage back into the matrix occurs, and results in partially coupled oxidative phosphorylation. It has been well established that this physiological process is a natural pathway that operates in intact cells and tissues, such as hepatocytes, thymocytes and intact skeletal muscle cells (235).

Mitochondrial uncoupling proteins have attracted major interest recently due to their role in several human disease processes such as diabetes (106), I/R injury (29) and ageing (35). Uncoupling protein-1 (UCP-1) is present in a high concentration, up to 10% of the membrane proteins. Basically, UCP-1 uncouples substrate oxidation from phosphorylation of ADP to ATP, leading to fast $O_2$ consumption and heat production. Although uncoupling of oxidative phosphorylation might be viewed as a negative response to UCPs, under certain circumstances it can be physiologically beneficial as it attenuates mitochondrial ROI generation at the expense of a small decrease in ATP synthesis (37). Moreover, mild uncoupling which implies a limited increase in proton conductance resulting in an increase in respiration and a decrease in ATP production is different from full uncoupling, where ATP synthesis is totally abolished and respiration is maximal.

### *1.13.12 Mitochondrial role in apoptosis*

Although mitochondria are essential for life due to their ability to produce metabolic energy in the form of ATP, they also play a key role in controlling the pathways that lead to cell death (139; 146; 162). This latter role of mitochondria is more than just a

'loss of function' resulting in an energy deficit but is an active process involving different mitochondrial proteins. Cyt c was the first mitochondrial factor shown to be released from the mitochondrial intermembrane space and to be actively implicated in apoptotic cell death. Other mitochondrial proteins, such as apoptosis inducing factor (AIF), Smac/DIABLO and endonuclease G are also released during apoptosis and have been implicated in various aspects of the mitochondrial apoptosis pathway. Members of the Bcl-2 protein family control the integrity and permeability of the mitochondrial membrane to apoptotic factors and orchestrate the response of mitochondria to apoptotic signals (189).

A breakthrough in the recognition of the central role of mitochondria in apoptosis came from the finding that cyt c is involved in caspase-9 activation. In this context, cyt c release occurs as a result of the effect of Bcl-2 on mitochondrial inner-membrane permeability. Subsequently, Bcl-2 moves into the cytosol where it reacts with Apaf-1 and ATP to form the apoptosome which in turn converts pro-caspase-9 into caspase-9 thus activating the intrinsic apoptotic pathway (146).

## 1.14 Apoptosis

### *1.14.1 Morphological features of apoptosis*

Programmed cell death (apoptosis) is an active form of cell death that plays a crucial role in a wide range of physiological processes during foetal development as well as in adult life. Early during apoptosis, swelling of the outer mitochondrial membrane

and release of cyt c and AIF from the mitochondrial intermembrane space occurs. Other morphological features of apoptotic cell death include chromatin condensation, nuclear fragmentation (pyknosis), blebbing of the plasma membrane and cell shrinkage (Fig 1-14). Ultimately, the dying cell fragments into membrane-bound apoptotic bodies that are cleared rapidly by phagocytosis (Fig 1-14).

### *1.14.2 Caspases and their role in apoptosis*

Cysteine aspartyl proteases (caspases) are a group of heterotetramer proteins which contain two subunits, small and large, consisting of two active sites per molecule and exist as in-active zymogens (69). In mammalian tissues, 14 caspases have been discovered which are converted into their active states by proteolytic cleavage of their aspartic acid residues upon receiving an apoptotic signal or when there is shortage of growth factors (67).

Similar to their mechanism of activation, caspases cleave their substrates at aspartic acid residues (67). Therefore, it has been postulated that caspases might actually activate each other in the same way that they cleave their substrates. These apoptotic mediators are classified into two groups, up-stream (initiator) and down-stream (effector) caspases (69). Down-stream caspases are totally dependent on upstream members for their activation and therefore, the cleavage sites that separate the small and large subunits of the effector caspases exactly fit the tetra-peptide specifications of the initiator caspases.

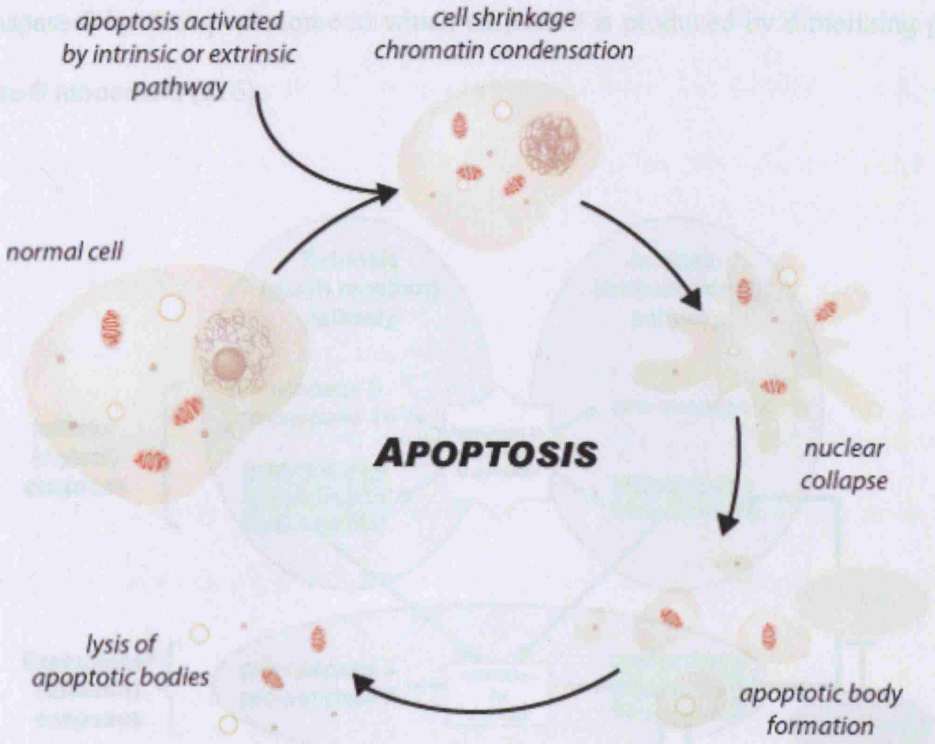



**Figure 1-14 Morphological features of apoptotic cell death adapted from BioTeach Co. Web page:**

**http://www.bioteach.ubc.ca/CellBiology/Apoptosis/.**

Two models for the conversion of pro-caspases into their active counterparts have been suggested (300). In the induced proximity model, the inactive form of the caspases is not totally inactive but it possesses a very low caspase activity (1%). Therefore, when the zymogens come in close proximity to each other, they can trans-activate each other to produce the fully-active caspases. Recently, a new model of caspase activation has been suggested in which no cleavage is necessary. In this model, aggregation of pro-caspase zymogens occurs followed by dimerization to assume an active conformation state (271). An example of this is the activation of

pro-caspase-9 by the apoptosome in which caspase-9 is produced by dimerizing pro-caspase-9 monomers (216).

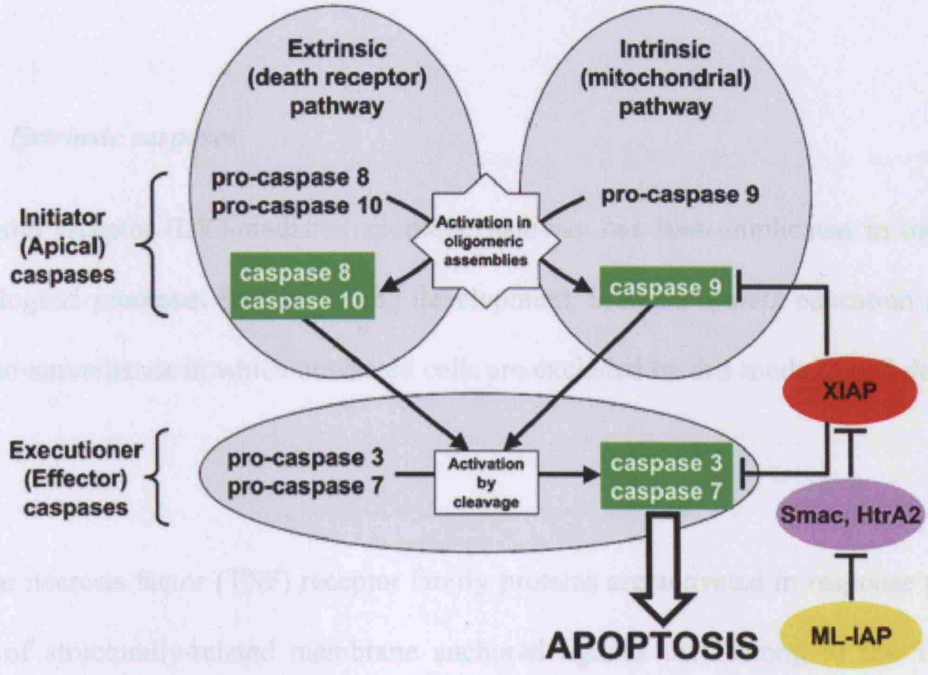



**Figure 1-15 Mechanisms of apoptosis adapted from the Biochemical Journal (2005) Volume 384. Web page: http://www.biochemj.org/bj/384/0201/bj3840201f03.htm. XIAP; X-linked inhibitor of apoptosis, HtrA2: high temperature requirement A2, ML-IAP; member livin of the inhibitor of apoptosis protein.**

Two pathways for caspase activation have been characterized, the extrinsic (death receptor) and the intrinsic (mitochondrial) pathway (Fig 1-15) (300). These pathways are described as separate but in fact a lot of cross-talk has been demonstrated between the two. In the extrinsic pathway, several signal protein interactions aid in the conversion of apoptotic signals received through death receptors to culminate in the activation of pro-caspase-8 (300). In the intrinsic pathway, however, pro-caspase-9

activation via the induced proximity mechanism results when cyt c is released from mitochondria and subsequently binds to pro-caspase-9, the cytosolic factor Apaf-1 and ATP/dATP to form the apoptosome which leads to the production of caspase-9 (300).

### *1.14.3 Extrinsic caspases*

The death receptor (DR)-mediated apoptotic pathway has been implicated in many physiological processes such as during development, immune system education and immuno-surveillance in which unwanted cells are excluded by this mode of cell death (73).

Tumour necrosis factor (TNF) receptor family proteins are activated in response to a group of structurally-related membrane anchored ligands that belong to the TNF family. In the cytoplasm, the DR contains a death domain (DD) which links them to the effector caspases (279). This death machinery starts by activation of DR of the (TNFR1) such as Fas (CD95 or Apo-1) (17). Upon activation by a death stimulus, conformational changes of the Fas cytosolic compartment occur that allow a death effector domain (DED) Fas associated death domain (FADD) to be recruited (17). FADD in turn interacts with pro-caspase-8 zymogens through its DED to form the death-inducing signal complex (DISC) which induces activation of pro-caspase-8 into the active form through the induced proximity model (32). The regulation of caspase-8 activation is mediated by the FADD (FLICE)-like inhibitory protein (FLIP) molecule which is a caspase-8 homolog that is devoid of cysteine and thus lacks

proteolytic activity. It has been shown that FLIP inhibits caspase-8 binding to DISC in a competitive manner only at high concentrations while at low concentrations it enhances its activity (51).

### *1.14.4 Intrinsic caspases*

Cells die by the intrinsic pathway in many pathological conditions such as exposure to UVA irradiation, cancer chemotherapy and states that lead to mitochondrial damage. In response to a death stimulus, the mitochondria become more permeable to cyt c which recruits and activates pro-caspase-9. Pro-caspase-9 is composed of monomers and is activated when they dimerize (230). However, unlike pro-caspase-8, cleavage of the linker region is neither enough nor necessary for the activation of pro-caspase-9.

The mitochondrion plays a very crucial role in the intrinsic cell death machinery due to its ability to orchestrate the release of both pro-apoptotic and anti-apoptotic proteins within its intermembrane space (175). The release of cyt c from the mitochondria is a central event in the process of apoptotic cell death. Cyt c binds to Apaf-1, ATP and pro-caspase-9 to form the apoptosome which leads to the activation of pro-caspase-9 into caspase-9 (175).

### *1.14.5 Executioner caspases*

In contrast to the initiator caspases, the executioner caspase-3 and -7 zymogens are located in the cytosol as inactive dimers (31) which are activated by limited

proteolysis within their inter-domain linker by an initiator caspase or occasionally by other proteases. The activation of these caspases is the "point of no return" for apoptotic cells because these proteins degrade vital cell components such as DNA and proteins.

## *1.15* Regulation of apoptosis

### *1.15.1 Death domain proteins*

Death domains play an important role in death-receptor mediated apoptosis. Many death receptors such as TNFR1, Fas (Apo1), DR3 (Apo2), DR4, DR5 and DR6 contain DD in their cytosolic part. The TNF family receptors, TNFR1, DR3 and DR6 bind an adaptor protein TNFR1–associated death domain (TRADD) via their DD (208). This factor has the capability to interact with other DD proteins such as the adaptor protein FADD which uses its DD to interact with TRADD or with TNFR family cytosolic DD.

The regulation of TNF death receptor family involves several mechanisms. The first one involves the silencer of death domains (SODD) (127) which has the ability to interact with DD of TNFR1 and DR3 preventing the activation of these death receptors in the absence of ligand.

DEDs exist in the initiator caspases and in caspase-8 and caspase-10 in humans. Examples of DED include FLICE-associated huge protein (FLASH) which mediates

Fas-mediated caspase-8 activation. Likewise, Fas-mediated apoptosis is mediated by death effector domain-containing testicular molecule (DEFT) domains which modulate Fas-mediated apoptosis by translocating to the nucleus and shutting off ribosomal RNA gene transcription (268). Not all DEDs enhance apoptosis, for example the DED, FLIP, which bears structural similarity to pro-caspase-8 and -10 (285) has strong binding capacity to FADD in a competitive manner to pro-caspase-8 and -10.

### *1.15.2 Caspase-recruitment domain proteins*

Caspase-recruitment domain (CARD) proteins are composed of 6 α-helices and exist in the N-terminal of various caspases in humans and mice such as caspase -1, -2, -4, -5, -9 and caspase -1, -2, -9, -11 and -12 respectively. These proteins have been established as key regulators of cell death by regulating the association between adaptor proteins and caspases or TNF-R family members (271).

Several CARD family proteins are critical components of the conserved cell death machinery which, when dysregulated, promote tumourigenesis and contribute to cancer resistance to chemotherapy (71). Examples of CARD proteins include the pro-apoptotic protein Apaf1, which is inactivated in some cancers (158).

### *1.15.3 Inhibitors of apoptosis proteins*

Inhibitors of apoptosis (IAPs) are Baculovirus IAP repeats (BIR) which contain proteins that suppress apoptosis in mammals, insects, and certain animal viruses. In

addition to BIR, IAPs contain RING zinc fingers, CARDs, ubiquitin conjugating enzymes (E2s) or putative nucleotide-binding domains. The relationship between cell turnover and IAP has been established. IAPs have been found to selectively bind certain caspases such as caspase-3, -7 and -9 and inhibit apoptosis induced by the intrinsic and extrinsic pathways (238).

Abnormalities in the expression of IAPs have been implicated in several disease processes such as in spinal muscular dystrophy (239) and cancer (57). In fact, modulators of IAPs have been identified recently such as SMAC/DIABLO which binds multiple IAPs allowing caspases to be activated (78).

### *1.15.4 Bcl-2 family proteins*

Bcl-2 proteins are pro-apoptotic or anti-apoptotic factors which exist in various organisms ranging from *Caenorhabditis elegans (C.elegans)* to mammals and constitute key regulators of mitochondrial dependent apoptosis (39). Several factors affect the relative ratio of pro-apoptotic to anti-apoptotic Bcl-2 factors including growth factors, hypoxia, radiation, anti-neoplastic drugs, oxidative agents and calcium. Due to their crucial role in regulating apoptosis (Fig 1-16), defects in Bcl-2 expression have been associated with multiple disease states such as Hodgkin's lymphoma (286), autoimmune diseases such as SLE (172) and I/R injury such as in stroke (108).

Examples of the anti-apoptotic Bcl-2 proteins include BclxL, Mcl-1, Bfl-1(A1), Bcl-w and Boo (Diva) whereas pro-apoptotic factors include Bax, Bak, Bock, Bad, Bid, Bim, Bik, Hrk, Bcl-Xs. As all Bcl-2 proteins are encoded by only 20 different genes, one Bcl-2 gene might encode more than one Bcl-2 protein sometimes with opposite effects such as BclxL and Bclxs. In addition, some Bcl-2 proteins might induce pro-apoptotic or anti-apoptotic effects according to the cell context (122).

Although most Bcl-2 proteins are located on the mitochondrial membrane, some of them, like Bcl-2, Bcl-xL, Bid, bim and Bad are located in the cytoplasm but can be recruited to the mitochondria. For example, Bim is inserted into the mitochondrial membrane through direct interaction with microtubules. Thereafter, it interacts with the Bcl-2 protein on the surface of the mitochondrial membrane. In addition, phosphorylation of Bcl-2 members has been reported to mediate recruitment of these proteins to the mitochondria. For example, BAD is phosphorylated and translocated from the cytoplasm to the mitochondria where it causes caspase activation (189). Conformational changes in Bcl-2 proteins might aid its incorporation into the mitochondrial membrane. An example of this is the Bax protein which controls apoptosis through modulating cyt c release (201).

The mechanisms that regulate cyt c release are not yet clear. It has been suggested that Bcl-2 proteins affect mitochondrial channel pore permeability, lead to swelling or even rupture of the mitochondria resulting in cyt c release (146).

## 1.16 Hypothesis

In this study, we tested the hypothesis that induction of HO-1 by the two ITCs, BTCC and PTCC, would protect against cisplatin and other nephrotoxic agent-induced renal failure.

## 1.17 Objectives

-To study the effects of ITCs on HO-1 in LLC-PK1 cells and the mechanisms and pathways activated during this process.

-To investigate the potential protective effects of HO-1 modulation by ITCs against CP-mediated renal failure and the role of the HO products in mediating this effect.

-To search for novel HO-1 inducers which could be used therapeutically to maintain renal function during administration of nephrotoxic agents.

# 2 MATERIALS AND METHODS

## 2.1 Materials

Male Sprague-Dawley or Wistar rats (for the CP experiments only) were used in our experiments. The rats were raised in the specific pathogen-free (SPF) unit of (Northwick Park Hospital, Harrow, Middlesex, UK). Anaesthetics used were: Hypnorm 0.7 mg/kg (Janssen Pharmaceuticals Ltd., Oxford, UK) and diazepam 2.5 mg/kg (Phoenix Pharmaceuticals Ltd., Gloucester, UK).

### *2.1.1* Reagents

All the reagents used were purchased from (Sigma-Aldrich Co., Poole, Dorset, UK) unless otherwise specified.

BTCC [S-(N-benzylthiocarbamoyl)-L-cysteine], PTCC [S-(N-3-phenylpropyl thiocarbamoyl)-L-cysteine and the guanylate cyclase inhibitor ODQ (1H-Oxadiazole [4, 3-a] quinoxaline-1-ore) were obtained form Axxora Corporation, Bingham, Nottingham, UK). Haemin (ferriprotoporphyrin IX chloride) was obtained from (Porphyrin Products Inc., Logan, Utah, USA). The HO-1 monoclonal antibody was purchased from (Stressgen, Victoria, Canada). The HO blocker (SnPPIX) was from (Frontier Scientific, Carnforth, Lancashire, UK).

## 2.2 Preparation of liver microsomal fraction

Male Sprague Dawley rats (250-300 g) were obtained from the SPF unit of (Northwick Park Hospital). The animal was allowed free access to commercial rat chow and tap water and housed in 12h/12h light dark cycle at 24 °C. The rat was anaesthetized using Hypnorm 0.7 mg/kg i.m. and diazepam 2.5 mg/kg i.p.After midline laparotomy incision was performed, the hepatic vein was cannulated with 22 G catheter (Venflon, Helsingborg, Sweden). Perfusion of the liver was performed in situ by using 50 ml cold saline (0.9% NaCl). The liver was then harvested, weighed and transferred into 10 ml Potter-Elvehjem glass Teflon homogenizer (Jencons Ltd., Leighton, Buzzard, Bedfordshire, UK) on ice containing 5 volumes of cold buffer (0.25 M sucrose, 50 mM tris-HCl, pH 7.4, 4 °C) and was homogenised at 200-300 rpm using an electric rotor (Glass-col, Terre Haute, Indiana, USA). The homogenate was transferred into a centrifuge tube and was centrifuged for 20 min at 27000xg, 4 °C using a swing bucket rotor centrifuge (LE-80, Ultracentrifuge, Beckman Instruments Inc, Palo, Alto, California, USA). The supernatant was transferred into a fresh tube and was centrifuged at 105000xg for 60 min. The supernatant was discarded and the pellet was resuspended in 1 ml cold buffer (0.1 M $KH_2PO_4$, 2 mM $MgCl_2$, pH 7.4) and aliquots were kept at -80 °C to be used for determination of HO activity biochemical assay as a source of biliverdin reductase,

## 2.3 Preparation of rat liver cytosol fraction

The rats used here were the same as in the previous section. One day before the procedure the rat was administered haemin 50 mg/kg i.p. On the following day the rat

was terminally anaesthetized with Hypnorm 0.7 mg/kg im. Mid-laparotomy incision was performed and the liver was immediately harvested and gently perfused in situ with cold KCl-Tris HCl (20 mM, pH 7.4) solution. The liver was cut into four equal pieces and transferred into separate polycarbonate tubes (Sarstedt Ltd., Beaumont Leys, Leicester, UK). Two to three volumes of the cold KCl-Tris HCl solution was added to each tube. The liver pieces were finely homogenized by using electric rotor (Glass-col, Terre Haute, Indiana, USA). The homogenate was transferred into centrifugation tubes and centrifuged at 5000xg for 20 min at 4 °C in a swing-bucket rotor centrifuge (LE-80). The supernatant was collected and transferred into another fresh tube and centrifuged at 105000xg for 80 min at 4 °C. The lipid top layer was removed and aliquots of the supernatant were kept at -80 until needed.

## 2.4 Cell culture

### *2.4.1 Cell culture and maintenance of cell line*

Porcine renal tubular epithelial (LLC-PK1) cells were obtained from the European Collection of Animal Cell Culture (Salisbury, Wiltshire, UK). The cells were grown in DMEM supplemented with 10% foetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin and 0.1 mg/ml streptomycin. Cells were grown in 75 $cm^2$ tissue culture flasks (Sarstedt Ltd.) and maintained at 37 °C in a humidified atmosphere of 95% $O_2$ and 5% $CO_2$. Confluent cells were passaged three times weekly by trypsinization. Confluent cells were incubated with agents using DMEM

supplemented with 10% FBS in all experiments except in LDH and Alamar Blue assay in which the cells were incubated using DMEM supplemented with 1% FBS.

### *2.4.2 Incubating cells with agents*

Treatment agents were prepared and diluted in the growth medium to the required final concentration for incubation with cells. 10 ml of incubation medium was added to flasks which were returned to the incubator for the time-course of the experiment. For cells cultured in 24-well and 6-well plates, the agents were diluted to the required concentrations and a total of 1 ml medium was added to each well.

### *2.4.3 Preparation of LLC-PK1 cell microsomal fraction*

At the end of the experimental incubation, LLC-PK1 cells were washed with ice cold PBS (Invitrogen Life Technologies, Paisley, UK) and the cells were manually scraped off the tissue culture flask base with a cell scraper (Sarstedt Ltd.) in 5 ml PBS. The cell suspension in PBS was transferred into a fresh tube. To ensure that all of the cells were transferred, the flask was washed another time with 4 ml PBS and transferred into the tube. The 9 ml total volume was centrifuged at 2000xg for 5 min at 4 °C in a centrifuge (Harrier 15180 MSE, Sanyo, Japan). The supernatant was discarded and the pellet was either resuspended in 550 µl PBS containing 2 mM $MgCl_2$ (for HO assay) or in 250 µl PBS solution containing 1% Triton x100 (for Western blot). Samples were kept at -80 °C until needed for HO activity assay or Western immunoblotting.

## 2.5 Animals and treatment

Male Wistar rats weighing 300-400 g were obtained from the animal facility of Northwick Park Hospital and were used in all CP experiments. A total of 55 rats were used in our experiments. For the ITCs-CP study, the rats were divided into five groups ($n$=5 in each group). Group 1 (control) received sterile water i.p. daily for 4 days but on day two they received equivalent volume of DMSO to the CP group. To induce renal failure in the rats, group 2 (CP) received a single i.p. dose of CP (7.5 mg/kg), a dose which has been shown previously to induce renal dysfunction in rats (508). Since no previous studies have tested an HO-1-inducing dose of these two ITCs in rats, group 3 (BTCC or PTCC) were started on 25 mg/kg dose of BTCC or PTCC i.p. daily for 4 days. To test whether these two compounds would protect against CP-mediated renal failure, group 4 (BTCC or PTCC plus CP) received 25 mg/kg BTCC or PTCC i.p. for 4 days starting 24 h prior to a single dose of CP 7.5 mg/kg i.p. To determine whether HO would be involved in the reno-protective effect of these two ITCs, group 5 (BTCC or PTCC+CP+SnPPIX) received daily injections of BTCC or PTCC (25 mg/kg i.p.) with SnPPIX (10 mg/kg i.p.) to inhibit HO-1 (131) for 4 days starting 1 day before the CP dose.

For the CORM-3-CP study, rats were divided into 6 groups ($n$=5 each): Group 1 and group 2 were the same as above. Group 3 received CORM-3 (10 mg/kg i.p.) daily for 4 days. Group 4 and group 5 received CORM-3 (10 mg/kg i.p.) or iCORM-3 (10 mg/kg i.p.), respectively, daily starting 24 h prior to CP treatment and continuing for 3 days thereafter. To examine a possible involvement of HO-1 induction following

CORM-3 treatment (292), group 6 received CORM-3 (10 mg/kg i.p.) in combination with SnPPIX (10 mg/kg i.p.) daily starting 24 h prior to CP treatment and continuing for 3 days thereafter.

At the end of the treatment, blood samples were taken by cardiac puncture to measure serum urea and creatinine and the animals were sacrificed by decapitation, the right kidney was gently collected and fixed immediately with buffered formalin for further analysis. The kidneys were embedded in paraffin, sectioned and then stained with the TUNEL technique for detection of apoptotic activity.

### 2.6 Haem oxygenase biochemical assay

Frozen microsomal fractions of LLC-PK1 cells were thawed for 5 min at 37 °C in a water bath and returned to the freezer for 20 min. This freezing/thawing cycle was repeated another two times. Protein concentration of samples was measured by using Bio-Rad protein assay kit (Bio-Rad Laboratories Ltd., Hemel Hampstead, Hertfordshire, UK). For the HO activity measurement, the following reagents were added in sequence to a glass tube:

300 µl PBS containing 2 mM $MgCl_2$.

3 mg rat liver cytosol fraction.

50 µl 2 mM glucose-6-phosphate (G6PD).

15 µl 0.2 U/l glucose-6 phosphate dehydrogenase (G6PDH).

30 µg liver microsomal fraction.

25 µl 2 mM haemin.

25 µl 0.8 mM NADPH.

400 µl sample.

The tubes were mixed for 2 min, sealed with Para-film (American National Cat, Greenwich, Connecticut, USA) and wrapped with aluminium foil to allow the reaction to take place in the dark. The tubes were left for 60 min in a water bath at 37 °C and the reaction was terminated with 1 ml chloroform. The tubes were mixed vigorously for 3 min before they were centrifuged at 900xg for 5 min. The tubes were then briefly mixed for 5 sec to mix the layers and they were centrifuged again at 1100xg for 5 min. The extracted BR was measured in 550 µl of the chloroform layer of each tube in a glass cuvette (Sigma-Aldrich Co.) and absorbance was determined at 464 nm and 530 nm against a blank which contained 1 ml chloroform, using a spectrophotometer (Uvikon 810p Spectrophotometer, Kontron Instruments Ltd., Watford, Hertfordshire, UK). HO activity was measured by calculating the difference between absorbance at 464 nm and 530 nm using the following equation:

$OD/40x10^6$ = pmoles bilirubin/mg protein/ h in 400 ml suspension.

## 2.7 Western blot analysis

Protein concentration was determined at the beginning of the procedure as described above. After determination, the relevant sample volumes were diluted in Laemmli buffer (62.5 mM Tris-HCl, pH 6.8, 10% v/v glycerol, 2% w/v SDS, 2.5% v/v ß-mercaptoethanol, 0.06% w/v Bromophenol Blue). The samples were then heated for 10 min at 100 °C in a dry heating block (Thermostat 5320, Eppendorf Netheler-Hinz-

GmbH, Hamburg, Germany). Thirty microgram protein from each sample were loaded into a 15% w/w SDS-polyacrylamide resolving gel with 4% w/v polycrylamide stacking gel (Bio-Rad Laboratories Ltd.). Electrophoresis of the samples was performed in running buffer (1XSDS PAGE Tank buffer "Electran" (BDH Merck Ltd., Poole, Dorset. UK). The gel was allowed to run in a MiniPROTEAN 2 Electrophoresis Cell (Bio-Rad Laboratories Ltd.) at 125 V until the bromophenol blue marker band had migrated to the bottom of the gel. A gel sandwich was assembled for the purpose of transferring the proteins to a nitrocellulose membrane (pore size 0.45 μM) (Amersham Plc., Little Chalfont, Buckinghamshire, UK). The sandwich was composed of six layers ordered as: sponge, Whatman paper, gel, nitrocellulose membrane, Whatman paper and sponge. The sandwich was then placed in a clean tank containing transfer buffer (running buffer containing 20% v/v methanol). The electrophoresis transfer was allowed to take place at 4 °C overnight at 30 V and good transfer was assessed by visualizing the bands on the membrane. After the transfer process had finished, non-specific binding was prevented by using a blocking solution which contained 20 ml PBS solution (0.1 M Phosphate buffer, 27 mM KCl pH 7.4 and 0.139 M NaCl pH 7.4) containing 0.05% (v/v) polyoxyethylenesorbitan monolanurate (Tween 20) (PBS-T) and 5% w/v non-fat dried milk for 2 h at room temperature on an orbital shaker (Luckham R100 Rotatest Shaker, Denley Instruments, West Sussex, UK). The membrane was washed once for 5 min with PBS before it was probed with mouse anti-HO-1 monoclonal antibody (Stressgen, Victoria, Canada) diluted 1 in 1000 in TBS (50 mM Tris, 138 mM NaCl, 2.7 mM KCl, pH 7.4) for 2 h at room temperature with continuous shaking. The

membrane was then washed once with PBS-T for 5 min and 2 washes with TBS were carried out. Thereafter, the membrane was incubated for 1 h with biotin-conjugated rabbit anti-mouse antibody (Sigma-Aldrich Co.) diluted 1 in 1000 in TBS. After 1 h the membrane was washed 3 times with 10 ml TBS before it was incubated for another 1 h in extravidine alkaline phosphatase antibody (Sigma-Aldrich Co.) diluted 1 in 1000 TBS after which the membrane was washed 3 times with TBS. The membrane was then incubated for 10 min with substrate mixture composed of 0.1 M Tris HCl pH 8.2, containing 20 ml/ml of 20 mM naphthol AS-BI phosphate and 1 mg/ml fast red RC salt. Finally the membrane was washed 3 times with distilled water and was kept wet for scanning.

### 2.8 LDH assay

LLC-PK1 cells were grown in 24-well plates (Sarstedt Ltd.). At the end of the experimental procedure, the medium was collected and centrifuged at 500xg for 5 min at 4 °C. The supernatant was used to determine LDH release by using the cytotoxicity detection kit "LDH" (Roche, Lewes, East Essex, UK) according to the manufacturer's instructions. The procedure for determination of LDH release is based on the reduction of pyruvate into lactate in the following equation;

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \rightleftharpoons \text{L-Lactate} + \text{NAD}$$

During the reduction of pyruvate to lactate, an equimolar amount of NADH is oxidized into NAD and results in reduction of absorbance at 340 nm which is directly

proportional to the amount of LDH activity in the sample. The percentage of LDH release in the control and treated cells was calculated as a percentage of that released in the 5% Triton-X100-extracted cells.

### 2.9 Alamar Blue assay

Cell metabolism was determined by the Alamar Blue assay kit (Serotec Inc., Kidlington, Oxford, UK). The oxidized, blue, nonfluorescent Alamar Blue is reduced to a pink fluorescent dye in the medium by cell activity (likely to be by oxygen consumption through metabolism) which in turn reflects cell viability (204). Cells were grown in 24-well plates and at the end of the incubation with the agents, the Alamar Blue stock solution was diluted 1:10 (v/v) according to the manufacturers' instructions in the growth medium; 500 µl was transferred into each well and left to incubate for 4 h in an incubator or until a colour change was seen. After incubation, absorbance at 570 and 600 nm was read in a microtitre plate reader (VersaMax Molecular Devices, Sunnyvale, CA, USA). Relative metabolic activity was determined by subtracting the background absorbance measured at 600 nm from the absorbance at 570 nm.

### 2.10 Caspase-3 activity assay

Caspase-3 specific activity was measured by using the caspase assay system colorimetric™ kit (Promega, Southampton, Hampshire, UK). LLC-PK1 cells were grown in 6-well plates (Sarstedt Ltd.). The assay was performed according to the manufacturer's instructions with minor modifications. Briefly, cells were gently scraped in their own medium using a cell scraper and collected by centrifugation at

500xg for 10 min. The pellet was gently washed with cold PBS and resuspended in 110 µl cell lysis buffer (312.5 mM HEPES, pH 7.4, 31.25% w/v sucrose and 0.3125% w/v CHAPS (3-[(cholamidopropyl) dimethylammonio 1 propane sulfonate) (Promega). To ensure complete cell lysis, the samples were subjected to 3 cycles of freezing/thawing before they were centrifuged again at 15000xg for 20 min. The supernatant was collected to measure protein concentrations. Equal concentrations of protein were used (20 µg) to measure caspase-3 activity using the caspase-3 substrate Ac-DEVD-pNA. Anti-Fas (Abcam plc., Cambridge, Cambridgshire, UK) was used as a positive control and DEVD-fmk (Promega) was used as a negative control. In this assay, the colorimetric substrate (Ac-DEVD-pNA) is labelled with the chromophore *p*-nitroaniline (pNA) which is released from the substrate upon cleavage by DEVDase (caspase-3). Free pNA produces a yellow colour that is monitored by a spectrophotometer at 405 nm. The amount of yellow colour produced upon cleavage is proportional to the amount of caspase-3 activity in the sample. Caspase-3 activity was calculated according to the manufacturer's instructions as pmol pNA liberated/h/µg protein.

### 2.11 TUNEL assay

DNA fragmentation as an indicator of necrosis and apoptosis was detected by TdT-mediated dUTP nick end-labeling (TUNEL) assay using the DeadEnd colorimetric TUNEL assay kit (Promega). LLC-PK1 cells were grown to confluence in 4-well glass slide chambers (Fisher, Loughborough, Leicestershire, UK). TUNEL assay was performed according to the manufacturer's instructions. Briefly, the medium was

removed and the cells were fixed in situ with buffered formalin for 25 min at room temperature to enhance the detection of apoptotic cells by this method. Cells were washed with PBS prior to permeabilization with 0.2% Triton-X100 solution in PBS. The cells were then equilibrated for 10 min with equilibration buffer (200 mM cacodylate [pH 6.6 at room temperature (RT)], 25 mM Tris-HCl [pH 6.6 at RT], 0.2 mM DTT, 0.25 mg/ml BSA and 2.5 mM cobalt chloride). The cells were then incubated with a mixture of equilibration buffer, terminal deoxynucleotidyl transferase (TdT) and biotinylated nucleotide mix for 60 min in humidified chamber at 37 °C. The reaction was terminated with 2x standard saline citrate (2xSSC) solutions. Endogenous peroxidases were blocked with 0.3% $H_2O_2$ solution. Then the cells were incubated with streptavidin horse radish peroxidase-labelled (HRP) solution for 10 min prior to staining with a mixture of diaminobenzidine (DAB) substrate, deionized water, 20x chromogen and 20% $H_2O_2$ for 10 min. The chambers were removed and cover slips were mounted on the slides by using depex polystyrene (DPX) mounting medium (Raymond A Lamb Ltd., Eastbourne, East Sussex, UK). Apoptotic cells were counter stained dark brown and observed with a light microscope.

For paraffin-embedded renal sections (see below), tissues were deparaffinized by immersing them in xylene substitute (Accustain) (Sigma-Aldrich Co.) in a coplin jar (Sigma-Aldrich Co.). Then the tissues were rehydrated by immersing them in graded alcohol solutions (100%, 95%, 85%, 70% and 50%). Tissues were subsequently refixed in situ with buffered formalin solution. Thereafter, they were incubated with

proteinase K solution for 30 min, to ensure sufficient permeabilization of cells, prior to fixation again with buffered formalin. Tissues were then incubated with a mixture of equilibration buffer, terminal deoxynucleotidyl transferase (TdT), and biotinylated nucleotide mix for 60 min in a humidified chamber at 37 °C. Subsequently, the tissue sections were treated like cultured cells. The necrotic and apoptotic changes were assessed by a renal pathologist.

### 2.12 Paraffin-embedding and sectioning of renal tissue

The kidneys were allowed to fix for 48 h in 10% formalin and after 24 h the fixative was replaced with fresh one. The kidneys were cut longitudinally into two equal pieces and each piece was placed in an embedding cassette. The tissues were allowed to be processed overnight using Tissue-TEK VIP Miles scientific tissue processor (Sakura Finetec Europe B.V., Zoeterwoude, Netherlands) and then embedded in wax. Excess wax was trimmed and 5 μm sections were made using Shandon A5325 Rotary Microtome (Thermo Electron Corporation, Waltham, MA, USA). The slides were then baked overnight at 40 °C in a lamp hotplate (Jepson Bolton & Co. Ltd., Watford, Herts, UK) after which were ready for staining.

### 2.13 Renal histological evaluation

At the end of the experimental procedures, the kidneys were fixed in 10% formalin, processed to paraffin wax, sectioned and stained with haematoxylin and eosin (H & E) for light microscopic analysis.

Histopathological evaluation was carried out by a histopathologist blinded to the experimental procedures. In each kidney section, a total of 60 high power fields (HPF) were examined for morphological changes in four histological regions of the kidney: cortex (proximal convoluted tubules), outer stripe of outer medulla (mainly the straight portion of the proximal convoluted tubules), inner stripe of outer medulla (mainly the thick ascending limb of the loop of Henle) and inner medulla (collecting ducts). A qualitative histopathological evaluation was carried out using a scoring system which was reported after assessing the histological changes in kidneys of 5 different rats that were treated in different weeks. The following parameters were evaluated: glomerular necrosis, tubular necrosis (by visualizing necrotic changes in the cells such as swelling or rupture of cells and blebbing of the plasma membrane), tubular dilatation, epithelial sloughing vascular congestion and extravasation. All these parameters were assessed according to the degree of changes involved using a 0 to 4 grading system. A score of 0 corresponded to the absence of change, a score of 1 indicated marginal changes, a score of 2 corresponded with minimal change and 3 and 4 indicated moderate and severe changes, respectively.

### 2.14 Isolation of rat kidney mitochondria

Male Sprague-Dawley rats (400-500g) were obtained from the animal facility of Northwick Park Hospital. The rats were initially sacrificed by cervical dislocation and the kidneys were immediately removed and gently flushed with cold saline. The cortex was visually distinguished and the medulla was removed. The cortex was minced in high EDTA-containing buffer (210 mM mannitol, 70 mM sucrose, 50 mM

Tris-HCl and 10 mM EDTA, pH 7.4). The cortex was homogenized using a glass Teflon Potter homogenizer (Glass-col) and the lysate was re-suspended in 50 ml high EDTA-containing buffer and centrifuged at 1000xg for 10 min at 4 °C (Beckman Avanti TM30 centrifuge, Fullerton, CA, USA). The supernatant was centrifuged again at 10,000xg for 10 min to obtain a first mitochondrial pellet which was re-suspended in low EDTA-containing buffer consisting of (225 mM mannitol, 75 mM sucrose, 10 mM Tris and 0.1 mM EDTA, pH 7.2). The pellet was further homogenized using a glass Teflon homogenizer and the volume of the homogenate was adjusted to 50 ml with low EDTA-containing buffer prior to centrifugation at 10,000xg for 10 min. The mitochondrial pellet was finally re-suspended in 500 µl low EDTA-containing buffer and kept on ice until use.

### 2.15 Measurement of mitochondrial respiration

All experiments were carried out using a 2 ml capacity incubation chamber maintained at 30 °C and a Clark electrode (Yelow Springs Instruments, Ohio, USA) attached to an oxygen monitoring system model 5300 (USI Inc., Madison, CT, USA) connected to a Servoger 124 flatbed chart recorder (LEM Ltd., Skelmersdal, Lancashire, UK). The buffer in the chamber was stirred constantly using an electromagnetic stirrer. All experiments were carried out using freshly isolated mitochondria. Protein assay of the sample was measured by using a protein assay kit (Bio-Rad Laboratories Ltd.) and a total protein concentration of 0.5 mg was used. The mitochondrial sample was re-suspended in a respiratory buffer containing: 100 mM KCL, 75 mM mannitol, 25 mM sucrose, 50 µM EDTA, 10 mM Tris-HCl and 10 mM

$KH_2PO_4$-Tris supplemented with 0.5 mg BSA. Mitochondrial respiration was measured using malate (5 mM) and glutamate (5 mM) as substrates. The following mitochondrial functional parameters were measured: 1) state 3, which is the active state of respiration representing oxygen uptake during oxidative phosphorylation or the production of ATP from ADP; 2) state 4, the rate of oxygen uptake after the complete exhaustion of ADP and represents the fraction of oxygen consumed by the mitochondria but not used for ATP synthesis; and 3) the respiratory control index (RCI), which is the ratio of $O_2$ utilization between state 3 and state 4 and indicates the tightness of coupling between respiration and phosphorylation. State 3 respiration was initiated by adding ADP 1 min after addition of CO-RM and state 4 respiration was measured after all the ADP was converted to ATP.

### 2.16 Statistical analysis

Differences in the data among different groups were analyzed by one way analysis of variance (ANOVA) combined with the Bonferroni test. Values were expressed as mean ± SEM and the differences were considered to be significant at $P< 0.05$.

# 3 ISOTHIOCYANATES MODULATE HO-1 EXPRESSION IN RENAL EPITHELIAL (LLC-PK1) CELLS

## 3.1 Introduction

HO-1 is induced in a number of experimental injuries and diseases of various organs including pleurisy (306), congestive heart failure (221), renal I/R injury (222), pancreatitis (249), carbon tetrachloride-induced liver injury (253), corneal inflammation (155), neurodegenerative diseases (210) and ischaemic stroke (209). Since the induction of HO-1 has been shown to protect against various forms of tissue injuries, the specific activation of the highly inducible *ho-1* gene by pharmacological modulation may represent a novel target for therapeutic intervention of various debilitating human diseases (181). An ideal HO-1 inducer would be a non-toxic, naturally occurring and available compound that could be used as a supplement in our diet to enhance defence against various toxic stimuli.

Increasing experimental and epidemiological evidence has recently shown the beneficial effects of several naturally-occurring compounds in preventing various pathological conditions (93). In particular, naturally occurring plant-derived compounds would have significant advantages as they are widely available and have acceptance among public. Therefore, extensive work has been conducted in our laboratory, to study the stimulatory effects of various plant-derived phenols such as

curcumin, CAPE, rosolic acid and 2-hydroxychalcone on HO-1 (93; 181). In this context, Motterlini *et al* have shown that the anti-inflammatory and chemprotective compound curcumin significantly induced HO-1 in endothelial cells. Moreover, the authors have demonstrated that this HO-1-stimulating effect played the major role in protecting the cells against oxidative stress (181). In another study, Balogun *et al* have indicated the important protective role of curcumin-induced HO-1 stimulation in renal epithelial cells against hypothermic storage-mediated injury (24).

Not only do Phase II detoxifying genes provide a major mechanism by which cells combat the toxicities of ROI but their induction also is highly effective in protecting cells against oxidative stress as well as the toxic and neoplastic effects of many toxicants and carcinogens (173). Many naturally-occurring chemopreventive phytochemicals are known to increase the transcriptional activation of these phase II genes and this has been shown to contribute to their overall cytoprotective effects (68). HO-1, with its mediated synthesis of ferritin, CO, BV and BR, has emerged as an important phase II gene with anti-oxidant and tissue-protective effects. HO-1 knockout studies have clearly shown that this enzyme plays a central role in cellular anti-oxidant defence and, specifically, in vascular protection (171).

ITCs have been found to exert chemprotective and cytoprotective effects via inducing various phase II enzyme genes (283). In this process, ITCs utilize several signalling pathways such as MAPK, PI-3K and PKC to increase the transcriptional expression of these highly protective enzymes (132). Initially, Nrf2 is phosphorylated and is

released from its binding site in the cytoplasm (Keap1) (141). Subsequently, Nrf2 is translocated into the nucleus where it interacts with the promoter region of various phase II genes that contains the ARE/EpRE and enhances the transcriptional activation of these genes (123).

## 3.2 Objectives

In this study, we utilized LLC-PK1 cells to examine the effect of the two synthestic and commercially available ITCs, [S-(N-benzylthiocarbamoyl)-L-cysteine] (BTCC) and [S-(N-3-phenylpropyl thiocarbamoyl)-L-cysteine (PTCC), on HO-1 and the mechanisms that underlie this effect. Moreover, we wanted to characterise the signal transduction pathways that are used by these two compounds to modulate HO-1.

## 3.3 Materials and methods

### *3.3.1 Chemicals and reagents*

BTCC and PTCC were purchased and prepared as previously outlined (see chapter 9). N-acetylcysteine (NAC), cycloheximide (CHX), actinomycin D (act D) and all other chemicals were obtained from (Sigma-Aldrich Co.).

### *3.3.2 Cell culture*

LLC-PK1 cells were used in all experiments and were obtained and maintained as stated in chapter 9.

### *3.3.3 Experimental protocol*

To examine the effect of BTCC and PTCC on HO-1 in LLC-PK1 cells, cells were exposed to increasing concentrations of BTCC or PTCC starting from a low concentration (2.5 μM) and a relatively short incubation time (6 h). Then we increased the concentration and time of incubation gradually to 10, 20 and 40 μM and the time of incubation to 18 and 36 h respectively to test whether this effect would be concentration- and time-dependent. Control cells were incubated with an equal amount of vehicle. At the end of the treatment, the cells were harvested manually and analyzed for HO activity or HO-1 protein expression by Western immunoblotting technique.

The participation of the MAPK pathways in the induction of HO-1 by these two ITCs was examined by pre-incubating the cells with highly specific inhibitors of the different MAPK pathways (168). Cells were pre-treated with 10 μM PD 098059 (ERK pathway inhibitor), 10 μM SP600125 (JNK pathway inhibitor) or 20 μM SB203580 (p38 pathway inhibitor) for 30 min prior to the exposure to 10 μM BTCC or PTCC for 6 h (a concentration and time which were shown to cause a significant HO-1 stimulation with no toxicity) in the inhibitor-containing medium. To evaluate the contribution of PI-3K and PKC pathways, the cells were pre-incubated with 40 μM LY294002 which is a specific PI-3K pathway inhibitor (168) or 5 μM chelerythrine chloride (Ch.Cl) which is an inhibitor of the A and B isoform PKC pathway (58) for 30 min prior to the addition of 10 μM BTCC or PTCC to the inhibitor-containing growth medium for an additional 6 h.

To examine the role of oxidative stress as a result of ROI generation in BTCC- and PTCC- mediated induction of HO-1, the cells were co-incubated with the radical scavenger NAC (2.5 mM) and BTCC or PTCC (10 μM) for 6 h. To establish whether new mRNA and HO-1 protein synthesis were required for HO-1 induction by BTCC and PTCC, cells were pre-incubated with 1 μg/ml of the transcriptional inhibitor act D (54) or 5 μg/ml of the protein synthesis inhibitor CHX (307) for 30 min prior to the addition of 10 μM BTCC or PTCC to the act D- or CHX- containing medium for additional 6 h.

### *3.3.4 Assay for HO activity*

Haem oxygenase activity was determined at the end of each treatment as previously described in chapter 9.

### *3.3.5 Western immunoblotting for HO-1 protein*

Samples of LLC-PK1 cells treated for HO activity assay were also analyzed by Western immunoblotting technique as described previously in chapter 9.

### *3.3.6 LDH assay*

To measure the capability of PTCC and BTCC to cause necrosis in LLC-PK1 cells, LDH assay was preformed as reported in chapter 9.

### *3.3.7 Alamar Blue assay*

Cell metabolism as an indicator of cell viability was determined by the Alamar Blue method as indicated in chapter 9.

## 3.4 Results

### *3.4.1 Effect of BTCC and PTCC on LLC-PK1 cell viability*

The percentage of LDH release was used as a measure of cell injury after incubation of cells with increasing concentrations of BTCC or PTCC for 24 h. LDH release noticeably increased in LLC-PK1 cells treated with 20, 40 and 60 μM BTCC or PTCC (Fig 3-1A). At low concentration (10 μM), both ITCs failed to induce any increase in LDH release. However, at concentrations above 20 μM, BTCC caused a concentration-dependent increase in LDH release but when the same concentration of PTCC were used an initial increase of LDH release was noticed at 40 μM but started to decrease thereafter.

Alamar Blue assay was used as an indicator of cell metabolic activity and viability (204). After exposure to both ITCs, cellular activity started to decrease significantly when the cells were incubated with 20 μM BTCC and dramatically decreased when the cells were exposed to 40 μM BTCC or PTCC (Fig 3-1B). Overall, BTCC seemed to be cause more toxicity to the cells than PTCC. For instance, at 40 μM, BTCC caused a complete shut down of cell metabolic activity. Notably, BTCC and PTCC, at concentrations below 10 μM, actually enhanced cellular metabolic activity and seemed to increase cell viability.

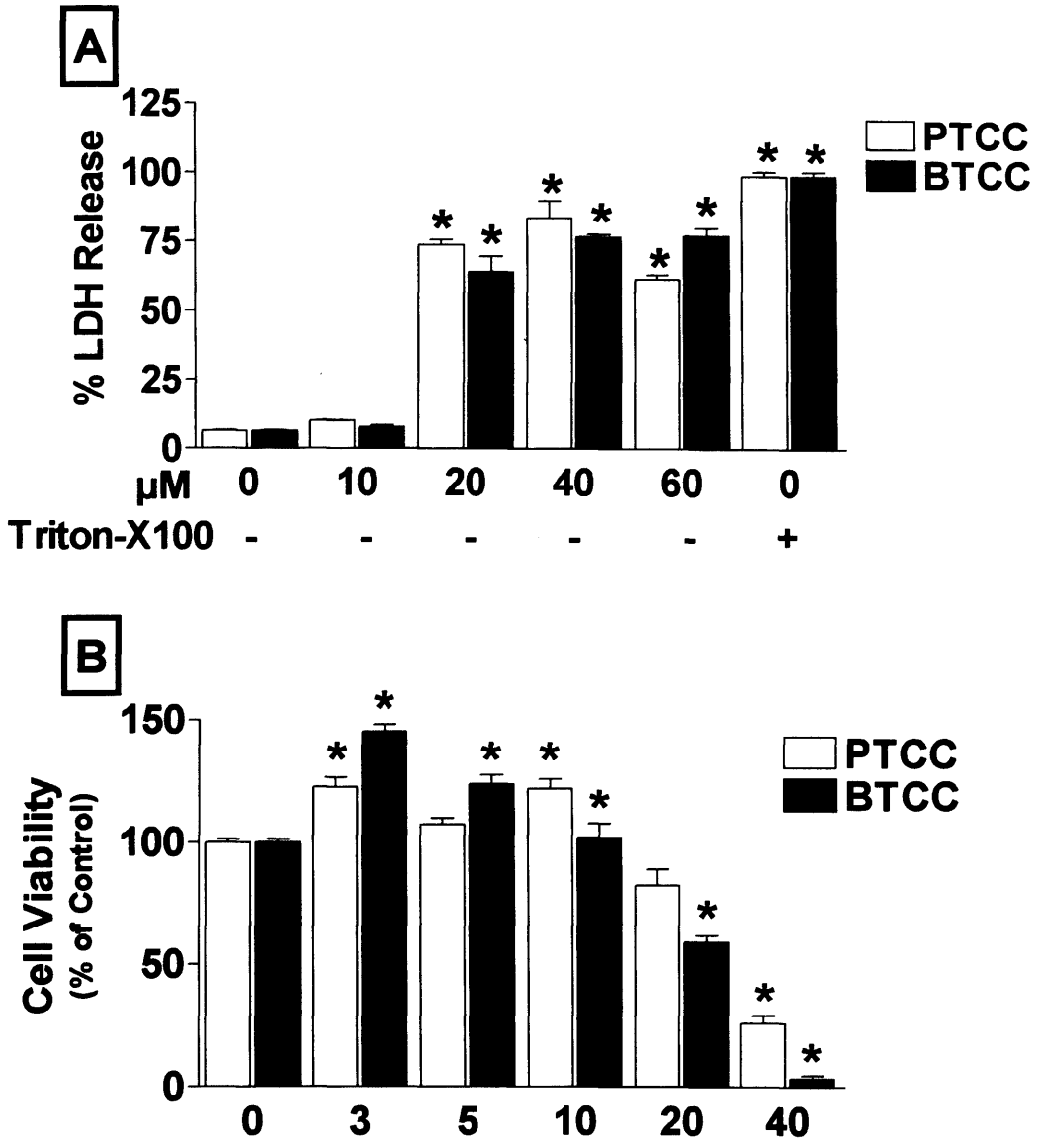



**Figure 3-1 Effect of BTCC and PTCC on LDH release and metabolic activity as an indicator of cell viability in LLC-PK1 cells.**

Cells were incubated with the indicated concentrations of BTCC or PTCC for 24 h. At the end of the incubation, an aliquot of supernatant was used for detection of LDH release and cell injury which was expressed as % of total LDH release of the 5% Triton-X100-treated group **(A)** or cell viability was measured with the Alamar Blue method **(B)** at the end of the incubation period as described in Materials and Methods. Bars represent the mean ± S.E.M. of 5 independent experiments. * $P<0.05$ vs vehicle-treated group.

### *3.4.2 BTCC and PTCC increase HO activity and HO-1 protein expression in LLC-PK1 cells*

To determine whether BTCC and PTCC induce HO-1, LLC-PK1 cells were exposed to increasing concentrations of BTCC or PTCC (Fig 3-2). To determine whether these two ITCs induces HO-1 in a concentration- and time-dependent manner cells were exposed to increasing concentrations (2.5-40 μM) and for three different periods (6, 18 and 36 h) respectively. After 6 h incubation, a concentration-dependent increase in HO activity and HO-1 protein expression were observed which reached the maximum at 20 μM concentration in the BTCC-treated cells and at 10 μM in the PTCC-treated cells. While at 40 μM, HO activity started to decrease. Noticeably, the potency of both compounds to induce HO-1 was almost equal at concentrations below 10 μM. However, at 20 and 40 μM, BTCC was shown to be a stronger HO-1 inducer.

A time course analysis in cells treated with 20 μM BTCC or PTCC showed a time-dependent increase in HO activity that reached the peak after 18 h but after 36 h a dramatic decrease in HO-1 activity was noticed which is mainly related to the cytotoxicity of these two compounds (Fig 3-3). The difference in HO activity between (Fig 3-2) and (Fig 3-3) could be related to two factors: the use of a new patch of cells which could have shown less HO-1-stimulating effect in response to BTCC and PTCC and the use of 2 independent groups of cells only which could have increased the variations in the results.

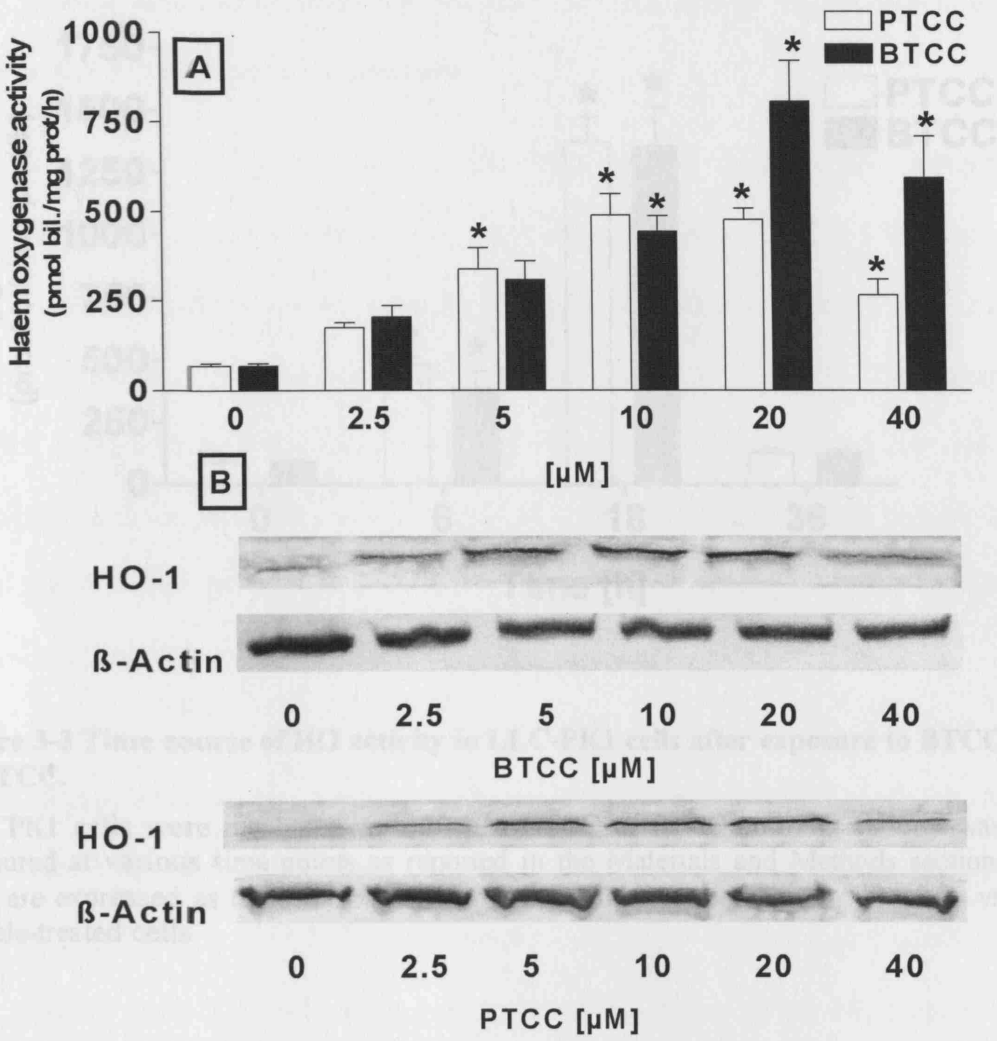



**Figure 3-2 PTCC and BTCC increase HO activity and HO-1 protein expression in LLC-PK1 cells.**

**(A)** Effect of various concentrations of BTCC and PTCC on HO activity. The cells were exposed to increasing concentrations of BTCC or PTCC (0-40) µM for 6 h and the enzyme activity was measured spectrophotometrically. Each bar represents the mean ± SEM of 5 different groups of cells of a minimum of 2 independent experiments. * $P< 0.05$ vs vehicle-treated cells. **(B)** Western blot analysis showing HO-1 protein expression in LLC-PK1 cells after exposure to increasing concentrations of BTCC or PTCC. The samples were probed with HO-1 primary antibody and ß-Actin was used as loading control.

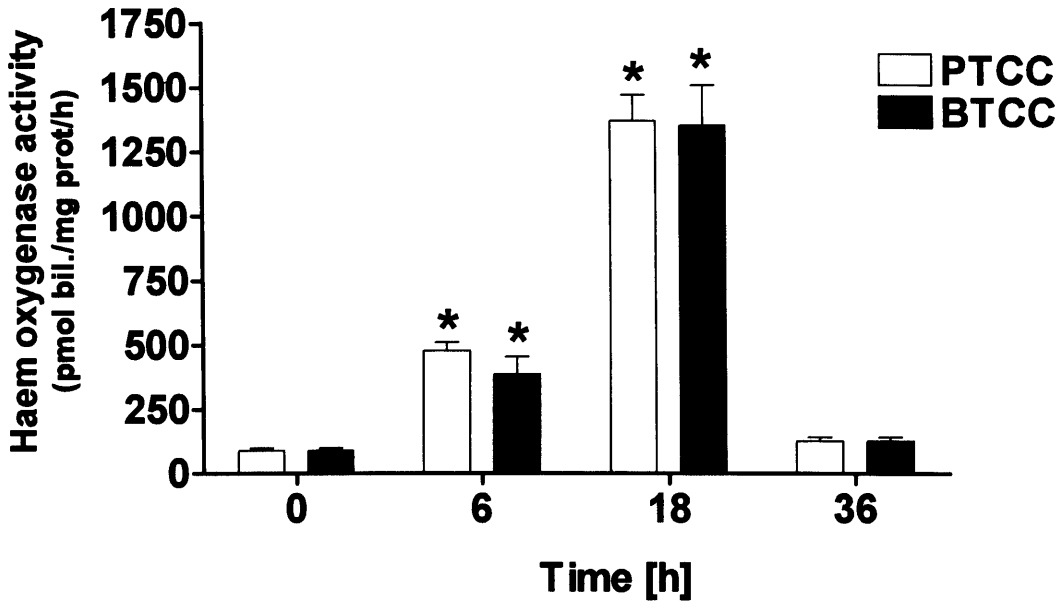



**Figure 3-3 Time course of HO activity in LLC-PK1 cells after exposure to BTCC or PTCC.**

LL-CPK1 cells were incubated with 20 μM BTCC or PTCC and HO activity was measured at various time points as reported in the Materials and Methods section. Data are expressed as the mean ± SEM of 5 independent experiments. * $P< 0.05$ vs vehicle-treated cells.

### *3.4.3 BTCC- and PTCC-mediated increase in HO activity is prevented by actinomycin D and cycloheximide*

To verify whether exposure to BTCC and PTCC results in new HO-1 mRNA synthesis, cells were pre-incubated with act D before addition of 10 μM BTCC or PTCC, which has been shown to cause a significant increase in HO-1 with no toxicity, to the act D-containing medium for additional 6 h (Fig 3-4). This agent prevents transcription by forming strong bonds with DNA and stabilizing cleavable complexes of topoisomerases I and II with DNA (140). Our results showed that act D completely blocked HO induction by BTCC and PTCC indicating that new mRNA synthesis is required for these two ITCs to up-regulate HO-1.

To investigate whether the increase in HO activity mediated by BTCC and PTCC is dependent on new protein synthesis, CHX was added to the cells prior to the addition of BTCC or PTCC to the CHX-containing medium (Fig 3-4). This agent interferes with protein synthesis by interfering with peptidyl transferase on the 60S ribosome (170).Our results showed that HO activity and HO-1 protein expression were totally blunted by this protein synthesis inhibitor suggesting that the BTCC- and PTCC-stimulated increase in HO activity requires new protein synthesis.

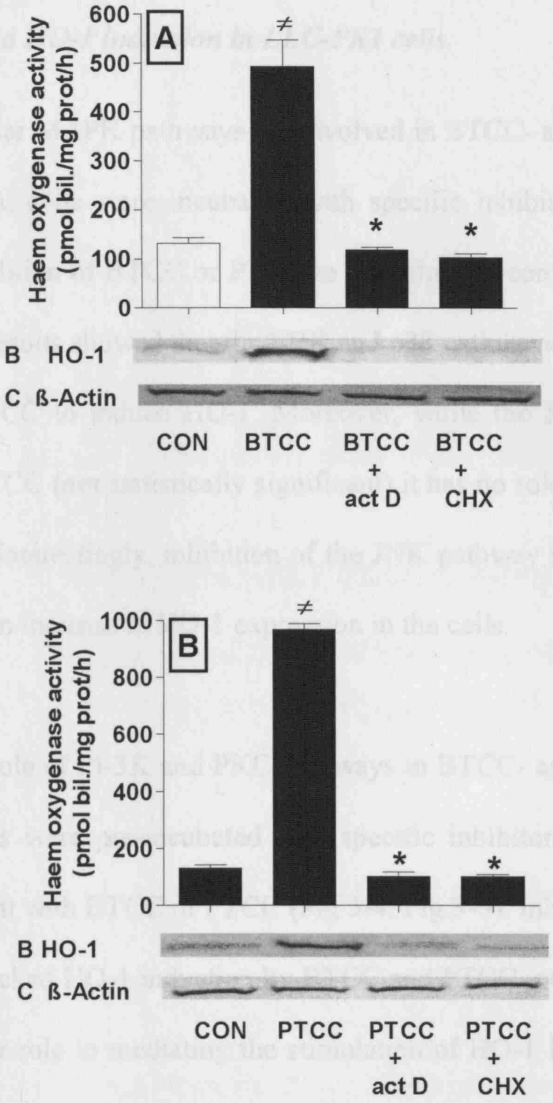



**Figure 3-4 BTCC and PTCC mediated induction of HO is prevented by actinomycin D and cycloheximide**

LLC-PK1 cells were exposed to act D or CHX for 30 min prior to exposure to (10 μM) BTCC **(A)** or PTCC **(B)** for 6 h, which has been shown to induce a significant increase in HO-1 expression with no toxicity. HO activity and HO-1 protein expression were measured as reported in the Materials and Methods section. Each bar represents the mean ± SEM of 5 groups of cells of 2 independent experiments. * $p<0.05$ vs BTCC or PTCC-treated group, ≠ $p< 0.05$ vs vehicle-treated group.

### *3.4.4 The role of MAPKs, PI-3K and PKC pathways in BTCC- and PTCC-mediated HO-1 induction in LLC-PK1 cells.*

To study whether MAPK pathways are involved in BTCC- and PTCC- mediated HO-1 up-regulation, cells were incubated with specific inhibitors of MAPK pathways prior to the addition of BTCC or PTCC to the inhibitor-containing medium (Fig 3-5, Fig 3-6). The results showed that the ERK and p38 pathways are partially required for BTCC and PTCC to induce HO-1. Moreover, while the JNK pathway is required partially by BTCC (not statistically significant) it has no role in PTCC-mediated HO-1 stimulation. Interestingly, inhibition of the JNK pathway in the PTCC-treated cells caused a sudden increase in HO-1 expression in the cells.

To assess the role of PI-3K and PKC pathways in BTCC- and PTCC-mediated HO-1 activation, cells were pre-incubated with specific inhibitors of these two pathways before treatment with BTCC or PTCC (Fig 3-4, Fig 3-5). Inhibition of PI-3K pathway completely blocked HO-1 induction by BTCC and PTCC indicating that this pathway plays the major role in mediating the stimulation of HO-1 by these two compounds. On the other hand, the A and B isoforms of the PKC pathway were found to be required partially for PTCC (but not BTCC) to enhance HO-1 in LLC-PK1 cells. It is noteworthy that when the ERK and p38 pathways were inhibited, a more dramatic decrease in HO-1 was noticed in the PTCC-treated than the BTCC-exposed cells indicating that these two MAPKs are more strongly implicated in PTCC-mediated HO-1 up-regulation.

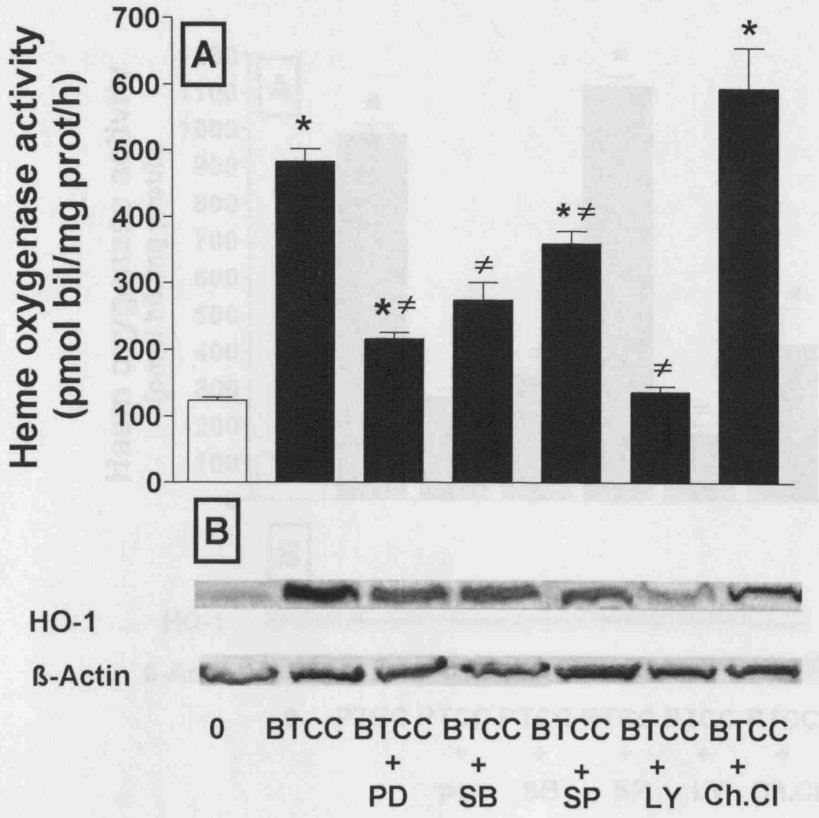



**Figure 3-5 The role of MAPK, PI-3K and PKC signal transduction pathways in the induction of HO-1 by BTCC.**

(A) LLC-PK1 cells were pre-incubated with 10 μM PD098059 (PD), 20 μM SB203580 (SB), 40 μM LY 294002 (LY) or 5 μM (Ch.Cl) for 30 min before BTCC (10 μM) was added to the inhibitor-containing medium. After 6 h, HO activity was measured. Data represent the mean ± SEM of 5 independent experiments. * $P<0.05$ vs vehicle-treated group. ≠ $p< 0.05$ vs BTCC-treated group.(B) Samples of cells treated as above were probed with anti-HO-1 antibody and ß-Actin was used as a loading control.

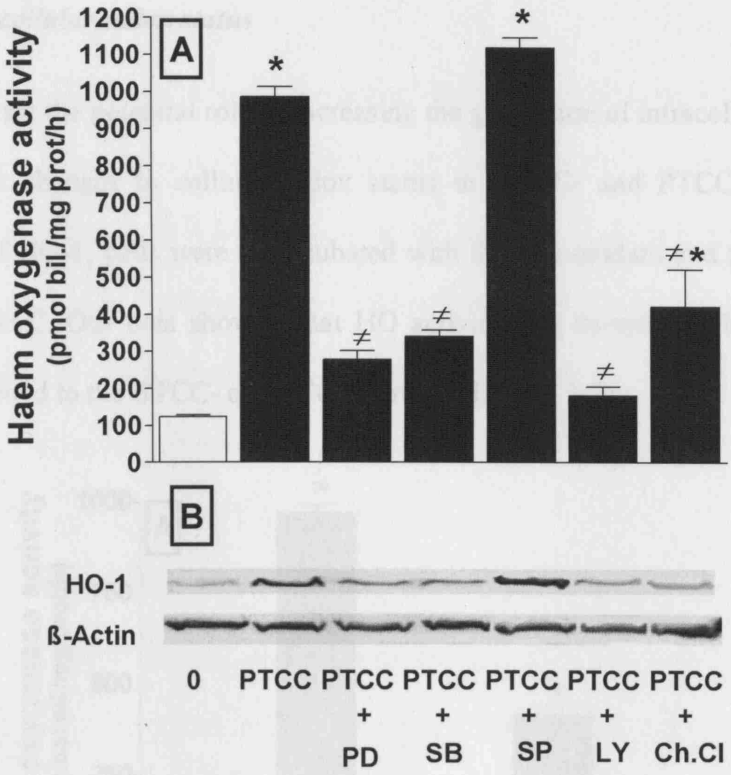



**Figure 3-6 Effect of induction of the MAPK, PI-3K and PKC pathways on the induction of HO-1 by PTCC.**

**(A)** LL-CPK1 cells were pre-incubated with 10 μM PD098059 (PD), 20 μM SB203580 (SB), 40 μM LY 294002 (LY) or 5 μM chelerythrine chloride (Ch.Cl) for 30 min prior to the addition of PTCC (10 μM) to the inhibitor-containing medium. After 6 h, HO activity was measured. Data represent the mean ± SEM of 5 independent experiments. * $p<0.05$ vs vehicle-treated group, ≠ $P<0.05$ vs PTCC-treated group. **(B)** Samples of cells treated as above were probed with anti-HO-1 antibody to detect HO-1 protein by Western immunoblotting technique. ß-Actin was used as a loading control.

### *3.4.5 BTCC- and PTCC- mediated stimulation of HO-1 involves changes in the intracellular redox status*

To demonstrate the potential role of increasing the generation of intracellular ROI and the resultant changes in cellular redox status in BTCC- and PTCC-induced up-regulation of HO-1, cells were co-incubated with the anti-oxidant and potent radical scavenger NAC. Our data showed that HO activity was completely blocked when NAC was added to the BTCC- or PTCC- treated cells (Fig 3-7).

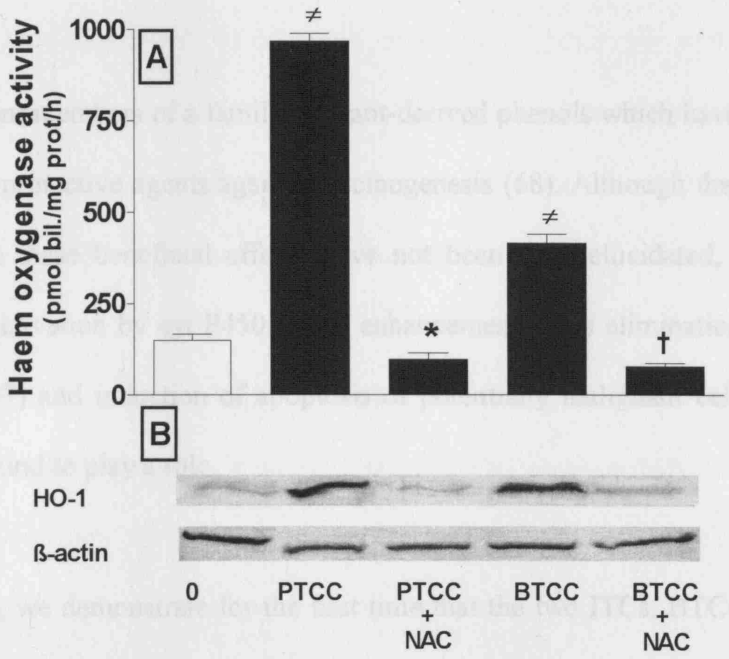



**Figure 3-7 Effect of increasing oxidative stress as a result of ROI generation on the induction of HO-1 by BTCC and PTCC in LLC-PK1 cells.**

**(A)** LLC-PK1 cells were coincubated with NAC and BTCC or PTCC for 6 h and then HO activity was measured as described in the Materials and Methods section. **(B)** Samples of cells treated as above were probed with anti-HO-1 antibody to detect HO-1 protein by Western blotting. ß-Actin was used as a loading control. Each bar represents the mean ± SEM of 5 independent experiments. * and † $P<0.05$ vs PTCC or BTCC groups respectively, $\neq p < 0.05$ vs vehicle-treated group.

### 3.5 Discussion

In recent years, there has been an increasing interest in identifying new therapeutic strategies to enhance defence mechanisms in cells by activating cellular cytoprotective enzymes. HO-1 represents an efficient anti-oxidant system that could be modulated pharmacologically to treat various oxidative and inflammatory disorders due to its characteristic sensitivity to various stimuli and the various positive influences of its reaction products (181).

ITCs represent members of a family of plant-derived phenols which have been shown to be potent protective agents against carcinogenesis (68). Although the mechanisms that underlie these beneficial effects have not been fully elucidated, inhibition of carcinogen activation by cyt P450 (190), enhancement of its elimination by phase II enzymes (137) and induction of apoptosis of potentially malignant cells (138; 316) have been found to play a role.

In this study, we demonstrate for the first time that the two ITCs, BTCC and PTCC, induce HO-1 in a time and concentration-dependent manner. This HO-1 stimulation was dependent on new HO-1 mRNA and protein synthesis because HO-1 activity was completely blocked by act D and CHX respectively.

There is a considerable evidence to support the vital role of HO-1 in suppression of inflammation (306), suppression of the hypertensive response (182), prevention of

cardiac graft rejection (261) and protection against oxidative and nitrosative stress (95). This heat shock protein is induced by a large number of stress conditions such as its substrate haem, metal ions, sulfhydryl compounds and UV irradiation (165). The efficacy of HO-1 in promoting cytoprotection is mediated by the wide range of beneficial effects of its products and by-products. Particularly, CO exerts anti-inflammatory, anti-proliferative, anti-apoptotic, vasoactive and activities in several cell types and has been shown to act as a neurotransmitter (50; 179; 205; 240; 246; 293). Moreover, BR has been shown to possess potent anti-oxidant and anti-inflammatory activities (269). Our current findings that ITCs also induce HO-1 adds to the previous work in our laboratory showing that the plant-derived compounds curcumin, CAPE, 2-hydroxychalcone and rosolic acid are potent inducers of HO-1 and that this effect might play a role in their overall cytoprotective effects (93; 181).

The potency of BTCC and PTCC to activate HO-1 in LLC-PK1 cells seems to be associated with an imbalance in cellular redox status because HO-1 activation was totally blocked when the cells were co-incubated with BTCC or PTCC and NAC. These findings are expected because during their absorption, ITCs bind to GSH via a reaction catalyzed by GST causing a significant decrease the availability of GSH to scavenge ROI. Furthermore, ITCs can be a source of toxic radicals because their –N=C=S group can undergo spontaneous hydrolysis leading to the formation of $O_2^{\cdot-}$ and $H_2O_2$ (324). This sudden oxidative imbalance in the cells induces the expression of anti-oxidant genes particularly HO-1. Our results have shown also that at higher concentrations or after long incubation times, these two ITCs did not cause any

further increase in HO activity. Furthermore, after 24 h, both compounds caused a dramatic increase in LDH release at 20 μM concentration indicating cell death by necrosis. Although BTCC caused a concentration-dependent increase in LDH release we could not find an explanation for the sudden decrease in LDH release at 60 μM after an initial peak. Our data indicated also that these two compounds enhanced cell cellular metabolic activity at low concentrations. However, a sudden decline in cell activity was noticed at 40 μM concentration. The observation that a lot of LDH release was noticed after 24 h incubation with 20 μM concentration but their was no significant drop in cell viability could be related to the fact that cell viability was measured 4 h after removal of BTCC or PTCC from the growth medium and addition of the Alamar Blue and growth medium mixture which could have been a long time for the cells which have survived the acute damage inflicted by these two ITCs to recover and start proliferating again. Therefore, and based on these findings, we speculated that at lower concentrations, ITCs induced a small rise in oxidative stress and a parallel increase in HO-1. However, at higher concentrations or after long times of exposure these compounds result in a greater increase in oxidative stress that overrides the increase in HO-1 and ultimately leads to cell death.

Several lines of evidence have demonstrated the importance of MAPKs, PI-3K and PKC signal transduction pathways for the induction of phase II enzymes of detoxification (186). Indeed, phosphorylation of the transcriptional factor Nrf2 leads to its release from its binding site in the cytoplasm (Keap1) after which it is translocated into the nucleus where it interacts with the promoter region of various

anti-oxidant protective enzyme genes. Specifically, Nrf2 activates ARE/EpRE leading to the transcriptional activation of several phase II enzyme genes.

Our results indicate that while the JNK, ERK and p38 MAPK pathways appear to mediate HO-1 activation by BTCC. However, only the latter two are required by PTCC to induce HO-1 in LLC-PK1 cells. Balogun *et al* have identified a role for the p38 pathway in the stimulation of HO-1 by curcumin in LLC-PK1 cells (25) whereas Hou *et al* have shown that the COX inhibitor celecoxib utilizes the JNK pathway to induce HO-1 in mesangial cells (116). On the other hand, Martin *et al* have indicated a role for ERK, p38 and JNK pathways in HO-1 stimulation by carnosol in rat pheochromocytoma (PC12) cells (168). So it appears that although MAPKs are important signal transduction pathways in the activation of HO-1 in response to various agents, the use of one MAPK rather than the other seems to be agent and/or cell specific.

In addition to the MAPK pathways, PI-3K pathway has been shown under our experimental conditions to play the major role in HO-1 induction by these two ITCs since the PI-3K inhibitor was the most effective blocker of HO activity and HO-1 protein expression in BTCC- and PTCC-treated cells. Interestingly, our data indicate that PKC pathway is partially required by PTCC, but not BTCC, to up-regulate HO-1 expression indicating that the pathways activated by different ITCs is agent-specific. Our results are in line with the recent report by Martin and co-workers who have shown that PI-3K is activated in carnosol-treated rat pheochromocytoma cells in

conjunction with the activation of MAPKs (168). Moreover, in support of our results about the role for PKC in HO-1 activation, Wung *et al* have recently identified a role for PKC in HO-1 induction by the plant-derived piceatannol while MAPKs and PI-3K had a minor role (309).

Our data and the findings of others indicate that various signalling pathways are activated during HO-1 stimulation by several agents. Moreover, it seems plausible that in most cases a combination of pathways is switched on in response to a specific agent emphasizing the fundamental role of the cross-talk between these pathways in mediating the induction of this cytoprotective enzyme.

### 3.6 Conclusions

Taken together, we report here for the first time that the two ITCs, BTCC and PTCC up-regulate HO-1 in LLC-PK1 cells at low non-toxic concentrations. The mechanisms that lead to this effect and the intracellular signal transduction pathways that are utilized by these compounds have been delineated. It needs to be emphasized that ITCs and other plant-derived constituents which are part of the human diet especially cruciferous vegetables can be utilized as natural non-toxic supplements to induce HO-1 and other intracellular defensive systems.

# 4 EFFECT OF BTCC AND PTCC ON CELL DEATH IN LLC-PK1 CELLS

## 4.1 Introduction

Recent evidence has shown that apoptosis, a physiological mode of cell death in which the cell executes the programme for its own demise, is an extremely important strategy used by various chemopreventive compounds. Therefore, the importance of apoptosis in chemoprevention has been an active field of research worldwide by scientists engaged in the search for cancer chemopreventive agents. Numerous studies demonstrated that evasion of apoptosis is one of the most important mechanisms of uncontrolled growth of tumour cells (283). Hence, apoptosis of initiated and/or neoplastic cells represents a protective mechanism against neoplastic transformation through elimination of genetically damaged cells or cells that may have been inappropriately induced to divide by mitogenic and proliferative stimuli (305). Therefore, considerable attention has been directed recently on the modulation of apoptosis as a novel and promising strategy for cancer chemoprevention (113; 149).

The mechanisms that mediate the chemprotective effects of ITCs have not been fully elucidated. However, the induction of apoptosis in deleterious cells has been found to play a major role (278). Although the potency of apoptosis induction by ITCs depends on the agent and/or the cell used (149), some ITCs are devoid of any apoptotic

potential (245). Since ITCs accumulate to a high level after they form conjugates with GSH (320), with a resultant depletion of this anti-oxidant, this leads to a decrease in scavenging ROI in the cell. As ROI may act as signalling molecules in different biological processes including initiation and execution of apoptosis (48), modulation of the intracellular redox environment may be pivotal in ITC-induced apoptosis.

The explanation of how ITCs are cytoprotective and at the same time cause apoptosis has been outlined by Kong and co-workers who have proposed a pattern of signal transduction events that are induced by ITCs and other chemopreventive compounds. According to the authors, at low concentrations induction of protective genes such as c-Fos, c-jun, GST, and QR occurs resulting in the enhancement of various cytoprotective and survival mechanisms. However, when the concentration of these compounds increases, caspases-3 activation and subsequent induction of apoptotic cell death ensues. This proposal was supported by Yu and colleagues who showed that PEITC at concentrations of 5–10 μM strongly activated JNK in a time- and concentration-dependent manner whereas increasing the concentration of this ITC from 10 to 20 μM strongly activated caspase-3-like protease activity in HeLa cells (316).

The involvement of MAPKs in ITCs-mediated apoptosis has been demosntrated previously in many studies (55; 308). However, it has been shown that the activation of a specific signalling pathway is agent and/or cell specific (52; 310). Moreover,

other signal transduction pathways have been implicated in inducing apoptosis by ITCs such as PI-3K (124) and PKC (18).

## 4.2 Objectives

The present study has been undertaken to examine the effects of BTCC and PTCC on cell death in LLC-PK1 cells and to study the role of several signal transduction pathways in these effects.

## 4.3 Materials and methods

### *4.3.1 Reagents*

BTCC and PTCC were purchased and prepared as demonstrated in chapter 9.

### *4.3.2 Cell culture*

LLC-PK1 cells were obtained and maintained as previously mentioned in chapter 9.

### *4.3.3 Caspase-3 activity assay*

Caspase-3 specific activity was measured as indicated in chapter 9.

## 4.4 Results

### *4.4.1 BTCC and PTCC induce apoptosis in LLC-PK1 cells*

Confluent cell monolayer was exposed to increasing concentrations of BTCC or PTCC (5-50 μM) for two different time periods (12 and 16 h) (Fig 4-1 and Fig 4-2). We selected 12 and 16 h incubation times because Anti-Fas, which was used as a positive control in these experiments, causes maximum apoptosis in this range of incubation time. Apoptotic cell death was assessed by measuring caspase-3 activity in the cell fractions. After 12 h and 16 h, there was a concentration- and time-dependent increase in caspase-3 activity. However, after 16 h and at 50 μM concentration both compounds failed to induce any further increase in caspase-3 activity indicating cell death by necrosis. Interestingly, at 5 and 10 μM concentration, a reduction in caspase-3 activity was observed (more at 10 μM). Although this was not statistically significant, it indicates that the cells might have developed resistance against apoptosis. This could be correlated to the data obtained in chapter 3 which showed that, at low concentrations (<10 μM), BTCC and PTCC failed to induce LDH release or to decrease cellular metabolic activity even after longer incubation periods (24 h) (Fig 4-1A and Fig 4-1B). However, with increasing time (>16 h) and concentration (>20 μM) these two ITCs enhance apoptotic cell death (Fig 4-1). As the time of incubation with high concentrations is increased further (24 h) more damage ensues and cells die by necrosis (Fig 4-1A).

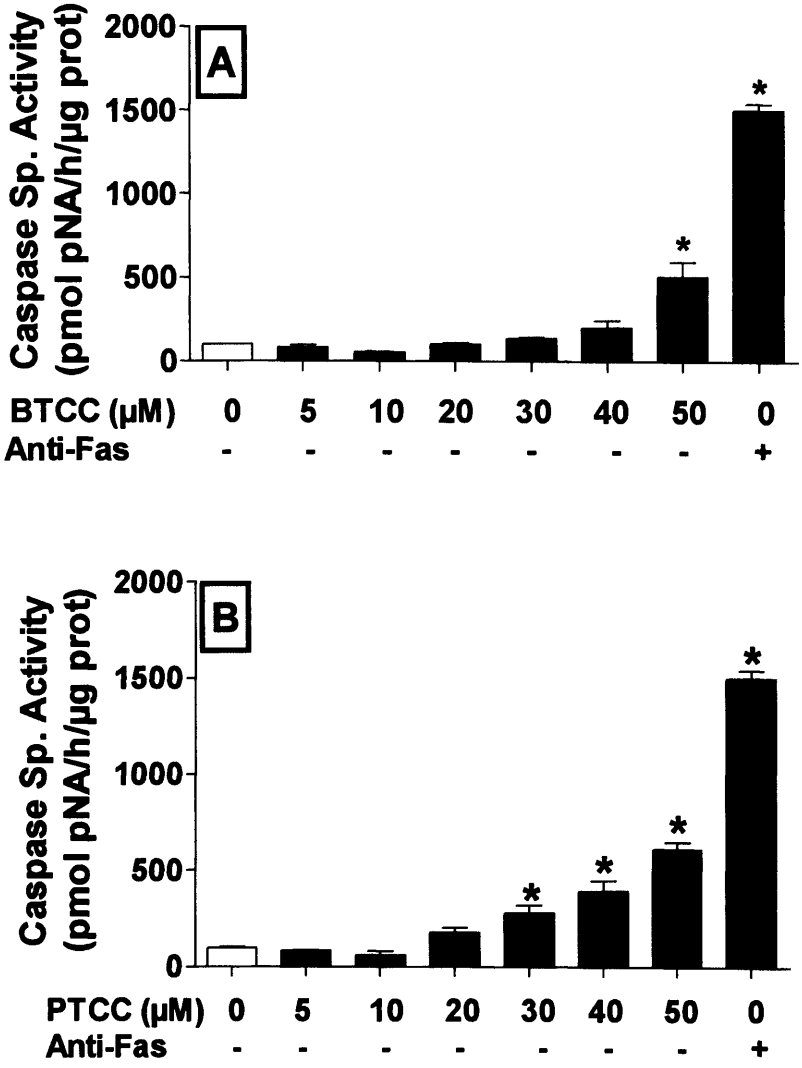



**Figure 4-1 Effect of BTCC and PTCC on apoptosis in LLC-PK1 cells as assessed by caspase-3 activity after 12 h incubation.**

Confluent cell monolayer was exposed to increasing concentrations of BTCC (**A**) or PTCC (**B**) (5-50 µM) for 12 h and then caspase-3 activity was measured in cellular lysates as an indicator of apoptotic cell death as described in the Materials and Methods. Anti-Fas (0.4 µg/ml) was used as a positive control. Each bar represents the mean ± SEM of 5 independent experiments. * $P<0.05$ vs. vehicle-treated group.

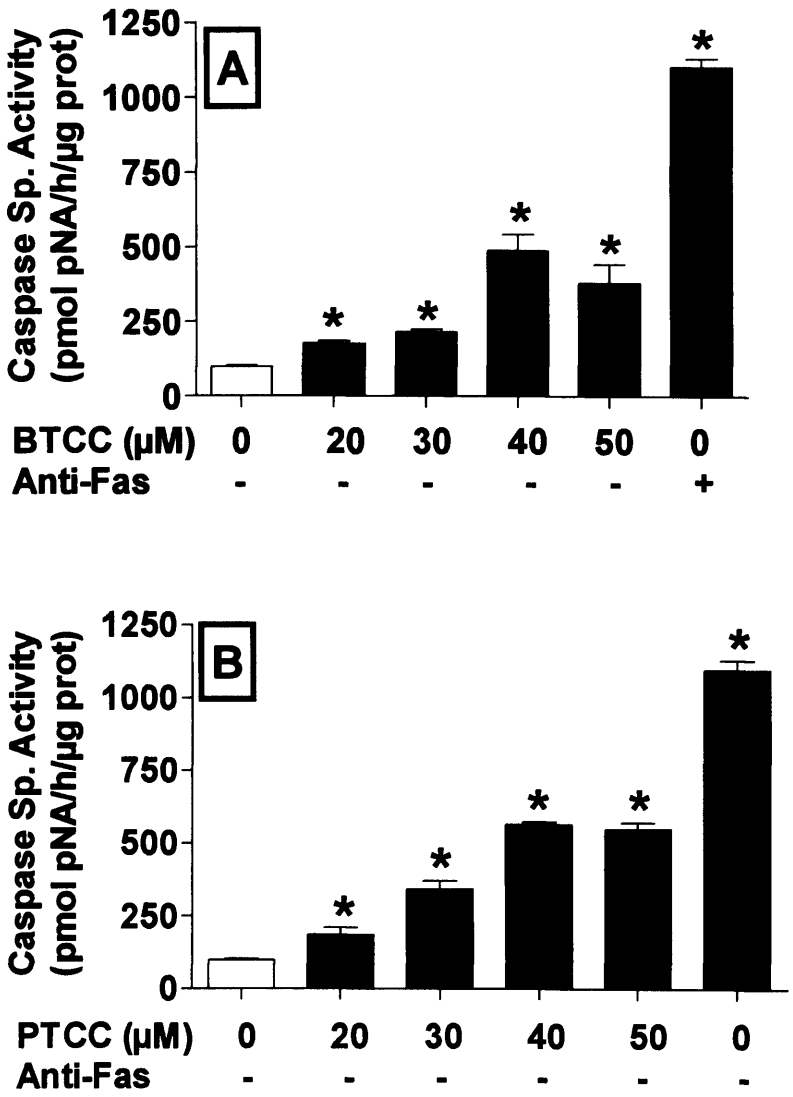



**Figure 4-2 Effect of BTCC and PTCC on apoptosis in LLC-PK1 cells as assessed by caspase-3 activity after 16 h incubation.**

Confluent cell monolayer was exposed to increasing concentrations of BTCC (**A**) or PTCC (**B**) (20-50 µM) for 16 h and then caspase-3 activity was measured in cellular lysates as an indicator of apoptosis as described in the Materials and Methods. Anti-Fas (0.4 µg/ml) was used as a positive control. Each bar represents the mean ± SEM of 5 independent experiments. * $P<0.05$ vs. vehicle-treated group.

### *4.4.2 Signal transduction pathways activated in BTCC- and PTCC-induced apoptosis in LLC-PK1 cells*

The role of different signal transduction pathways in BTCC- and PTCC-mediated apoptosis in LLC-PK1 cells was investigated by using highly specific blockers of the ERK, JNK and p38 MAPK pathways (168), PI-3K (244) and the A and B isoforms of the PKC pathways (58) (Fig 4-3, Fig 4-4). The cells were pre-incubated with the inhibitors for 30 min prior to the addition of BTCC or PTCC to the inhibitor-containing medium. While the ERK pathways played a major role in BTCC-mediated apoptosis, the JNK pathway had no role and the p38 pathway was shown to be partially required. However, the PI-3K and the A and B isoforms of the PKC inhibitor had no role in the apoptotic-stimulating effect of this ITC.

PTCC seemed to induce apoptosis in LLC-PK1 cells via mechanisms that partially involve the p38 MAPK pathway but the ERK and JNK pathways had no role. While the PI-3K pathway had no role in the apoptotic-stimulating effect of PTCC, the A and B isoforms of the PKC pathway seem to have contribution to this effect. In accordance with previous studies (55; 56), our results have shown that the JNK pathway has the major role in the apoptotic mechanisms caused by BTCC. Surprisingly, however, this pathway had no role in PTCC-mediated apoptosis and the p38 and the A and B isoforms of the PKC plays were the only pathways that were involved in this effect. Of great interest the finding that the ERK pathway, which is mainly activated in response to growth factors, plays a major role in BTCC-induced

apoptosis. The data presented here indicate that different ITCs activate different signalling transduction pathways to induce apoptosis.

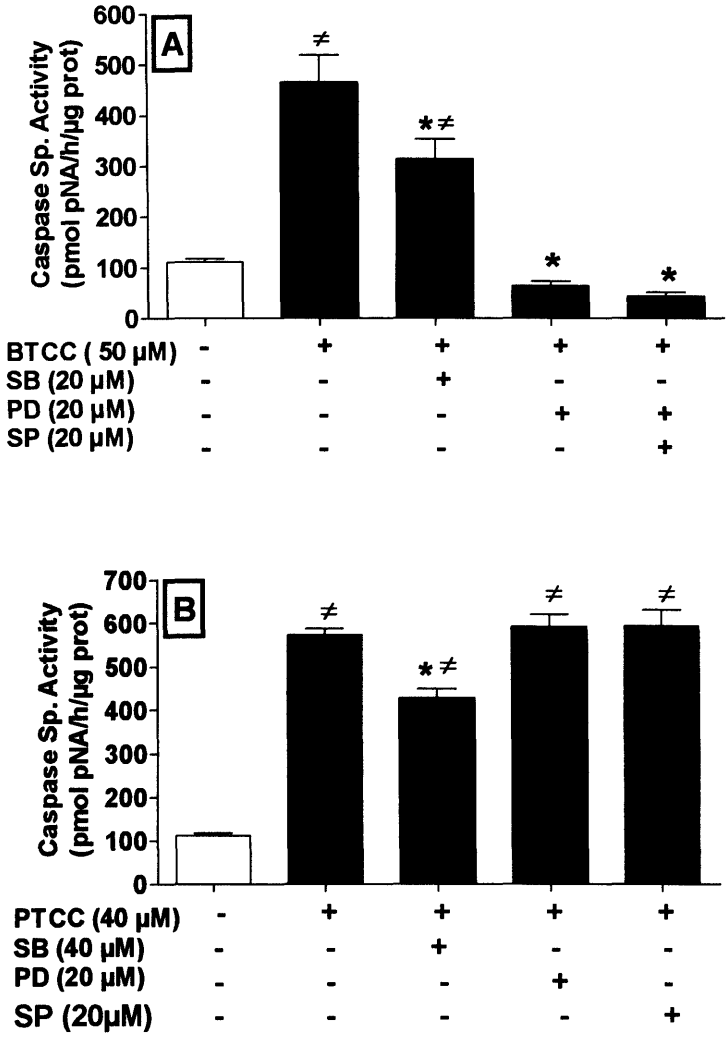



**Figure 4-3 Effect of MAPK pathway inhibitors on BTCC- and PTCC-induced apoptosis in LLC-PK1 cells.**

LLC-PK1 cells were stimulated with 50 µM BTCC (**A**) or 40 µM PTCC (**B**) in the presence or absence of the indicated concentrations of the ERK inhibitor (PD98059), p38 inhibitor (SB203580) or the JNK inhibitor (SP600125). Apoptotic cell death was then assessed by performing caspase-3 specific activity assay on the cell lysates as reported in the Materials and Methods. Each bar represents the mean ± SEM of 5 independent experiments. * $P<0.05$ vs. BTCC or PTCC groups, ≠ $P<0.05$ vs. vehicle-treated group.

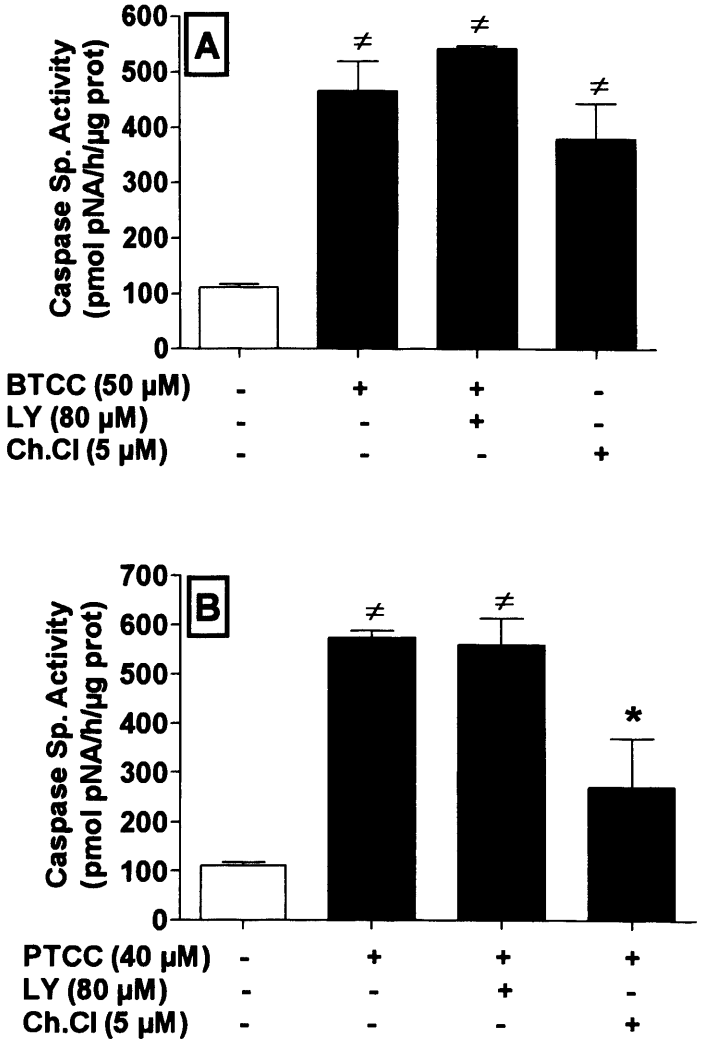



**Figure 4-4 The effect of PI-3K and PKC pathway inhibitors on caspase-3 activation in LLC-PK1 cells by BTCC and PTCC**

LLC-PK1 cells were exposed to 50 µM BTCC (**A**) or 40 µM PTCC (**B**) in the presence or absence of the indicated concentrations of PI-3K blocker (LY294200) or the A and B isoforms inhibitor of the PKC pathways (Ch.Cl) for 16 h. Caspase-3 assay was performed as reported in the Materials and Methods. Each bar represents the mean ± SEM of 5 independent experiments. * $P<0.05$ vs. BTCC groups. ≠ $P<0.05$ vs. vehicle-treated cells.

## 4.5 Discussion

Our diet contains several potential chemopreventive agents that comprise a safe, available and effective method to reduce the risk of developing cancer. ITCs are among the agents that are widely available in many edible plants especially cruciferous vegetables (86). These compounds are easily absorbed to high concentrations and exert potent chemprotective effects through modulating gene expression of several drug metabolizing enzymes (186).

Apoptosis plays a very crucial role in cancer chemoprevention by excluding terminally changed cells that can develop into cancer. This process is driven by a group of pro-enzymes (pro-caspases) that exist in an inactive form under physiological conditions (67). However, when a death signal is received, these pro-enzymes are activated and result in several devastating cellular effects that culminate in cell death by apoptosis.

In the current investigation, we aimed to study the effect of BTCC and PTCC on the two forms of cells death (apoptosis and necrosis). Furthermore, we wanted to study the different signal transduction pathways that are utilized by these two ITCs to induce apoptosis.

We report here that BTCC and PTCC induce apoptosis in LLC-PK1 cell in a concentration- and time-dependent manner after relatively short incubation times (12 and 16 h) (Fig 4-1 and Fig 4-2). Our data seem to show, however, that these

compounds after longer incubation periods (24 h) (Fig 2-1A) or at relatively higher concentrations (above 50 μM) cause necrosis as indicated by failure to induce further increase in caspase-3 (Fig 4-2) activity and a significant increase in LDH release (Fig 2-1A). We demonstrated in chapter 3 that BTCC and PTCC induce necrosis at concentrations above 20 μM after 24 h incubation (Fig 2-1A). The fact that these two ITCs induce necrosis at these concentrations and time point although significant apoptosis was observed at 16 h could be explained by the fact that apoptotic cells could develop secondary necrosis (289). In addition, the cells which survive the apoptotic effects of BTCC and PTCC undergo necrosis causing an increase in LDH release.

Under physiological conditions, ITCs react with GSH to form DTC through a reaction stimulated by GST (323). Rapidly thereafter, the ITCs-GSH conjugates are absorbed and then exported outside the cells resulting in GSH depletion (322). Increasing concentrations of GSH have been found to be associated with cellular proliferation and a reduction in apoptosis while GSH efflux mechanisms have been implicated in apoptosis (218). The involvement of redox mechanisms in BTCC and PTCC-mediated apoptosis seems possible. Particularly, the ability of ITCs to increase oxidative stress through depleting intracellular GSH, enhancing the generation of ROI and damaging mitochondria with subsequent leakage of cyt c could contribute to their pro-apoptotic effects. In this context, Nakamura *et al* have demonstrated that exposure of rat liver epithelial (RL34) cells resulted in a significant increase in ROI generation which was accompanied by mitochondrial damage, release of cyt c and apoptosis

(191). Moreover, recent evidence has indicated that ITCs lead to the accumulation of the pro-apoptotic Bcl-2 protein Bax which binds to the inner mitochondrial membrane and increases its permeability to cyt c thus initiating the mitochondrial apoptotic pathway (237).

Of great interest, BTCC and PTCC at non-toxic HO-1 stimulatory concentrations decreased the release of caspase-3 activity after a relatively short incubation time (12 h) in comparison with control cells. Although this decrease in caspase-3 activity was not statistically significant, it indicates that cells showed resistance against apoptosis. These data emphasize that although ITCs are toxic at higher concentrations or after longer incubation periods by causing apoptosis or necrosis, at low concentrations these compounds enhance the expression of defensive genes such as HO-1 which might protect cells against apoptosis.

Several reports have revealed that modulation of the MAPK pathways plays an important role in ITCs-mediated apoptosis (55; 56; 308). Moreover, in chapter 3 we showed that MAPKs are required by BTCC and PTCC to induce HO-1 in LLC-PK1 cells (Fig 2-5 and Fig 2-6). Therefore, in this study we wanted to investigate the possible role of the different MAPKs in the apoptotic effect of these two ITCs. We provide evidence here that BTCC utilizes JNK, ERK and to a much lesser extent p38 pathway to induce apoptosis in LLC-PK1 cells. On the other hand, the latter played a minor role in PTCC-mediated increase in caspase-3 activity while PKC played the major role. Chen *et al* have found that several ITCs cause concentration-dependent

apoptosis in various cell lines and have shown also that activation of JNK pathway is the major pathway that is activated in apoptotic cells in response to treatment with these compounds (52). In another study, Chen *et al* have indicated that JNK activation, together with the suppression of phosphatase activity of this pathway, may be the possible molecular events required for PEITC-induced apoptosis suggesting that JNK is a critical signalling mediator of apoptosis induced by this ITC (55). Studies by Xu *et al*, however, demonstrated a role for ERK pathway activation in ITCs-mediated apoptosis in PC-3 cells (310). So, based on our observations and the findings of others it seems that the modulation of different MAPKs during activation of the apoptotic mechanisms in response to ITCs is an important event during this process and might be agent and/or cell specific.

## 4.6 Conclusions

Taken together, we report here that the two ITCs, BTCC and PTCC, induce apoptosis in LLC-PK1 cells in a concentration- and time-dependent manner. Despite the fact that at low concentrations or after relatively short incubation times ITCs conferred resistance to apoptosis in the cells, at higher concentrations these two compounds resulted in necrotic cell death. While JNK and ERK played the major role in BTCC-mediated apoptosis, p38 had a minor role. PTCC, however, required PKC partially to induce caspase-3 while p38 had a much lesser role. Our data provide evidence that the signal transduction pathways activated during ITCs-mediated apoptosis are agent-specific.

# 5ISOTHIOCYANATES PROTECT AGAINST CISPLATIN-INDUCED NEPHROTOXICITY

## 5.1 Introduction

Cisplatin (*cis*-dichlorodiammineplatinum II, CP) is a potent anti-neoplastic agent that is particularly effective for the treatment of solid tumours (101). However, its clinical use is limited by its nephrotoxic effects which occur even after a single dose (14). CP accumulates preferentially in the S3 segment of the proximal tubules of the kidney where it forms adducts with DNA (314), inhibits protein synthesis, damages mitochondria (272) and decreases GSH thus making cells vulnerable to the cytotoxic effects of ROI and markedly enhances its renal damaging effects (281). Although the detailed mechanisms of CP-mediated nephrotoxicity have not yet been fully elucidated, numerous reports point to the crucial role of free radical-mediated necrosis and apoptosis in renal proximal cells (200). CP like many other anti-neoplastic agents causes cancer cell death by induction of apoptosis (13). Therefore, special attention has been directed recently to this effect and the possible contribution of apoptosis caused by CP accumulation in the proximal tubule cells to its overall nephrotoxic effects (227).

Drug-induced nephrotoxic injury is one of the oxidative stress conditions that lead to the release of free haem from haemoproteins. Due to its strong oxidant properties,

haem is rapidly metabolized by the HO enzyme (165). The kidney is particularly vulnerable to the toxicity of increasing haem. This has been demonstrated in several models of acute renal failure such as I/R injury and drug-induced nephrotoxicity (3; 258).

A growing body of evidence has demonstrated that HO-1 induction serves as an immediate protective response against renal damage inflicted by CP while its abrogation worsens renal failure (4). For instance, CP administration results in HO-1 induction in a time- and dose-dependent manner and the administration of the HO-1 inhibitor (SnPPIX) aggravated CP-induced renal injury (3). In addition to the CP model of AKI, other studies using knockout mice have been used to characterize HO-1 role in protecting against several forms of renal injury. In this context, Nath *et al* have demonstrated that when CP was administered to HO-1 knockout mice, they had a more severe rise in renal function and tubular injury which were accompanied by 100% mortality when compared to wild-type mice indicating the crucial role of HO-1 as a reno-protective enzyme against the damaging effects of this agent on the kidny (197).

## 5.2 Objectives

In chapter 3, we demonstrated that BTCC and PTCC induce HO-1 in LLC-PK1 cells. In the present study, we utilized *in vitro* and *in vivo* models to investigate the hypothesis that these two ITCs would protect against CP-induced renal failure and to test the possible contribution of their HO-1 stimulation in this effect.

## 5.3 Materials and methods

### *5.3.1 Reagents*

CP was purchased from (Sigma-Aldrich Co.). Stock solution of 5 mM CP was freshly prepared in DMSO on the day of the experiment. BTCC and PTCC were prepared as described in chapter 9. SnPPIX was prepared in distilled water.

### *5.3.2 Cell culture and experimental protocol*

LLC-PK1 cells were purchased and maintained as previously described in chapter 9. Cells were exposed to CP only (50 μM) for 16 h, which has been shown previously to induce apoptosis in renal epithelial cells (211), or pre-incubated with BTCC or PTCC (5 or 10 μM) for 6 h to induce HO-1 (Fig 2-2). Thereafter the medium which contained BTCC or PTCC was removed and the cells were exposed to CP only (50 μM) for an additional 16 h. To verify the contribution of HO-1 to the potential protective effect of BTCC and PTCC against CP-induced apoptosis, another group of cells was pre-incubated with BTCC or PTCC in the presence of the HO-1 inhibitor (SnPPIX) (10 μM) for 6 h prior to exposure to CP only for another 16 h. At the end of the incubation, caspase-3 activity assay and TUNEL technique were performed to detect the extent of apoptosis.

### *5.3.3 Animals and treatment*

Male Wistar rats were grouped as explained in chapter 9.

### *5.3.4 Caspase-3 activity assay*

Caspase-3 specific activity was measured as shown in chapter 9.

### *5.3.5 TUNEL assay*

The TUNEL technique was performed as indicated in chapter 9.

## 5.4 Results

### *5.4.1 BTCC and PTCC protect against CP-induced apoptosis in LLC-PK1 cells via mechanisms that involve HO-1*

Exposure of cells to CP (50 μM) for 16 h resulted in significant ($p<0.05$) apoptotic cell death as evidenced by increasing cell detachment from the monolayer (>70%), significant increase in caspase-3 specific activity as compared to control cells (Fig 4-1) and a remarkable increase in the number of apoptotic cells as detected by TUNEL assay (Fig 4-2). Pre-incubation of the cells with BTCC or PTCC prior to CP treatment resulted in a remarkable concentration-dependent decrease in apoptotic cell death based on all apoptosis parameters tested (Fig 5-1, Fig 5-2).

To examine the possible contribution of HO-1 induction in this anti-apoptotic effect of BTCC and PTCC, cells were pre-incubated with BTCC or PTCC in the presence of SnPPIX prior to exposure to CP. Inhibition of HO caused CP-induced caspase-3 activation to increase significantly to the level of CP-treated cells (Fig 5-1 and Fig 5-2).

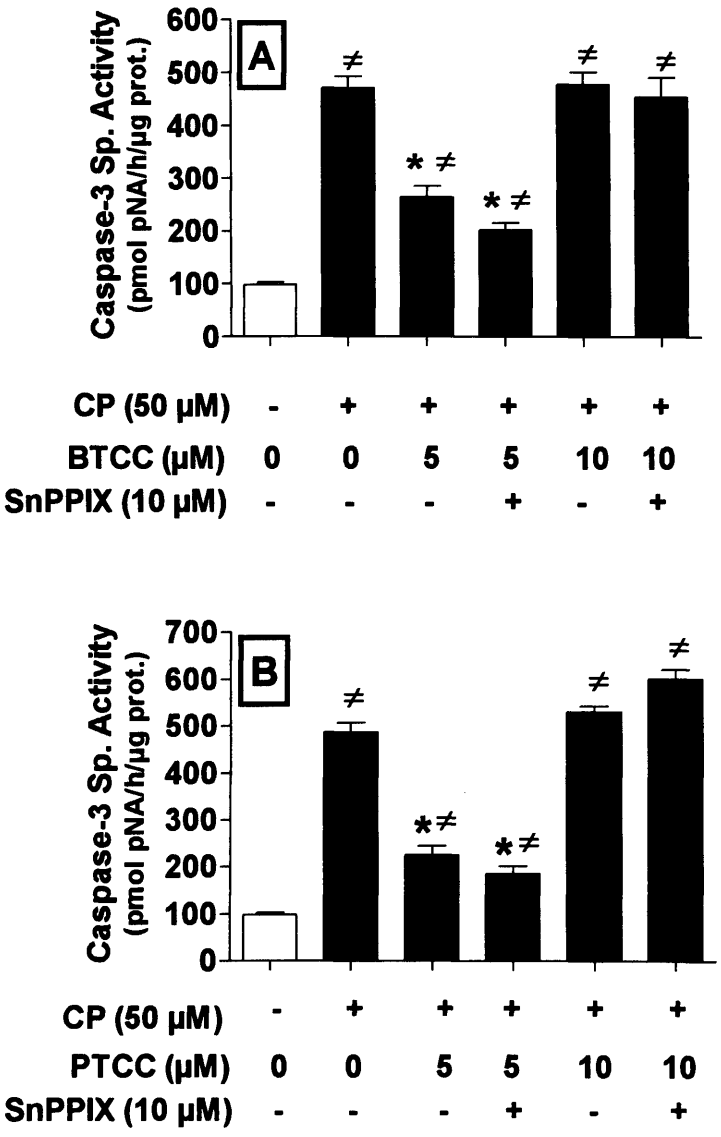



**Figure 5-1 BTCC and PTCC protected LLC-PK1 cells against CP-induced apoptosis via mechanisms mediated by HO-1.**

Confluent cells were incubated with DMSO only, CP 50 µM for 16 h or pre-incubated with 5 or 10 µM BTCC **(A)** or PTCC **(B)** in the presence or absence of the HO inhibitor SnPPIX (10 µM) prior to exposure to CP. Then caspase-3 activity measurement was performed as described in the Materials and Methods section. Each bar represents the means ± SEM of 5 independent experiments. * $p<0.05$ compared to CP-treated cells, ≠ $p<0.05$ vs. vehicle-treated group.

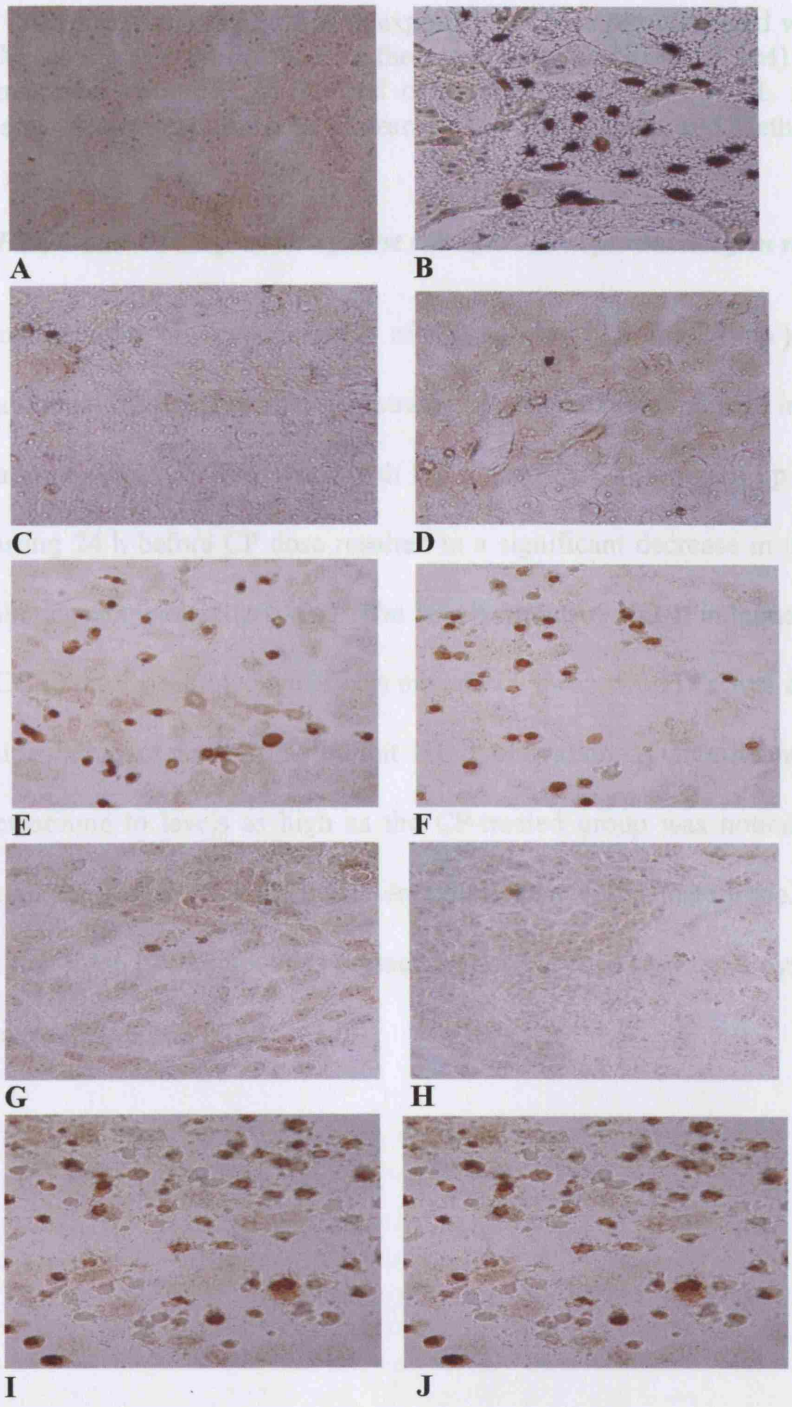



**Figure 5-2 Effect of BTCC and PTCC on CP-induced apoptosis in LLC-PK1 cells as detected by the TUNEL technique.**

Cells were incubated with DMSO only **(A)** (x20), CP 50 μM **(B)** (x20), or pre-incubated with BTCC 5 or 10 μM for 6 h **(C, D)** (x20) or in combination with

SnPPIX (10 μM) **(E, F)** (x20) prior to exposure to CP or pre-incubated with PTCC 5 or 10 μM for 6 h **(G, H)** (x20) or in the presence of SnPPIX (10 μM) **(I, J)** (x20) before treatment with CP. At the end of the treatment, the TUNEL method was performed to detect apoptotic cells as described in the Materials and Methods.

### *5.4.2 BTCC and PTCC protect against CP-induced nephrotoxicity in rats*

The administration of a single dose of CP to rats (7.5 mg/kg i.p.) resulted in significant renal dysfunction as demonstrated by a remarkable increase in serum urea and creatinine (Fig 5-3). Treatment with BTCC or PTCC (25 mg/kg i.p.) daily for 4 days starting 24 h before CP dose resulted in a significant decrease in the measured renal function parameters ($p<0.05$). The involvement of HO-1 induction by BTCC and PTCC in the reno-protective effects exerted by these two ITCs was demonstrated by administration of SnPPIX to inhibit HO-1 activation. A significant increase in serum creatinine to levels as high as the CP-treated group was noticed. However, serum urea increased to a much lesser extent than serum creatinine. These data indicate that these two compounds protect against CP-mediated renal dysfunction via mechanisms that induce HO-1.

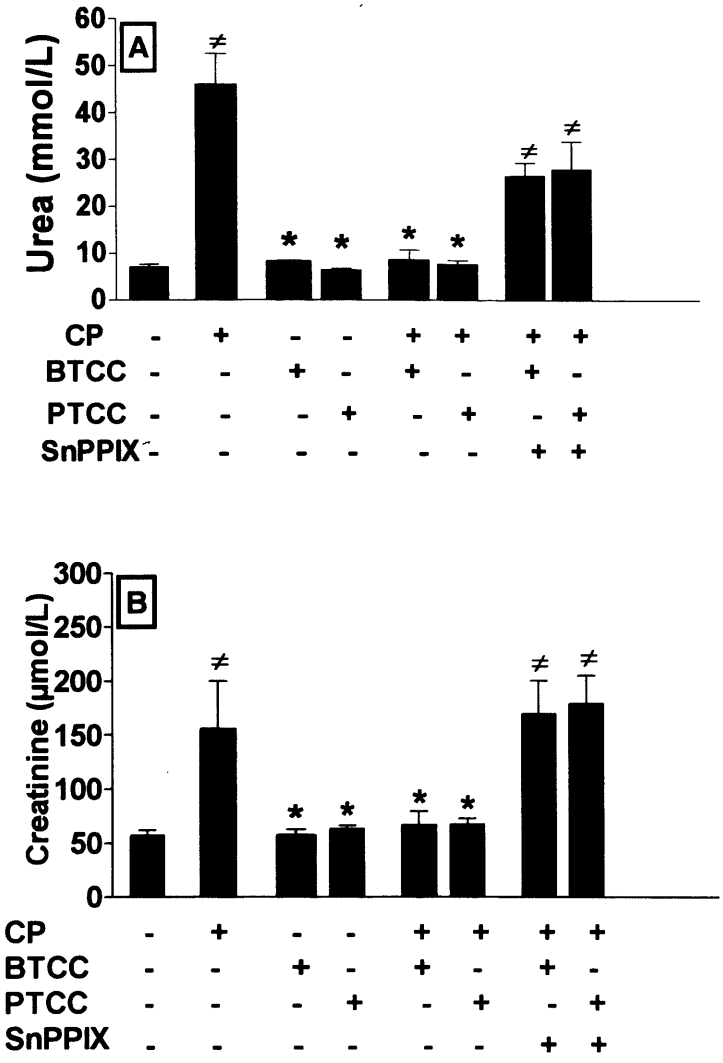



**Figure 5-3 Effect of BTCC and PTCC on renal function in CP-treated rats as measured by serum urea and creatinine.**

Renal function was assessed in the control and treated groups by measuring serum urea **(A)** and creatinine **(B).** Control rats showed normal renal function. Administration of CP 7.5 mg/kg i.p. resulted in a significant elevation in serum urea and creatinine. Treatment with 25 mg/kg BTCC or PTCC daily starting 24 h before CP resulted in a remarkable improvement in renal function. Administration of SnPPIX to BTCC- or PTCC- and CP-treated rats significantly increased serum creatinine and to a much lesser extent urea indicating diminishing this BTCC and PTCC protective effect. Each bar represents the means ± SEM for 5 independent experiments. * $p<0.05$ compared to CP. ≠ $p<0.05$ compared to vehicle-treated rats.

### *5.4.3 BTCC and PTCC alleviate CP-induced necrosis in renal proximal cells of rat kidney*

To investigate the potential protective effect of BTCC and PTCC against cell death in kidneys of CP-treated rats and the possible involvement of their HO-1-stimulating effect, paraffin-embedded tissue sections from control and treated kidneys were stained with the TUNEL technique for the detection of apoptosis and necrosis (Fig 5-4). In agreement with the data obtained by Sheikh-Hamad *et al* (257), our results have demonstrated that the CP-treated rats showed extensive necrosis associated with dispersed areas of apoptosis in the proximal tubules (probably the S3 segment) and may be the LOH (Fig 5-4B). The dense dark staining in (Fig 5-4B) indicated that the TUNEL reagents might have reacted in a non-specific manner with the necrotic tubular cells. Moreover, the presence of collections cell debris might have added to the density of the brown staining. Administration of BTCC or PTCC to CP-treated rats resulted in a significant reduction in the number of necrotic cells (Fig 5-4C and Fig 5-4E, respectively) but there were still some apoptosis. Co-administration of SnPPIX, which has been shown previously to inhibit HO-1 in a renal failure model (3), with BTCC or PTCC to CP-treated rats failed to increase the extent of necrotic cell death (Fig 5-4D and Fig 5-4F, respectively). This could be due other mechanisms, other than the induction of HO-1, which could be used by BTCC and PTCC to exert their reno-protective effects *in vivo*.

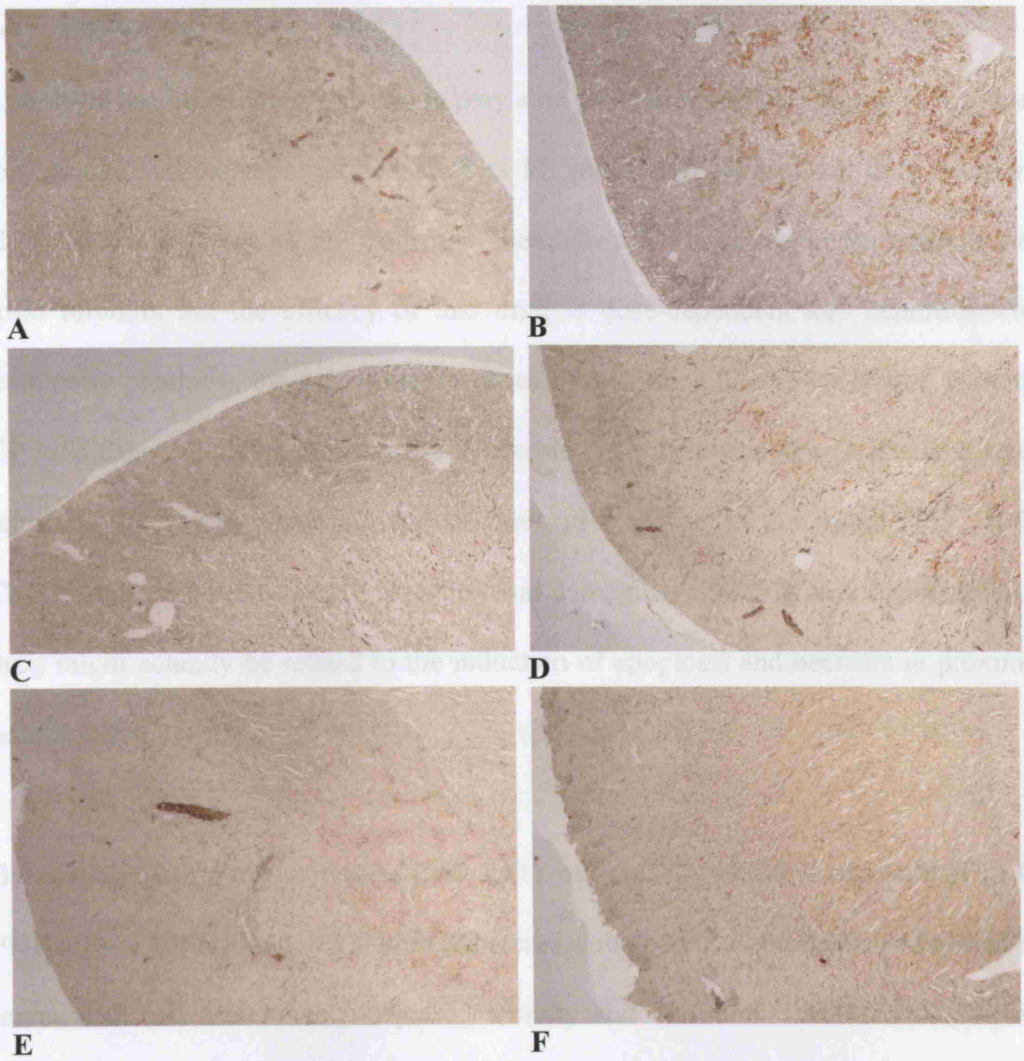



**Figure 5-4 Effect of BTCC and PTCC administration on cell death of proximal tubules in CP-treated rats.**

Formalin fixed paraffin-embedded kidneys were stained with the TUNEL method for the detection of apoptosis and necrosis in control, CP-treated, BTCC or PTCC plus CP-treated groups in the presence or absence of SnPPIX. Control kidneys showed no necrotic or apoptotic activity **(A)** (x40). CP treatment caused extensive necrotic and dispersed areas of apoptotic cell death in the proximal tubules and LOH **(B)** (x40). Administration of BTCC **(C)** (x40) or PTCC **(E)** (x40) (25 mg/kg i.p.) resulted in a significant reduction in CP-induced necrosis. However, administration of SnPPIX failed to abolish this protective effect of BTCC **(D)** (x40) and PTCC **(F)** (x40).

## 5.5 Discussion

Apoptosis has been recently shown to play a major role in renal cell injury in response to various renal damaging effects such as I/R injury and nephrotoxic drug reactions (103; 136). CP is one of the front-line chemotherapeutic agents in the treatment of solid tumours but the efficacy of this drug is dose-dependent and nephrotoxicity frequently precludes the use of higher doses to maximize its anti-neoplastic activity. CP kills cancer cells by induction of apoptosis but it has been shown also that renal tubular cells die by apoptosis and necrosis in response to this agent (159; 305). Therefore, it has been thought that the renal damaging effects of this anti-neoplastic drug might actually be related to the induction of apoptosis and necrosis in proximal tubular cells.

By analogy with previous reports (159; 211), our data showed that exposure of LLC-PK1 cells to CP resulted in a significant increase in apoptotic cell death as detected by increase in cell detachment, caspase-3 activity and the number of TUNEL-positive cells. We report here for the first time that low concentrations of BTCC and PTCC protected against CP-mediated apoptosis because when LLC-PK1 cells were pre-incubated with these two ITCs prior to exposure to CP they showed marked resistance to the apoptotic effects of this anti-cancer agent. Moreover, we showed that this anti-apoptotic effect of BTCC and PTCC was mediated by their HO-1 induction because inhibition of HO completely abolished this effect. To further investigate this protective effect *in vivo*, we utilized a rat model of CP-induced renal failure. In this study, a single dose of CP (7.5 mg/kg i.p.) resulted in a significant elevation of serum

urea and creatinine and a marked increase in necrotic cell death of the proximal tubular epithelial cells. However, when CP-administered rats were pre-treated with BTCC or PTCC, a remarkable improvement in renal function and reduction in the number of necrotic cells in renal tubules was noticed. In agreement with the data obtained by Sheikh-Hamad *et al* (257), the degree of necrosis in renal cells correlated with the extent of renal failure and the improvement in renal function after treatment with BTCC or PTCC paralleled the necrotic changes emphasizing the importance of this mode of cell death in CP-mediated renal injury.

CP accumulates preferentially in the proximal tubules of the kidney where most of its damaging effects occur (150). Upon accumulation, CP depletes several anti-oxidant proteins leading to an increase in the oxidative stress in renal cells. Therefore, several anti-oxidants such as CAPE, vitamin C, vitamin D, and melatonin have been shown to exert protective effects against CP-induced nephrotoxicity (81; 225; 255). Although several ITCs have been found to possess potent anti-oxidant properties, under our experimental conditions, the anti-oxidant effects of ITCs were unlikely to be involved in their protective effect since their anti-apoptotic effects lasted for several hours after BTCC and PTCC were removed from the incubating medium. Alternatively, our results have shown that these two ITCs might provide their anti-apoptotic effects indirectly through up-regulating HO-1 in renal cells.

In the light of the importance of oxidative stress and cell death in CP-induced nephrotoxicity, HO-1 and its reaction products have been thought to serve as a

potential strategy to protect against CP-induced renal failure. In line with our findings, Baliga *et al* have demonstrated that inhibition of HO-1 in CP-treated rats aggravated renal dysfunction (23). In another study, Shiraishi *et al* have shown that pre-induction of HO-1 by haemin and over-expression of the *ho-1* gene remarkably ameliorated CP-induced renal damage. Furthermore, the authors have indicated that HO-1 knockout mice showed high sensitivity to CP-mediated toxic effects (260).

Although inhibition of HO-1 significantly worsened renal failure (especially serum creatinine), it did not show a parallel increase in proximal tubular necrosis indicating that induction of HO-1 by these compounds might not play a role in this reno-protective effect of BTCC and PTCC against CP-mediated necrosis *in vivo*. Alternatively, other mechanisms could be utilized by these two ITCs to exert their reno-protective effect. In particular, their known inhibition of phase I enzymes and their anti-inflammatory effects mediated by inhibition of NFkB activation, which both have been implicated to play a role in CP-mediated renal failure, might contribute to their observed reno-protective effect (161; 223).

## 5.6 Conclusions

Altogether, our results seem to indicate that BTCC and PTCC protect against CP-induced renal failure *in vitro* via mechanisms that involve HO-1. However, *in vivo* other mechanisms might be utilized by these two ITCs to mediate this effect. The

present study emphasizes the potential therapeutic use of BTCC and PTCC in protecting the kidney against CP-mediated damaging effects.

# 6PROTECTION AGAINST CISPLATIN-INDUCED NEPHROTOXICITY BY CORM-3

## 6.1 Introduction

Oxidative stress caused by increased generation of free radicals and caspase-mediated apoptosis play a major role in nephrotoxicity and renal dysfunction that progressively develop in response to CP treatment (81). This anti-neoplastic agent is particularly useful for the treatment of solid tumours and is one of the few curative chemotherapeutic agents used in clinical practice. Therefore, particular attention has been devoted to understanding the mechanisms of the nephrotoxic effects of this agent to develop therapeutic strategies that could counteract this side effect and allow the use of higher doses to maximize its chemotherapeutic activity.

Kidney, like other organs, is well-equipped with diverse inducible anti-oxidant and anti-apoptotic enzymes which, as part of a ubiquitous and intrinsic natural defence, are engaged to detoxify cells from the stress inflicted by nephrotoxic drugs and other xenobiotics introduced into the body. In higher organisms, HO-1 has been widely studied as a model for redox-regulated gene expression (275). This inducible gene responds to chemical and physical agents that directly or indirectly generate ROI or deplete cellular GSH (11).

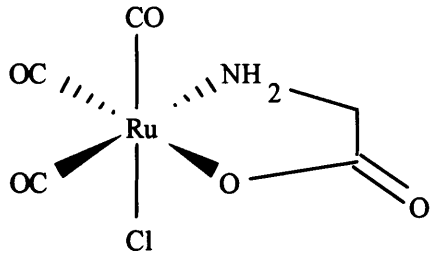
The HO-1/CO system has been demonstrated to counteract several pathological conditions that affect the kidney including I/R injury (169), glomerular inflammation (298), renal failure (258) and angiotensin-mediated hypertension (5; 34). Notably, the induction of HO-1 has been reported to prevent nephrotoxicity as well as renal tubule apoptosis mediated by CP (3; 260), although the protective contribution of CO and BV remains to be fully investigated.

Based on the attractive hypothesis that stimulation of the HO-1/CO pathway could be exploited for therapeutic purposes, we have developed a technology that enables CO to be delivered in a controlled fashion to biological systems. This can be achieved by certain transition metal carbonyls and other carbonylating agents, a novel class of compounds that we initially characterized and termed CO-releasing molecules (CO-RMs) (180; 183). Extensive research has been conducted in our laboratory in order to improve the chemical and pharmacological features of these CO carriers (184). CORM-3 ($Ru(CO)_3Cl(glycinate)$) (Fig 5-1) is a recently synthesized water-soluble compound that, in a similar manner to CO gas, has been shown to possess potent vasorelaxant properties (92), promote cardio-protection against I/R injury (64) and exert anti-inflammatory activities (250). Furthermore, our group has recently shown that CORM-3 and CORM-A1 exerted remarkable protective effects against cold preservation and I/R injury in isolated perfused kidneys (247). In support of these findings, Vera *et al* have shown in a recent study that CORM-3 administered 1 h before the onset of ischaemia significantly decreased the level of plasma creatinine 24 h after reperfusion as compared with control mice. From a pathophysiological point of

view, renal I/R injury and drug-induced nephrotoxicity bear a great level of similarity (202). Therefore, we hypothesized that CO released from CORM-3 would protect renal tissues against the renal damaging effects of CP.

## 6.2 Objectives

In chapter 5, we have shown that through stimulating HO-1, BTCC and PTCC protected against CP-mediated renal injury. In the current investigation, we wanted to identify which one of HO pathway products or by-products is the actual mediator of this reno-protective effect. Therefore, we examined the effect of CORM-3 (as a CO donor), BV and BR on CP-mediated apoptosis in LLC-PK1. Moreover, we utilized a rat model of CP-induced renal failure to explore the potential therapeutic benefits of CO released from CORM-3 against nephrotoxicity and renal dysfunction.




Tricarbonylchloro(glycinato)ruthenium(II )
$[Ru(CO)_3Cl(glycinate)]$

**CORM-3**

**Figure 6-1 Chemical structure of CORM-3.**

## 6.3 Materials and methods

### *6.3.1 Reagents and solutions*

CP, BV, BR and all other reagents were purchased from (Sigma-Aldrich Co.) unless otherwise specified. Stock solutions of CP (5 mM) were freshly prepared in DMSO on the day of the experiment. CORM-3 solution (2.5 mM) was synthesized and prepared freshly in distilled water (or sterile water for injection if used in animals) as previously reported by our group (64). An inactive compound (iCORM-3) was prepared by adding CORM-3 (2.5 mM) to a phosphate buffer solution, left in an open container at room temperature for 48 h to ensure complete dissociation of CO and finally bubbled with nitrogen to remove the residual solubilized CO (64; 92). SnPPIX was purchased and prepared as previously described in chapter 5 (182). The guanylate cyclase inhibitor (1H-Oxadiazole [4, 3-a] quinoxaline-1-ore) (ODQ) was obtained from (Alexis Corporation).

### *6.3.2 Caspase-3 activity assay*

Caspase-3 specific activity was measured indicated in chapter 9.

### *6.3.3 TUNEL assay*

TUNEL technique was performed as demonstrated in chapter 9.

### *6.3.4 Cell culture and experimental protocol*

LLC-PK1 cells were cultured as described in chapter 9. Confluent cells were exposed for 16 h to CP (50 μM) alone or in combination with increasing concentrations of either CORM-3 or iCORM-3 (1, 5, 10 or 50 μM). At the end of the incubation period, caspase-3 activity was measured as a marker of apoptosis. To investigate the possible involvement of cGMP, the inhibitor of guanylate cyclase ODQ (10 μM) was added to the cell culture medium 30 min prior to addition of CP and CORM-3. Since BV and BR are generated alongside CO during haem degradation, additional experiments were performed to examine the effect of BV (0.5-10 μM) and BR (1, 5 and 10 μM) on CP-mediated caspase-3 activation by activating CP-treated cells with the indicated concentrations of these two bile pigments. We started with a smaller concentration of BV (0.5 μM) because this bile pigment has been shown to exert a more protective response against Cu(II)-mediated DNA breakage when compared to BR in a previous study (16).

### *6.3.5 Animal studies and experimental protocol*

Rats were grouped as previously described in chapter 9.

### *6.3.6 Histopathological scoring*

Histopathological scoring was assessed as reported in chapter 9.

## 6.4 Results

### *6.4.1 CORM-3 protects against CP-mediated apoptosis in LLC-PK1 cells via mechanisms that involve cGMP*

LLC-PK1 exposed to CP for 16 h displayed a significant increase in caspase-3 activity (Fig 6-2A). This effect was accompanied by severe damage as evidenced by an increased number of cells floating in the culture media which was estimated to be >70% compared with the control cells (no detachment). Addition of CORM-3 (1-50 μM), which rapidly releases CO in the cell media (64; 184), prevented the increase in caspase-3 activity in a concentration-dependent manner. CORM-3 was very effective in protecting cells from CP-mediated damage since both the increase in caspase-3 activity and cell detachment were completely abolished at concentrations as low as 10 μM (Fig 6-2A). Interestingly, an inactive form of CORM-3 (iCORM-3), which is incapable of liberating CO, did not have any effect on cell disruption and caspase-3 activation caused by CP. The protective effect of CO appears to be mediated by cGMP because when CORM-3 was added to the cells in the presence of ODQ (10 μM) it actually increased caspase-3 activity induced by CP (Fig 6-2B). This increase was statistically significant ($p<0.05$) at 1, 5 and 50 μM CORM-3.

BR caused a non-significant decrease in caspase-3 activity ($p>0.05$) at 1 and 5 μM concentrations (Fig 6-3A) whereas BV caused a statistically significant increase in caspase-3 activity at all concentrations used (Fig 6-3B) indicating that CO plays the major role in the prevention of nephrotoxicity inflicted by this anti-neoplastic agent.

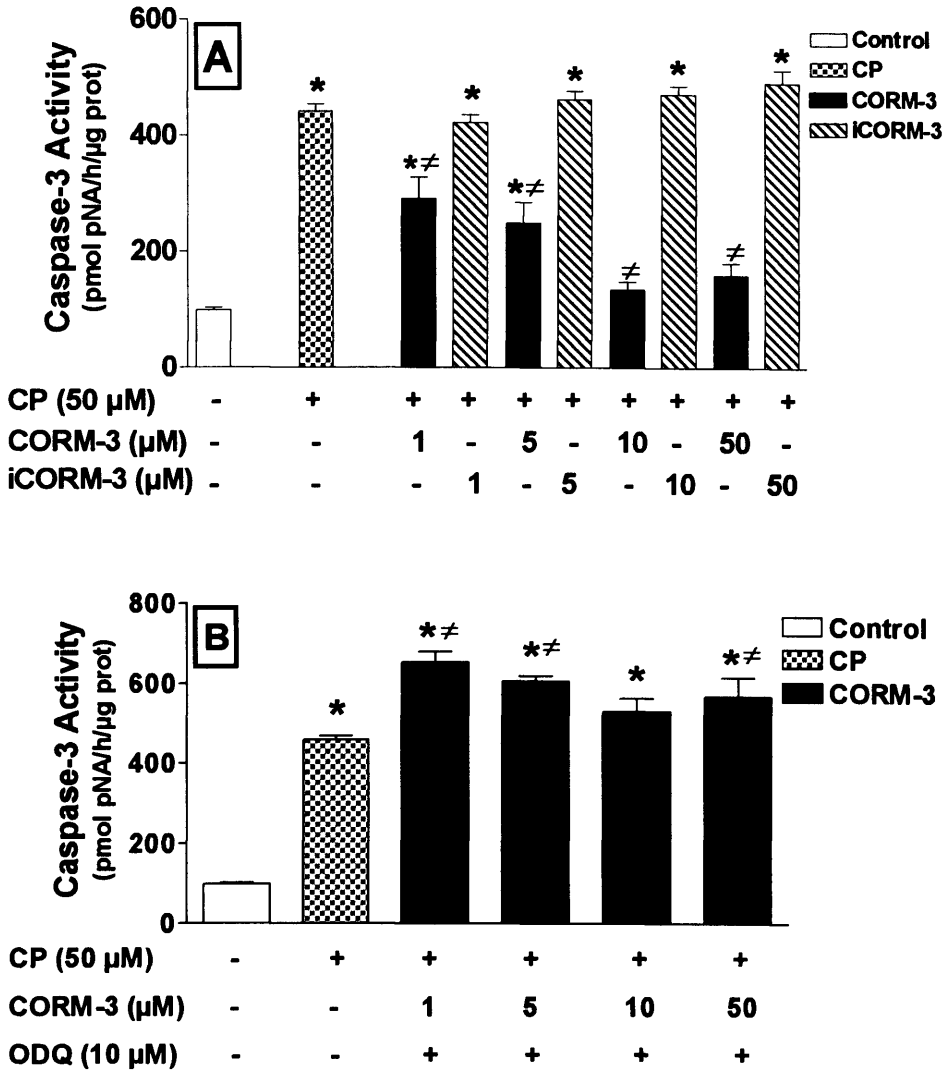



**Figure 6-2 Inhibition of CP-induced caspase-3 activation by CORM-3 in renal tubule cells involves cGMP.**

**(A)** Confluent LLC-PK1 cells were exposed to CP (50 µM) alone or in the presence of increasing concentrations of CORM-3 (1-50 µM). The inactive compound iCORM-3 was used as a negative control. After 16 h incubation, caspase-3 activity was measured as described in the Materials and Methods section. Each bar represents the mean ± SEM of 5 different experiments. **(B)** Cells were treated with CP (50 µM) plus CORM-3 (1-50 µM) in the presence of 10 µM ODQ, an inhibitor of GC, and caspase-3 activity was measured after 16 h incubation. * $p<0.05$ vs. vehicle-treated cells; ≠ $p<0.05$ vs. CP-treated cells.

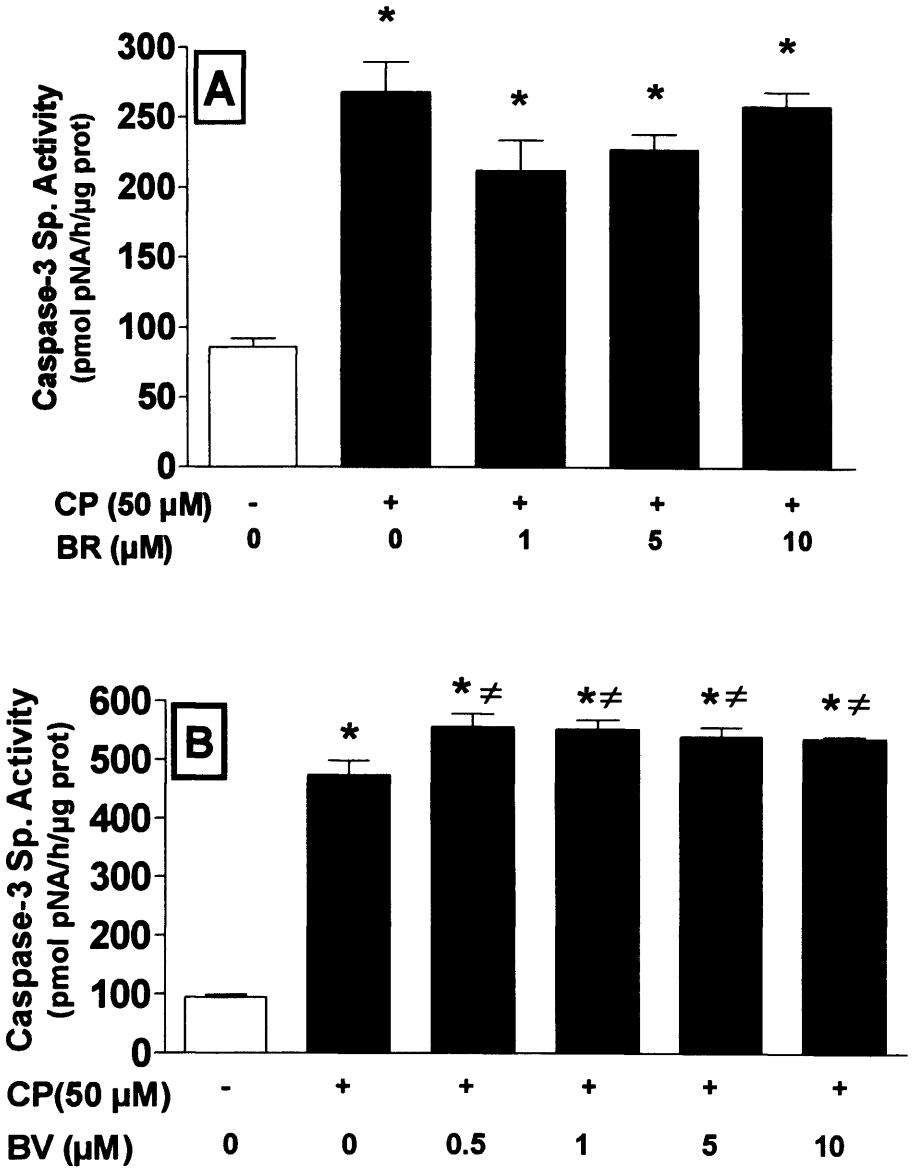



**Figure 6-3 Effect of BR and BV on CP-induced caspase-3 activation in LLC-PK1 cells.**

Confluent LLC-PK1 cells were exposed to CP (50 µM) alone or in the presence of increasing concentrations of BR (1, 5 or 10 µM) **(A)** or BV (0.5-10 µM) **(B)**. After 16 h incubation, caspase-3 activity was measured as described in the Materials and Methods section. Each bar represents the mean ± SEM of 5 different experiments. *$p<0.05$ vs. vehicle-treated cells, ≠ $p<0.05$ vs. CP-treated cells.

### *6.4.2 CORM-3 alleviates CP-mediated acute renal failure in a rat model*

We further tested whether similar results would be reproduced *in vivo*. Administration of CORM-3 completely prevented acute renal failure and kidney tissue damage induced by CP. Specifically, CP caused a significant ($p<0.05$) increase in plasma urea (from 7.0±0.5 to 46.0±6.5 mmol/l) and creatinine (from 57±5 to 179±49 μmol/l) levels; this effect was totally prevented by CORM-3 as plasma urea (5.3±0.5 mmol/l) and creatinine (62±3 μmol/l) concentrations were reduced to levels similar to the control group (Fig 6-5A and Fig 6-5B). The protective action of CORM-3 is mainly mediated by CO because when the rats were treated with the negative control (iCORM-3) in combination with CP serum creatinine increased to the level of the CP only-treated group whereas serum urea partially increased in response to CP. This negative control by itself did not have any effect on the basal levels of urea and creatinine (data not shown).

To test the possible contribution of HO-1 induction by CORM-3 in its reno-protective effects, another group of rats was administered SnPPIX in combination with CORM-3 and CP. Our results have shown that SnPPIX only partially restored the increase in urea and creatinine levels following CP treatment suggesting that renal HO-1 induction in response to CORM-3, which has been demonstrated in a previous study (292), might contribute to the observed reno-protective effect of this CO donor, albeit partially. However, since HO-1 inhibition by SnPPIX failed to increase renal function to CP levels, we conclude that CO released from CORM-3 primarily contributes to its observed renal protection (Fig 6-5A and Fig 6-5B). It has to be noted also that the

difference in weight before and after the various treatments directly correlated with the extent of renal dysfunction. Specifically, treatment with CP resulted in a 7.6±1.2% weight loss which was significantly ($p$<0.05) attenuated by CORM-3 (1.7±1.9%), but not iCORM-3 (7.1±2.0%) (Fig 6-4).

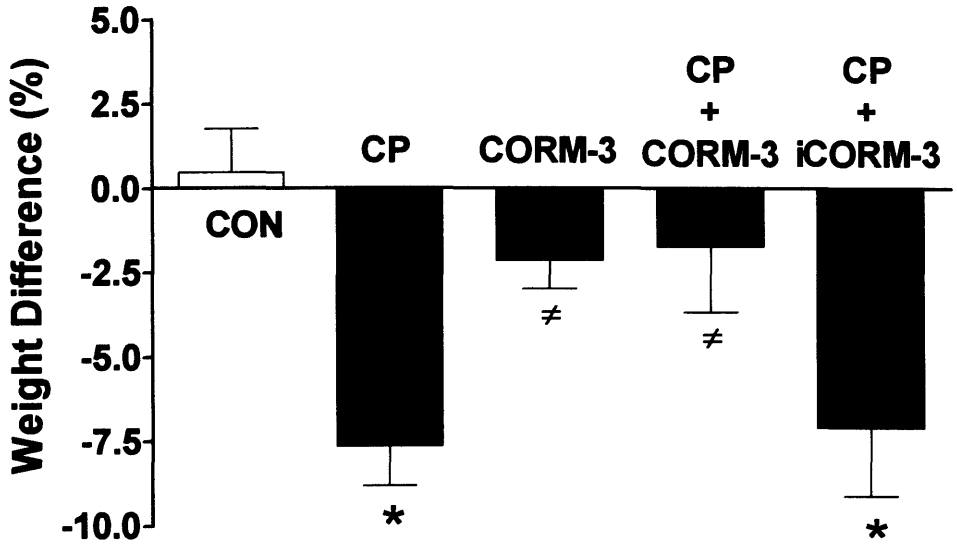



**Figure 6-4 Effect of CORM-3 on rat body weight following CP-induced nephrotoxicity.**

Rats were divided into groups as previously described in the Materials and Methods. The rats were weighed before treatment and immediately before sacrifice in order to compare the effect of CP and CORM-3 treatment on body weight. Each bar represents the mean ± SEM of 5 independent rats.* $p$<0.05 vs. CON (vehicle-treated rats); ≠ $p$<0.05 vs. CP group.

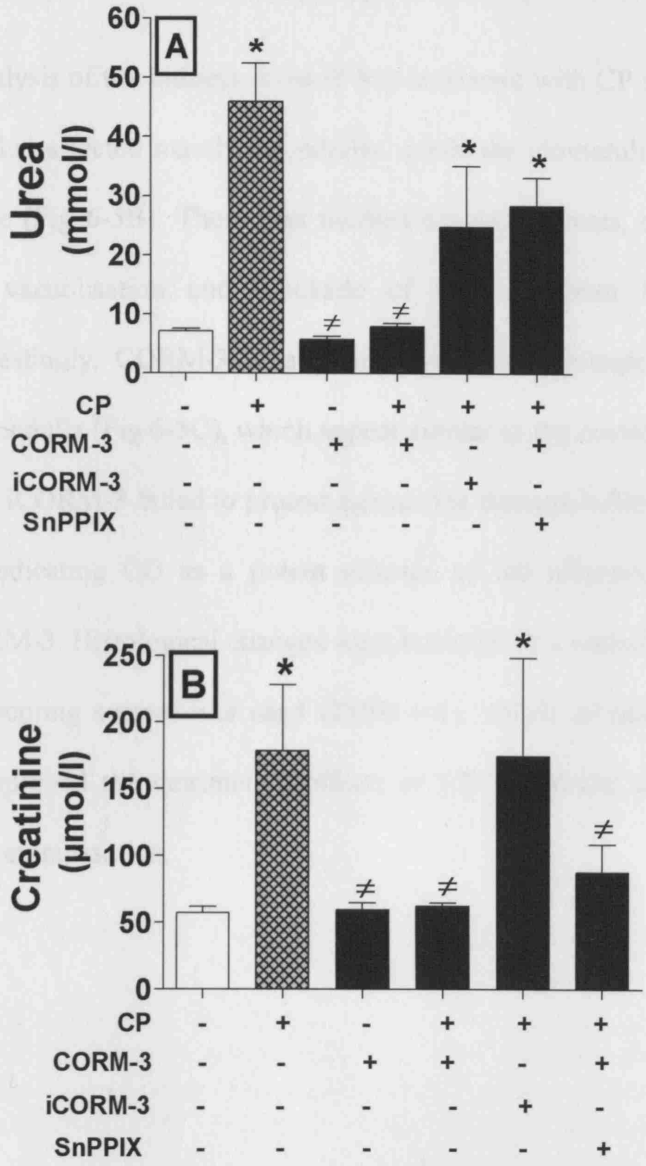



**Figure 6-5 Effect of CORM-3 on serum urea and creatinine *in vivo* following CP-induced nephrotoxicity.**

Acute renal failure in rats was induced by a single ip injection of CP (7.5 mg/kg b.w.). CORM-3 (10 mg/kg i.p.) was administered daily to animals starting 1 day prior to CP treatment and continuing for 3 days thereafter. Plasma urea **(A)** and creatinine **(B)** were measured at the end of treatment. Each bar represents the mean ± SEM of 5 independent rats.*$p<0.05$ vs. vehicle-treated rats; ≠$p<0.05$ vs. CP or iCORM-3 groups.

### *6.4.3 CORM-3 improves renal histological profile in response to CP*

Histological analysis of the kidneys showed that treatment with CP resulted in severe tissue damage that affected mainly the tubules, while the glomeruli maintained their normal structure (Fig 6-5B). There was marked tubular necrosis, sclerosis, cellular desquamation, vacuolisation and blockade of tubular lumen by proteinacious materials. Interestingly, CORM-3 greatly improved the histological profile of the cortex and the medulla (Fig 6-5C), which appear similar to the control kidneys (Fig 6-5A) (table 6-1); iCORM-3 failed to protect against the damage inflicted by CP (Fig 6-5D) strongly indicating CO as a potent effector of the pharmacological activity elicited by CORM-3. Histological changes were assessed by a pathologist in a blinded manner and a scoring system was used (Table 6-1), which revealed that CORM-3 significantly improved the detrimental effects of CP on tubular necrosis and to a lesser extent on extravasation.

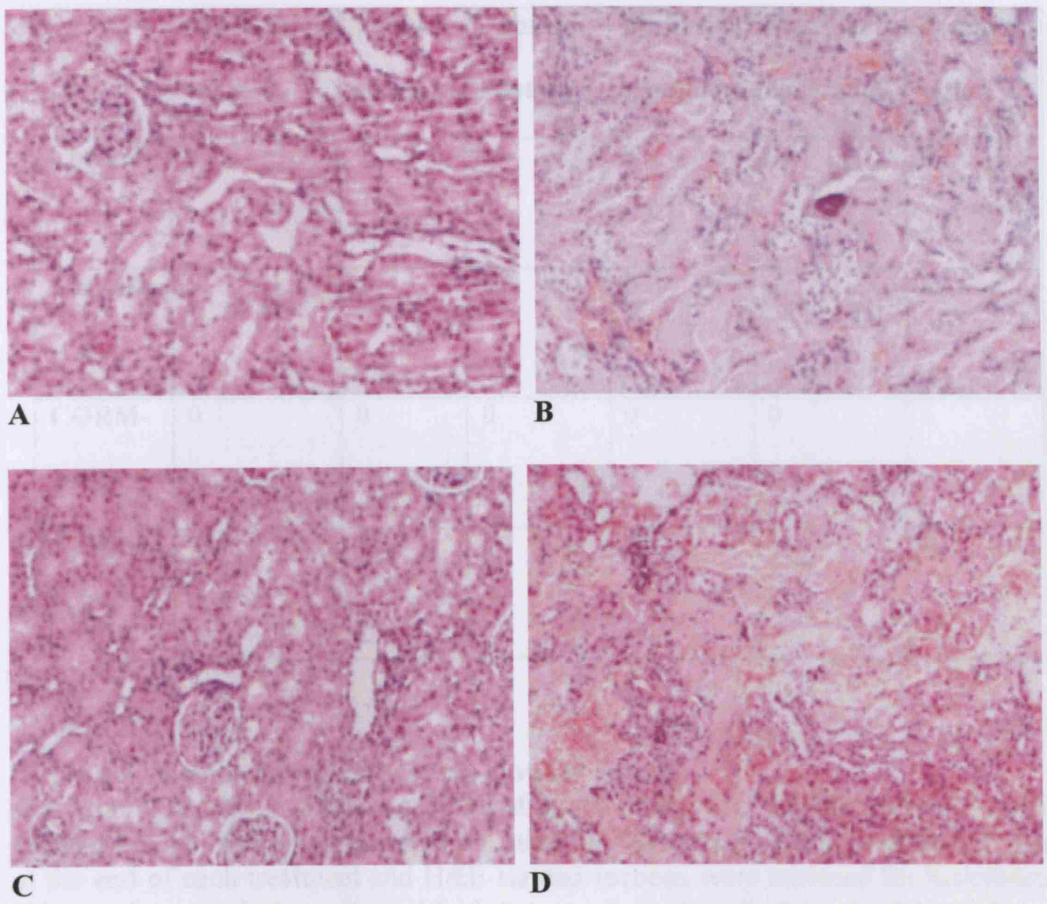



**Figure 6-6 Effect of CORM-3 on kidney morphology *in vivo* following CP treatment.**

Rats were treated as described in Fig 6-5. At the end of each treatment, renal tissue was collected for histological examination (see the Materials and Methods). As shown, while the control group displayed normal kidney histology **(A)** (x200), extensive tubule epithelial cellular necrosis, desquamation, vacuolization and swelling were observed in CP-treated kidneys **(B)** (x200). Administration of CORM-3 resulted in a remarkable improvement in the histological appearance and reduction in tubule cell damage **(C)** (x200), whereas the negative control iCORM-3 resulted in only a decrease in vascular congestion and a slight reduction in extravasation **(D)** (x200).

| | Glomerular necrosis | Tubular necrosis | Tubular dilatation | Epithelial sloughing | Vascular congestion | Extravasation |
|---|---|---|---|---|---|---|
| **Control** | 0 | 0 | 0 | 0 | 0 | 0 |
| **Cisplatin (CP)** | 0 | 4 | 0 | 0 | 3 | 3 |
| **CORM-3+CP** | 0 | 0 | 0 | 0 | 0 | 1 |
| **iCORM-3+CP** | 3 | 4 | 3 | 3 | 0 | 2 |

**Table 6-1 Histopathological scoring of renal tissue.**
Cisplatin (CP, 7.5 mg/kg i.p.), CORM-3 (10 m/kg i.p.) or the inactive control (iCORM-3, 10 mg/kg i.p.) were administered to Wistar rats. Kidneys were harvested at the end of each treatment and H&E stained sections were assessed for histological changes by a pathologist in a blinded manner as described in the Materials and Methods. Values are expressed as the estimated average of 5 independent experiments.

### *6.4.4 CORM-3 decreases CP-induced necrosis in renal tubular cells*

To investigate the effect of CORM-3 on CP-mediated necrosis and apoptosis in rat kidney, the TUNEL assay was performed on paraffin-embedded renal sections. Treatment with CP caused extensive necrotic cell death associated with dispersed areas of apoptosis which mainly affected the S3 segment of the proximal tubule epithelial cells and possibly the LOH. The dense staining in Fig 6-7B could be due to a non-specific reaction of the TUNEL reagents with the necrotic tubular cells and as a result of the formation of dead cell debris. Administration of CORM-3 completely

prevented CP-mediated necrosis (Fig 6-7C). Nevertheless, when CP-treated rats were administered iCORM-3 there was cortical proximal tubular necrosis with absence of the medullary damage seen in kidneys of CP-treated rats indicating that this compound decreased CP-mediated necrosis in the proximal tubules in the medullary region that was seen in CP-treated group but it caused a new form of renal damage which mainly targeted the outer cortex (Fig 6-7D).

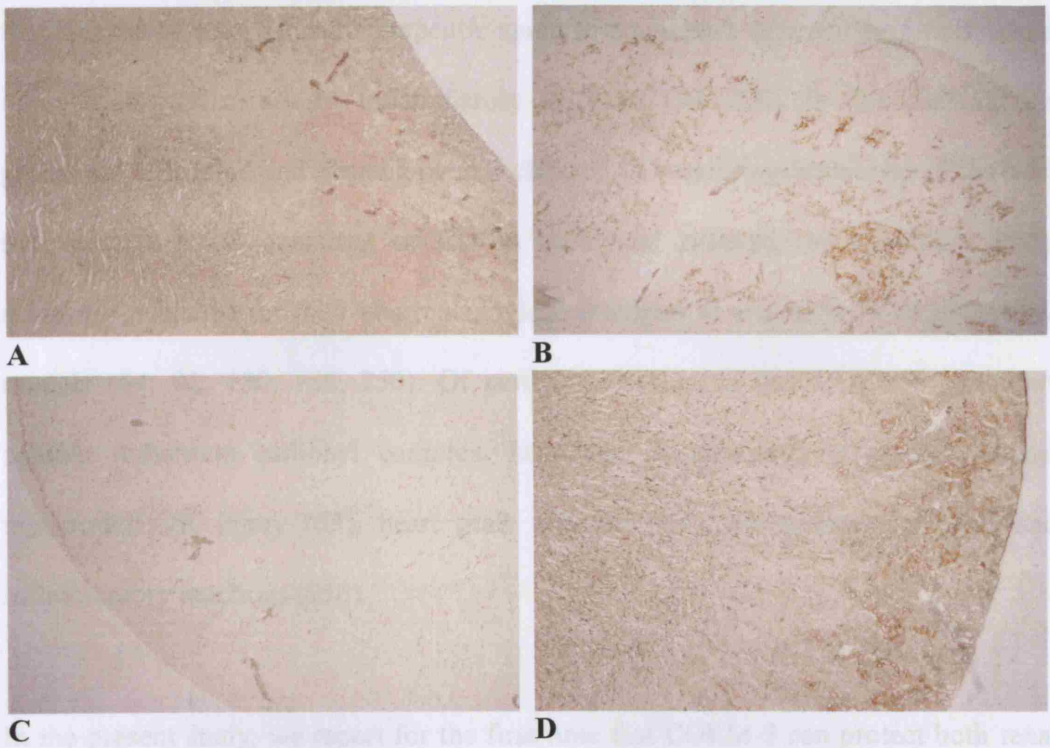



**Figure 6-7 Effect of CORM-3 on renal tubule necrosis *in vivo* following CP treatment.**

Rats were treated as described in Fig 6-5. At the end of each treatment, renal tissue was collected for the assessment of necrosis and apoptosis using the TUNEL assay. Kidneys from the control group displayed very little necrosis and apoptosis **(A)** (x40). Treatment with CP significantly increased the number of necrotic tubular cells mainly in the S3 segment and LOH **(B)** (x40) whereas CORM-3 administration significantly reduced this effect **(C)** (x40). The negative control (iCORM-3) resulted in superficial cortical proximal tubular necrosis in response to CP **(D)** (x40).

## 6.5 Discussion

The multiplicity of the beneficial effects elicited by CO, a signalling mediator generated by constitutive (HO-2 and HO-3) and inducible (HO-1) haem oxygenase enzymes (94), encompasses vasodilatory, anti-inflammatory and anti-apoptotic activities (121; 182; 306). Its key involvement in the regulation of important cellular processes in physiology and disease has been underlined by recent studies reporting that CO could be used as a therapeutic agent to counteract vascular dysfunction and other conditions driven by inflammatory reactions (94; 205). In this context, our group has identified and characterized a class of CO-releasing molecules (CO-RMs) that liberate small quantities of CO in biological systems and provided strong scientific evidence for their pharmacological activities in a number of experimental models (64; 92; 180; 184; 250). Of particular interest is that CORM-3, a water-soluble ruthenium carbonyl complex, has been demonstrated to protect against myocardial I/R injury (63), heart graft rejection (64), vasoconstriction (92) and inflammatory reactions (250).

In the present study, we report for the first time that CORM-3 can protect both renal tubule epithelial cells and kidneys against the structural and functional impairment induced by CP, a widely used anti-neoplastic drug that is also known for its nephrotoxic side effects (14; 157). Specifically, our main findings show that: 1) CORM-3 suppressed the CP-mediated increase in caspase-3 activation and cell detachment in cultured LLC-PK1 cells, an effect that was completely abolished by inhibition of the GC pathway (Fig 6-1A, Fig 6-1B); 2) administration of CORM-3 *in*

*vivo* prevented acute renal failure caused by CP as evidenced by a significant reduction in rat serum urea and creatinine levels Fig 6-5A and Fig 6-5B); 3) the protective effects of CORM-3 against renal dysfunction inflicted by CP *in vivo* was associated with preservation of kidney morphology (Fig 6-6C) (Table 6-1), decrease in the number of the necrotic tubule epithelial cells (Fig 6-7C) and a significant prevention of weight loss (Fig 6-4). Specifically, assessment of H&E stained sections of the kidneys revealed a marked protective effect by CORM-3 against CP-induced tubular necrosis, which has been shown previously to be the main damaging renal damaging effect caused by CP (257), and to a lesser extent against extravasation. The fact that iCORM-3, an inactive negative control that does not release CO, failed to prevent both caspase-3 activation *in vitro* and nephrotoxicity *in vivo* strongly implicates CO as mediator of the observed pharmacological effect. We cannot exclude a possible contribution of endogenously generated CO following CORM-3 treatment since previous studies have shown that this transition metal carbonyl complex induces HO-1 protein expression and increases HO activity *in vitro* and *in vivo* (250; 292). In fact, our results show that inhibition of HO by SnPPIX in combination with CORM-3 only partially restored the increase in urea (56%) and to a much lesser extent in creatinine (26%) levels following CP treatment. These data indicate that CORM-3-mediated activation of HO-1, and by inference increased endogenous CO, participates in the improved outcome of kidney function following CP-induced acute renal failure. However, our results demonstrate that CO liberated from CORM-3 has a marked additive effect to the protection elicited by HO-1

activation and that CO-RMs could be used therapeutically as an additive to HO-1 inducers for the protection against CP-induced nephrotoxicity.

Our data are in line with previous reports demonstrating that induction of HO-1 protects human renal tubule cells from CP-mediated cell death and preserves renal haemodynamic functions in rats treated with this chemotherapeutic agent and other clinically used drugs (4; 260). In the present study we also showed that CP-mediated caspase-3 activation is not affected by BV or BR and that the anti-apoptotic effect of CORM-3 is completely abolished by inhibition of the cGMP pathway, highlighting the specificity of HO-derived CO to counteract CP-mediated renal toxicity. This is consistent with the findings showing that CO gas protects vascular smooth muscle cells against caspase-3 activation and apoptosis via a cGMP-dependent mechanism (163). The fact that GC is a preferential target for the vascular activity of CO liberated from CORM-3 also supports this concept (92). Since apoptosis caused by CP is associated with an increased production of free radicals (296), we cannot exclude a priori that the anti-apoptotic effects of CORM-3 may also involve the suppression of crucial pathways that trigger oxidative stress (274).

Our data on the reno-protective effects of CORM-3 against CP-mediated increase in creatinine and urea levels emphasize the potential use of CO carriers for therapeutic purposes. The concentration of plasma urea and creatinine were dramatically reduced to basal levels by CORM-3 in CP-treated rats, an effect that was accompanied by a complete suppression of tubular cell necrosis and preservation of the renal tubule

morphology. Our results are consistent with previous studies revealing a pharmacological effect on kidney by all CO-RMs that were originally identified by our group (64; 180; 184). In fact, both water-soluble (CORM-3) and lipid-soluble (CORM-1 and CORM-2) CO-releasing agents markedly improved renal haemodynamic in rats (15) and limited renal damage in a mouse model of ischemia-induced acute renal failure (292). Moreover, we have recently produced data showing that addition of CO-RMs to cold storage solutions during the preservation of kidneys improves mitochondrial respiration at reperfusion (247). Thus, CO-RMs appear to be excellent candidates in the development of pharmaceuticals for the treatment of nephrotoxicity and renal dysfunction (183; 185).

The observation that iCORM-3 *in vivo* caused a different pattern of renal damage that was characterized by superficial cortical necrosis with no medullary damage could indicate the possibility that this compound might interact with CP or is metabolized in the kidney resulting in the production of a new agent that could cause the new damage. Moreover, the fact that iCORM-3 significantly decreased CP-induced medullary necrosis indicated that, in addition to CO, other molecules that form CORM-3 including heavy metals might on the one hand contribute to its reno-protective effects against CP-mediated damage and on the other hand it might inflict different form of cortical damage to the kidneys of CP-treated rats.

It should be noted, in addition, that the observed decrease in CP-mediated medullary necrosis might indicate the possibility that not all CO had been released during the

preparation of iCORM-3 and that more time should have been allowed or a another method of inducing CO release from CORM-3 should be attempted such as treating this compound with different buffer other than PBS to enhance CO release before use.

## 6.6 Conclusions

In summary, our results demonstrate for the first time that the injurious effects mediated by CP on renal tissue can be mitigated by delivering small amounts of CO into the organism suggesting that CO-RMs could be utilized as effective adjuvant to prevent nephrotoxicity in cancer patients undergoing treatment with platinum-derived chemotherapeutic agents.

# 7 CORM-3 MODULATES MITOCHONDRIAL RESPIRATION IN RAT KIDNEY

## 7.1 Introduction

Mitochondria play a crucial role for sustaining life by producing the majority of cell energy in the form of ATP. In this process, electrons flow through sequential cytochrome-containing complexes (complex I to IV) in the inner mitochondrial membrane and the energy created is used by ATP synthase to produce energy for the cell. Although the protonmotive force that is set up by proton pumping is the main driving force of ATP synthesis, electrons my leak back across the inner membrane and divert the conserved energy away from ATP synthesis into heat production and react inappropriately with $O_2$ to form $O_2^{\cdot-}$ (41). Under physiological conditions, most of the ROI produced in the mitochondria are detoxified by the SOD and other mitochondrial anti-oxidant enzymes. However, after extreme stress conditions such as during excessive exercise, the high level of free radicals produced overwhelms the mitochondrial detoxification capacity and results in severe cellular stress and ultimately cell death.

Mild uncoupling of oxidative phosphorylation, which might result in a mild decrease in ATP synthesis, has been found to reduce both membrane potential and pH gradient and very effectively decrease mitochondrial ROI generation (35). Brand and

colleagues have shown that ROI generated from the mitochondrial complex I and complex III induce the generation of UCPs that significantly reduce oxidative stress by causing mild uncoupling of oxidative phosphorylation (36). This natural anti-oxidant machinery allows decreasing oxidative stress at the expense of a slight reduction of ATP synthesis (36).

The exact mechanism of CP-mediated renal damage has not been fully elucidated but oxidative stress as a result of ROI release has been shown to play a major role. The toxic effects of CP in the kidney are characterized by signs of oxidative injury such as GSH depletion and lipid peroxidation (302). Moreover, this anti-cancer agent has been shown to cause a fall in the level of anti-oxidants in cancer patients (304). Baliga *et al* have demonstrated that $Fe^{+2}$ released during treatment with CP induces the release of $OH^{\cdot}$ and thus exacerbates renal damage and showed also that $Fe^{+2}$ chelators remarkably improved renal function of CP-treated rats (22). In another study, Kruidering and co-workers have examined the relationship between mitochondrial damage, ROI and cell death due to CP exposure (148). The authors have demonstrated that this agent reduced ATP synthesis by decreasing the activity of complex I-IV of the respiratory chain, significantly decreased GSH and resulting in an increase in ROI. Based on the above-mentioned studies, mitochondrial generation of ROI might play a significant role in CP-mediated nephropathy.

## 7.2 Objectives

In the light of the strong reno-protective effects of CORM-3 that we have demonstrated *in vitro* and *in vivo* by using CP-induced apoptosis in LLC-PK1 cells and in a rat model of CP-induced nephrotoxicity, the major purpose of this study was to investigate the effect of CORM-3 on mitochondrial respiration and the possible effect of this CO donor on uncoupling of oxidative phosphorylation in order to explore the possible contribution of such possible anti-oxidant effect to the protection against CP-induced renal damage exerted by this CO donor.

## 7.3 Materials and methods

### *7.3.1 Chemicals and reagents*

CORM-3 and its inactive counterpart (iCORM-3) were synthesized and prepared as previously reported by our group (64). ADP was obtained from (Boehringer Mannheim, Lewes, UK) and all other reagents were obtained from (Sigma-Aldrich Co.) unless otherwise specified.

### *7.3.2 Isolation of rat kidney mitochondria*

Isolation of renal mitochondria was performed as described in chapter 9.

### *7.3.3 Measurement of respiration in isolated mitochondria*

Mitochondrial respiratory parameters were measured as indicated in chapter 9.

## 7.4 Results

### *7.4.1 Effect of CORM-3 on mitochondrial respiration*

The exposure of isolated cortical mitochondria to increasing concentrations of CORM-3 (10, 50 and 100 μM) affected all mitochondrial respiratory parameters. That is, there was a significant decrease in state 3 in a concentration-dependent manner (Fig 6-1). For instance, there was 43.5% ± 2.6% decrease in state 3 when mitochondria were incubated with 100 μM CORM-3 (Fig 6-1). The corresponding negative control (iCORM-3) caused a minor decrease in state 3 (not statistically significant) (Fig 6-1). A similar non-statistically significant decrease in the respiratory control index (RCI), the ratio of state 3/state 4 which indicates the tightness of oxidation and phosphorylation, was observed (Fig 6-3). State 4 respiration was measured after all ADP had been consumed and was significantly affected by CORM-3 but iCORM-3 had no effects on this mitochondrial parameter. There was a significant increase in state 4 respiration when mitochondria were exposed to 50 and 100 μM CORM-3 ($p<0.05$ vs. the negative control iCORM-3) but no changes were observed at 10 μM (Fig 6-2). As a result of the decrease in state 3 and the increase in state 4 respiration, CORM-3 resulted in a concentration-dependent and a statistically significant reduction in the calculated RCI values. For instance, treatment with CORM-3 decreased the RCI values to 3.7 ± 0.2 at 10 μM, 1.8 ± 0.15 at 50 μM and 1.2 ± 0.07 at 100 μM (Fig 6-3).

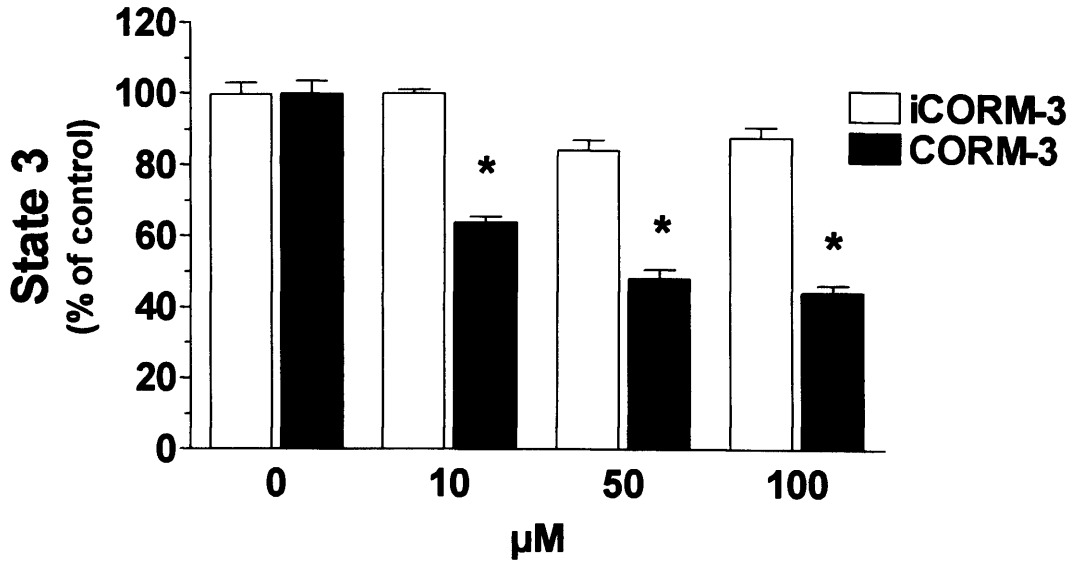



**Figure 7-1 The effect of CORM-3 on mitochondrial state 3 respiration.**

Kidney mitochondria were isolated from non-treated rats. The respiratory activity from isolated renal cortex mitochondria incubated for 1 min with CORM-3 was assessed in the presence of ADP, malate and glutamate as substrates. Each bar represents the mean ± SEM of 5 renal mitochondrial extracts from different rats on different occasions. * $p<0.05$ vs. iCORM-3-treated group.

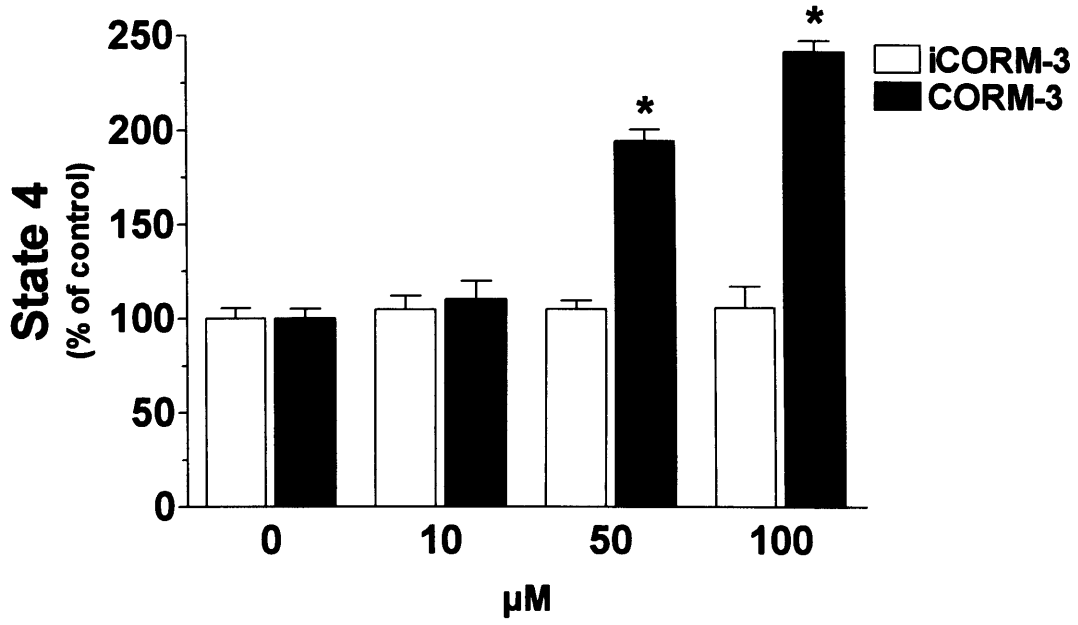



**Figure 7-2 The effect of CORM-3 on mitochondrial state 4 respiration rate.**

The respiratory activity from isolated renal cortex mitochondria was assessed in the presence of ADP, malate and glutamate. Each bar represents the mean ± SEM of 5 different experiments using renal mitochondria extracted from 5 different rats on different occasions. * $p<0.05$ vs. iCORM-3-treated group.

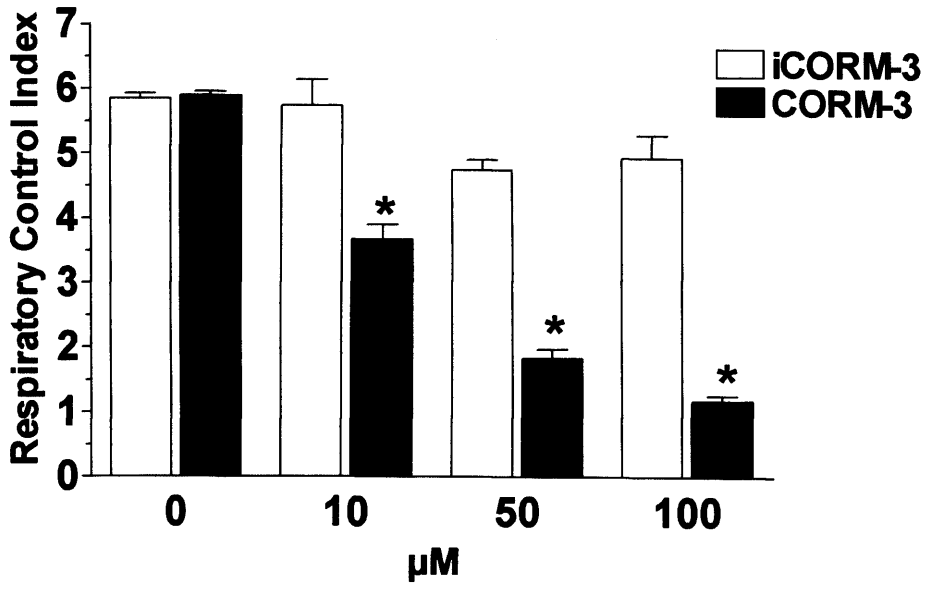



**Figure 7-3 The effect of CORM-3 on mitochondrial respiratory control index (RCI).**

The respiratory activity from isolated renal cortex mitochondria was assessed in the presence of malate and glutamate. Isolation was described in the Materials and Methods section. Each column represents the mean ± SEM of 5 independent experiments in each group using renal mitochondria extracted from kidneys of different rats on different occasions. * denotes statistical significance at $p<0.05$ vs. iCORM-3-treated group.

## 7.5 Discussion

Mounting evidence suggests that endogenously generated CO as a result of haem degradation by HO plays a key part in the modulation of various physiological and pathological processes (121; 181; 258). This observation has been further investigated by the use of CORMs which are able to carry and deliver CO in biological systems in a controllable fashion in a similar fashion as the endogenously produced CO (180) (185). Due to its high affinity for haem-containing proteins, we hypothesized that CO would bind the mitochondrial chain complexes and modulate their respiratory function parameters. Indeed, the major mechanism of CO toxicity in cells is related to its binding to haem proteins in a competitive manner to $O_2$ resulting in generalized tissue hypoxia.

Oxidant mechanisms have been previously shown to play an important role in CP-mediated renal injury (81). Since mitochondria are a major source of ROI generation in the living cell (36), in the current study, we investigated the effect of CORM-3 on the mitochondrial respiration in rat kidney in an effort to examine whether the anti-oxidant effects of CORM-3 by modulating the mitochondrial respiratory function would contribute to its reno-protective effects that we have demonstrated in chapter 6.

Our data indicated that low concentrations of CORM-3 modulate the respiratory activity of mitochondria. Specifically, by using malate, glutamate and ADP as substrates for ATP synthesis, the RCI was significantly reduced. This was associated with a significant decrease in state 3 (active respiration in the presence of ADP)

indicating that oxygen uptake and oxidative phosphorylation had been affected. Moreover, state 4 which reflects proton flow across the mitochondrial inner membrane and the rate of oxygen utilization after ADP consumption was remarkably reduced indicating that more oxygen had been used whereas no ATP was produced. In all cases, these mitochondrial respiration parameters were not affected by the treatment with iCORM-3. Thus our data give evidence that CORM-3 works as an uncoupling agent in ATP synthesis.

As a matter of fact, the majority of reports on CO highlight its potential toxicity and the ability to modulate mitochondrial function. For instance, CO has been shown to impair energy metabolism in rat brain and to decrease oxidative phosphorylation and cyt c oxidase in CO poisoning (10; 246). CO binds and inactivates cyt c oxidase (complex IV), stops electrons from flowing through the respiratory chain and thus markedly decreases ATP production.

Generally, CO produces an overall decrease in mitochondrial respiratory chain function and might result in apoptotic cell death as a consequence of ATP depletion. Energy depletion leads to cellular injury or dysfunction especially in high metabolic tissues such as in central nervous system, heart and skeletal muscles. Indeed, muscle weakness and heart failure have been reported in cases of CO poisoning (10).

The negative effect of CO on mitochondrial function results in interruption of electron flow through the mitochondrial complexes and diverts them towards the production of

ROI such as $OH^{\cdot}$. In a recent study, it has been shown that CO targets NADPH oxidase and other cytochrome-containing complexes that are strictly associated with its anti-proliferative effects in the human airway smooth muscle cells (274). Although mitochondria are an important source of ROI in several cell types (36), they comprise one of the targets for the damaging effects of free radicals that result in mitochondrial swelling, lipid peroxidation, inactivation of electron transport chain and inhibition of mitochondrial respiration (220). Overall, CO acts as an un-coupling agent and results in suppression of mitochondrial respiratory function.

Our results leave open the question of what benefit CO modulation of mitochondrial function might have *in vivo*. Our data seem to indicate reduction in RCI by CORM-3 treatment that is associated with uncoupling of phosphorylation through enhanced proton leakage across the inner mitochondrial membrane.

Although uncoupling of phosphorylation seems to be a negative effect as it deprives cells of energy, a reversible controlled modulation of respiratory coupling may be sometimes helpful because it reduces the ROI load in cells at the expense of a small decrease in ATP synthesis (35). Therefore, it is tempting to investigate the hypothesis that CO utilizes this mechanism to exert its protective effects against damage due to inflammation and I/R injury (7; 250). Moreover, it seems possible that CO might exert the reno-protective effects that we have shown in chapter 6 in a CP-model of acute renal failure through this mechanism.

## 7.6 Conclusions

Taken together, we have shown here that CO released from CORM-3 produced an uncoupling effect on isolated renal mitochondria as measured by a decrease in state 3 and RCI and an increase in state 4. Since controlled uncoupling of oxidative phosphorylation reduces ROI generation at the expense of a small loss of energy, we speculate that this mechanism might play a role in the anti-apoptotic and the reno-protective effect of CORM-3 we have shown in CP-mediated renal injury.

## 7.7 Acknowledgment

The data presented in this chapter were obtained from a shared project with Dr Ashraf Sandouka. In this project Dr Sandouka and I worked all experiments together in a project which aimed to delineate the effects of CORM-3 on renal mitochondrial respiratory function.

# 8 GENERAL DISCUSSION

The living cell has been equipped with several anti-oxidant enzymes that allow it to acclimatize under continuous exposure to endogenous and exogenous toxic stimuli. During oxidative stress, the pro-oxidant haem is released from haemoglobin and other haem-proteins and is degraded by HO into biologically active molecules that include CO, BV and $Fe^{2+}$ (165). This enzyme exists in constitutive (HO-2 and HO-3) and inducible (HO-1) isoforms and provides a unique model for studying the mechanisms by which alterations in cellular redox potential result in the modulation of gene expression (181). Indeed, HO-1 represents a highly refined inducible gene that is extremely sensitive to a large number of stimuli. Together with the diverse biological and physiological effects of its reaction products, HO-1 affords cytoprotection to cells against a wide range of tissue-damaging insults.

Although this enzyme performs one function in the cell, the catabolism of haem, the products and by-products of this metabolic reaction are far more significant. Not only does HO-1 provide cytoprotection in oxidative stress, the release of BR and BV with their potent anti-oxidant properties add remarkably to this effect (91; 135). Moreover, CO has been shown to possess vasodilatory (180), anti-proliferative (179), anti-inflammatory (207) and anti-apoptotic effects (163). Although $Fe^{2+}$ is produced during haem degradation, HO-1 has been found to stimulate the synthesis of ferritin which sequesters and thus inhibits the oxidative effects of this molecule (27).

In view of the increasing evidence that HO-1 provides cytoprotection against oxidative stress, the idea of pharmacological modulation of HO-1 gene expression has gained particular interest recently (181). In particular, the identification of naturally-occurring, non-toxic inducers of HO-1 could be utilized for the treatment of various debilitating human diseases (93).

In this study, we report that the two ITCs, BTCC and PTCC, significantly stimulate HO activity and HO-1 protein expression in porcine renal tubular epithelial (LLC-PK1) cells in a time and concentration-dependent manner.

In line with our data, several naturally occurring compounds such as curcumin (25; 181), 2-hydroxycalcone (93), rosolic acid (93) and CAPE (251) have been shown by our group to induce HO-1 and this has been suggested to contribute to their overall cytoprotective effects. In this context, Motterlini *et al* have shown that HO-1 induction by curcumin in bovine aortic endothelial cells had a major contribution to the protective effects of this naturally-occurring compound when the cells were exposed to hypoxia (181). Furthermore, Balogun *et al* have demonstrated a significant contribution of HO-1 stimulation by curcumin in conferring protection to renal epithelial cells against cold storage-mediated damage (24).

Among the plant-derived compounds, ITCs, the metabolic products of glucosinolates in various cruciferous vegetables, have gained special interest due to their potent anti-cancer properties (96; 111; 137; 256). The positive effects of ITCs result from their

induction of phase II drug metabolizing enzymes and inhibition of the pro-carcinogen activating phase I enzymes (cyt P450) (190; 325). In addition to their promising cancer-preventive properties, ITCs have been shown to confer anti-oxidant effects (166) through mechanisms that include transcriptional activation of multiple anti-oxidant proteins (174). In this context, Manesh *et al* have demonstrated that allyl ITC and phenyl ITC resulted in significant anti-oxidant effects in rats manifested by a remarkable decrease in $O_2^{\cdot-}$ generation from peritoneal macrophages and increased $OH^{\cdot}$ scavenging *in vitro* (166). Our novel findings that BTCC and PTCC up-regulate HO-1 extend our knowledge about the mechanisms that contribute to the cytoprotective and anti-oxidant effects of these chemoprotective compounds which might include HO-1 stimulation.

During the examination of the intracellular events mediating the stimulatory effects of HO-1 by these two ITCs, our study has revealed that the observed up-regulation of HO-1 was strictly associated with a rapid change in the intracellular redox status. Apparently, pre-incubation of the cells with NAC prior to exposure to BTCC or PTCC completely prevented HO-1 induction by these ITCs. It seems that these two compounds caused disruption of cellular redox balance resulting from an increase in ROI generation or a decrease in cellular oxidative buffering systems. Nakamura *et al* have reported that exposure of rat liver epithelial (RL34) cells to benzyl ITC resulted in an immediate and significant increase in ROI generation which was further enhanced by a remarkable decrease in cellular GSH an effect which was thought to account for GST stimulation (193). Thus it seems plausible that HO-1 induction by

BTCC and PTCC represents an adaptive response to an increase in cellular oxidative stress elicited by these two compounds.

The critical role of HO-1 as a cellular anti-oxidant protein that is up-regulated rapidly in response to rise in cellular oxidants has been well-established (27). Poss *et al* have shown that exposure of HO-1 deficient cells to oxidative challenge resulted in an increase in ROI generation while exposure of HO-1 null mice to endotoxins caused severe hepatic necrosis indicating the crucial role of HO-1 stimulation in the protection against oxidative stress (217).

The involvement of redox mechanisms in HO-1 stimulation by ITCs was expected because despite the fact that ITCs exert anti-oxidant and free radical scavenging properties, they can be a source of ROI because their –N=C=S group can undergo spontaneous hydrolysis leading to the formation of $O_2^{\cdot-}$ and $H_2O_2$ (324). Most importantly, ITCs lead, at least initially, to GSH depletion, via a reaction mediated by GST. Thereafter, ITCs accumulate rapidly and efficiently in cells to high levels due to their ability to react with GSH (thiocarbamoylation reaction) to form DTC (320). The depletion of GSH by ITCs might be marked and efficient and undoubtedly renders cells susceptible to stress-induced damage (311). Moreover, ITCs might increase the generation of ROI by causing mitochondrial damage. Recent evidence has suggested that the treatment of RL34 cells with ITCs resulted in inhibition of mitochondrial respiration, swelling of mitochondria and release of cyto c (191). Our data contrast

with those reported by Scapagnini *et al* who have claimed no role for GSH depletion or redox modulation in curcumin-mediated up-regulation of HO-1 (251).

Under our experimental conditions, BTCC and PTCC failed to increase HO-1 expression at high concentrations and after long incubation periods. This was associated with a decline in cell viability as observed in the Alamar Blue assay and a significant increase in LDH release indicating cell death by necrosis. In agreement with our results, Motterlini *et al* have shown that curcumin produced maximum HO-1 activation in endothelial cells at 15 μM concentration whereas at 30 μM it decreased cell viability and failed to stimulate further increase in HO-1 expression indicating a similar response (181). Furthermore, a similar pattern was demonstrated when Scapagnini *et al* studied the effect of CAPE and curcumin on HO-1 in astrocytes (251). Therefore, we would suggest a model in which at low concentrations, ITCs confer protection against oxidative stress by inducing cytoprotective enzymes whereas at high concentrations or after longer incubation periods, ITCs cause oxidative stress that overrides the anti-oxidant capacity and thus leads to cell death.

It is becoming increasingly apparent that the modulation of HO-1 occurs at the transcriptional level in which the interaction between Nrf2 and ARE/EpRE plays a pivotal role (6; 8). In searching for the downstream targets of BTCC and PTCC in HO-1 modulation, we tried to delineate the contribution of several signalling cascades including MAPKs, PI-3K and PKC pathways. The present investigation has shown that kinases of the ERK and p38 pathways are involved in the stimulation of HO-1

expression in LLC-PK1 cells in response to treatment with BTCC and PTCC. However, under our experimental conditions, the JNK pathway was not required by BTCC to stimulate HO-1 and similarly had no role in PTCC-mediated HO-1 induction. The role of MAPKs in HO-1 induction in response to various agents has been demonstrated in many studies and in various cells lines (9; 53; 318). By analogy with the data presented here, Chen *et al* have shown that NO induces HO-1 expression in HeLa cells by mechanisms that require the activation of the ERK and p38 pathways (53) whereas Balogun *et al* have reported that curcumin utilizes the p38 pathway to up-regulate HO-1 in LLC-PK1 cells (25). Other studies, however, have shown that either all MAPKs are required for HO-1 induction such as in lung I/R injury (318) or have a minor role in response to other inducers as in the case of rosolic acid and 2-hydroxychalcone (93). Our data have demonstrated also that inhibition of the PI-3K pathway completely blocked HO-1 stimulation in BTCC or PTCC treated cells. In line with our findings, several studies have emphasized the role of PI-3K pathway in HO-1 induction by different agents. Martin *et al* have indicated that in addition to MAPKs, PI-3K pathway played a significant role in HO-1 induction by the plant-derived compound carnosol (168). Although few agents utilize the PI-3K pathway only to exert their HO-1 stimulatory effect (297). In agreement with our observations, most HO-1 inducers have been reported to require the PI-3K in combination with the MAPKs pathways (116; 168).

In addition to the MAPKs and PI-3K, we report here that the PKC pathway is utilized for HO-1 up-regulation by PTCC only, indicating that the signalling pathways used

by these two HO-1 inducers are agent-specific although both belong to the same family. Few other studies have revealed the role of the PKC pathway in HO-1 induction (282; 309). Recently, Wung *et al* have provided evidence of the indispensability of PKC in HO-1 modulation by piceattanol (282).

We report here that various signal transduction pathways are switched on to mediate HO-1 induction by BTCC and PTCC. Collectively, there has been a common consensus that the cross-talk between different signalling pathways facilitates the translation of extracellular signals to intracellular responses. Based on the abovementioned data, we propose a model for the intracellular events that mediate HO-1 stimulation by BTCC and PTCC in LLC-PK1 cells. The exposure of LLC-PK1 cells to BTCC and PTCC increases cellular oxidative stress by depleting cellular GSH, damaging mitochondria and stimulating the release of ROI. These events activate ERK, p38, PI-3K and PKC pathways (in the case of PTCC) which in turn enhance the release Nrf2 from Keap1 facilitating its interaction with ARE/EpRE in the promoter region of HO-1 gene leading to an increase in its expression.

Induction of apoptosis is one of the mechanisms that underlie the cancer-preventive effects of ITCs. Since chemoprevention is considered one of the most strategic approaches to the prevention of cancer, ITCs with their anti-cancer properties and widespread availability have attracted particular attention recently. Apoptosis-stimulating potency of ITCs seems to correlate, at least in part, with their chemopreventive efficacy. For example, 6-phenylhexyl ITC, which was the most

potent inhibitor of lung tumours in F344 rats (61) was also the most potent apoptosis inducer in HeLa cells (316). On the contrary, phenyl ITC, which showed minimal anti-carcinogenic activity as compared to other ITCs, did not induce apoptosis (316).

In this study, we have investigated the apoptotic potential of BTCC and PTCC in LLC-PK1 cells and we have delineated the signalling pathways that mediate this effect. Our results indicated that these two ITCs stimulated concentration- and time-dependent apoptotic cell death in LLC-PK1 cells. Furthermore, we showed that at high concentrations or after long time of exposure these compounds induced necrotic cell death.

The mechanisms of ITCs-mediated apoptosis are still not fully elucidated but oxidative stress by depletion of intracellular GSH, mitochondrial damage and free radical generation may contribute to the subsequent events leading to apoptosis. In support of this concept, Kim *et al* have reported that sulforaphane caused GSH depletion in HepG2 cells and the authors hypothesised that this effect might be responsible for its pro-apoptotic effects (138).

In an effort to delineate the signalling transduction pathways activated during induction of apoptosis by these two ITCs, we showed that BTCC requires the JNK, the ERK and p38 MAPKs while PTCC activates the PKC pathway and to a much lesser extent p38 pathway to exert its pro-apoptotic effects.

Several reports have demonstrated the apoptotic properties of ITCs and showed that the signal transduction pathways that underlie this effect are cell and/or agent specific. Chen *et al* have demonstrated that the apoptotic potential of various ITCs requires the activation of JNK pathway (56). Nevertheless, Jakubikova *et el* have tested the different signalling pathways mediating apoptosis induced by different ITCs and have shown that PI-3K and JNK pathways were activated during induction of apoptosis in response to these agents (124).

Renal tubular cells (RTC) die by apoptosis and necrosis in response to various renal damaging stimuli such as drug-induced nephrotoxicity and I/R injury (136; 206). However, the relative contribution of apoptotic cell death to the development of renal failure is still under debate because apoptotic cells are rapidly cleared by phagocytosis which makes their quantification practically difficult.

CP is an extremely useful anti-neoplastic agent that is particularly effective for the treatment of solid tumours but its therapeutic activity is limited by its nephrotoxic effects that restrict increasing its dose to maximize its anti-cancer activity (159). The renal-damaging effects of CP have been attributed to its preferential uptake by proximal cells of the cortico-medullary region especially the S3 and S1 segments of the proximal tubule (147). Kronong *et al* have shown that the sensitivity of different parts of the nephron to the toxic effects of CP is different. Specifically, the cytotoxic effects of CP were most marked in S1 then S3 then the DCT cells (147).

CP causes apoptosis or necrosis in renal tubular cells depending on the concentration and duration of exposure but the underlying mechanisms that lead to apoptosis in response to CP are still controversial (159). Both death receptor and mitochondrial-mediated apoptotic pathways have been reported in response to CP. Razzaque *et al* have indicated the importance of death receptor stimulation in CP-induced apoptosis in renal tubular cells (227). However, Park *et al* have shown that CP caused apoptosis in renal cells via the mitochondrial pathway (211).

In agreement with previous reports (103; 159; 206), our data have indicated that exposure of LLC-PK1 cells to CP resulted in a time- and concentration-dependent apoptosis. However, at higher concentrations (>100 μM) CP failed to cause any further increase in caspase-3 activity indicating death by necrosis. In support of these findings, Liberthal *et al* have studied the effect of CP on renal cells and showed that at high concentrations (800 μM) necrotic cell death occurred over a few hours. However, at lower concentrations (8 μM) apoptosis occurred over few days (159).

The major objective of the current study was to investigate the hypothesis that modulation of HO-1 by ITCs would protect against drug-induced nephrotoxicity. Moreover, we wanted to verify which of the HO pathway products and by-products actually mediate this effect.

Oxidative stress plays an important pathophysiological role in CP-mediated renal dysfunction and several anti-oxidants have shown promise in protecting against CP-

mediated renal injury (81; 255). Durak *et al* have reported that CP administration caused a significant decrease in the anti-oxidant enzyme level such as SOD and catalase and an increase in MDA. Furthermore, treatment with anti-oxidants restored renal function and strengthened the renal anti-oxidant system (81).

Previous studies examined the potential protective effects of various phenols and plant-derived agents against CP-induced renal damage. Rao *et al* have indicated that various phenolic anti-oxidants caused a remarkable reduction in CP-mediated cytotoxicity and renal failure and emphasized the role of radical scavenging properties of these compounds in their reno-protective effects (225).

Increasing experimental evidence has suggested that HO-1 is induced in response to several renal damaging effects such as I/R injury and drug-induced nephrotoxicity (206; 260). Agarwal *et al* have shown that HO-1 is up-regulated in response to glycerol, gentamicin and CP administration indicating that the induction of this stress protein might play an important role in reducing renal injury (4). Shiraishi *et al* have demonstrated the importance of HO-1 induction in the protection against CP-mediated renal failure in HO-1 null mice and compared them to wild type mice (260). In this study, HO-1 gene ablation resulted in more severe renal failure in response to CP treatment. The reno-protective role of HO-1 was further emphasized when HO-1 induction by haemin in human renal proximal tubular cells significantly attenuated CP-induced apoptosis and necrosis, whereas inhibition of HO-1 enzyme activity reversed these protective effects.

In line with these observations, we report here for the first time that pre-incubation of LLC-PK1 cells with non-toxic HO-1 stimulating concentrations of BTCC and PTCC significantly decreased apoptosis in response to CP. Of great significance, this anti-apoptotic effect of BTCC and PTCC was completely abolished when SnPPIX was added to the culture medium of cells treated with these two ITCs prior to CP exposure indicating that this anti-apoptotic effect of BTCC and PTCC is mediated by their HO-1 stimulation. To further test this hypothesis *in vivo* we utilized a rat model of CP-induced AKI. In accord with previous reports (22; 255), the administration of a non-lethal dose of CP to rats resulted in overt renal failure as indicated by a significant increase in serum urea and creatinine. Moreover, CP administration stimulated extensive necrosis in the S3 segment of the proximal tubular cells as evidenced by the TUNEL technique. Once again, the administration of BTCC or PTCC to CP-treated rats caused a significant reduction in renal function and rendered renal tubular cells resistant to CP-mediated necrosis. The administration of SnPPIX to rats treated with BTCC or PTCC and CP resulted in a significant elevation of serum urea and creatinine but failed to increase necrosis. Notably, as previously demonstrated by Sheikh-Hamad *et al* (257), the extent of renal dysfunction in rats treated with CP correlated well with the degree of necrosis indicating a clear association between this mode of cell death and renal failure.

Although ITCs have strong anti-oxidant properties, according to our results it would be unlikely that the direct radical scavenging properties of ITCs were implicated in their reno-protective effects because their anti-apoptotic effect lasted for several hours

after removal of BTCC and PTCC from the culture medium. Alternatively, the anti-oxidant properties of HO-1 played a significant part in BTCC and PTCC reno-protective effects because inhibition of HO-1 in the presence of these two ITCs completely abolished their anti-apoptotic effect. This is in agreement with the findings by Schaaf *et al* who showed that HO-1 induction protected against CP-induced damage in LLC-PK1 cells by maintaining cellular redox balance (252).

Our *in vivo* data indicated that HO-1 inhibition failed to increase CP-mediated necrosis in kidneys of rats treated with CP. Given the various mechanisms that mediate CP-induced nephrotoxic effects that include ROI generation, stimulation of lipid peroxidation, mitochondrial injury and inflammatory reactions several mechanisms of protection by BTCC and PTCC seem possible such as inhibition of cyt P450 enzymes. In this context, Liu *et al* have shown that cyt P450 null mice exhibited exaggerated renal tubular cell apoptosis in response to CP indicating a role of these enzymes in CP-mediated damage (161).

Recently, the role of inflammation in response to CP has emerged as an important mediator of its nephrotoxic effects. Ramesh *et al* have highlighted the role of TNF-α in CP-mediated renal injury in a study which showed that TNFR2 null mice were resistant to CP-mediated renal damage and displayed reduced levels of renal TNF-α, ICAM-1 and serum TNF-α (223). On the other hand, Deng *et al* have demonstrated that the anti-inflammatory cytokine (IL-10) conferred reno-protective effects against I/R injury and CP-mediated renal failure by decreasing TNF-α and ICAM-1 (75).

More recently, Ramesh *et al* have demonstrated that salicylates protected against CP-mediated renal failure by decreasing the inflammatory response and inducing HO-1 (224).

ITCs have been reported to possess potent anti-inflammatory effects. Murakami *et al* have investigated the anti-inflammatory properties of benzyl ITC in LPS-activated murine Raw macrophages which revealed that this ITC effectively prevented the degradation of IkappaB which leads to TNF-α release (187). Therefore, we hypothesize that the inhibition of cyt P450 enzymes and the anti-inflammatory properties of ITCs could play a role in their protection against CP-mediated toxicity which could be the focus of future studies.

Three major pathways have been proposed to account for the anti-apoptotic effects of HO-1 which are decreased pro-oxidant level, increased BR level and increased CO generation. HO-1 decreases oxidative stress by degrading haem, over-expressing ferritin and stimulating an ATP-dependent $Fe^{2+}$ pump which exports $Fe^{2+}$ out of the cell. Since cellular oxidants are known to induce apoptosis, it has been thought that an HO-1 mediated decrease in oxidative stress might play a role in its anti-apoptotic effects. Baliga *et al* have demonstrated the important role of $Fe^{2+}$ released in CP-mediated renal failure. The authors have reported that treatment of LLC-PK1 cells with CP resulted in a significant increase in $Fe^{2+}$ content and $OH^{\cdot}$. Furthermore, they have demonstrated that treatment with $OH^{\cdot}$ scavengers and $Fe^{2+}$ chelators significantly alleviated CP-mediated cellular toxicity (22). Since HO-1 induction has

been shown in our study to exert reno-protective effects we propose that ferritin and the resultant $Fe^{2+}$ sequestration stimulated as a result might play a role in the HO-1 reno-protective effects.

Our next step was to examine the role of the different HO-1 products and by-products in its observed reno-protective and anti-apoptotic effects. Since many anti-oxidants have been demonstrated to alleviate CP-mediated renal toxicity, we wanted to verify if BR or BV with their anti-oxidant properties play a role in the HO-1-mediated alleviation of the apoptotic and nephrotoxic effects of CP. Our data have shown that BR and BV had a minor role in this regard because when the cells were incubated with these two HO-1 products in the presence of CP they failed to confer any anti-apoptotic effects. The data presented here are in contrast with the findings obtained by Tanaka *et al* who have shown that the anti-apoptotic properties of HO-1 in rat hepatoma cells were partially mediated by the anti-oxidant effects of BR (277). Therefore, we hypothesized that CO might play the major role in the HO-1 reno-protective and anti-apoptotic effects. Petrache *et al* have indicated that the induction of HO-1 resulted in reduction of apoptosis in murine L929 fibroblasts. Moreover, they confirmed that this effect was mediated by CO because the administration of this gas to the cells reproduced these anti-apoptotic effects (213). Furthermore, Brouard *et al* have demonstrated that HO-1 inhibits apoptosis in endothelial cells and that the generation of CO is an important mediator of its anti-apoptotic effect (42).

Our group produced several CO-RMs which contain transition metal carbonyl molecules that can carry and deliver CO in a controllable fashion in biological systems (180). The first group of chemically produced CO-RMs like CORM-1 and CORM-2 were lipophilic. However, our group succeeded in producing CORM-3 (tricarbonylcholro(glycinato)-ruthenium (II)) which is the first water-soluble CORM. This novel compound has been shown to liberate CO *in vitro*, *in vivo* and *ex vivo* (184). Since its introduction, several studies have been undertaken to examine the pharmacological and biological properties of CORM-3 which showed that this promising compound exerts protective effects against cardiac I/R injury (64), possesses potent anti-inflammatory (250) and vaso-active properties (92). Since analogous mechanisms are believed to contribute to renal tissue injury after I/R and CP treatment, it is possible that CO released from CORMs is able to activate similar pathways to protect the kidney against CP-mediated injury (202). Although these pathways were not examined in the current study, they are important areas to which future studies could be directed.

In this study, we aimed to verify whether the HO-1-mediated reno-protective effects elicited by BTCC and PTCC were related to CO release by using CORM-3 as a CO-donor. For this purpose, LLC-PK1 cells were exposed to CP in the presence of increasing concentrations of CORM-3. Interestingly, the cells showed exceptional resistance to CP-mediated apoptosis in a concentration-dependent manner. To verify whether this effect was due to CO release we exposed another group of cells to CP in the presence of iCORM-3, which is devoid of CO release, no protection was noticed.

Furthermore, we have investigated the potential protective effects of CORM-3 *in vivo* by utilizing a rat model of CP-induced renal failure. Administration of CORM-3 to rats normalized renal function, improved the histological picture of renal sections and remarkably alleviated CP-mediated necrotic cell death in the proximal tubular cells. However, iCORM-3 caused a cortical necrosis in the proximal cells and significantly reduced the extent of cortical damage caused by CP. This reno-protective effect of iCORM-3 could be related to the possibility that CO might have not been sufficiently released before use. However, why iCORM-3 resulted in a new distribution of CP-mediated damage could be explained by assuming that the loss of CO from iCORM-3, even if it was partial, resulted in the production of a new compound which could either directly or indirectly cause the damage.

In accordance with our finding that CO is the actual mediator of HO-1 reno-protective effects, Chow *et al* have shown that the anti-oxidant quercetin exerted protective effects against $H_2O_2$-mediated apoptosis in macrophages via mechanisms that involve HO-1 stimulation and CO was the only HO-1 product that reproduced these anti-apoptotic effects (60). More recently, Sandouka *et al* have examined the possible beneficial effects of CO liberated from CO-RMs on renal damage caused by cold storage as well as I/R in isolated perfused kidneys and found that CO released from CO-RMs protected kidneys against the damage inflicted by these two renal-damaging stimuli (247). In addition, Vera *et al* have shown that CORMs alleviated ischaemia-induced renal damage as evidenced by a remarkable improvement in renal function (292). Although in the current investigation we have revealed an important role for

the anti-apoptotic effects of CO in protecting renal cells against CP-mediated damage, other mechanisms such as anti-oxidative and anti-inflammatory properties of CO cannot be excluded.

Since it has been reported previously that CORM-3 stimulates HO-1 in renal cells, we wanted to examine the contribution of endogenously released CO as a result of HO-1 induction in the observed reno-protective effect of CORM-3. In agreement with these reports, we showed that administration of CORM-3 in the presence of SnPPIX partially increased renal function in response to CP administration but did not cause the renal function level to reach the values observed in the CP-treated only rats. Although our data have identified a possible role of HO-1 induction by CORM-3 and the consequent increase in endogenous CO release in the improved outcome of renal function following CP-induced renal failure, our results demonstrated that CO liberated from CORM-3 has a marked additive effect to the protection elicited by HO-1 activation and that CO-RMs could be used therapeutically as an adjuvant to HO-1 inducers for the prevention of drug-induced nephrotoxicity. These results are in contrast with those reported by Vera *et al* who have shown that although the administration of CORM-3 induced HO-1 expression this had no role in its reno-protective effects (292).

CO exerts vascular activities via cGMP-dependent mechanisms (92) and for its anti-inflammatory effects this gaseous molecule utilizes the p38 pathway (205). Little information is known, however, about the pathways that mediate the anti-apoptotic

effects of CO. Our data provided evidence that CO modulates apoptosis via mechanisms that involve cGMP signalling pathway because inhibition of GC completely abolished CORM-3 anti-apoptotic effects. Our findings, along with the observations of others, indicate that the signaling pathways utilized by CO to exert its anti-apoptotic effects might be cell specific.

Since apoptosis caused by CP is associated with an increased production of free radicals (81) we cannot exclude the possibility that the anti-apoptotic effects of CORM-3 may also involve the suppression of crucial pathways that trigger oxidative stress especially the mitochondrial electron transport chain. Recently, ROI generated during oxidative phosphorylation, especially $O_2^{\cdot-}$ , have been shown to stimulate a group of cellular uncoupling proteins (UCP1 and UCP2) which enhance the uncoupling process and decrease generation of ROI at the expense of a small decrease in ATP synthesis (35; 36).

The overall negative effect of CO on mitochondria has been emphasized in several reports. Rogatsky *et al* have shown that CO poisoning suppressed energy production in rat brain (234). Moreover, Alonso *et al* have studied the effects of CO on mitochondrial complexes in muscle tissues and reported that this gas specifically targets cyt c oxidase (complex IV) resulting in a significant decrease in its activity (10).

In the current study, we examined the effect of CORM-3 on mitochondrial function by exposing rat renal mitochondrial extracts to increasing concentrations of CORM-3. Our data have indicated that CORM-3 produced an overall negative effect on mitochondrial function by acting as an uncoupling agent. We report here that low concentrations of CORM-3 modulate mitochondrial oxidative phosphorylation. Specifically, CORM-3 caused significant decrease in RCI which was associated with a marked decrease in state 3. Moreover, exposure of the mitochondria to CORM-3 caused a significant increase in state 4 indicating that this CO carrier caused interruption of electron flow through the mitochondrial transport chain and acted as an uncoupling agent of oxidative phosphorylation. This effect was due to CO release since iCORM-3 had no effect on these mitochondrial indices in all experiments.

A reversible controlled modulation of respiratory coupling reduces ROI load in cells and might actually contribute to the cytoprotective effects of CO. Of great interest, it seems possible that CO might stimulate the anti-apoptotic effects that we have shown in a CP-model of acute renal failure through its anti-oxidant mechanisms that involve uncoupling of oxidative phosphorylation. In support of our data, Taille *et al* have shown that CORM-2 inhibits proliferation in airway smooth muscle cells by modulating mitochondrial oxidative phosphorylation. Moreover, these authors have demonstrated the involvement of increased mitochondria-derived ROI generation as NAC partially inhibited the effects of CORM-2 (274).

In conclusion, in this study we have provided evidence that BTCC and PTCC stimulate HO-1 expression in LLC-PK1 cells in a time- and concentration-dependent manner via mechanisms that involve the activation of MAPK, PI-3K and PKC pathways. These two ITCs, however, induce apoptosis in LLC-PK1 cells in a concentration-dependent manner an effect which requires the activation of MAPK and PKC. Our data indicate a role of HO-1 stimulation by BTCC and PTCC to protect renal cells against CP-mediated apoptosis. These data were reproduced in an *in vivo* model of CP-mediated renal failure in which the administration of BTCC or PTCC to rats significantly improved renal function and reduced the number of apoptotic cells. In searching for the actual mediators of this anti-apoptotic and reno-protective effect of HO, our data have indicated that while BR plays a minor role BV had no contribution to this effect. However, by using CORM-3 as a CO donor we showed that this protective HO-1 effect is actually mediated by CO in which cGMP was used as a signalling pathway. This data was reproduced *in vivo* in which the administration of CORM-3 to CP-treated rats normalized renal function, markedly improved histological picture and abolished apoptosis in renal tubular cells.

Future research projects would include studying other mechanisms used by ITCs and CORM-3 to protect against drug-induced nephrotoxicity. That is, in addition to the anti-apoptotic effects, the contribution of the anti-inflammatory effects of ITCs and CORM-3 which have been demonstrated in previous studies (192; 250) to this reno-protective effect could be examined. CP has been shown to induce TNF-α release and this has been related to its renal damaging effects (223). The effect of ITCs and

CORM-3 on TNF-α release in renal cells could be studied and the reno-protective effects of these compounds could be compared to their anti-inflammatory effects. Moreover, the involvement of other phase II drug-metabolising enzymes in the protective effect of ITCs could be tested. Particularly, how GST stimulation by these ITCs would contribute to this beneficial effect would be a good field of study. Given that there is a lot of controversy about the role of GSH in CP-mediated nephrotoxic affects, this could be an interesting area of research in the future. Although GSH has been shown to exert a protective effect against CP-mediated renal injury (77) while others showed that it actually enhances CP-renal damaging effects by converting it into a nephrotoxin in the proximal tubular cells (284). The role of GSH in the protection of ITCs and CORM-3 against CP-mediated renal failure could be tested by comparing the level of GSH in CP-, ITCs- or CORM-3- and CP-ITCs- or CP-CORM-3-treated cells or rats. Moreover, the effect of GSH could be further confirmed by inhibiting GSH in the treated groups by GSH inhibitor such as buthionine sulfoximine.

CP causes other side effects besides its nephrotoxic effects. Future research could be directed to the potential of ITCs and CORM-3 to protect against CP-mediated ototoxicity and neurotoxicity, another two important toxic effects of this anti-neoplastic agent. Moreover, the mechanisms of this protection could be studied especially by investigating the contribution of the anti-oxidant, anti-apoptotic and anti-inflammatory effects of these two classes of compounds.

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