THE DEVELOPMENT OF AN IN VITRO MODEL FOR STUDYING MECHANISMS OF NEPHROTOXICITY AS AN ALTERNATIVE FOR ANIMAL EXPERIMENTS

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Proefschrift
ter verkrijging van de graad van doctor
in de landbouw- en milieuwetenschappen
op gezag van de rector magnificus,
dr. H.C. van der Plas,
in het openbaar te verdedigen
op vrijdag 11 januari 1991
des namiddags te vier uur in de Aula
van de Landbouwuniversiteit te Wageningen.

STELLINGEN

I

De LLC-PK1 cellijn is niet in het bezit van een probenecide gevoelig para-amino-hippuurzuur-transportsysteem .

Dit proefschrift.

Rabito C.A. Am. J. Physiol., 250, F734-F743 (1986).

II

De afwezigheid van een probenecide-gevoelig organisch-aniontransportsysteem in de LLC-PK1 cellijn vormt in principe geen belemmering voor de bruikbaarheid van deze cellijn als modelsysteem voor het bepalen van niertoxische eigenschappen van glutathion- en cysteineconjugaten van haloalkenen.

Dit proefschrift.

III

De door Chu et al gerapporteerde sexeverschillen in morfologische veranderingen in de nier na blootstelling aan 1,2,4,5-tetrachloorbenzeen, kunnen zeer waarschijnlijk verklaard worden vanuit een "protein droplet nephropathy" zoals die optreedt bij bepaalde lager gechloreerde benzenen.

Chu I. et al, J. Toxicol. Environ. Health, 11, 663-677 (1983).

Bomhard E. et al, Arch. Toxicol., 61, 433-439 (1988).

Charbonneau M. et al, Toxicol. Appl. Pharmacol.,99, 122-132 (1989).

IV

Gezien de wijze waarop "protein droplet nephropathy" ontstaat, is inzicht hierin niet alleen van belang bij de beoordeling van sexeverschillen in niertoxische effecten, maar mogelijk ook bij de beoordeling van dergelijke verschillen met betrekking tot de biotransformatie van chemicaliën in de lever.

V

Er is aanleiding de normstelling ten aanzien van cadmium bij te stellen.

Buchet J.P. et al, The Lancet, 336, 699-702 (1990).

VI

Validering van alternatieven voor dierproeven in het toxicologisch onderzoek dient te geschieden op basis van mechanistisch inzicht in de werking van stoffen.

VII

Gebrek aan inzicht in werkingsmechanismen van stoffen vormt de belangrijkste belemmering voor een adequate extrapolatie van toxiciteitsproeven in het kader van de risicoevaluatie van stoffen ten aanzien van de gezondheid van mens en milieu.

VIII

In onze samenleving bestaan geen stelselmatige voorzieningen voor een adequate publieksvoorlichting over risico's van stoffen.

IX

De uitvoering van de wetgeving met betrekking tot de milieuverontreiniging in ons land stagneert omdat onvoldoende voortgang wordt geboekt bij het stellen van milieukwaliteitseisen via Algemene Maatregelen van Bestuur.

X

Het gebruik van de term "in vitro" als tegengesteld aan "in vivo", maakt cellen "in vitro" niet minder levend.

XI

Voor het behoud en herstel van vrede is inzicht in elkaars gevoeligheden van cruciaal belang.

XII

Bij het ontwerpen van een "eerste pagina" ten behoeve van het TELEFAX-verkeer is het drukken van de verzendkosten niet van doorslaggevende betekenis geweest.

XIII

Arbeidstijdverkorting levert banen op.

"Dit proefschrift"

Jos J.W.M. Mertens
The development of an in vitro model for studying mechanisms of nephrotoxicity as an alternative for animal experiments.
11 januari, 1991, Wageningen.

Voor mijn ouders

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VOORWOORD

Voor U ligt het tastbare resultaat van ruim vier jaar onderzoek binnen een project gefinancierd door het Ministerie van Onderwijs en Wetenschappen. Het onderzoek is voor het grootste deel uitgevoerd op de vakgroep Toxicologie van de Landbouwuniversiteit Wageningen. Bij de totstandkoming van dit proefschrift ben ik velen erkentelijk voor hun bijdrage en interesse. Zonder iemand te kort te willen doen, wil ik een aantal personen met name noemen, die van bijzondere betekenis zijn geweest.

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In het onderzoek beschreven in dit proefschrift is gebruik gemaakt van diverse modelstoffen, diverse conjugaten. De synthese daarvan is vaak moeilijk en arbeidsintensief. Mijn dank gaat dan ook uit naar Bert Spenkelink voor zijn steun. Ook Dr. Ben van Ommen van de afdeling Biologische Toxicologie van het TNO-CIVO te Zeist wil ik op deze plaats bedanken voor zijn hulpvolle ideeën bij de diverse syntheses. Van de afdeling Organische Chemie van de Landbouwuniversiteit wil ik met name Cees Theunis en Bep van Veldhuizen bedanken voor de hulp bij de identificatie van diverse modelstoffen.

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CONTENTS

PART I

INTRODUCTION

CHAPTER 1

Introduction

1.1 GENERAL INTRODUCTION

Presently in our society animal tests still form the main starting point for the assessment of the possible risk of chemicals with regard to human and animal health. Especially during the last three decades a series of more or less standardized whole-animal tests with a variety of laboratory animals has been devised under the auspices of agencies as OECD (Organization for Economic Cooperation and Development) and EEC (European Economic Community). These models are aimed at the identification of toxic properties of chemicals, both qualitative and quantitative, and form an obligatory element within the legal framework through which pesticides, drugs, food additives, and most other chemicals are registered. In most countries attempts are made continuously by toxicologists and other scientists to improve the predictive value and the cost-effectiveness of toxicity test systems. In this connection various initiatives have been taken to introduce in vitro models and some of them have indeed led to a succesful addition.

In the development of new and useful in vitro model systems a number of mutagenicity tests should be mentioned especially (for review: Hollstein et al, 1979). Examples of such biological systems using various bacteria, fungi, and mammalian cells are the Ames Salmonella/microsome test (Ames et al, 1975), Neurospora crassa tests (Brockman et al, 1984) , and mouse lymphoma L5178Y(TK $^{+/-}$ /TFT) assay (Clive et al, 1979). Early validation studies showed that these tests seemed to predict carcinogenic potential also, and therefore they were adopted by the chemical industry for screening purposes. Subsequently, they were incorporated as an integral part into requirements for screening mutagenic and carcinogenic potential as laid down in national legislations . The developments in the genetic toxicology have stimulated considerably the attempts to develop in vitro models which could replace whole-animal studies, throughout the different areas of toxicological and other biomedical research. National and international organizations as FRAME (Fund for the Replacement of Animals in Medicinal Experiments) and ERGATT (European Research Group for Alternatives in Toxicity Testing), specialized centers as the John Hopkins Center for Alternatives to Animal Testing have been founded, and even a journal on development and use of alternatives to animal testing, ATLA (Alternatives To Laboratory Animals) has been launched in 1973. In the mean time the increasing ethical concern about the use of animals in our society influenced and supported these initiatives .

Ever since the development of the Ames test and the growing awareness about its limited value in predicting carcinogenicity due to the complexity of mechanisms and organisms (Douglas et al, 1988; Ashby and Tennant, 1988), the search for alternatives to animal testing has followed two main strategies. First, initiatives have been taken to replace animal screening tests by in vitro screening tests. This

resulted in some interesting achievements. Several alternatives for the heavily criticized Draize rabbit's eye irritancy test, which was developed in the early 1940's, have been proposed (ECETOC, 1988), including the HET-CAM test (Hen's Egg Test- ChorioAllantoic Membrane)(Luepke and Kemper, 1986) and some very promising cell systems using BALB 3T3 cells (Bracher et al, 1987) and red blood cells (Pape et al, 1987). Although such in vitro tests are currently only used as prescreening tests, a real replacement of the Draize test seems possible once sufficient validation data have been obtained. Progress has also been made in the development of in vitro test systems for the assessment of teratogenicity. A large number of in vitro tests has been described using vertebrate and invertebrate embryo, organ, and cell cultures (for review see: Faustman, 1988). Several tests as MOT (Mouse Ovarian Tumor cells), HEPM (Human Emryonic Palatal Mesenchymal cells), and assays with primary cultures of rat embryo midbrain cells and limb bud cells have been proposed for screening purposes. As a substitute for the classical LD50 (dose which produces 50% mortality in a population of animals) test; batteries of cytotoxicity tests are being developed. Although some reasonable in vitro/in vivo correlations have been obtained between cytotoxicities to 3T3-L1 cells and mouse i.p. LD50 values (Clothier et al, 1987; Clothier et al, 1988), these tests will probably reflect no more than a rough estimation of a direct toxic potential. Even when acute in vitro toxicity tests may be useful in comparing related compounds, the unawareness about the mechanisms of toxicity will limit their predictive value. In the search for alternatives to animal testing, this limitation of most in vitro screening tests caused a gradual shift to a second strategy: one of studying mechanisms of toxicity and the factors which influence it, leading to the development of rational in vitro models. Following this strategy, the present thesis aims at contributing to this development.

Several cell systems are currently used and improved for studying mechanisms of toxicity (for review see: Stammati et al, 1981). In the target organ toxicity the liver has received most emphasis. This is understandable because of its crucial role in biotransformation of chemicals. Freshly isolated hepatocytes are widely and succesfully used for studying mechanisms of toxicity (Guillouzo and Guguen-Guillouzo, 1986). However, due to the short-life span of metabolic enzymes isolated hepatocytes can only be used for a short period of time. The maintenance of this biotransformation capacity limits the use of hepatic cell lines and primary cultures of hepatocytes. As a consequence, much effort is put in the abolishment of this most severe short-coming of hepatic culture and cell culture in general. A major break-through seems to be offered by the application of recombinant DNA technology (Phillips et al, 1987). Cell lines have been created with a stable activity of P450 isoenzymes (Doehmer et al, 1988; Dogra et al, 1990).

In view of the importance of the kidney it is surprising that to date relatively few efforts have been made to develop cell models for studying mechanisms of renal toxicity. This organ, more specifically the proximal tubule, has been chosen in this thesis as the subject for development of such cell models.

In the development of a cell model for nephrotoxicity, we have chosen to study mechanisms of nephrotoxic glutathione conjugates. These model compounds enable an investigation of the functional presence of specific enzyme and transport systems in the cell model. The possible role of these systems in nephrotoxicity of chemicals in general and of glutathione conjugates derived from haloalkenes and halobenzenes in particular, will be dealt with in this introductory chapter.

1.2 STRUCTURE AND FUNCTION OF THE KIDNEY, ASPECTS OF NEPHROTOXICITY

The kidneys are paired organs, highly dynamic and complex, whose normal functions are to regulate the composition and volume of our body fluids. Processes as filtration, reabsorption, excretion and metabolism, perform a crucial role in this homeostasis. The function of the kidney is a result of collaboration within a complex anatomical organization of numerous smaller units: the nephrons , broadly situated in cortex and medulla as indicated in figure 1.1. Each nephron consists of glomerulus, surrounded by a double-walled epithelial capsule (Bowman's capsule), proximal tubule, Henle's loop, and distal tubule. At the glomerulus the arterial blood is filtered into the proximal tubule. This, originally subdivided into pars convoluta and pars recta, can be subdivided ultrastructurally into three segments (Pfaller et al, 1985). The first segment (S1) comprises roughly half of the convoluted portion and changes gradually into the second segment (S2). The second segment includes the very beginning of the pars recta. The third segment (S3) represents the rest of the straight part. Through this long tubule formed by the proximal tubule and the remaining parts of the nephron the filtrate reaches the collecting tubule. Several nephrons deliver their fluid in a collecting tubule. These join each other into larger straight tubules: the papillary ducts of Bellini. As urine it reaches the bladder via the ureter.

From the point of filtration to where it leaves the kidney as urine, the filtrate undergoes drastic changes. The nephrons are supplied with an intricate network of capillaries, which provide a tight contact between the bloodstream and the epithelial cells lining the tubules. Water and substances that are useful for body metabolism are reabsorbed from the tubular lumen across the epithelium into the blood circulation, and waste products are excreted , both possibly accompanied with metabolism. In the end , less than 1% of the volume that is filtrated remains as urine.

The high blood flow of 25% of the resting cardiac output together with the specialized functions and the high metabolic activity, makes the kidney vulnerable to xenobiotics. Different parts of the kidney can be affected. Damage to the glomerulus has been reported for e.g. mercury, gold, and D-penicillamine (Weening, 1989). Cyclosporin A has presumably a main effect on the vascular system (Dieperink et al, 1989). Most frequently, the tubules (e.g. aminoglycosides (Walker and Duggin, 1988)) and/or the interstitium (e.g. lead (Goyer, 1989)) are involved. Of the nephrotoxic chemicals reported in the proceedings of the Third

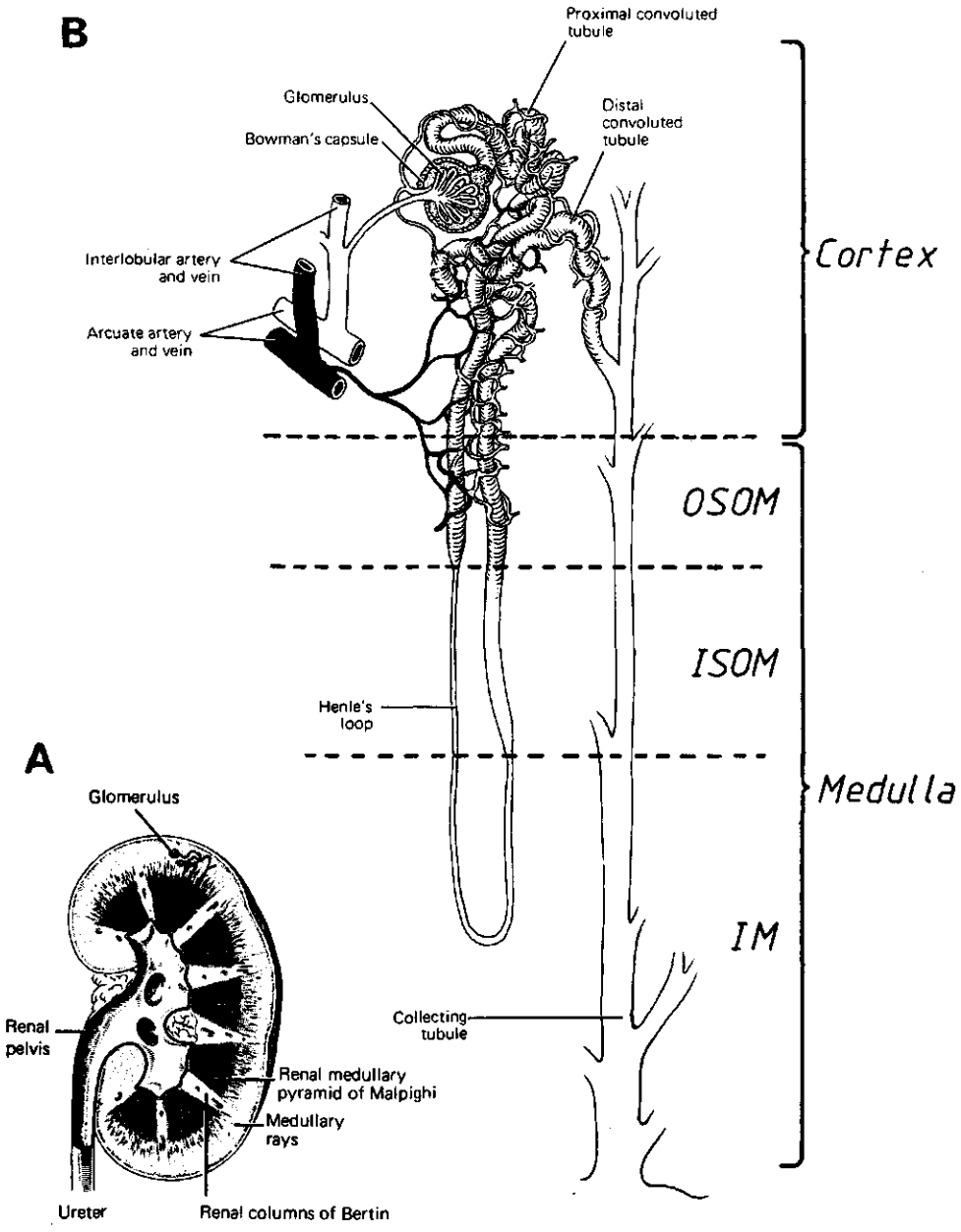

Figure 1.1 The general organization of a kidney (A), and a nephron with its vascular supply (B). IM, inner medulla; ISOM, inner stripe of the outer medulla; OSOM, outer stripe of the outer medulla.(Adapted from Junqueira et al, 1977 and Pfaller et al, 1985).

Table 1.1 A selection of chemicals with nephrotoxic properties which primarily affect the proximal tubule.

CHEMICAL	DOSE/SPECIES	SITE	REFERENCES
Acetaminophen	750 mg/kg s.c. male F344	PT(S3)	McMurtry et al,1978
Bromobenzene	4.85 mmol/kg i.p. male C57Bl/6J	PT	Reid, 1973
Cadmium chloride	10 ppm in water o. ad libitum, 24 wks male Wistar King A	PT(S3)	Nishizhumi, 1972
Cephaloridine	400 mg/kg s.c. guinea pig	PT	Tune and Fravert, 1980
Chlorobenzene	6.75 mmol/kg i.p. male C57Bl/6J	PT	Reid, 1973
Chlorotrifluoroethylene	220 ppm inh. 4 h. male F344	PT	Potter et al, 1981
1,2-Dibromo-3-chloropropane	40 mg/kg/day s.c. 4 days, male F344	PT(S3)	Kluwe. 1981
1,2-Dichlorobenzene	10 mmol/kg i.p. male SD/C57Bl/6J	PT	Reid, 1973
1,4-Dichlorobenzene	150 mg/kg/day o. 4 wks, male F344	PT	Bomhard et al, 1988
Dimethylnitrosamine	60 mg/kg i.p. female Wistar	PT(S2)	Hard et al, 1984
Gentamicin	15 mg/kg s.c. male + female Harlan rats	PT	Welles et al, 1973
Hexachlorobutadiene	15.6 mg/kg/day o. 12 wks female Wistar(TNO)	PT(S2,S3)	Harleman and Seinen, 1979
Hexafluoropopene	470 ppm inh. 4 h. male F344	PT	Potter et al, 1981
3,5-N-(Dichlorophenyl)succinimide	0.4 mmol/kg i.p. male F344/SD	PT	Yang et al, 1985
Lead acetate	2% lead acetate o. 10 wks, male + female Wistar	PT	Hirsch, 1973
Ochratoxin A	5 ppm in diet o. 90 days, male + female rats	PT	Munro et al, 1974
Pentachlorobenzene	71 mg/kg/day o. 28 days, male SD	PT	Chu et al, 1983
1,2,4,5-Tetrachlorobenzene	3.4 mg/kg/day o. 28 days, male SD	PT	Chu et al, 1983
Tetrafluoroethylene	6000 ppm inh. 6 h male Alderley Park	PT(S3)	Odum and Green, 1984
Tobramycin	15 mg/kg s.c. male + female Harlan rats	PT	Welles et al, 1973

F344, Fisher 344 rats ; inh., inhalation; i.p., intraperitoneal; o., oral; PT, proximal tubule; s.c., subcutaneous; SD, Sprague Dawley rats.

International Symposium on Nephrotoxicity , more than 70% affect the proximal tubule. A selection of such chemicals is presented in table 1.1. The reasons for the selectivity of the nephrotoxicity can be manifold. One of them is the presence of different transport systems and another is the metabolic activity in the kidney.

1.3 TUBULAR TRANSPORT SYSTEMS IN RELATION TO NEPHROTOXICITY

The physiological role of the proximal tubule is one of excretion and resorption, schematically represented in figure 1.2. Glucose, amino acids, proteins and about 85% of the sodium chloride and water are reabsorbed from the glomerular filtrate. Waste products which are not glomerularly filtrated are exreted through the tubule. As a consequence of this functional orientation, the presence of uptake mechanisms capable of transporting xenobiotics across the luminal or serosal membrane can be of importance for a possible nephrotoxic effect. Aminoglycosides as gentamycin, tobramycin, and amikacin, are glomerularly filtrated. At the proximal tubular brush border electrostatic binding can occur ensued by a concentration dependent pinocytotic uptake (Porter and Bennett, 1989). Recently a multidrug transporter, known as P-glycoprotein or P170, was found concentrated on the luminal membrane of the proximal tubule (Thiebaut et al, 1987). This transporter seems to play an important role in protecting cells by extruding xenobiotics as actinomycin D, vinblastine, and colchicine. Interference with this transporter might cause toxic effects (Gottesman and Pastan, 1988). Transport systems often mentioned in relationship with a proximal tubular nephrotoxicity are the organic anion and cation transporter.

Most thoroughly studied is the organic anion transporter. It has an extensive variation in substrates, but all have an organic backbone and a negative charge in

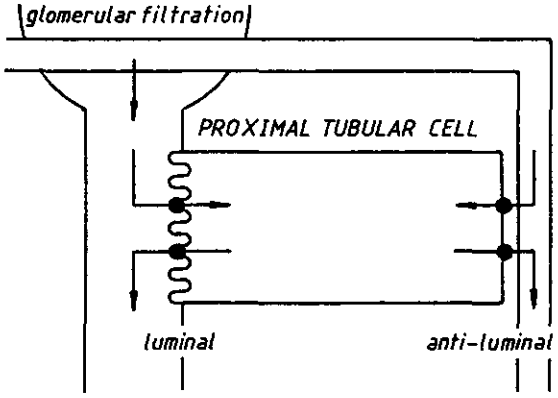

Figure 1.2 Schematic representation of excretion and resorption by the proximal tubular cell.

common (Møller and Sheikh, 1983; Pritchard, 1987; Ullrich et al, 1987a ; 1987b ; 1987c). Differences in patterns of inhibition of the transport of the different anions resulted in a subdivision in at least three transport systems termed as: sulfate, dicarboxylate and para-aminohippurate transport . However, this subdivision is relative; there is an overlapping specificity, and organic anions can be recognized by all three units (Fritzsch et al, 1989).

In general the organic anion transporter is typified by the transport of para-aminohippurate (PAH), a model organic anion. In the mammalian kidney this transport is situated in the proximal tubule. Which segment is the most active in the PAH transport depends on the species involved (Møller and Sheikh, 1983). PAH transport occurs in the serosal and the luminal membrane. At the serosal membrane organic anions are actively transported into the proximal tubular cell. The exact driving force is still under debate. The sodium dependence of the PAH transport suggested before appears to be indirect. Presumably , a (Na^+,K^+)-ATPase causes a sodium gradient across the serosal membrane. A dicarboxylate is cotransported with sodium into the cell, and is subsequently exchanged against PAH (Burckhardt and Ullrich, 1989) . At the luminal membrane PAH is transported outside the proximal tubular cell via diffusion, possibly carrier mediated . At both membranes probenecid is a competitive inhibitor of the PAH transport (Pritchard, 1987).

Compared to the organic anion transport, relatively little is known about the organic cation transporter. The overall direction is from serosal to luminal membrane. Early studies , using tetraethylammonium (TEA) and N-methylnicotinamide as model ions, suggested that organic cations are transported across the luminal as well as the serosal membrane (Rennick, 1981). However, it now appears that the active step is located at the luminal membrane and seems to be driven by countertransport with protons secondary to Na^+ ,H^+-exchange (Rennick, 1981; Wright, 1985; Rafizadeh et al, 1987). A potent inhibitor of TEA-transport is quinine (Rafizadeh et al, 1987).

Dependent on the rate of transport at the luminal and serosal membrane high intracellular concentrations of xenobiotics can arise. The group of cephalosporin antibiotics seems to be transported by organic anion transporters. Probenecid decreases their nephrotoxicity (Tune and Fravert, 1980).The most nephrotoxic representative of this group is cephaloridine. Unlike the other cephalosporins, cephaloridine is not able to cross the luminal membrane by diffusion. Due to the positive charge at the quaternary nitrogen of the pyridinium substituent , the limited luminal transport seems to be mediated by an organic cation transporter, as evidenced by an increase in nephrotoxicity by other cations (Wold et al, 1979). Organic anion transport also seems involved in the nephrotoxicity of cisplatin (Fillastre and Raguenez-Viotte, 1989), and mycotoxins like ochratoxin A and citrinin. Probenecid decreases the clearance of cisplatin (Caterson et al, 1983), the mortality of rats after exposure to citrinin, as well as citrinin's renal accumulation and urinary excretion (Berndt and Hayes, 1982). In in vitro studies probenecid inhibits the accumulation of radiolabeled citrinin in cortical slices

(Berndt, 1983). In the nephrotoxicity of ochratoxin A probenecid has contradictory effects: It decreases the clearance of ochratoxin A, but it increases its accumulation and nephrotoxicity (Stein et al, 1985).

One of the early indications that organic anion transport is also involved in the nephrotoxicity of haloalkenes was the observation that there were differences in organic ion accumulation in cortical slices of rats treated with hexachlorobutadiene (HCBD) compared to the controls. The organic anion transport, as measured by means of PAH accumulation, was lowered, whereas the organic cation transport, as measured by means of TEA accumulation, was not affected (Berndt and Mehendale, 1979; Hook et al, 1982). Later studies showed that prior administration of probenecid protects rats against the nephrotoxicity produced by HCBD (Lock and Ishmael, 1985).

In contrast to e.g. the cephalosporins, it appears that a metabolite of the haloalkenes is transported via an organic anion transporter . Together with the presence of transport systems, renal as well as extrarenal biotransformation thus is an important factor in targetting the nephrotoxic action of many xenobiotics to the proximal tubule.

1.4 BIOTRANSFORMATION IN RELATION TO NEPHROTOXICITY

The kidney is a metabolically active organ. Different xenobiotic-metabolizing enzymes are unevenly distributed within the kidney. Mixed-function oxidases exhibit a cortico-papillary gradient with the highest activity in the cortex (Zenser et al, 1978). In addition, the cytochrome P-450 present in the proximal tubule has several isoenzymes differentially distributed among the different cell types. In the inner medulla the prostaglandin endoperoxidase synthetase system has its highest activity (Christ and van Dorp, 1972).

Examples of xenobiotics which need renal metabolic activation before becoming nephrotoxic are dealt with in several reviews (Hook et al, 1979; Rush et al, 1984a; Walker and Duggin, 1988). Of those leading to toxicity in the proximal tubular epithelium, only haloalkenes and halobenzenes will be discussed below, since representatives of glutathione conjugates derived from these compounds are used in this thesis.

1.4.1 Haloalkenes

Haloalkenes appear as byproducts or are used as intermediates in the manufacture of plastics. They have been used as soil fumigants, are present in mixtures of pesticides, and are persistent environmental contaminants. Several haloalkenes are known nephrotoxins in experimental animals, and cause selective damage to the proximal tubule . Although chronic low-level exposure does occur in the work place, little information is available concerning a nephrotoxic risk for man, owing to the functional reserve of the kidney and the difficulties to relate a

possible nephrotoxic effect to exposure to one solvent (Bernard and Lauwerys, 1989).

Although effects of HCBD in experimental animals have been observed in convoluted as well as in straight limbs (Harleman and Seinen, 1979; Stott et al, 1981), the S3 segment is the most vulnerable part. The necrosis is often accompanied by numerous casts in the loop of Henle and distal tubule, and is visible as a distinct band of damage in the outer stripe of the outer medulla with some extension into the medullary rays (Hook et al, 1983). Selective damage of the S3 segment was also reported for chlorotrifluoroethylene (Potter et al, 1981) and tetrafluoroethylene (Odum and Green, 1984), whereas hexafluoropropene resulted in necrosis throughout pars recta and pars convoluta (Potter et al, 1981). The mechanism of this nephrotoxicity has been subject of investigations for several years. Although considerable progress has been made, demonstrating a complex cooperation of extra- and intrarenal metabolism (for reviews see: Lock, 1988; Anders et al, 1988), the exact reason for the site selectivity of the nephrotoxicity is still not clear.

Exposure of male and female rats to HCBD resulted in a marked depletion of non-protein sulfhydryl groups in the liver, whereas renal levels were influenced only in female rats (Lock and Ishmael, 1981; Hook et al, 1983). This suggested the formation of glutathione conjugates by glutathione S-transferases (GST) in the liver. Microsomal and cytosolic studies demonstrated the formation of such glutathione conjugates of HCBD (Wolf et al, 1984). Usually conjugation with glutathione is regarded as the first step in a detoxication patway (Fig. 1.3). After subsequent hydrolysis or transamination by γ-glutamyltranspeptidase of the glutathione conjugate, and removal of the glycine moiety by dipeptidases, a cysteine conjugate is formed. This is acetylated into a mercapturic acid and excreted via the urine. However, for several haloalkenes glutathione conjugation can result in an activation followed by nephrotoxicity.

Analysis of the bile of rats treated with HCBD, revealed the presence of both S-(1,2,3,4,4-pentachlorobutadienyl)glutathione (PCBD-GSH) and S-(1,2,3,4,4-pentachlorobutadienyl)cysteinylglycine (PCBD-CYSGLY). Biliary cannulation before administration of HCBD completely protected the rats from HCBD nephrotoxicity; however, dosing the rats orally with the bile caused the nephrotoxicity (Nash et al, 1984). So apparently an enterohepatic cycle is involved in the nephrotoxicity of haloalkenes.

The existence of an enterohepatic cycle enables the delivery of different metabolites of the mercapturic acid pathway to the kidney (Fig. 1.4). This aspect of xenobiotic metabolism has been reviewed previously (Inoue, 1985). Glutathione conjugates formed in the liver can be translocated to the caval perfusate as well as to the bile , dependent on the size of the molecule and the rate of glutathione conjugation (Wahlländer and Sies, 1979). At a low rate of synthesis, excretion in the bile is the predominant route . On their way to the intestinal lumen glutathione conjugates are metabolized into cysteinylglycine or cysteine conjugates. The responsible enzymes, γGT and dipeptidases, are present on canalicular (Inoue et

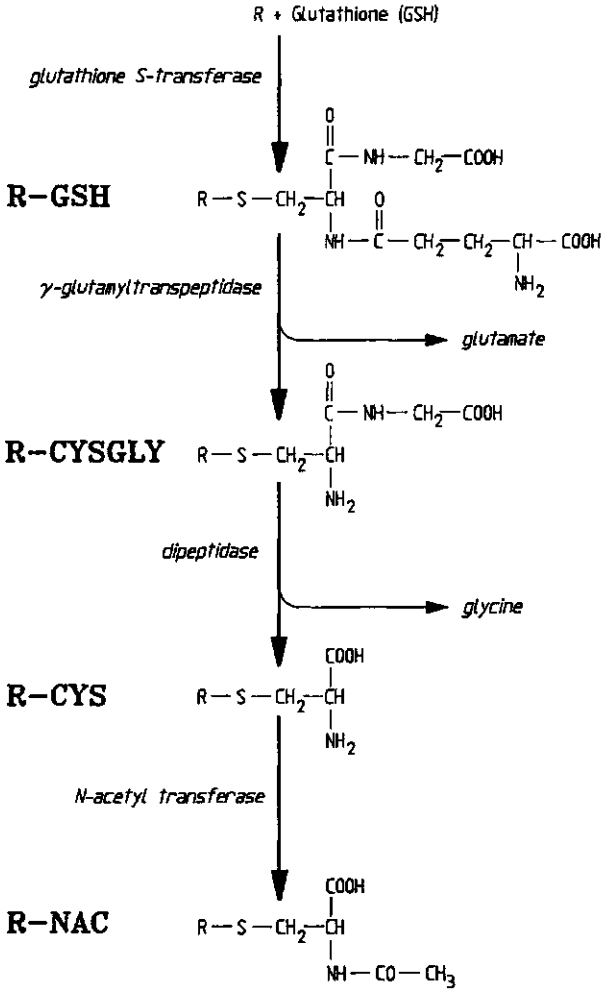

Figure 1.3 Detoxication of xenobiotics via the mercapturic acid pathway.

al, 1983) and ductal membranes, in the bile and pancreatic juice, as well as on the luminal membrane of small-intestinal mucosal cells (Grafström et al, 1980) . After reabsorption , the cysteine conjugates are transferred to the kidney, directly or after acetylation in the intestine or liver. As a consequence of this enterohepatic cooperation, glutathione, cysteine, and N-acetylcysteine conjugates may reach the kidney.

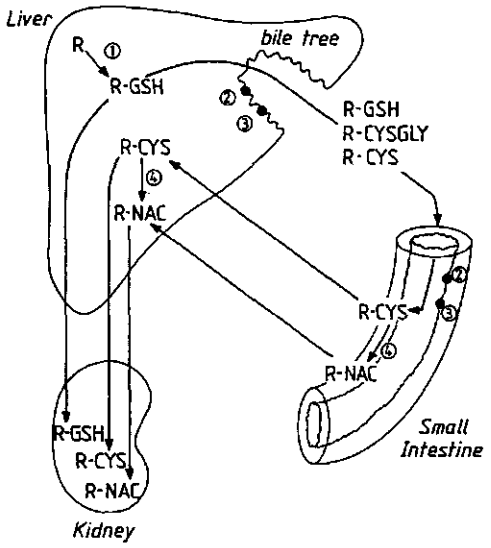

Figure 1.4 Enterohepatic cooperation in the biotransformation of xenobiotics leading to renal delivary of several conjugates. Enzymatic reactions are indicated: 1. Glutathione S-transferase, 2. γ-Glutamyltranspeptidase, 3. Dipeptidase, and 4. N-Acetyltransferase (Modified from Inoue, 1985).

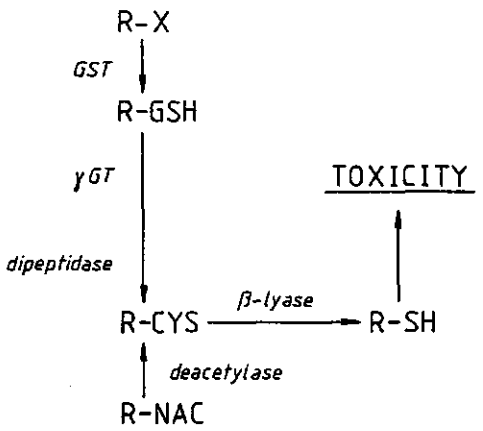

Figure 1.5 Activation via mercapturic acid-, and β-lyase pathway.

At present several glutathione conjugates of haloalkenes and their metabolites are known nephrotoxicants. A key enzyme in this nephrotoxicity is the pyridoxal phosphate-dependent β-lyase. Metabolism by this enzyme causes the generation of pyruvate , ammonia and a reactive thiol (Fig. 1.5) (Lock, 1988; Anders et al, 1988). The nature of the ultimate intermediate which is responsible for the cytotoxicity is still under investigation. Chlorofluorothionoacyl fluoride (Dekant et al, 1987) and difluorothionoacyl fluoride (Commandeur et al, 1989) have been suggested as reactive intermediates of S-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine and S-(1,1,2,2-tetrafluoroethyl)-L-cysteine respectively.

Before a cysteine conjugate can become nephrotoxic it has to be delivered to the interior of the renal cell. For this there are two possibilities. Either the cysteine conjugate becomes available to β-lyase after deacetylation of the N-acetyl-cysteine conjugate, or it is transported into the cell after being formed extra-cellularly by metabolism of the glutathione conjugate. The responsible enzymes, γGT and dipeptidases, are predominantly present on the luminal membrane of the proximal tubular cell (Tate and Maack, 1985). The nephrotoxicity of the N-acetyl-cysteine conjugate of HCBD and its accumulation in renal cortical slices can be inhibited by probenecid (Lock and Ishmael, 1985; Lock et al, 1986). Apparently a serosal organic anion transporter is responsible for the renal uptake. This transporter has also been suggested for the uptake of S-(1,2-dichlorovinyl)-L-cysteine (Lash and Anders, 1986).

1.4.2 Halobenzenes

Halobenzenes are used for various purposes, among them: dye carriers, pesticides, flame retardents, and intermediates in the manufacture of other chemicals. In contrast to several haloalkenes, e.g. HCBD, the toxic effects of halobenzenes are not limited to the kidney only. Effects on liver, lungs, and thyroid have also been reported (Reid, 1973; Chu et al, 1983). About the mechanism of nephrotoxicity of several halobenzenes less is known than about the mechanism of haloalkenes. GSH conjugates appear to be of importance. However, recently some halobenzenes have been reported to cause a nephrotoxicity, known as protein droplet nephropathy (PDN). Since an unawareness of this phenomenon might obscure the comparison of in vivo data of different halobenzenes, attention will be paid to PDN , before going into the mechanism which is subject of investigation in this thesis.

Protein Droplet Nephropathy

Several chemicals , among them halobenzenes, cause a nephrotoxicity known as Protein Droplet Nephropathy (PDN) (Table 1.2). It appears that this PDN is unique to the male rat. Kidneys of female rats, and of mice and dogs of both sexes are not affected. One of the reasons for this specificity is the presence of a protein : the α_{2u} -globulin.

The α_{2u} -globulin is a low molecular weight protein and was described by Neuhaus and Roy (for references see: Kanerva et al, 1987b) in the late sixties and

seventies. It is synthesized in the male rat by hepatic parenchymal cells, and transferred to the kidney. After glomerular filtration, α_{2u} -globulin is poorly reabsorbed. Once absorbed α_{2u} -globulin can accumulate in the proximal tubular (S2) epithelium, due to poor hydrolysation. It is visible as hyaline droplets. Repeated testosterone injections can induce the formation of hyaline droplets also in ovarioectomized female rats (Kanerva et al, 1987b). The physiological function of α_{2u}-globulin is presumably that of a pheromone carrier.

The pathology of the nephrotoxicity after exposure to the chemicals summarized in table 1.2 , is similar. The diameter of the hyaline droplets in the proximal tubular epithelium increases and the inclusions become more angular and crystalloid (Stone et al, 1987). At the junction of the inner and outer bands of the outer zone of the medulla granular casts appear , followed by nephron obstruction and chronic nephrosis (Kanerva et al, 1987a). From studies by Lock and Charbonneau it seems that the α_{2u} -globulin functions as a carrier and targets the xenobiotic or a metabolite to the proximal tubule. After dosing the rats with trimethylpentane the hyaline droplets consisted of a reversibly bound complex of α_{2u} -globulin and 2,4,4-trimethyl-2-pentanol (Charbonneau et al, 1987; Lock et

Table 1.2 Chemicals or mixtures of chemicals that induce a Protein Droplet Nephropathy specific for the male rat.

CHEMICALS	REFERENCES
60 Solvent	Carpenter et al, 1975a
70 Solvent	Murty et al, 1988
t-Butylcyclohexane	Henningsen et al, 1987
C_{10} -C_{11} Isoparaffinic solvents	Phillips and Egan, 1984
Decalin	Kanerva et al, 1987a
p-Dichlorobenzene	Bomhard et al, 1988
N,N-Diethyl-m-toluamide	Bomhard et al, 1989
Diisobutyl ketone	Murty et al, 1988
Isophorone	Murty et al, 1988
Jet fuels (RJ-5, JP-4, JP-5, JP-7, JP-10)	Parker et al, 1981; Murty et al, 1988
d-Limonene	Kanerva and Alden, 1987
Methyl isobutyl ketone	Murty et al, 1988
Naphtas (various)	Murty et al, 1988
Pentachloroethane	Goldsworthy et al, 1988
Perchloroethylene	Goldsworthy et al, 1988
Stoddard Solvent	Carpenter et al, 1975b
Tetralin	Murty et al, 1988
1,3,5-Trichlorobenzene	Bomhard et al, 1989
2,2,4-Trimethylpentane	Stonard et al, 1986
o-Xylene	Bomhard et al, 1989
Unleaded gasoline	Olson et al, 1987

al, 1987). Probably, this binding causes a structural alteration of the protein, which influences its reabsorption and degradation.

Recently Olson et al (1990) made a comparison between α_{2u}-globulin and human urinary proteins. Although traces of proteins similar to α_{2u}-globulin (α_1-acid glycoprotein, α_1-microglobulin) are present in human urine, the very low urinary protein content, the relatively low amount of cationic proteins, and the high molecular weight of the most abundant urinary proteins , make this type of nephrotoxicity of no relevance for man.

Of the halobenzenes, only chlorinated benzenes up to three chlorine atoms have been studied for their ability to induce PDN, and only *p*-dichlorobenzene and 1,3,5-trichlorobenzene were positive (Bomhard et al, 1988; 1989) In this light , the male rat-specific nephrotoxicity reported for 1,2,4,5-tetrachlorobenzene, pentachlorobenzene and hexachlorobenzene (Chu et al, 1983; 1984) has to be regarded with some prudence.

Glutathione conjugates

Of the compounds which have been shown to be negative in the ability of inducing PDN, bromobenzene, chlorobenzene , and o-dichlorobenzene (Cameron et al 1937; Reid, 1973; Rush et al, 1984b) cause an extensive necrosis of the proximal convoluted renal tubules. Covalent binding in the rat kidney 6 h after dosage of 1 mmol/kg [^{14}C]-chlorobenzene was 4 fold higher than after an equal dose of [^{14}C]-bromobenzene. In addition, the covalent binding as a result of 0.5 mmol/kg [^{14}C]-o-dichlorobenzene was comparable with that of the labeled bromobenzene. After 24 h covalent binding was increased with 20% when bromobenzene was used, whereas covalent binding after exposure to chlorobenzene and o-dichlorobenzene decreased with 24% and 50% respectively. Inhibition of metabolism by piperonyl butoxide , an inhibitor of cytochrome P-450, prevented the nephrotoxicity, whereas phenobarbital mediated induction had the opposite effect (Reid, 1973). These studies indicated that a metabolite formed by cytochrome P-450 (presumably in the liver) is responsible for the nephrotoxic effect. The nature of this metabolite remained unclear for a long time . Sipes et al (1974) suggested biliary excretion of a bromobenzene glutathione conjugate followed by intestinal reabsorption. However, the proposed structure was not of importance for the nephrotoxicity. It was the metabolite o-bromophenol that reproduced the same nephrotoxicity even at a five fold lower dose (Lau et al, 1984a). Liver microsomal incubations with bromobenzene and o-bromophenol demonstrated the formation of a bromohydroquinone. In addition, this was detected as a conjugate in the urine of rats treated with bromobenzene or o-bromophenol. Administration of bromohydroquinone (i.p.) to rats caused an identical nephrotoxicity as after o-bromophenol or bromobenzene exposure; however, the dose required was less than 10% of that of bromobenzene (Lau et al, 1984b). Liver microsomal incubations with bromohydroquinone resulted in the formation of several mono- and disubstituted glutathione conjugates, and two of them were identified as 2-bromo-3-(glutathion-S-yl)hydroquinone (2-Br-3-(GSyl)-

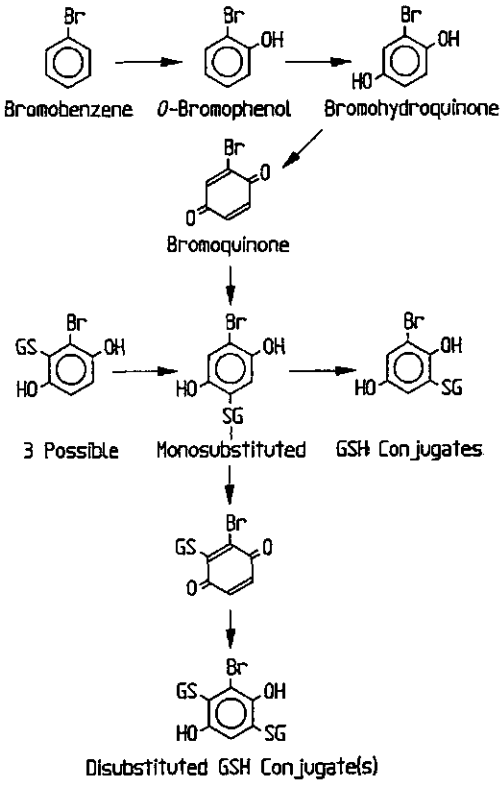

Figure 1.6 Proposed pathway of bromobenzene metabolism to nephrotoxic glutathione conjugates. (Adapted from Monks et al, 1985).

HQ) and 2-bromo-(diglutathion-S-yl)hydroquinone (2-Br-(diGSyl)HQ (Monks et al, 1985). Figure 1.6 summarizes the results of these studies in a postulated pathway of bromobenzene metabolism responsible for nephrotoxicity. Administration (i.v.) of glutathione conjugated (bromo)hydroquinones caused the same nephrotoxicity as after dosing bromobenzene (Monks et al, 1985; Monks et al, 1988a; Lau et al, 1988a). The exact mechanism of nephrotoxicity of glutathione conjugated halohydroquinones is not fully understood. Several in vivo experiments indicate that, similar to glutathione conjugates of haloalkenes , γGT is an important factor. Pretreatment of rats with acivicin (AT-125), an irreversible inhibitor of γGT (Allen et al, 1980; Schasteen et al, 1983) , can partly inhibit the nephrotoxicity of bromohydroquinone (Monks et al, 1985), 2-Br-(diGSyl)HQ (Monks et al, 1988a), and (tri-glutathion-S-yl)hydroquinone (triGSyl)HQ (Lau et al, 1988a). Also in vitro experiments using rat renal slices suggested a role for γGT (Lau et al, 1988b).

A role for β-lyase in the nephrotoxicity of glutathione conjugated

hydroquinones seems to be absent. In vivo experiments showed only minor or no effects of inhibition of β-lyase by aminooxyacetic acid on the nephrotoxicity of 2-Br-(diGSyl)HQ (Monks et al, 1988a) and (triGSyl)HQ (Lau et al, 1988a). Oxidation of the hydroquinone into the corresponding quinone is a more likely mechanism of nephrotoxicity, since thiophenols lacking the hydroquinone moiety cause no nephrotoxicity (Monks et al, 1988b).

1.5 SCOPE OF THE THESIS

A usefull cell model for studying mechanisms of toxicity requires the presence in vitro of characteristics which may play a role in this toxicity in vivo, and the possibility of demonstrating their involvement. From the foregoing it will be clear that nephrotoxic chemicals mostly affect the proximal tubule, and that transport- and metabolic enzyme systems play an important role.

In chapter 2, monolayers of LLC-PK1 - and of rat renal cortical cells , which are used in this thesis as in vitro model systems for the proximal tubule, are described. Since the mechanism of nephrotoxicity of HCBD is relatively well known, the glutathione conjugate of HCBD and its derivatives are used in part II as model compounds to investigate the usefulness of both in vitro systems for studying mechanisms of nephrotoxic glutathione conjugates. In chapter 3 and 4 this is done for the LLC-PK1 cell line, and in chapter 5 the primary cultures of rat renal cortical cells are dealt with.

In part III glutathione conjugates derived from halogenated hydroquinones are subject of investigation. In a first attempt to validate the in vitro test system and to see whether the mechanism of nephrotoxicity of glutathione conjugated bromohydroquinones is more generally valid, other halogenated (GSyl)hydroquinones (dichloro-(GSyl)hydroquinone and trichloro-(GSyl)hydroquinone) were studied in vivo (Chapter 6). The information obtained in part II was used in the decision which cell system to use in investigating the role of γGT in the toxicity of glutathione conjugates derived from halogenated hydroquinones (Chapter 7).

Finally, in part IV, a summary and concluding remarks are given in both English (chapter 8) and Dutch (chapter 9).

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CHAPTER 2

Monolayers of renal cortical cells as an in vitro model for proximal tubular epithelium

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Bruggeman I.M., Mertens J.J.W.M., Temmink J.H.M., Lans M.C., Vos R.M.E., and van Bladeren P.J. *(1989) Toxic. in Vitro, 3, 262-269.*

Monolayers of renal cortical cells as an in vitro model for proximal tubular epithelium

2.1 INTRODUCTION

Several in vitro models (e.g.: membrane vesicles, slices, isolated tubules and cells) , have been used to investigate the physiology of the kidney. Some of them have also been used in studying mechanisms of nephrotoxicity . For studying initial interactions and mechanisms at the cell membrane, isolated membrane vesicles are very useful (Ishikawa et al, 1985; Inui et al, 1988). However, several experimental difficulties can occur: problems involving cell fractionation, alterations in membrane structure, interactions with other cellular components during isolation, and lack of vesicle homogeneity. An advantage of the use of slices often referred to is that the histological organization of the kidney stays intact and as a consequence the targetting of the toxicity can be studied . Although over the years the technique of preparing slices has significantly improved (Deutsch, 1936; Kacew and Hirsch, 1981; Ruegg et al, 1987) some of the disadvantages Chahwala and Harpur (1986) summarized , may still occur. Most of the cells are not directly exposed to the suspending medium, and the immediate environment of many cells is affected by the rates of diffusion of substances through the extracellular space of the slice. Many intracellular binding sites are exposed on the cut surfaces of the slice. Medium can penetrate only difficultly into the tubular lumen. The latter can also be a problem when isolated tubules are used. In addition, isolated tubules have only a limited lifespan (1-6 h). Several methods of isolating renal tubules have been used (Schlondorff, 1986). The most widely used methods are based on enzymatic digestion of the tissue which might cause cellular damage. This counts also for suspensions of cells , either freshly isolated from a kidney or of cell lines . However, culturing these cells gives them time to recover from trauma and minor damage as a result of the isolation procedure. Their use has been subject of several reviews (Handler et al, 1980; Handler, 1983; Wilson, 1986; Sakhrani and Fine, 1983; Rabito, 1986).

The structure and function of the kidney in general and of the proximal tubule in particular, requires that an in vitro model should allow to pay attention to the fact that in vivo luminal as well as serosal exposure of the proximal tubular cell can occur. Using monolayers of renal cortical cells offers a possibility to approximate this in vivo situation as much as possible by culturing them on porous membranes. Although this technique has been used before in more physiological studies, it has not been used in renal toxicological studies. In the present investigations monolayers of LLC-PK1- and rat renal cortical cells have been used. The cells were cultured either conventionally on a solid support, or on a porous support so that apical (luminal) and basolateral (serosal) membranes are accessible.

2.2 MONOLAYERS OF LLC-PK1 CELLS

Very few kidney cell lines are of a proximal tubular origin. Sofar the LLC-PK1 cell line is the best characterized. It originated in 1958 and was derived from a juvenile male Hampshire pig (Hull et al, 1976). In culture the cells maintain their typical epithelial pattern and have numerous microvilli. When a culture reaches confluency , domes arise (Fig.2.1A). At those places the monolayer detaches from the substrate, and fluid accumulates between the cell layer and the substrate. Formation of these domes, enabled by junctional complexes (Fig.2.1B), was the first indication of the presence of transport systems in this cell line. Although some characteristics can be associated with other parts of the nephron, most characteristics indicate that the probable site of origin is the proximale tubule (Table 2.1). The reason of these functional dissociations is not known. Its possible that as a result of the repeated culturing cells may express or lose several functions. However, a more likely explanation is that the LLC-PK1 cell line exists of several cell types. Wohlwend et al (1986) demonstrated such functional an morphological heterogeneity. Cells different in their response to calcitonin and in their ability to form domes, were cloned into three subpopulations. Another clone (LLC-PK1A) shows a 250% increase in sodiumhydrogen exchange activity (Viniegra and Rabito, 1988).

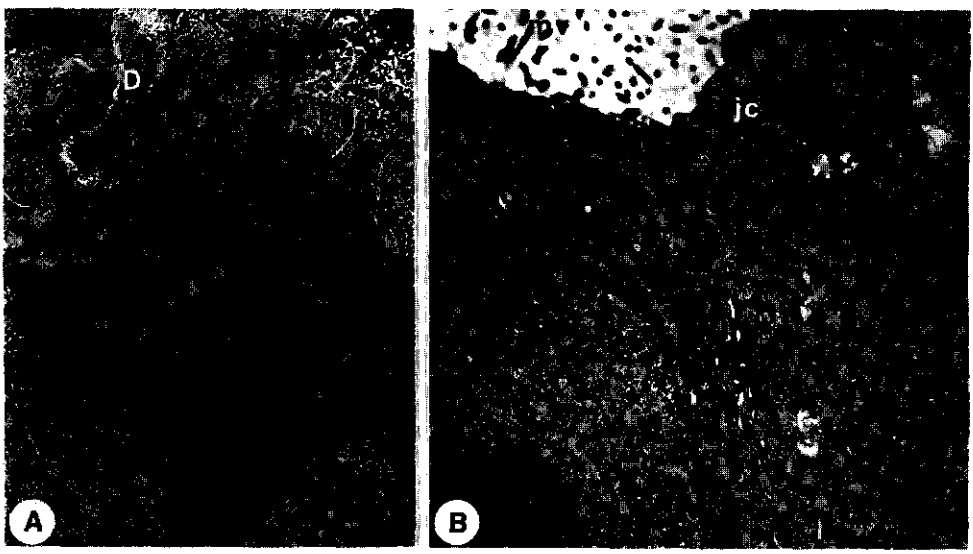


Figure 2.1 Scanning (A) and transmission (B) electron micrograph of LLC-PK1 monolayers. *Reaching confluency LLC-PK1 monolayers form domes (D) enabled by apical junctional complexes* (jc). Note the numerous microvilli (mv) . (A, x 1520; B, x 6,300).

Table 2.1 Processes and enzymes identified in the LLC-PK1 cell line.

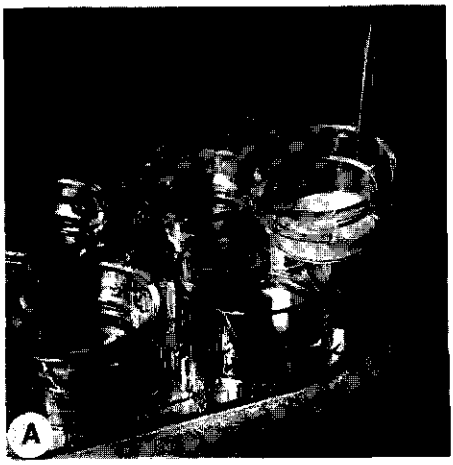
CHARACTERISTIC	KIDNEY LOCATION	REFERENCES
Alkaline phosphatase	PT	Gstraunthaler et al, 1985
Amino acid transport System A	PT and DT	Rabito and Karish, 1982
Amino acid transport System ASC	PT and DT	Rabito and Karish, 1982 Sepúlveda and Pearson, 1982
Amino acid transport System L	PT and DT	Rabito and Karish, 1982 Sepúlveda and Pearson, 1982
cAMP respons to calcitonin and vasopressin	MTAL	Golding et al, 1978 Sakhrani and Fine,1983
Calcium transport	PT	Parys et al, 1986
1,25-Dihydroxyvitamin D3 receptor mediated 24-hydroxylase activity	PT	Colston and Feldman,1982
Electrical resistance 200-400 $\Omega.cm^2$	DT	Sakhrani and Fine,1983
γ-Glutamyltranspeptidase	PT	Perantoni and Berman,1979
Leucine aminopeptidase	PT	Perantoni and Berman,1979
α-Methyl-D-glucoside transport	PT	Mullin et al, 1980 Rabito and Ausiello,1980
Oxidative 11β-hydroxysteroid dehydrogenase	PT	Korbmacher et al, 1989
Phosphate transport	PT	Rabito, 1983 Biber et al, 1983
Sodium-hydrogen antiport	PT	Moran et al, 1986

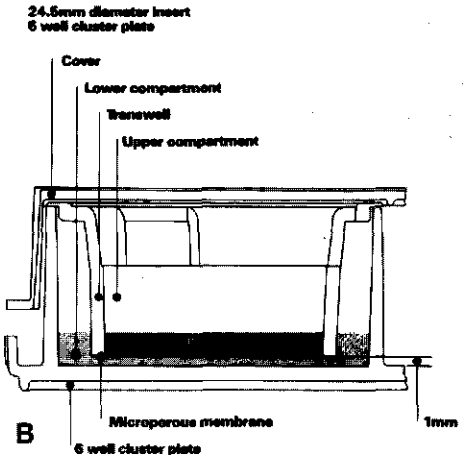
DT, Distal tubule; MTAL, Medullary Thick Ascending Limb; PT, Proximal Tubule

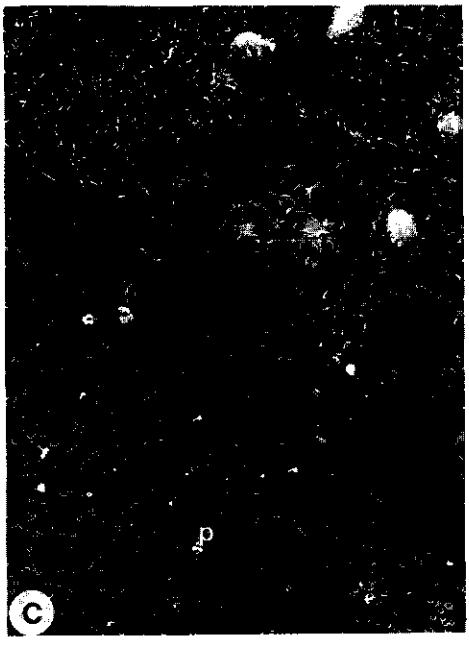
LLC-PK1 cells were cultured in Williams Medium E supplemented with 10% fetal calf serum (GIBCO, Glasgow, Scotland), penicillin (50 IU/ml, Gist-Brocades, Delft, The Netherlands) , and streptomycin (100 mg/l, Gist-Brocades) in culture flasks (Costar, Badhoevedorp, The Netherlands) at 37 ^{o} C in a humidified atmosphere containing 5% CO_2 . Stock cultures, seeded at 0.8-1.0x10 ^{4} cells/cm ^{2} , were subcultured every 3-4 days by trypsinization with 0.25% trypsin (Difco, Detroit, MI) and 0.05% EDTA (Merck, Darmstadt, FRG). LLC-PK1 cells turned out to be rather insensitive to trypsinization. The best results were obtained when cells were subcultured before the stage the domes had appeared. Cells were rinsed with Hanks' Balanced Salt Solution without Ca^{2+} and Mg^{2+} (HBSS), and 4 ml of the trypsinizing solution was added to a flask of 75 cm ^{2} . The trypsinization was followed microscopically at room temperature. Before the cells became detached the trypsinizing solution was gently removed , and subsequently the cells were incubated at 37 ^{o} C until all cells were detached. Experiments were performed with cells at passage 193-220.

Experiments where only apical exposure was of importance were conducted with monolayers in 24-well tissue culture plates (Costar Mark II) . On day 0, cells were seeded at 1.5x10 ^{5} cells/well, and on day 3 the monolayers were ready for use. At this time numerous domes had formed.

To enable seperate exposure from either apical or basolateral side, monolayers were cultured on porous supports of tissue culture transwell inserts







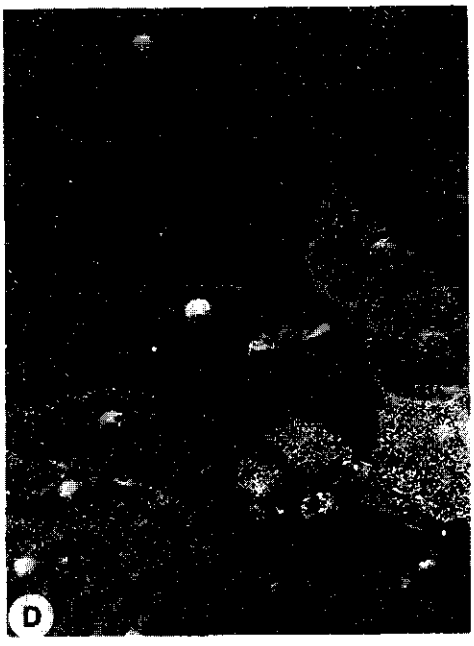


Figure 2.2 LLC-PK1 cells cultured on porous membranes of transwell inserts. The porous membrane of the transwell (A,B) is the only possibility for exchange between the apical (upper) and basolateral (lower) compartment. LLC-PK1 cells were seeded on day 0 at 0.8×10^{6} cells/insert. After 1 day (C) a few places were not fully covered and pores (p) are visible. Within 3 days (D) a confluent monolayer had formed. Microvilli are especially visible on cells not fully expanded. (C, x 1520; D, x 800).

(Costar). Cells were seeded on day 0 at 0.8-1.0x10^6 cells/insert. Already within three days the monolayers were confluent (Fig. 2.2) (Mertens et al, 1989).

2.3 MONOLAYERS OF RAT RENAL CORTICAL CELLS

Renal cortical cells were isolated according to a modified method of Jones et al (1979) and cultured according to Bruggeman et al (1989) . Female Wistar rats (170-190 g, Centre for Laboratory Animals of the Agricultural Univerity Wageningen) were injected i.p. with sodium pentobarbital and heparin (6 mg/100g and 250 IU/100g, respectively). The abdomen was opened and the aorta and vena cave were separated from the surrounding tissue caudal and rostral of the renal arteries and veins. An arterial clamp was placed on the aorta rostral of the renal arteries. A catheter (I.D. 0.95x51 mm; Terumo Corporation, Tokyo) was inserted into the aorta, caudal from the renal arteries, and secured with a Buldog vascular clamp. The kidneys were perfused in situ with a Hanks' Balanced Salt Solution without Ca^{2+} and Mg^{2+} (HBSS), containing 20 mM HEPES and 0.8 mM EDTA (flow-rate 10 ml/min, Gilson Minipuls 2). The buffer was oxygenated (95% O_2 , 5% CO_2) and kept at 37 ^{o} C. A vein in the abdomen was cut. Within 1 min from the start of the perfusion the kidneys started to become pale. Perfusion was continued for 10-20 min.

Kidneys were excised following perfusion, decapsulated, and the papillae were removed. The cortex was minced into 1-2 mm pieces and incubated in 0.125% trypsin- 0.05% EDTA in HBSS at 37 ^{o} C in a gently shaking water bath for 20-30 min. At 5 min intervals pieces were drawn through a 10-ml pipette, to help to dissociate the cells. The suspension was centrifuged (400 g, 3 min) and the pellet was resuspended in a solution of 12.5 mg collagenase (Clostridium histolyticum; Boeringer, Mannheim, FRG), 0.8 mg DNase (Grade II; Boehringer, Mannheim) , and 31.5 mg $CaCl_2$ in 50 ml of HBSS and placed in a gently shaking water bath for 10-30 min. At 5-min intervals the suspension was drawn through a 5-ml pipette. The suspension was centrifuged (400 g, 3 min) and the cells were resuspended in 80 ml of Williams Medium E supplemented with 10% fetal calf serum , penicillin (50 IU/ml), and streptomycin (100 µg/ml). The cells were counted in a haemocytometer and cell viability was determined by trypan blue exclusion. The isolation yielded a suspension of 6-10x10^6 single cells per kidney with a viability of more than 85%. In addition, cell clumps and some small tubule fragments were present in the suspension. Of this suspension 0.5 ml /well was plated in a 24-well tissue culture plate or 2 ml/insert in a tissue culture transwell plate. These concentrations were optimal for obtaining a confluent monolayer within 3-5 days (Fig. 2.3A and 2.4). Colonies mostly derived from attached cell clumps. This was also observed in other studies (Kedinger et al, 1987; Yang et al, 1987). Most of the cells in the culture were identified as proximal tubular cells . Histochemical detection of γ-glutamyltranspeptidase (Rutenburg et al, 1969)

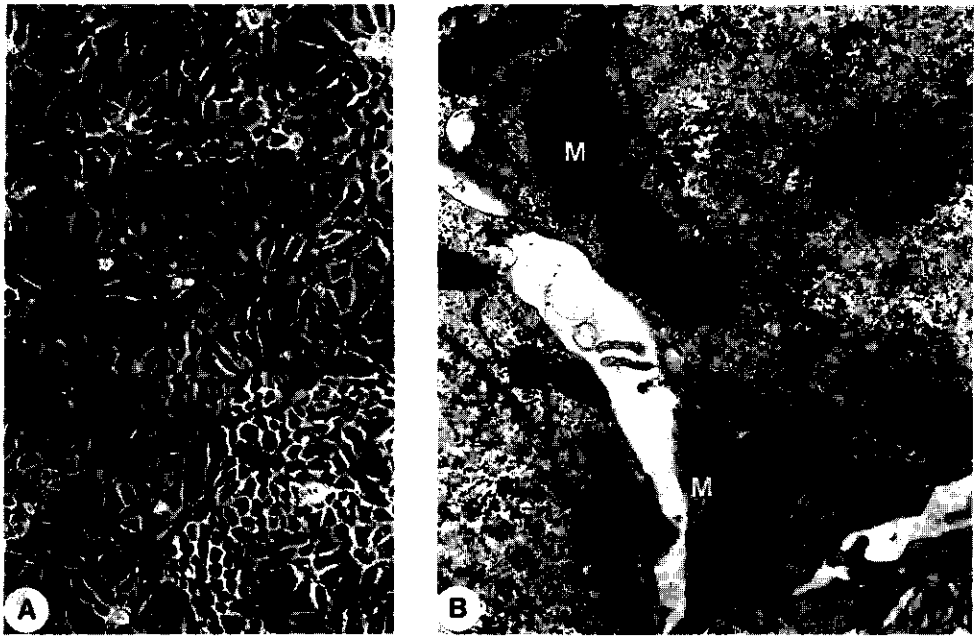


Figure 2.3 Primary culture of rat renal cortical cells. A phase-contrast micrograph of a 4-days culture monolayer (A), and a transmission electron micrograph of parts of two adjacent proximal tubular cells (B). The size of the mitochondria (M) and the electron transparancy of the nucleoplasma (N) are similar to those in proximal tubular cells in vivo (Mansbach, 1973). (A, x 160; B, x 13,200).

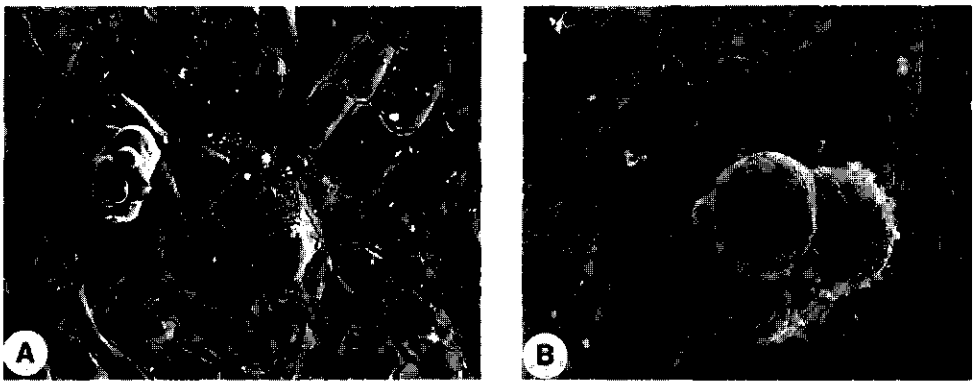


Figure 2.4 Scanning electron micrographs of a 4-day old primary culture of rat renal cortical cells on porous membranes. . Microvilli on fully expanded cells differ in number and length from those on the cell clump (CC) from which the former derive. (A, x 320; B, x 1080).

showed bright red areas demonstrating the presence of the proximal tubular brushborder enzyme (not shown). Transmission electron microscopy (Fig. 2.3B) revealed, a relative electron transparancy of the nucleoplasma and number, and size of mitochondria , similar to those in proximal tubular cells in vivo (Mannsbach, 1973) . Since no special purification preceeded the culture, cells of different origin (distal tubular, connective tissue) were present too.

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PART II

INVENTARISATION OF MONOLAYERS OF RENAL CORTICAL CELLS FOR STUDYING MECHANISMS OF NEPHROTOXICITY OF GLUTATHIONE CONJUGATED HALOALKENES AND THEIR DERIVATIVES

CHAPTER 3

Differential toxicity as a result of apical and basolateral exposure of LLC-PK1 monolayers with S-(1,2,3,4,4-pentachlorobutadienyl)glutathione and N-acetyl-S-(1,2,3,4,4-pentachlorobutadienyl)-L-cysteine

Mertens J.J.W.M., Weijnen J.G.J, van Doorn W., Spenkelink A, Temmink J.H.M., and van Bladeren P.J. *(1988) Chem.-Biol. Interactions, 65, 283-293.*

Differential toxicity as a result of apical and basolateral treatment of LLC-PK1 monolayers with S-(1,2,3,4,4-pentachlorobutadienyl)glutathione and N-acetyl-S-(1,2,3,4,4-pentachlorobutadienyl)-L-cysteine

SUMMARY

Monolayers of LLC-PK1 cells, a cell line with features typical of proximal tubular epithelial cells, were treated at the apical and basolateral side with S-(1,2,3,4,4-pentachlorobutadienyl)glutathione (PCBD-GSH) and N-acetyl-S-(1,2,3,4,4-pentachlorobutadienyl)-L-cysteine (PCBD-NAC).

Apical treatment with PCBD-GSH (>20 μM) resulted in cytotoxicity , which could be inhibited by acivicin and aminooxyacetic acid (AOAA), inhibitors of γ-glutamyltranspeptidase (γGT) and β-lyase respectively. In contrast apical treatment with PCBD-NAC was only toxic at high concentrations (>850 μM), and this effect could hardly be inhibited by AOAA.

Basolateral treatment of confluent LLC-PK1 monolayers , grown on porous membranes, with PCBD-GSH gave a much smaller respons than apical treatment, consistent with the fact that γGT is predominantly present at the apical side. Basolateral treatment even with high concentrations of PCBD-NAC (1.1 mM) did not show an increase in cytotoxicity when compared to the effect after apical treatment.

These results suggest the absence of an organic anion transporter, by which these conjugates in vivo are transported into the cells from the basolateral side. This supposition was substantiated in a study of transcellular transport of the model ions tetraethylammonium (TEA) and para-aminohippurate (PAH) , in LLC-PK1 monolayers , grown as indicated above. No active PAH transport could be demonstrated , whereas an active TEA transport was present.

The absence of an organic anion transporter limits the usefulness of LLC-PK1 cells for the study of nephrotoxicity of compounds , like PCBD-NAC, needing this transport to enter the cells. However, the finding of an active basolateral organic cation transporter, together with the presence of γGT, dipeptidase and β-lyase, makes this system especially interesting for testing all compounds that use this transporter or these enzymes in order to elicit toxicity.

ABBREVIATIONS

AOAA, aminooxyacetic acid; DCV-CYS, S-(1,2-dichlorovinyl)-L-cysteine; DCV-GSH, S-(1,2-dichlorovinyl)glutathione; EBSS, Earle's Balanced Salt Solution; EDTA, ethylenediaminetetraacetic acid; FCS, fetal calf serum; γGT, γ-glutamyltranspeptidase; GSH, glutathione; HBSS, Hanks' Balanced Salt Solution; HCBD, hexachlorobutadiene; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid; HPLC, high performance liquid chromatography; LDH, lactate dehydrogenase;

PAH, para-aminohippurate; PCBD-CYS, S-(1,2,3,4,4-pentachlorobutadienyl)-L-cysteine; PCBD-NAC, N-acetyl-S-(1,2,3,4,4-pentachlorobutadienyl)-L-cysteine; PCBD-GSH, S-(1,2,3,4,4-pentachlorobutadienyl)glutathione; TEA, tetraethylammonium; WE, Williams Medium E.

INTRODUCTION

The kidney is a rather heterogeneous organ containing numerous cell types. For investigations on mechanisms of nephrotoxicity, a stable in vitro system of homogeneous cells, representative for the site of action of the nephrotoxicant, is of considerable importance . As such a model the LLC-PK1 cell line has been used by several authors (Hori et al, 1984; Saito et al, 1986; Troyer et al, 1986; Stevens et al, 1986). This cell line is derived from a normal Hampshire pig kidney (Hull et al, 1976); it expresses a number of features typical of the proximal tubular epithelium, while also maintaining the polarity typical of epithelial cells. It exhibits high activities of proximal brush border membrane enzymes , e.g. γ-glutamyl-transpeptidase (γGT) (Perantoni and Berman, 1979; Rabito et al, 1984) and possesses a number of the membrane transport systems found in vivo (Rabito and Ausiello, 1980; Mullin et al, 1980; Misfeldt and Sanders, 1981; Rabito and Karish, 1982; Rabito and Karish, 1983; Sepúlveda and Pearson, 1982; Rabito, 1983; Biber et al, 1983).

Recently a number of glutathione (GSH) conjugates have been reported to be nephrotoxic. Although GSH conjugation is generally accepted as one of the major pathways of detoxification, GSH- and/or cysteine conjugates of e.g. trichloroethylene (Hassal et al, 1983), chlorotrifluoroethene (Dohn et al, 1985), hexachlorobutadiene (HCBD) (Jaffe et al, 1983; Nash et al, 1984), and 2-bromohydroquinone (Monks et al, 1985) have been found to be responsible for the nephrotoxicity of these compounds. Their major site of action is the proximal tubulus. The initial step in the metabolic activation is thought to be the hydrolysis of the GSH conjugate by γGT at the apical membrane. After subsequent removal of the glycine by a dipeptidase, the corresponding cysteine conjugate is not acetylated by N-acetyltransferase, to give the mercapturic acid, but converted by a β-lyase, to a reactive thiol which is thought to be responsible for the ultimate toxic effect (Elfarra and Anders, 1984), (Fig. 3.1).

On the other hand, basolateral transport systems such as organic anion transporters may play a decisive factor in the nephrotoxicity of these compounds. The N-acetylcysteine conjugate of e.g. HCBD (N-acetyl-S-(1,2,3,4,4-pentachlorobutadienyl)-L-cysteine (PCBD-NAC) has been reported to be nephrotoxic (Nash et al, 1984). This conjugate apparently can be transported into the cell through the basolateral membrane by an organic anion transporter , since PCBD-NAC accumulation in renal cortical slices is inhibited by probenecid (Lock et al, 1986). The mercapturic acid may either leave the cell at the apical (luminal) side into the tubular lumen , or may be deacetylated yielding the cysteine conjugate which again can be metabolized to the reactive thiol (Fig. 3.1).

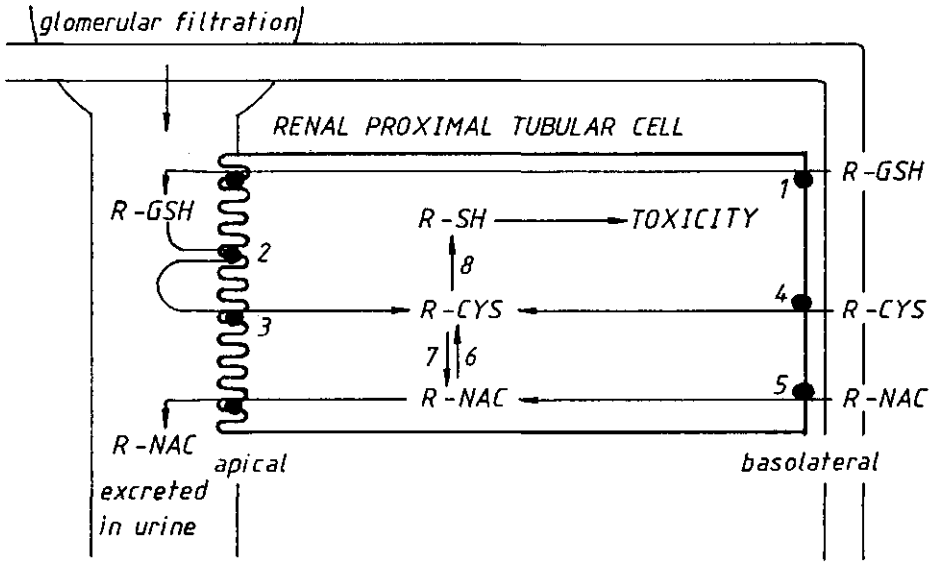

Figure 3.1 Schematic representation of renal glutathione conjugate metabolism. Glutathione conjugates (R-GSH), either directly from the glomerular filtrate or indirectly after probenecid sensitive basolateral transport through the epithelial cell to the filtrate, enter the proximal tubular lumen (1). They are metabolized by γGT (2) and a dipeptidase (3) to the corresponding cysteine conjugates (R-CYS), which enter the cell at the apical side. Basolaterally a probenecid sensitive transport of R-CYS (4) and the mercapturic acid (R-NAC) (5) has been postulated. R-NAC can be excreted into the urine or be deacetylated (6) to R-CYS. This derivative can be acetylated by N-acetyltransferase (7) or metabolized by β-lyase (8) to a reactive thiol, resulting in toxicity.

Basolateral transport systems may also play a role in the access to the proximal tubular cell of cysteine conjugates and of the glutathione conjugates themselves: Probenecid sensitive uptake of S-(1,2-dichlorovinyl)glutathione (DCV-GSH) (Lash and Jones, 1985), and probenecid inhibition of S-(1,2-dichlorovinyl)-L-cysteine (DCV-CYS) toxicity (Lash and Anders, 1986), have been reported in basolateral vesicles as well as in isolated kidney cells .

In the present study the LLC-PK1 cells were grown on porous membranes in transwell cell culture chambers. Using this system the role of apical and basolateral transport systems in the toxicity elicited by the GSH and N-acetylcysteine conjugates of HCBD was studied. For a complete picture organic anion and cation transport was also investigated with the model ions para-aminohippurate (PAH) and tetraethylammonium (TEA).

MATERIALS AND METHODS

Chemicals

[Glycyl-1- ^{14} C]PAH, spec. act. 42.7 mCi/mmol and [1- ^{14} C]TEA, spec. act. 4.8 mCi/mmol were purchased from New England Nuclear, Boston, MA, USA. Glutathione , HCBD , Aminooxyacetic acid (AOAA) and quinine were obtained from Janssen, Beerse, Belgium. Acivicin was a gift from Dr. Wendell Wieringa from The Upjohn Company, Kalamazoo, Michigan, USA. Probenecid was bought from Sigma, St Louis, MO , USA. Sodium pyruvate was obtained from BDH Chemicals Ltd, Poole, England and NADH from Boehringer, Mannheim, FRG. Triethylamine was purchased from Merck-Schuchardt, Hohenbrunn, FRG. All other reagents, when not specified, were obtained from commercial sources and were of the highest purity available.

Syntheses

S-(1,2,3,4,4-pentachloro-1,3-butadienyl)-glutathione (PCBD-GSH) was synthesized by adding 3.32 g hexachloro-1,3-butadiene to a solution of 1 g glutathione in 100 ml of dry methanol to which was added 5 ml of triethylamine. The reaction mixture was stirred for 6 days, the solvent evaporated in vacuo, and the residue dissolved in 50 ml of 0.5 M $NaHCO_3$. The aqueous phase was extracted repeatedly with diethylether and then adjusted to pH 3.5 with 1% H_3PO_4. The precipitate formed was collected by centrifugation, and washed several times by stirring with water/ethanol (95:5 v/v) followed by centrifugation. Drying in vacuo (KOH) yielded 399 mg (23.1%) of a yellow material (mp. 186-190 ^{o} C; lit. Nash et al, 1984: 186-187 ^{o} C). The identity of the product was confirmed by field desorption mass spectrometry on a MS 902 equipped with a VG-ZAB console (signal at emitter current 24-30 mA (only ^{35} Cl-peak is given), m/e 530 (MH^+) (Tunek et al, 1980). The purity was checked by thin layer chromatography (TLC) (n-propanol/acetic acid/H_2O: 16/3/5; Rf 0.54; visualization after spraying with ninhydrin).

PCBD-NAC was synthesized according to Nash et al (1984). Purification of the crude product was achieved by high performance liquid chromatography (HPLC) using a reversed phase preparative column (250x12 mm, Lichrosorb 10RP-18) (Chrompack, Middelburg, The Netherlands) eluted isocratically with methanol/water (1% acetic acid) (80:20 v/v) at a flow rate of 5 ml/min. Purity exceeded 98% as judged by HPLC using a reversed phase analytical column (150x4.6 mm; Lichrosorb 5RP-18) (Chrompack, Middelburg, The Netherlands) and methanol/water (1% acetic acid) (70:30 v/v) as mobile phase at a flow rate of 1 ml/min . The UV-spectrum (methanol) was determined with a Beckman DU-8 spectrophotometer (Beckman Instruments Inc, Fullerton, USA) and showed λ_{max} at 283 nm. The identity was further confirmed by nuclear magnetic resonance analysis and mass spectral analysis (only ^{35} Cl-peaks are given) using both field desorption mass spectrometry (signal at emitter current 24-30 mA, m/e 385 (M^+)) and electron impact mass spectrometry (70 eV, m/e 385 (M^+, 1%),

350 (M^+-Cl, 1%), 340 (M^+-COOH, 3%), 305 (M^+-COOH-Cl, 3%), 298 (M^+-COOH-COH_2, 5%), 255 ($C_4Cl_5S^+$, 9%), 220 ($C_4Cl_4S^+$, 100%).

Cell strain and culture

Epithelial pig kidney cells (LLC-PK1) (Flow, Irvine, Scotland)were grown in William's Medium E (WE)(Flow, Irvine, Scotland), supplemented with 10% fetal calf serum (FCS) (GIBCO, Glasgow, Scotland), 100 IU of penicillin, and 100 mg/l of streptomycin (Gist-Brocades , Delft, The Netherlands) in culture flasks (Costar, Badhoevedorp, The Netherlands) at 37 ^{o} C in a humidified atmosphere, containing 5% CO_2 . Stock cultures, seeded at 0.8-1.0 x10 ^{4} cells/cm ^{2} , were subcultured every 3 to 4 days by trypsinization with 0.25% trypsin (Difco, Detroit, Mich. USA) and 0.05% EDTA (Merck, Darmstadt, FRG), after being rinsed with Hanks' Balanced Salt Solution (HBSS). Experiments were performed with cells at passage 193-220.

Cytotoxicity assay

For experiments where monolayers were exposed only at the apical side cells were seeded into 24-well tissue culture plates (Costar) at 3.5x10 ^{5} cells/well. Within 2-3 days the monolayers were confluent and dome formation occurred. At the third day after seeding, the wells were rinsed with HBSS and the test compounds were added dissolved in 1.0 ml WE without FCS and antibiotics. The concentration of the test compounds was afterwards checked by a UV-scan, λ_{max}= 280-284 nm and ε=10500 (Reichert and Schütz, 1986). Whenever enzyme inhibitors were used, the cells were preincubated for half an hour with the inhibitors before the test compounds were added (for concentrations see the figure legends). After overnight incubation (16 hours) , 400 µl samples of the media were taken from each well and centrifuged. Lactate dehydrogenase activity (LDH) was measured (Mitchell et al, 1980) in 100 µl of supernatant . The maximal release (Rmax) of LDH was obtained by scraping control cells with a pipet tip in 1 ml of 0.5% triton X-100 in 0.1 M phosphate buffer, and measuring the LDH activity in the supernatant after sonication (5 min.) and centrifugation. Each experiment was performed in triplicate. Toxicity data are presented as percentage of Rmax and as the mean +/- S.D. for at least three experiments.

In experiments where apical treatment was compared with basolateral treatment, the cells were grown in transwell cell culture chambers (Costar) with an effective porous membrane area of 4.7 cm ^{2} . Cells were seeded at 0.8x10 ^{6} cells/membrane and the medium was renewed after 3-4 days. At the 6 th or 7 th day after seeding the cells were used for an experiment. Confluency was checked by scanning electron microscopy (not shown). After the cells were carefully rinsed with WE the membranes were returned to the wells, filled with 2.6 ml of WE, with or without the test compounds dissolved. Then the apical compartment was filled with 1.5 ml of WE, with or without test compounds dissolved. Using these volumes, an equal level of medium in both compartments was achieved. After overnight

incubation , the medium from the apical and basolateral compartments were added together and a 400 µl sample was taken. LDH activity was measured in the supernatant of the sample after centrifugation.

Transport assay

The transcellular transport experiments were performed with cells grown in transwell culture chambers as described above. After rinsing with Earle's Balanced Salt Solution (EBSS), without phenol red and supplemented with 2.4 mM HEPES (pH 7.5), the porous membranes were incubated in the same solution. Hydrostatic pressure was prevented by keeping the solution at either side at the same level . Before (20 min.) and during the experiment cultures were kept at 37 ^{o} C in a humidified atmosphere containing 5% CO_2 . The experiment was started by adding a solution of ^{14} C-PAH (42.7 mCi/mmol) or ^{14} C-TEA (4.8 mCi/mmol) in EBSS (-phenol red, + 2.4 mM HEPES) such that a final concentration of 6 µM and 25 µM respectively was reached, concentrations near the optimal concentration used with kidney tubules (Hassall et al, 1983). At different times 100 µl samples were taken at the transcellular compartment. Each volume of solution removed from a compartment was restored with an identical volume of the corresponding non-radioactive solution.

RESULTS

Toxicity as a result of apical treatment of LLC-PK1 monolayers with HCBD conjugates

When confluent monolayers of LLC-PK1 cells in multiwells are used, only the apical side of the cell is accessible. Overnight incubation of such LLC-PK1 monolayers with PCBD-GSH resulted in a dose related cytotoxicity starting at 20 µM, measured by LDH release (Fig.3.2A). Inhibitors of enzymes of the mercapturic acid pathway were used to determine the specificity of the toxic effect. As expected, inhibition of γ-GT (acivicin) and β-lyase (AOAA) prevents the toxicity . In figure 3.2B , the results are shown obtained when LLC-PK1 monolayers were treated with PCBD-NAC . No toxicity could be observed at concentrations up to 850 µM PCBD-NAC . The toxicity observed at higher concentrations was only slightly inhibited by AOAA.

Apical versus basolateral treatment of LLC-PK1 monolayers with PCBD-GSH and PCBD-NAC

Basolateral exposure of the LLC-PK1 cells was achieved by growing monolayers on porous membranes using a transwell cell culture chambers. After apical or basolateral treatment with PCBD-GSH , a large difference in toxicity, was observed, which disappeared as a result of acivicin (Fig. 3.3).

However , basolateral incubation with PCBD-NAC did not result in a higher toxicity than apical incubation, as would be expected if a PCBD-NAC transporter

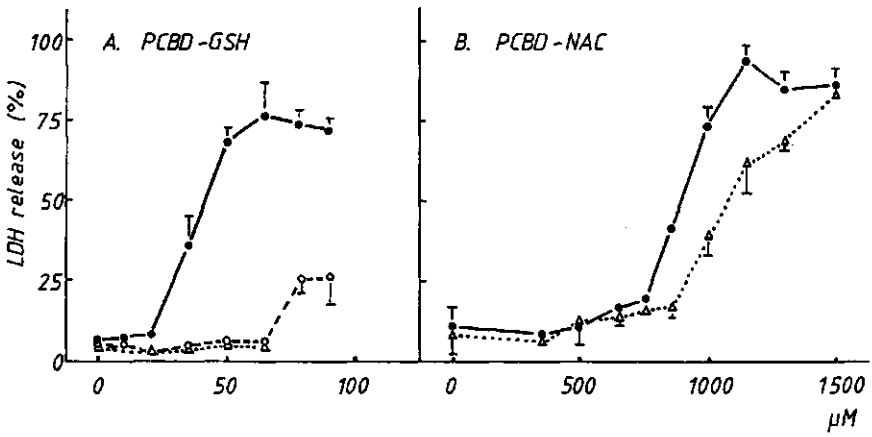

Figure 3.2 Effect of PCBD-GSH (A) and PCBD-NAC (B) on LLC-PK1 monolayers. Confluent monolayers of LLC-PK1 cells were treated apically for 16 h in the absence (●——●) or presence of inhibitors of γGT (acivicin, 0.5 mM; O- - -O) or β-lyase (AOAA, 0.5 mM; Δ ····· Δ). Note the difference in the scale of the abscissa. Each point represents the mean of at least three experiments.

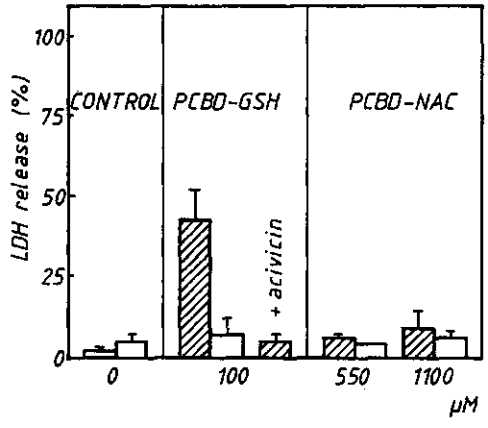

Figure 3.3 Apical versus basolateral treatment of LLC-PK1 monolayers with PCBD-GSH and PCBD-NAC. LLC-PK1 monolayers were grown in transwell cell culture chambers (see Methods) were treated apically (▨) or basolaterally (□) for 16 h. Acivicin was used at a concentration of 0.5 mM and added only at the apical side. Each bar represents the mean (±S.D.) of three membranes of one experiment typical for at least three.

at the basolateral side would be involved . Even when we increased the concentration up to 1.1 mM , no differences in toxic effects were observed. These results suggest the absence of an organic anion transporter in LLC-PK1 cells by which these conjugates are transported into the cell across the basolateral membrane in vivo.

Transepithelial organic cation (TEA) and anion (PAH) transport

The existence of both organic cation and anion transport systems was investigated using radioactive TEA and PAH. Figure 3.4 shows very clearly that TEA is transported from the basolateral side to the apical side. Both quinine (0.1 mM), a potent competitive inhibitor of organic cation transport, and ouabain (0.1 mM), which acts by inhibiting the Na^+,K^+ - ATPase, were able to inhibit this transport. In contrast no probenicid sensitive PAH transport was observed.

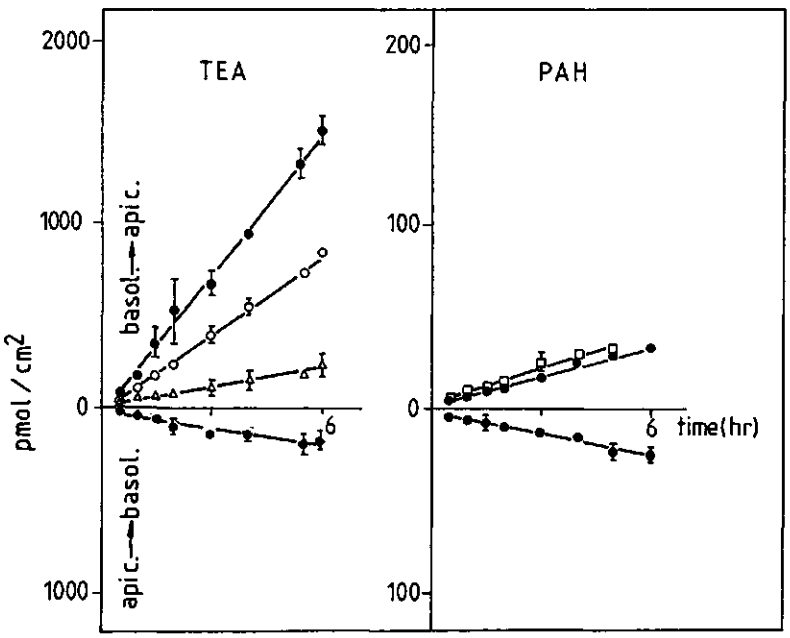

Figure 3.4 Transepithelial organic cation (TEA) and anion (PAH) transport. Monolayers were grown in transwell cell culture chambers (see Methods). Transport was measured from the apical to the basolateral side, and from the basolateral to the apical side, in the absence (●——●) or presence of inhibitors: ouabain, 0.1 mM (○——○); quinine, 0.1 mM (△——△) or probenecid, 1.0 mM (□——□). Note the scale difference in the ordinate. Each point represents the mean (±S.D.) of three membranes and the line was calculated by regression analysis of all points.

DISCUSSION

Organic anion transporters may play an important role in the nephrotoxicity of GSH conjugates and their metabolites. Probenecid, a well-known inhibitor of organic anion transport, has been reported to inhibit the nephrotoxicity of PCBD-GSH, PCBD-CYS, PCBD-NAC (Lock and Ishmael, 1985) and S-(2-chloroethyl)-DL-cysteine (Elfarra et al, 1985) in vivo, as well as the renal cortex slice accumulation of PCBD-NAC (Lock et al, 1986) in vitro. Experiments with freshly isolated rat kidney cells show the same effect of probenecid; the cytotoxicity of S-(1,2-dichlorovinyl)-L-cysteine (Lash and Anders, 1986) as well as the uptake of S-(1,2-dichlorovinyl)glutathione (Lash and Jones, 1985) was inhibited.

Since the organic anion transporter is situated at the basolateral side of the proximal tubular epithelium (see Fig.3.1), in the present study the cytotoxicity of the model compounds PCBD-GSH and PCBD-NAC as a result of either apical or basolateral treatment of LLC-PK1 monolayers was compared.

LLC-PK1 monolayers in culture plates only allow apical treatment . In this system PCBD-GSH elicited a toxic effect, that could be inhibited by acivicin , consistent with the fact that γGT is present in the apical membrane. Apical treatment with PCBD-NAC resulted in a small toxic effect. The slight effect of AOAA indicates that the toxicity is essentially not due to the reactive metabolite formed via the β-lyase pathway. Most likely at these high concentrations some general cytotoxic effect occurs, which has nothing to do with nephrotoxicity. These results are in agreement with the finding of Stevens et al (1986) that N-acetyl-S-(1,2-dichlorovinyl)-L-cysteine in a similar system also has low toxicity and confirms the finding that these substances are transported into the cell by an organic anion transporter (Lock et al, 1986). In order to further substantiate this we extended the test system and cultured monolayers of LLC-PK1 cells on porous membranes in a transwell system which allows selective basolateral treatment. Apical incubation with PCBD-GSH resulted in a much larger toxic effect than basolateral incubation, showing that 1) γGT is indeed predominantly present in the apical membrane and 2) transport of the GSH conjugate from the basolateral side through the cell followed by conversion via γGT on the apical side does not occur.

However , basolateral treatment with PCBD-NAC did not result in an increase in the toxic respons compared to apical treatment, as would be expected if a basolateral transporter were present . Since the monolayers on the porous membranes are slightly less sensitive to PCBD-GSH than the monolayers in the multiwells, the effect of PCBD-NAC was studied up to a concentration of 1.1 mM, but still no increase in toxicity due to basolateral treatment was detected. The absence of an increase in toxicity by basolateral treatment with PCBD-NAC may have two explanations. Either the LLC-PK1 cell is not capable of deacetylating PCBD-NAC, or it does not have a transport system by which PCBD-NAC can enter the cell. The absence of a transport system seemed the most likely explanation and was further investigated by measuring transcellular transport of radiolabeled PAH.

Indeed no probenecid sensitive transepithelial PAH transport was found. This

is in agreement with a recent report by Rabito (1986) who did not find any probenecid sensitive accumulation of PAH. In contrast, an active transcellular transport from the basolateral side to the apical side, of the cation TEA, was clearly present.

In conclusion, LLC-PK1 monolayers are a suitable model for testing glutathione conjugates such as PCBD-GSH. The role of a number of important enzymes of the mercapturic acid pathway as γGT and β-lyase, can then be studied. Because of the absence of an organic anion transporter no meaningful toxicity studies can be carried out with these cells when basolateral uptake via this organic anion transporter is a prerequisite for their toxic effect.

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CHAPTER 4

Cytotoxicity of S-(1,2,3,4,4-pentachlorobutadienyl)-L-cysteine after apical and basolateral exposure of LLC-PK1 monolayers. Involvement of an amino acid transport system

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Cytotoxicity of S-(1,2,3,4,4-pentachlorobutadienyl)-L-cysteine after apical and basolateral exposure of LLC-PK1 monolayers. Involvement of an amino acid transport system

SUMMARY

Glutathione conjugation and subsequent formation of cysteine conjugates are key steps in the nephrotoxicity of halogenated alkenes. In this metabolic activation several organs are involved. However little is known about the transporters responsible for the uptake of cysteine conjugates. Recent evidence suggest that amino acid transporters play a role in this uptake.

Monolayers of LLC-PK1 cells, a kidney cell line, were exposed to S-(1,2,3,4,4-pentachlorobutadienyl)-L-cysteine (PCBD-CYS). Cytotoxicity was used as a parameter for PCBD-CYS uptake. Basolateral exposure (1 h : 400 μM and 16 h : 25 μM) to PCBD-CYS resulted in a much higher aminooxyacetic acid inhibitable cytotoxicity than apical exposure, suggesting a preferential basolateral uptake of PCBD-CYS. Exposure to PCBD-CYS in the absence of sodium did not result in a decrease of the cytotoxicity, suggesting a sodium independency of the PCBD-CYS uptake. Amino acids and amino acid analogues were used as diagnostic compounds in the further identification of the PCBD-CYS transporter. In cis-inhibition experiments monolayers were co-incubated with PCBD-CYS and these diagnostic compounds during one hour. System L substrates such as 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid (BCH) and cycloleucine did not inhibit cytotoxicity. D-Tryptophan, a model inhibitor of System T, caused a strong inhibition. System L has, in contrast to System T, a high sensitivity to trans-stimulation. Pre-loading the monolayers with the diagnostic compounds should cause an increase in cytotoxicity when System L is involved. Neither System L substrates such as BCH and cycloleucine nor D-tryptophan increased cytotoxicity.

These results suggest a preferential basolateral uptake of PCBD-CYS in LLC-PK1 monolayers and involvement of an amino acid transporter with characteristics of System T.

ABBREVIATIONS

AIB, α-aminoisobutyric acid; AOAA, aminooxyacetic acid; BCH, 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid; BCO, 3-aminobicyclo[3,2,1]octane-3-carboxylic acid; CYS, cysteine; DCV-CYS, S-(1,2-dichlorovinyl)-L-cysteine; EBSS, Earle's Balanced Salt Solution; γGT, γ-glutamyl-transpeptidase; GSH, glutathione; HBSS, Hanks' Balanced Salt Solution; HCBD, hexachlorobutadiene; LDH, lactate dehydrogenase; MeAIB, α-(methylamino)isobutyric acid; PCBD-CYS, S-(1,2,3,4,4-pentachlorobutadienyl)-L-cysteine; PCBD-NAC, N-acetyl-S-(1,2,3,4,4-pentachlorobutadienyl)-Lcysteine; SBC, S-benzyl-L-cysteine; SEC, S-ethyl-L-cysteine; SMC, S-methyl-L-cysteine; WE, Williams Medium E.

INTRODUCTION

Several halogenated alkenes have been reported to elicit a specific proximal tubular nephrotoxicity (Kociba et al, 1977; Odum and Green, 1984). Responsible for this effect is a cysteine(CYS)-conjugate that is activated via β-elimination by β-lyase, an enzyme which is present in the renal proximal tubular epithelium. The CYS-conjugate is formed after a complex series of metabolic steps, involving several organs. The initial reaction is a glutathione (GSH) conjugation of the halogenated alkenes in the liver. The next steps are enzymatic conversion by γ-glutamyl-transpeptidase (γGT) and a dipeptidase leading to the CYS-conjugate. These steps can occur either intrarenally, after the GSH-conjugate has reached the brush border in the proximal tubulus, or extrarenally, in the bile duct and/or intestine. In the latter case the CYS-conjugate is absorbed from the intestine and can reach the kidney as it is, or after acetylation into the corresponding N-acetyl-cysteine conjugate in the liver. Deacetylation in the kidney then results in the reformation of the CYS-conjugate (for review see: Anders et al, 1988; Lock, 1988).

Due to the structure and function of the proximal tubule, conjugates can reach the epithelial cell from either the luminal or serosal side. It is evident that cellular uptake systems for these different conjugates in either membrane play an important role in the nephrotoxicity of halogenated alkenes. For the N-acetyl-cysteine conjugate of hexachlorobutadiene (HCBD) Lock et al (1985;1986) demonstrated involvement of an organic anion transporter in the renal uptake, probably located in the anti-luminal membrane. Less is known about the renal uptake of CYS-conjugates. Lash and Anders (1986) postulated involvement of an organic anion transporter in the uptake of S-(1,2-dichlorovinyl)-L-cysteine (DCV-CYS). Recently, Schaeffer and Stevens (1987a) found that DCV-CYS could be taken up in LLC-PK1 monolayers via an amino acid transporter. The LLC-PK1 cell line (Hull et al, 1976) is a convenient cell model for studying transport aspects of CYS-conjugates. It possesses a number of characteristics typical of proximal tubular epithelium, among them the enzymes, necessary for the activation of GSH- and CYS-conjugates (Stevens et al, 1986; Mertens et al, 1988) and several amino acid transporters (Rabito and Karish, 1982; Rabito and Karish, 1983; Sepúlveda and Pearson, 1982). It maintains the epithelial polarity, when cultured on a solid or porous support. In their investigations Schaeffer and Stevens (1987a) used LLC-PK1 monolayers on a solid support . This makes access of the CYS-conjugate to the basolateral membrane virtually impossible, thus obviating appraisal of the relative importance of apical versus basolateral uptake routes. Using a porous support for LLC-PK1 cells allows the investigation of apical and basolateral uptake processes (Mertens et al, 1988) and should give information about the relative role of amino acid transporters of both membrane systems in the cytotoxicity of CYS-conjugates in these cells.

The terminology of amino acid transporters has been proposed by Christensen and co-workers (Oxender and Christensen, 1963; Christensen et al, 1967; Vadgama and Christensen, 1985; Christensen, 1984). Within the group of neutral

amino acid transporters transport systems are distinguished by their sodium dependence, substrate specificity and exchange properties. System L is a sodium independent transport system that preferentially transports zwitterionic amino acids with branched and apolar side chains, like cycloleucine and 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid (BCH). It has a high sensitivity to trans-stimulation of substrate uptake, i.e. enhanced uptake of a substrate as a result of fast exchange with a previously loaded intracellular analogous substrate. System T resembles System L, it is also sodium independent, but has a preference for aromatic and bicyclic amino acids. It shows some sensitivity to trans-stimulation of substrate uptake and has D-trytophan as a model substrate. System ASC is a sodium dependent transporter with preference for zwitterionic amino acids with preferentially three to five carbon in a chain, like α-aminoisobutyric acid (AIB). It excludes N-methylated amino acids , like α-(methylamino)-isobutyric acid (MeAIB) and shows minor sensitivity to trans-stimulation. System asc is similar to ASC in substrate specificity, but differs from it by its sodium-independence, which is indicated by the use of the lower-case letters. It has valine as model substrate. System A is also a sodium dependent transporter of zwitterionic amino acids, but it accepts N-methylated amino acids like MeAIB and is sensitive to trans-inhibition: exchange inhibited by a pre-loaded analogous substrate.

In the present study we investigated in LLC-PK1 monolayers the uptake of S-(1,2,3,4,4-pentachlorobutadienyl)-L-cysteine (PCBD-CYS) at the apical and the basolateral membrane. Experiments of Schaeffer and Stevens (1987) demonstrate unequivocally that cytotoxicity data give reliable information about uptake. We therefore used cytotoxicity as a parameter for uptake. Furthermore the influence of amino acids and amino acid analogues on the cytotoxicity was studied. The results indicate preferential basolateral uptake and involvement of a sodium independent amino acid transporter with characteristics of System T in the cytotoxicity of PCBD-CYS .

MATERIALS AND METHODS

Chemicals

S-ethyl-L-cysteine (SEC), S-methyl-L-cysteine (SMC), S-benzyl-L-cysteine (SBC), BCH, AIB and D-tryptophan were purchased from Janssen Chimica (Beerse, Belgium). Aminooxyacetic acid (AOAA), MeAIB and cycloleucine were bought from Aldrich-Chemie (Steinheim, FRG), histidine and valine from Merck (Darmstadt, FRG).

Syntheses

PCBD-CYS was synthesized by hydrolysis of N-acetyl-S-(1,2,3,4,4-pentachlorobutadienyl)-L-cysteine, which was synthesized as described before (Mertens et al, 1988). Field desorption mass spectrometry on a MS 902 equipped

with a VG-ZAB console (signal at emitter current 24-30 mA) showed a 5 chlorine pattern at m/e 343 (M^+) confirming the identity of PCBD-CYS. Purity exceeded 98% as judged by HPLC.

Cell culture

Epithelial pig kidney cells (LLC-PK1) (Flow, Irvine, Scotland) were grown in culture flasks (Costar, Badhoevedorp, The Netherlands) at 37 °C in a humidified atmosphere containing 5% CO_2. The medium was William's Medium E (WE) (Flow) supplemented with 10% fetal calf serum (GIBCO, Glasgow, Scotland), penicillin (50 IU/ml), and streptomycin (100 mg/l) (Gist-Brocades, Delft, The Netherlands). Subconfluent cultures were subcultured every 3 to 4 days . Cells were rinsed with Ca^{2+} - and Mg^{2+}-free Hanks' Balanced Salt Solution (HBSS) and trypsinized with 0.25% trypsin (Difco, Detroit, Mich. USA) and 0.05% EDTA (Merck). Experiments were performed with cells at passage 193-220.

Cytotoxicity assay

LLC-PK1 monolayers were obtained by seeding 24-well tissue culture plates (Costar) with 3.5×10^5 cells/well or porous membranes of transwell cell culture chambers(Costar, no 3412) with 1.0×10^6 cells/membrane on day 0. Experiments were conducted at the third day after seeding. At this time numerous domes had formed in the monolayers in the culture plates. This culture period also was long enough to produce tightly confluent monolayers on the porous membranes as evidenced by scanning electron microscopy. In addition, the tightness was checked with the inhibitable transcellular transport of radiolabeled tetraethylammonium as described before (Mertens et al, 1988). Monolayers were washed with Earle's Balanced Salt Solution (10 mM $NaHCO_3$, 20 mM HEPES , pH 7.4) (EBSS), and treated with PCBD-CYS in EBSS during 1 or 16 h . Cytotoxicity was determined immediately after the 16 h exposure or after a subsequent overnight stay in EBSS in the experiments with 1 h exposure. LDH-leakage was used as a parameter for cytotoxicity (Mertens et al, 1988).

Experimental procedure

To see whether and which amino acid transporter is involved in the uptake and subsequent cytotoxicity of PCBD-CYS in LLC-PK1 monolayers, we conducted the following experiments:

Sodium dependence was investigated by exposing LLC-PK1 monolayers during one hour to PCBD-CYS either in EBSS in which the sodium was replaced by equimolar amounts of choline (EBSS-Na^+) or in EBSS in the presence of ouabain (5 µM and 1 mM). The monolayers were left overnight in EBSS before cytotoxicity was determined.

To see whether cis-inhibition occurred, monolayers were exposed during one hour to PCBD-CYS in EBSS in the presence of 10 mM of several different amino acids or amino acid analogues, which were used as diagnostic compounds. Only S-benzyl-L-cysteine (SBC) was used at lower concentrations due to its lower

solubility. The concentration of PCBD-CYS chosen was high enough to produce a strong toxic effect in the control cultures, enabling registration of inhibition of the toxicity as a result of the co-incubation with the diagnostic compound.

Trans-stimulation was determined by exposing monolayers overnight to PCBD-CYS after pre-loading the cells for one hour with 10 mM of the diagnostic compound. In this case the concentration of PCBD-CYS chosen was low enough to produce a small toxic effect in the control, enabling detection of a stimulation of the toxicity. Higher concentrations resulted in a high toxicity which was not inhibited by the use of the amino acid transporter substrates, indicating that metabolism by β-lyase was not influenced.

In the experiments where sodium dependence and cis-inhibition were investigated, we chose for a short term exposure of one hour. Long term exposure (e.g. 16 h) may obscure competitive inhibition of uptake because, this inhibition will probably only delay for a limited time , the reaching of a maximum and the ensuing toxic effects. Furthermore trans-stimulatory effects could mask cis-inhibition as Schaeffer and Stevens suggested (1987a).

RESULTS

Cytotoxicity after apical or basolateral exposure

Apical exposure of confluent LLC-PK1 monolayers in 24-well tissue culture plates to PCBD-CYS resulted in a cytotoxicity that could be inhibited by AOAA, a β-lyase inhibitor (Fig.4.1).

Monolayers in transwell cell culture chambers were exposed separately from the apical or basolateral side. As shown in figure 4.2, basolateral exposure gives a higher AOAA inhibitable toxicity than apical exposure, suggesting a preferential basolateral uptake of PCBD-CYS. Note the difference in sensitivity to apical exposure between monolayers in 24-well tissue culture plates and those on the porous membranes of the transwells (Mertens et al, 1988).

Sodium dependence

No sodium dependence could be demonstrated for the cytotoxicity of PCBD-CYS. One hour exposure to PCBD-CYS in EBSS without sodium or EBSS containing ouabain, an inhibitor of Na^+,K^+-ATPase, did not result in decreased cytotoxicity (Table 4.1). On the contrary, ouabain increased the cytotoxicity of PCBD-CYS. The sodium independence was found for both apical and basolateral exposure.

Cis-inhibition

As shown in table 4.2, the use of AIB and MeAIB, model substrates for discriminating between Systems A and ASC, did not result in a decrease of the cytotoxicity in the cis-inhibition experiments. Neither did cis-inhibition occur when substrates were used of System asc, like valine, or System L, like cycloleucine and

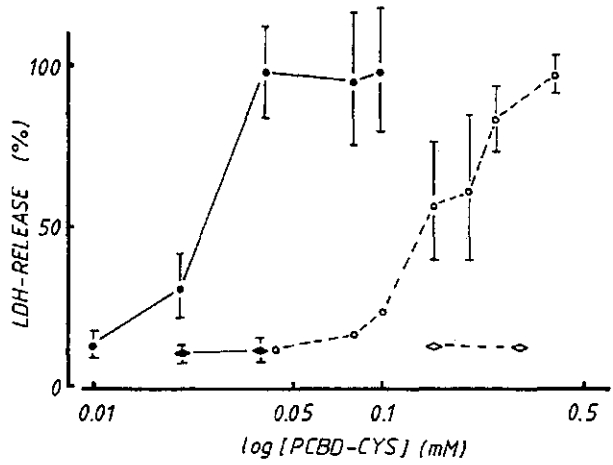

Figure 4.1 Cytotoxicity of LLC-PK1 monolayers as a result of PCBD-CYS exposure. LLC-PK1 monolayers in 24-well tissue culture plates were exposed to PCBD-CYS during 1 h (O---O) or 16 h (●——●). AOAA was used at a concentration of 0.5 mM (◇ , ◆) LDH-leakage was used as parameter and was measured after 16 h (see materials & methods). Results are the mean (± S.D.) of 2-5 experiments in triplicate.

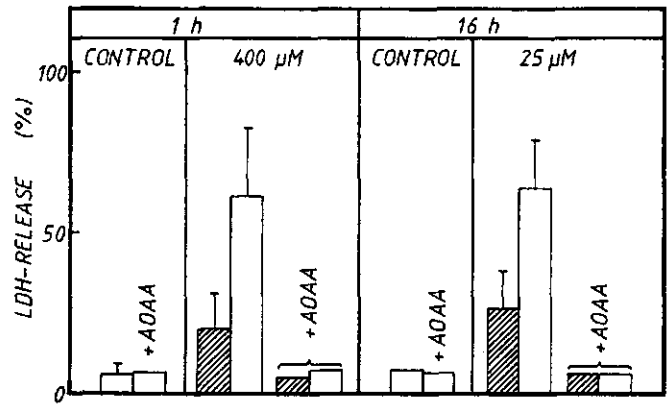

Figure 4.2 Cytotoxicity of PCBD-CYS after apical or basolateral exposure of LLC-PK1 monolayers. LLC-PK1 monolayers on porous membranes of transwell cell culture chambers were exposed during 1 or 16 h to PCBD-CYS either apically (▨) or basolaterally (□). AOAA was used at a concentration of 0.5 mM and added to both sides. LDH-leakage was used as parameter . Each bar represents the mean (± S.D.) of 2-6 experiments in triplicate.

Table 4.1 Na^+-dependence of PCBD-CYS toxicity. LLC-PK1 monolayers in 24-well tissue culture plates (apical) or on porous membranes of transwell cell culture chambers (basolateral) were exposed to PCBD-CYS during 1 h , under the indicated conditions. LDH-leakage data are the mean (± S.D.) of two experiments in triplicate.

	% LDH-release			
CONDITION	APICAL		BASOLATERAL	
	0 µM	150 µM	0 µM	250 µM
Control	6.3 ± 0.6	46.7 ± 15.1	5.9 ± 2.0	58.5 ± 4.4
Ouabain 5 µM	7.4 ± 2.1	62.6 ± 19.2	4.2 ± 0.1	78.7 ± 4.8
1 mM	7.3 ± 1.4	65.8 ± 7.4	9.8 ± 0.3	78.6 ± 2.4
EBSS-Na^+	7.8 ± 2.1	46.1 ± 6.3	5.6 ± 0.4	51.4 ± 3.3

Table 4.2 Inhibitory effect of various amino acid transporter substrates on the cytotoxicity of PCBD-CYS . LLC-PK1 monolayers were exposed apically (24-well tissue culture plates) with 0.2 mM or basolaterally (transwell cell culture chamber) with 0.4 mM PCBD-CYS in the presence of 10 mM diagnostic compound during 1 h. LDH-leakage data of co-incubated cells were expressed as frequency of LDH-leakage data of control cells. Percentage are the mean (± S.D.) of n experiments in triplicate. Asteriks indicate when inhibition was statistically significant (Wilcoxon, $p<0.05$) in each experiment.

DIAGNOSTIC COMPOUND	Percentage of control APICAL	n	BASOLATERAL	n
none	100 (79% ±20)[1]	10	100 (60% ±23)[1]	12
SMC	77 ± 7.8*	5	75 ± 30.7*	4
SEC	77 ± 9.4*	5	75 ± 23.6*	4
SBC[2]	99 ± 8.5	3	104 ± 14.8	2
AIB	115 ± 14.5	3	117 ± 19.8	2
MeAIB	107 ± 31.2	3	118 ± 23.1	4
cycloleucine	101 ± 21.8	4	136 ± 16.0	2
BCH	96 ± 16.0	2	123 ± 26.6	4
histidine	69 ± 17.0*	7	68 ± 18.8*	9
D-tryptophan	78 ± 7.2*	6	86 ± 18.2*	8
valine	89 ± 5.5	3	120 ± 41.0	3

1) LDH-release
2) 4 mM

BCH. A decrease in cytotoxicity did occur when histidine, SMC, SEC and the System T inhibitor D-tryptophan were used. No significant inhibition was visible with SBC. No clear cut differences could be seen between apical or basolateral exposure.

Trans-stimulation

Preloading the cells with amino acid transporter substrates resulted in a substantial stimulation of the cytotoxicity after apical or basolateral exposure to PCBD-CYS with SBC only (Table 3). Basolateral preloading with AIB also seemed to stimulate the PCBD-CYS toxicity, but the stimulation was not statistically significant.

Table 4.3 Stimulatory effect of various amino acid transporter substrates on the cytotoxicity of PCBD-CYS. After pre-loading with 10 mM diagnostic compound for 1 h, LLC-PK1 monolayers were exposed apically (24-well tissue culture plates) or basolaterally to 20 µM PCBD-CYS during 16 h. LDH-leakage data of pre-loaded cells were expressed as frequency of LDH-leakage data of control cells. Percentages are the mean (± S.D.) of n experiments in triplicate. Asteriks indicate when trans-stimulation was statistically significant (Wilcoxon, $p<0.05$) in each experiment.

DIAGNOSTIC COMPOUND	Percentage of control APICAL	n	BASOLATERAL	n
none	100 (20% ±8)[1]	10	100 (14% ±6)[1]	6
SMC	119 ± 9	4	92 ± 32	3
SEC	124 ± 2	3	118 ± 3	2
SBC[2]	178 ± 16*	5	248 ± 103*	6
AIB	111 ± 8	3	164 ± 18	2
MeAIB	123 ± 35	5	101 ± 28	3
cycloleucine	95 ± 6	3	101 ± 14	3
BCH	129 ± 37	6	85 ± 44	3
histidine	108 ± 14	5	109 ± 34	3
D-tryptophan	110 ± 16	4	131 ± 75	3
valine	111 ± 30	4	88 ± 28	2

[1]) LDH-release
[2]) 8 mM

DISCUSSION

As was demonstrated before , LLC-PK1 monolayers grown on porous membranes of transwell cell culture chambers allow separate exposure of the apical or basolateral side of the cell. Whereas apical treatment with PCBD-GSH was found to result in a higher toxicity than basolateral treatment (Mertens et al, 1988), the opposite is the case after exposure to PCBD-CYS, suggesting a preferential basolateral uptake of the cysteine conjugate.

Lock and co-workers demonstrated that the organic anion transporter is also the transporter of PCBD-NAC. Probenecid inhibited the accumulation of the radiolabeled PCBD-NAC in cortical slices (Lock et al, 1986) and the toxicity of the PCBD-NAC (Lock and Ishmael, 1985). This organic anion transporter has been

postulated by Lash and Anders (1986) to be of importance in the transport of cysteine-conjugates also. They showed inhibition of the toxicity of DCV-CYS by probenecid in a suspension of isolated kidney cells . Since an organic anion transporter is absent in the LLC-PK1 cell line (Mertens et al, 1988; Rabito, 1986), CYS-conjugates have to enter this cell by another route to become toxic. Amino acid transporters would seem likely candidates.

In our experiments we not only studied amino acid transport systems already described in the LLC-PK1 cell line (Rabito and Karish, 1982; Sepúlveda and Pearson, 1982), but also System asc and System T (Vadgama and Christensen, 1985; Rosenberg et al, 1980), which to our knowledge have not been investigated in this cell line before . The sodium independence of the cytotoxicity excludes the involvement of System A and System ASC (See also Table 4.4). This is further supported by the inability to inhibit the cytotoxicity of PCBD-CYS in monolayers co-incubated with AIB or MeAIB . Of the sodium independent amino acid transporters, the System asc seems to play no role in the transport of PCBD-CYS, since the specific substrate valine (Vadgama and Christensen, 1985) has no effect on the cytotoxicity .

System T is an amino acid transporter which has overlapping substrate specificity with System L. System L is characterized by a high sensitivity to trans-stimulation, a sodium independence and a weak pH dependence. System T is also sodium and pH independent, it apparently prefers among the classical System L substrates those with a benzene ring in the amino acid structure (Rosenberg et al, 1980), but it has a lower sensitivity to trans-stimulation

Table 4.4 Some characteristics of amino acid transport systems. (references: Vadgama and Christensen, 1985; Christensen, 1984; Rosenberg et al, 1980; and Gazzola et al, 1980).

System	Na^+-dependence	Substrate	Exchange properties
A	+	Zwitterionic amino acids. MeAIB is a model substrate.	Trans-inhibition
ASC	+	Zwitterionic amino acids with preferentialy three to five carbon in a chain. Excludes N-methylated amino acids.	Trans-stimulation
L	-	Zwitterionic amino acids with preferentially branched and apolar side chains. BCH is a model substrate.	Strong Trans-stimulation
asc	-	Same as ASC. A favourable substrate is valine.	?
T	-	Prefers among the classical System L substrates aromatic and bicyclic amino acids. D-tryptophan is a model substrate	Trans-stimulation but questionable

(Vadgama and Christensen, 1985) . Vadgama and Christensen found that substrates as BCH and BCO, which had been used as specific System L substrates, also are transported in part via System T (Vadgama and Christensen, 1985). Apart from the difference in sensitivity to trans-stimulation, the use of D-tryptophan makes it possible to distinguish System T from System L. This amino acid is an inhibitor of System T, but does not inhibit System L (Rosenberg et al, 1980).

On the basis of our results we propose that System T is involved in the uptake of PCBD-CYS in LLC-PK1 cells. Firstly, in our cis-inhibition experiments D-tryptophan induced inhibition of cytotoxicity. In contrast BCH and cycloleucine, which are primarily substrates for System L , did not show any inhibitory effect. Secondly, histidine also induced strong inhibition of cytotoxicity. Although it has been suggested that histidine shares System L and T, the inhibitory action of histidine on System L is very weak (Vadgama and Christensen et al, 1985; Rosenberg, 1982). Finally, if System L were responsible for the uptake of PCBD-CYS, a strong stimulation of toxicity should occur after pre-loading the LLC-PK1 monolayers with System L substrates. However, neither BCH nor cycloleucine showed any stimulation.

When we compare our results with those of Schaeffer and Stevens (1987a), there are some apparent discrepancies. In the trans-stimulation experiments they demonstrated an inversely proportional relationship of the ability of S-cysteine conjugates to trans-stimulate the uptake and toxicity of DCV-CYS to their hydrophobicity (SMC>SEC>SBC). Histidine and BCH also showed a substantial trans-stimulation. However in the results presented here, using PCBD-CYS, only SBC showed a strong trans-stimulation. Comparing the cis-inhibition experiments is only partly possible due to the differences in the protocol. Schaeffer and Stevens (1987a) found a strong stimulation of DCV-CYS cytotoxicity due to histidine, where they expected an inhibition. They suggested that in their protocol trans-stimulatory effects could mask cis-inhibition . Nonetheless a strong trans-stimulator as SMC and to a lesser extent BCH, did cause a substantial cis-inhibition in the DCV-CYS cytotoxicity. To prevent a possible masking effect we shortened the exposure time from 14 to 1 h . However in our experiments BCH and SBC were not inhibitory. Neither were they in previous experiments where we used a longer exposure period of 16 h (results not shown). Thus, these differences between our results and those of Schaeffer and Stevens (1987a) indicate that different amino acid transporters are responsible for the uptake of DCV-CYS and PCBD-CYS, possibly due to differences in molecular size.

In the LLC-PK1 cell line System T has not yet been described , but other amino acid transporters like System A, ASC and L are only or primarily present in the basolateral membrane (Rabito and Karish, 1982). This seems to hold for the PCBD-CYS transporter also: basolateral exposure of LLC-PK1 monolayers to PCBD-CYS leads to a higher LDH-release than apical exposure . However the trans-stimulated and cis-inhibited uptake was similar at either side. This apparant discrepancy is currently under investigation.

How important the renal uptake of CYS-conjugates via amino acid transporters in vivo is, remains unclear. Schaeffer and Stevens (1987b), using membrane vesicles of rat kidney cortex , found a Na^+ -stimulated apical DCV-CYS uptake that was not inhibited by BCH. However Lash and Anders (1989), using freshly isolated rat renal proximal tubular cells, found a Na^+ -dependent and a Na^+ -independent DCV-CYS uptake, inhibitable by respectively probenecid or AIB and BCH, suggesting a DCV-CYS uptake by an organic anion transporter and amino acid transporters. Recent results of Zhang and Stevens (1989) question the role of an organic anion transporter in the DCV-CYS uptake. They showed in proximal tubules an inhibition in uptake by probenecid only when using the N-acetyl-S-(1,2-dichlorovinyl)-L- cysteine , and not in the uptake of DCV-CYS. Further investigations using primary cultures might help to solve this matter.

In conclusion, our results show a cytotoxicity of PCBD-CYS in LLC-PK1 monolayers , indicate a preferential basolateral uptake and suggest involvement of an amino acid transporter with characteristics of System T .

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CHAPTER 5

The effect of S-(1,2,3,4,4-pentachlorobutadienyl)glutathione and its derivatives on primary cultures of rat renal cortical cells

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The effect of S-(1,2,3,4,4-pentachlorobutadienyl)glutathione and its derivatives on primary cultures of rat renal cortical cells

SUMMARY

Specific metabolic processes are necessary in the nephrotoxicity of several haloalkenes. Although renal uptake of metabolites is a prerequisite for their toxicity, little is known about the relative importance of organic anion and amino acid transporters in this process.

Apical exposure of monolayers of rat renal cortical cells during 16 h to S-(1,2,3,4,4-pentachlorobutadienyl)glutathione (PCBD-GSH)(20 μM), S-(1,2,3,4,4-pentachlorobutadienyl)-L-cysteine (PCBD-CYS)(20 μM), and N-acetyl-S-(1,2,3,4,4-pentachlorobutadienyl (PCBD-NAC) (200 μM) caused a specific cytotoxicity. This toxicity could be decreased by inhibition of the extracellular γ-glutamyltranspeptidase (γGT) and/or the intracellular pyridoxal phosphate-dependent β-lyase using 0.3 mM acivicin or aminooxyacetic acid (0.5 mM) respectively, indicating the essential role of these enzymes.

Culturing the monolayers on porous substrates enabled exposure of cells from the basolateral side. The presence of an organic anion and cation transporter in those monolayers was demonstrated by a probenecid-sensitive para-aminohippuric acid and a quinine-sensitive tetraethylammonium accumulation. In spite of the accessibility of the basolateral membrane, and the presence of the organic anion transporter, cytotoxicity of PCBD-CYS and PCBD-NAC could neither be inhibited by probenecid (0.5 mM) nor by the amino acid transporter substrates (10 mM) histidine and D-tryptophan.

These results indicate that the renal cellular uptake of conjugates is not the rate-limiting step in the mechanism of toxicity.

ABBREVIATIONS

AOAA, aminooxyacetic acid; CYS, cysteine; DCV-CYS, S-(1,2-dichlorovinyl)-L-cysteine; DCV-NAC, N-acetyl-S-(1,2-dichlorovinyl)-L-cysteine; EBSS, Earle's Balanced Salt Solution; EDTA, ethylenediaminetetraacetic acid; γGT, γ-glutamyltranspeptidase; GSH, glutathione; HBSS, Hanks' Balanced Salt Solution; HCBD, hexachlorobutadiene; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid; LDH, lactate dehydrogenase; NAC, N-acetylcysteine; PAH, para-aminohippuric acid; PCBD-CYS, S-(1,2,3,4,4-pentachlorobutadienyl)-L-cysteine; PCBD-GSH, S-(1,2,3,4,4-pentachlorobutadienyl)glutathione; PCBD-NAC, N-acetyl-S-(1,2,3,4,4-pentachlorobutadienyl)-L-cysteine; TEA, tetraethylammonium; WE, Williams Medium E .

INTRODUCTION

The nephrotoxicity of halogenated alkenes is a resultant of specific metabolic and transport processes in several organs . The first step is a conjugation with glutathione (GSH), a detoxification step which sometimes is the initial step in an activation route (van Bladeren, 1988). Metabolism by γ-glutamyltranspeptidase (γGT) and a dipeptidase results in the formation of a cysteine (CYS)-conjugate which can be acetylated to give a N-acetylcysteine (NAC)-conjugate. In the renal proximal tubular cell the pyridoxal phosphate-dependent β-lyase transforms the CYS-conjugate, formed either after intracellular deacetylation of the NAC-conjugate or extracellular metabolism of the GSH-conjugate, into a reactive thiol, leading to cytotoxicity (Anders et al, 1988; Lock, 1988).

Although several transport systems have been postulated to be responsible for the uptake of NAC-, and CYS-conjugates, little is known about the relative importance of those transporters for the nephrotoxicity. The transporter of NAC-conjugates is probably an organic anion transporter. Probenecid is often used as an inhibitor of this organic anion transport. The uptake of N-acetyl-S-(1,2,3,4,4-pentachlorobutadienyl)-L-cysteine (PCBD-NAC) in rat renal slices was probenecid sensitive (Lock et al, 1986), whereas a probenecid sensitive toxicity has only been demonstrated in vivo (Lock and Ishmael, 1985). Other NAC-conjugates are apparently transported by the same transporter. The uptake of N-acetyl-S-(1,2-dichlorovinyl)-L-cysteine (DCV-NAC) was probenecid sensitive in rat kidney proximal tubules (Zhang and Stevens, 1989) and in rabbit renal slices. The toxic effects of this compound in slices were also probenecid sensitive (Wolfgang et al, 1989). In the uptake of CYS-conjugates recent evidence indicates the involvement of amino acid transport systems. The toxicity of S-(1,2,3,4,4-pentachlorobutadienyl)-L-cysteine (PCBD-CYS) (Mertens et al, 1990) and S-(1,2-dichlorovinyl)-L-cysteine (DCV-CYS) (Schaeffer and Stevens, 1987a) in LLC-PK1 monolayers could be inhibited by amino acid transporter substrates. Influence of these substrates on the uptake of DCV-CYS has been demonstrated in LLC-PK1 cells (Schaeffer and Stevens, 1987a), rabbit renal slices (Wolfgang et al, 1989), and rat kidney cortex membrane vesicles (Schaeffer and Stevens, 1987b).

Since LLC-PK1 monolayers lack a probenecid sensitive organic anion transporter (Mertens et al, 1988; Rabito, 1986) primary cultures of rat renal cortical cells have been used in the present study to investigate the toxicity of a series of conjugates derived from hexachlorobutadiene (HCBD) and the relative importance of various uptake systems for their toxic effects.

MATERIALS AND METHODS

Chemicals

Probenecid was obtained from Sigma (St Louis, MO); acivicin from The Upjohn Company (Kalamazoo, MI); aminooxyacetic acid (AOAA) from Aldrich-Chemie

(Steinheim, FRG); D-tryptophan, and quinine from Janssen Chimica (Beerse, Belgium); histidine from Merck (Darmstadt, FRG); and para-[*glycyl*-1-C]amino-hippuric acid (PAH) (50 mCi/mmol), and [1-^{14}C]tetraethylammonium (TEA) (4.8 mCi/mmol) from New England Nuclear (Boston, MA).

Syntheses

S-(1,2,3,4,4-pentachlorobutadienyl)-L-glutathione (PCBD-GSH), PCBD-NAC, PCBD-CYS were synthesized as described previously (Mertens et al, 1988; Mertens et al, 1990).

Cell isolation and culture

The renal cortical cells were isolated according to Bruggeman et al (1989) with slight modifications. The kidneys were perfused in situ with a Hanks' Balanced Salt Solution without Ca^{2+} and Mg^{2+} (HBSS) containing 20 mM HEPES and 0.8 mM EDTA. After excision of the kidneys the cortex was removed, minced into 1-2 mm pieces, and incubated in 0.125% trypsin-0.05% EDTA in HBSS at 37 ^{o}C in a gently shaking water bath for 20-30 min . After centrifugation (400 g, 3 min) the pellet was resuspended in a solution of 12.5 mg collagenase (Clostridium histolyticum; Boehringer, Mannheim, FRG), 0.8 mg DNase (Grade II; Boehringer, Mannheim), and 31.5 mg $CaCl_2$ in 50 ml of HBSS and placed in a shaking water bath for 10-30 min. During both incubations , the suspension was drawn through a pipette of 10 and 5 ml, respectively at 5-min intervals . After a second centrifugation the pellet was resuspended in 80 ml of Williams Medium E (WE)(Flow Laboratories, Irvine, Scotland), supplemented with 10% fetal calf serum (GIBCO, Glasgow, Scotland), penicillin and streptomycin (50 IU/ml and 100 µg/ml respectively; Gist Brocades, Delft, The Netherlands). Cultures were started on day 0 by pipetting 0.5 ml of the suspension per well in a 24-well tissue culture plate or 2 ml/insert in a tissue culture transwell plate (3412; Costar, Badhoevedorp, The Netherlands). On day 4 the primary cultures were ready for use in the experiments (Bruggeman et al, 1989).

Organic ion transport

Both organic anion transport and organic cation transport was measured using radiolabeled PAH and TEA respectively as model ions . On day 5 the cultures on the porous membranes were gently rinsed with Earles Balanced Salt Solution with 10 mM $NaHCO_3$ and 20 mM HEPES, pH7.4 (EBSS). The inserts were transferred to a culture plate with 25 µM PAH or TEA (sp. act. 4.8 mCi/mmol) in EBSS with or without 0.5 mM probenecid or 0.1 mM quinine, an inhibitor of the organic cation transport. At different times the medium in the apical compartment was added to the one of the basolateral compartment, and the inserts were rinsed with a cold solution (200 mM LiCl, 20 mM Tris, 20 mM HEPES, pH 7.4) . 100 µl Samples of incubation medium, and the membranes of the inserts were transferred to scintillation vials. Before radioactivity was determined the inserts were solubilized for 1.5 h with soluene-350 (Packard, Brussels, Belgium) at 55 ^{o}C in a shaking

water bath. The uptake was expressed as a filter- to-medium ratio (F/M).

Cytotoxicity assay

On day 4 cultures were rinsed with EBSS and exposed to the conjugates dissolved in EBSS. When acivicin was used the cells were pre-incubated for 15 min with this γGT-inhibitor. A short-time exposure period was followed by an overnight incubation in WE before cytotoxicity was determined by measuring lactate dehydrogenase (LDH) leakage (Mertens et al, 1988) . Exposure of primary cultures on porous substrates was conducted two-sided, apically as well as basolaterally.

RESULTS

Apical exposure to conjugates

Primary cultures of rat renal cortical cells were sensitive to all three conjugates. After an overnight apical exposure to 20 μM of PCBD-GSH or PCBD-CYS cytotoxicity was evident. Exposure to PCBD-NAC also resulted in cytotoxicity, but higher concentrations were necessary to elicit the same effect (Fig. 5.1). Inhibition of the brushborder enzyme γGT by the irreversible inhibitor acivicin (0.3 mM) inhibited the cytotoxicity of PCBD-GSH (Fig. 5.2). As expected, the cytotoxicity of PCBD-CYS was not affected. Inhibition of the pyridoxal phosphate-dependent β-lyase by the competitive inhibitor AOAA (0.5 mM) inhibited

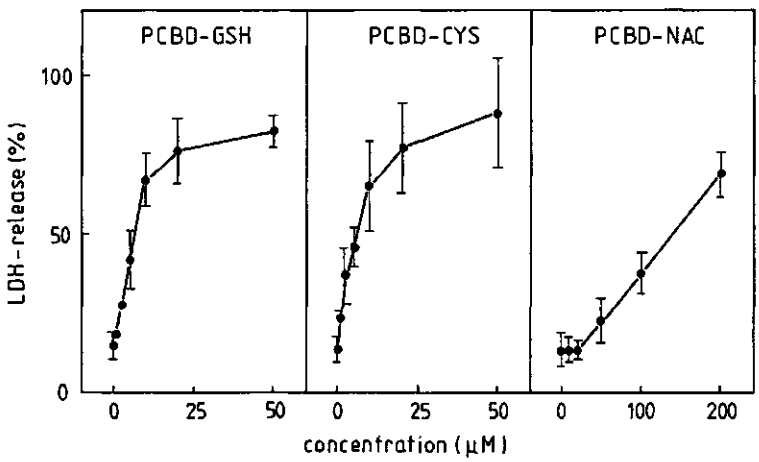

Figure 5.1 Apical exposure of monolayers of rat renal cortical cells to PCBD-GSH, PCBD-CYS and PCBD-NAC. Monolayers of rat renal cortical cells in 24-well tissue culture plates were exposed to PCBD-GSH, PCBD-CYS or PCBD-NAC dissolved in EBSS. After 16 h LDH-release was determined. Results are the mean (±S.D.) of at least three experiments in triplicate.

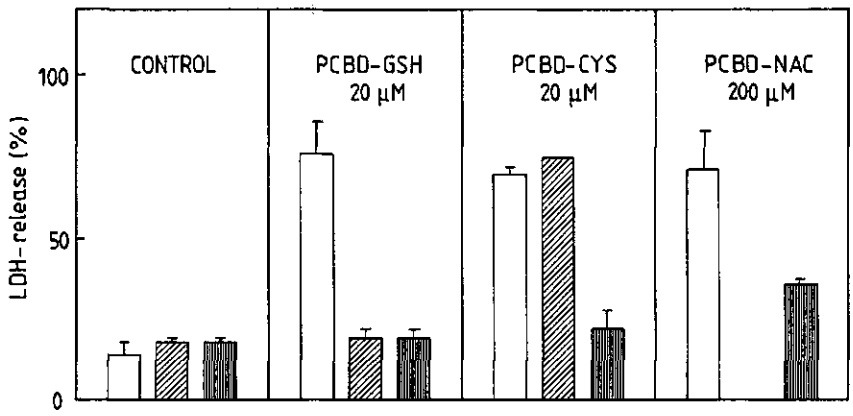

Figure 5.2 Effect of inhibitors of γGT and β-lyase on the toxicity after apical exposure of primary cultures of rat renal cortical cells to PCBD-GSH, PCBD-CYS, and PCBD-NAC. Monolayers of rat renal cortical cells in 24-well tissue culture plates were exposed to PCBD-GSH, PCBD-CYS or PCBD-NAC dissolved in EBSS in the absence (□) or presence of 0.3 mM acivicin (▨) or 0.5 mM AOAA (▥). After 16 h LDH-release was determined. Bars represent the mean (± S.D.) of at least two experiments in triplicate.

the cytotoxicity of all three conjugates, but could not completely prevent the cytotoxicity of PCBD-NAC.

Organic anion and cation transport

The presence of the organic ion transporters was demonstrated by an intracellular accumulation of both the radiolabeled PAH and TEA, in monolayers on porous supports (Fig.5.3). The accumulation of PAH could be inhibited for 40% (after 60 min) by an initial basolateral concentration of 0.5 mM probenecid. Quinine (0.1 mM) caused an inhibition of 52% of the TEA filter to medium ratio (after 60 min).

Investigating the presence of these organic transporters by measuring transcellular transport from the basolateral side to the apical side, as we did previously in LLC-PK1 monolayers (Mertens et al, 1988), proved to be impossible. Comparison in the same experiment of transcellular TEA transport in LLC-PK1 monolayers and in primary cultures on porous membranes, revealed an apparent 17-fold higher transcellular TEA transport in the primary culture than in the LLC-PK1 monolayer within 30 min. Since inhibition with quinine only occurred in the LLC-PK1 monolayers, transcellular transport in the primary cultures was apparently mainly a result of leakage (data not shown).

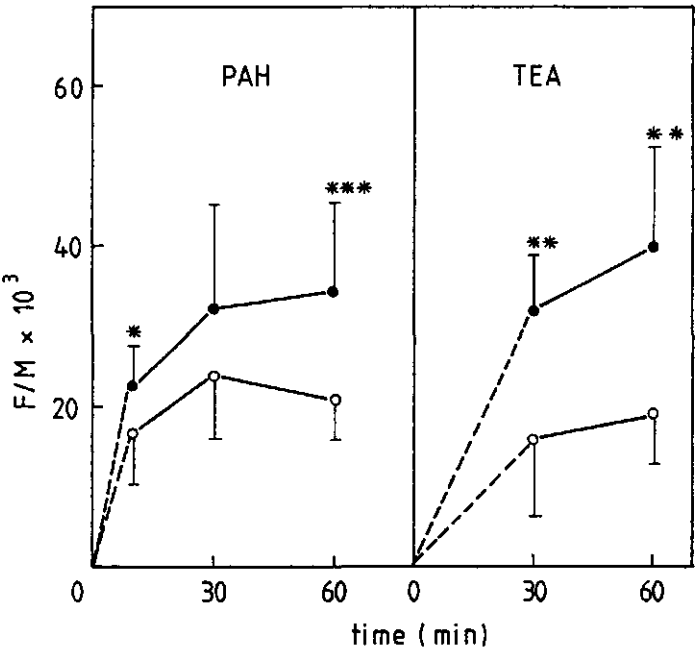

Figure 5.3 Accumulation of PAH and TEA in monolayers of rat renal cortical cells on porous membranes. Cultures were incubated basolaterally with 25 µM radiolabeled PAH or TEA (see methods) with (○) or without (●) inhibitor. Probenecid (0.5 mM) was used as inhibitor of PAH transport and quinine (0.1 mM) was used as inhibitor of TEA transport . Each point represents the mean (± S.D.) of at least three incubations . Values are significantly different at * $p<0.1$, ** $p<0.05$, or *** $p<0.01$ (Wilcoxon-Mann-Whitney).

Cytotoxicity in the presence of transporter substrates

PCBD-NAC has been postulated to be transported into the renal proximal tubular cell by an organic anion transporter. However, 0.1 mM probenecid resulted in an increase of PCBD-NAC toxicity after an overnight apical exposure of primary cultures in 24-well tissue culture plates (Fig.5.4). PCBD-NAC (0.5 mM) toxicity after a short exposure (0.5 or 1.0 h) of primary cultures on porous substrates, with the accessibility of a basolateral organic anion transporter, could not be inhibited by 0.5 mM probenecid (Fig.5.5). This lack of inhibition of toxicity was also found for 0.3 mM PCBD-NAC, the lowest concentration with an exposure time of 1 h that still caused a cytotoxic effect.

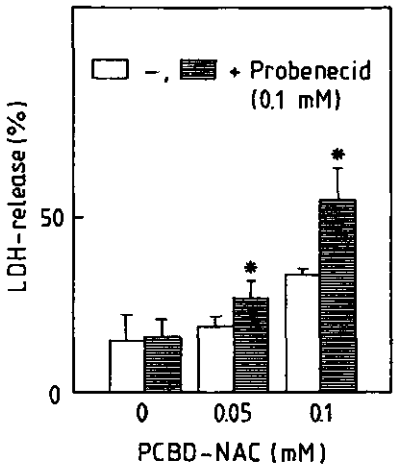

Figure 5.4 Influence of probenecid on the toxicity after apical exposure of monolayers of rat renal cortical cells to PCBD-NAC. Cultures of rat renal cortical cells in 24-well tissue culture plates were exposed apically to PCBD-NAC with or without 0.1 mM probenecid. After 16 h LDH-release was determined. Bars represent the mean (± S.D.) of two experiments in triplicate. Asteriks indicate statistically significant differences in both experiments ($p<0.05$, Wilcoxon-Mann-Whitney).

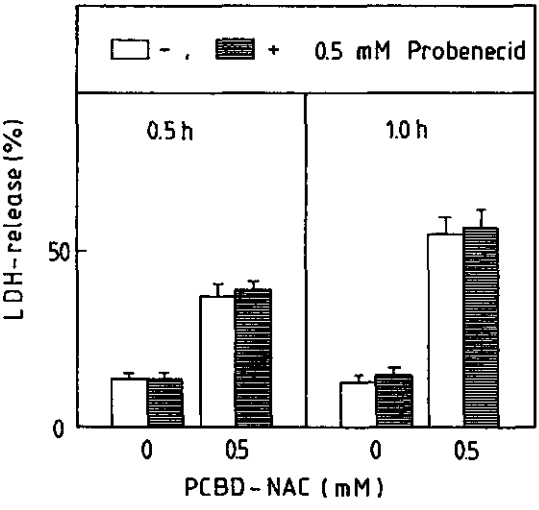

Figure 5.5 Absence of an effect of probenecid on the toxicity after simultaneous apical and basolateral exposure of monolayers of rat renal cortical cells to PCBD-NAC. Cultures of rat renal cortical cells on porous substrates were simultaneously exposed from apical and basolateral sides with or without 0.5 mM probenecid during 0.5 or 1.0 h. After a subsequent incubation without the conjugate in WE, LDH-release was determined. Bars represent the mean (±S.D.) of at least four incubations.

Exposure of monolayers on porous supports during 1 h to PCBD-CYS resulted in a cytotoxicity (46% LDH-leakage) at a concentration of 25 µM already. Neither probenecid (0.5 mM) nor the amino acid transporter substrates (10 mM) histidine and D-tryptophan caused any inhibition of cytotoxicity (data not shown).

DISCUSSION

Nephrotoxicity of PCBD-GSH and its derivatives is the resultant of a complex metabolic pathway and several transport processes. The primary cultures in the present study, obtained by a rapid and simple isolation and culture procedure of rat renal cortical cells (Bruggeman et al, 1989), have a sufficient level of the essential enzymes and uptake systems to elicit a specific cytotoxicity of these conjugates. The presence and involvement of γGT and β-lyase could be demonstrated by a decrease in cytotoxicity after the inhibition of these enzymes.

A transport system generally accepted to be of importance in the nephrotoxicity of HCBD is a basolateral probenecid-sensitive transporter, presumably an organic anion transporter. Lock and co-workers (Lock and Ismael, 1985; Lock et al, 1986) demonstrated inhibition of nephrotoxicity and accumulation of PCBD-NAC in cortical slices by the organic anion probenecid. The presence of both organic anion (PAH) and cation (TEA) transporters in the primary cultures used in the present study, could be demonstrated by measuring intracellular accumulation of the radiolabel, inhibited by probenecid and quinine respectively. In studies with LLC-PK1 monolayers , involvement of amino acid transport systems in cytotoxicity of haloalkene cysteine conjugates could be demonstrated using cytotoxicity as a parameter (Schaeffer and Stevens, 1987a; Mertens et al, 1990). However, in the present study, cytotoxicity due to a two-sided (apical and basolateral) exposure of monolayers to PCBD-CYS and PCBD-NAC could not be inhibited by transporter substrates . The reasons for this may be manifold. In contrast to LLC-PK1 monolayers, metabolism instead of cellular uptake may well be the rate-limiting step in the toxicity of PCBD-NAC and PCBD-CYS in these primary cultures. In addition, the total amount of conjugate metabolized by β-lyase may be influenced by apical carrier-mediated diffusion of conjugates out of the cell during and after the exposure period. The balance between acetylation and deacetylation seems to be of prime importance for toxic effects. N-acetyl-S-tetrafluoroethyl-L-cysteine and N-acetyl-S-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine were deacetylated to a larger extent and thus more cytotoxic than N-acetyl-S-(1,1,-difluoro-2,2-dichloroethyl)-L-cysteine and N-acetyl-S-(1,1,-difluoro-2,2-dibromoethyl)-L-cysteine in freshly isolated rat renal proximal tubular cells (Boogaard et al, 1989). Zhang and Stevens (1989) observed in isolated rat renal proximal tubules that after 20 min still 40% of the DCV-NAC taken up was present intracellularly. Studies with the isolated perfused rat kidney revealed an excretion of PCBD-NAC after perfusion with PCBD-CYS, demonstrating that not all PCBD-CYS is metabolized by β-lyase, but also is acetylated into PCBD-NAC and transported extracellularly (Schrenk et al, 1988). Exposure of rat kidney proximal tubules to radiolabeled DCV-CYS resulted in covalently bound fragments to cellular molecules, and in freely soluble label. Of the latter about 27% was present as DCV-NAC after 5 min (Zhang and Stevens, 1989). Also in freshly isolated rat renal proximal tubular cells S-(1,1-dichloro-2,2-difluoroethyl)-L-cysteine and S-(1,1-difluoro-2,2-dibromoethyl)-L-cysteine were

partly acetylated into the corresponding mercapturates (Boogaard et al, 1989). Thus a short period of time of partly inhibited uptake of PCBD-NAC or PCBD-CYS does not necessarily lead to a decreased cytotoxicity.

Several authors have reported a decreased in vitro toxicity caused by probenecid (Boogaard et al, 1989; Lash and Anders, 1986; Wolfgang et al, 1989). Although probenecid is commonly used as an inhibitor of organic anion transport, it is a rather unspecific inhibitor and has also been demonstrated to influence metabolism. It has been reported to inhibit glycine conjugation of *p*-aminobenzoic acid and *p*-aminosalicylate (Beyer et al, 1950), acetylation of sulfanilamide and choline (Kuriaki and Marumo, 1959), and protein synthesis (Wack et al, 1982). Probenecid itself can also be metabolized; it can be oxidized and glucuronidated. When rats were dosed with probenecid, only less than 1% of the excreted amount in the urine was unchanged parent compound (Emanuelsson and Paalzow, 1989). Thus the reported probenecid sensitivity of toxicity could well be caused by an influence on metabolism. In the present experiments in fact an increase in toxicity after an overnight exposure to PCBD-NAC in the presence of probenecid was observed, illustrating the variability in the effects of this compound. The in vivo inhibition by probenecid of the nephrotoxicity of HCBD, PCBD-GSH, PCBD-CYS, and PCBD-NAC (Lock and Ishmael, 1985) might well be a result of a combination of effects on hepatic metabolism, hepatic transport systems, renal transport systems, and renal metabolism.

In conclusion, the present study demonstrates the presence of the essential enzymes and uptake systems in primary cultures of rat renal cortical cells for a specific toxicity of PCBD-GSH and its derivatives. The absence of an inhibition of toxicity by transporter substrates indicates that the rate-limiting step in the mechanism is not the uptake, but may lie thereafter in the metabolism. Further studies will be necessary to identify this rate-limiting step and to investigate the exact effect of probenecid.

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PART III

ROLE OF γ-GLUTAMYLTRANSPEPTIDASE IN THE NEPHROTOXICITY OF GLUTATHIONE CONJUGATED HALOGENATED HYDROQUINONES

CHAPTER 6

Inhibition of γ-glutamyltranspeptidase potentiates the nephrotoxicity of glutathione conjugated chlorohydroquinones

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Inhibition of γ-glutamyltranspeptidase potentiates the nephrotoxicity of glutathione conjugated chlorohydroquinones

SUMMARY

Administration of either 2,5-dichloro-3-(glutathion-S-yl)-1,4-benzoquinone (DC-(GSyl)BQ) or 2,5,6-trichloro-3-(glutathion-S-yl)-1,4-benzoquinone (TC-(GSyl)BQ) to male Sprague-Dawley rats caused dose dependant (50-200 μmol/kg; i.v.) renal proximal tubular necrosis as evidenced by elevations in blood urea nitrogen (BUN), urinary lactate dehydrogenase (LDH) , γ-glutamyltranspeptidase (γGT) and glucose values as well as histological alterations. Administration of the corresponding hydroquinone conjugates (DC-(GSyl)HQ and TC-(GSyl)HQ), prepared by reducing the quinones with a threefold molar excess of ascorbic acid, resulted in a substantial increase in nephrotoxicity. Surprisingly, and in contrast to other glutathione (GSH) conjugated hydroquinones (Monks et al, 1988b, Lau et al, 1988a) , the nephrotoxicity of both DC-(GSyl)HQ and TC-(GSyl)HQ was potentiated when rats were pretreated with acivicin, an irreversible inhibitor of γGT. Neither quinone-GSH nor hydroquinone-GSH conjugates caused any effect on liver histology or serum glutamate-pyruvate transaminase levels.

The results suggest that coadministration of ascorbic acid with DC-(GSyl)BQ or TC-(GSyl)BQ decreases their interactions with extra-renal nucleophiles including plasma proteins, and thus increases the concentration of the conjugates delivered to the kidney, and hence toxicity. Furthermore the ability of γGT inhibition to potentiate the nephrotoxicity of DC-(GSyl)HQ and TC-(GSyl)HQ suggests that different renal transport mechanisms may be involved in the proximal tubular accumulation of GSH conjugated hydroquinones, and/or that metabolism by γGT can constitute a detoxication reaction for some of these conjugates.

ABBREVIATIONS

BUN, blood urea nitrogen; 2-Br-HQ, 2-bromohydroquinone; 2-Br-(diGSyl)HQ, 2-bromo-(diglutathion-S-yl)hydroquinone; 2-Br-(GSyl)HQ, 2-bromo-(glutathion-S-yl)hydroquinone; DCBQ, 2,5-dichloro-1,4-benzoquinone; DC-(GSyl)BQ, 2,5-dichloro-3-(glutathion-S-yl)-1,4-benzoquinone; DC-(GSyl)HQ, 2,5-dichloro-3-(glutathion-S-yl)-1,4-hydroquinone; DMSO, dimethyl sulfoxide; γGT, γ-glutamyltranspeptidase; GSH, glutathione; HPLC, high performance liquid chromatography; IM, inner medulla; i.p., intraperitoneal; i.v., intravenous; LDH, lactate dehydrogenase; OSOM, outer stripe of the outer medulla; PBS, phosphate buffered saline; SGPT, serum glutamate pyruvate transaminase; TC-(GSyl)BQ, 2,5,6-trichloro-3-(glutathion-S-yl)-1,4-benzoquinone; TC-(GSyl)HQ, 2,5,6-trichloro-3-(glutathion-S-yl)-1,4-hydroquinone; TeCBQ, tetrachloro-1,4-benzoquinone; (triGSyl)HQ, 2,3,5-(triglutathion-S-yl)hydroquinone.

INTRODUCTION

Several halogenated benzenes have been reported to cause nephrotoxicity (Reid, 1973; Chu et al, 1983). The mechanism underlying this effect has only been investigated in detail for bromobenzene: o-Bromophenol, a major metabolite of bromobenzene in rats, caused a more severe necrosis of the proximal tubule than bromobenzene itself (Lau et al, 1984a). The data suggested that o-bromophenol, or a metabolite thereof, was formed in the liver and subsequently transported to the kidney. 2-Bromohydroquinone (2-Br-HQ) was subsequently identified as a major in vivo and in vitro metabolite of o-bromophenol which could reproduce the renal proximal tubular necrosis caused by both bromobenzene and o-bromophenol (Lau et al, 1984b). However, the dose of 2-Br-HQ (0.80 mmol/kg; i.p.) required to cause toxicity was less than 10% of that required by bromobenzene. Further studies revealed the in vitro (Monks et al, 1985) and in vivo (Lau and Monks, 1990) formation of several mono- and di-substituted glutathione (GSH) conjugates of bromohydroquinone and that 2-Br-(diglutathion-S-yl)HQ (2-Br-(diGSyl)HQ) was a very potent nephrotoxicant . The corresponding mono-substituted GSH conjugates were considerably less toxic than the di-substituted analog. (Monks et al, 1985, 1988a). A similar correlation between toxicity and extent of GSH substitution was also found for GSH conjugated hydroquinones which lacked a halogen atom (Lau et al, 1988a).

GSH conjugation, as a rule a detoxication pathway, is for several classes of compounds an activation route (van Bladeren, 1988). GSH conjugation of haloalkenes results, after subsequent extracellular metabolism by γ-glutamyl-transpeptidase (γGT) and a dipeptidase , in the formation of cysteine conjugates . Intracellularly these are converted by cysteine conjugate β-lyase into a reactive thiol, which can rearrange to form a thioacylating intermediate such as a thioketene and/or a thionoacyl halide (Dekant et al, 1987, 1988; Commandeur et al, 1989). The covalent binding of these intermediates to cellular macromolecules probably leads to nephrotoxicity (Anders et al, 1988; Lock , 1988). The first step in this activation pathway, the metabolism by γGT, also appears to be of importance in the nephrotoxicity of GSH conjugated hydroquinones, since inhibition of γGT with acivicin (L-(αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid, AT-125) protected rats from both 2-Br-di(GSyl)HQ (Monks et al, 1988a) and 2,3,5-(triglutathion-S-yl)hydroquinone ((triGSyl)HQ) (Lau et al, 1988a) mediated nephrotoxicity.

At present, no chlorinated GSH conjugated benzo- or hydroquinones have been studied. In the present study we therefore investigated the toxicity of 2,5-dichloro-3-(glutathion-S-yl)benzoquinone (DC-(GSyl)BQ) , 2,5,6-trichloro-3-(glutathion-S-yl)benzoquinone (TC-(GSyl)BQ) and the corresponding hydroquinones. The results show a selective nephrotoxicity which , surprisingly, was potentiated by the acivicin mediated inhibition of γGT.

MATERIALS AND METHODS

Chemicals

GSH was obtained from Janssen (Beerse, Belgium). 2,5-Dichloro-1,4-benzoquinone (DCBQ) was purchased from Kodak (Rochester, NY, USA) and 2,3,5,6-tetrachloro-1,4-benzoquinone (TeCBQ) from Merck-Schuchardt (Hohenbrunn, FRG). Acivicin was a gift from the National Cancer Institute. All other chemicals were of the highest grade commercially available.

Synthesis of GSH conjugates

Quinone GSH conjugates were synthesized according to van Ommen et al (1988) with slight modifications. To a solution of 1.2 mmol TeCBQ in 0.4 l of methanol, a solution of 0.12 mmol GSH in 12 ml of destilled water was added dropwise with constant stirring. The reaction mixture was evaporated in vacuo, the residue was collected in water and ethyl acetate, and the aqueous phase was extracted repeatedly with ethyl acetate. After evaporating the remaining ethyl acetate, the aqueous phase was frozen in dry ice/acetone and lyophilized. Minor impurities appeared to be more water soluble than the major product. They were removed by washing the product with water. After centrifugation the supernatant was discarded and the remaining pellet was frozen and lyophilized. Purity of the product dissolved in DMSO exceeded 95% as determined by high performance liquid chromatography (HPLC) (Shimadzu LC-6A) using a Whatman ODS-3 reversed phase analytical column and a linear gradient of methanol/1 % acetic acid in distilled water (10:90 to 100:0) at a flow rate of 1 ml/min, over 60 min and monitored at 254 nm. The retention time of the product was 26.4 min.The UV-spectrum (200-600 nm) (Shimadzu UV-160) of the compound dissolved in distilled water showed five maxima at 472; 321; 287; 248 and 204 nm. Field desorption mass spectrometry on a MS 902 equipped with a VG-ZAB console showed a signal (m/e 518 (M^+ +3)) at emitter current 26-30 mA (only ^{35}Cl-peak is given), which is in agreement with the protonated 2,5,6-trichloro-1,4-benzoquinone GSH conjugate.

The 2,5,6-trichloro-3-(glutathion-S-yl)hydroquinone (TC-(GSyl)HQ) was prepared by reducing TC-(GSyl)BQ , dissolved in dimethyl sulfoxide (DMSO), using a threefold molar excess of ascorbic acid , dissolved in phosphate buffered saline (PBS) , immediately prior to administration to rats. The extent of reduction was determined by HPLC . Authentic TC-(GSyl)BQ had a retention time of 26.4 min which completely disappeared upon addition of ascorbic acid with the concomitant appearance of a new peak with a retention time of 25.0 min .

Synthesis of the DC-(GSyl)BQ was essentially the same as described above. A GSH solution in distilled water (0.34 mmol in 12 ml) was added dropwise to a solution of 3.4 mmol DCBQ in 0.4 l of methanol. After extraction with ethyl acetate the aqueous phase was frozen in dry ice/acetone and lyophilized. The purity of the product, dissolved in water, exceeded 95 % as determined by HPLC under the conditions as described above. The retention time of the product was

22.4 min. The UV-spectrum revealed maxima at 465; 278; 248 and 204 nm. Field desorption mass spectrometry showed a signal (m/e 484(M^++3)) at emitter current 28-32 mA, corresponding to the protonated form of DC-(GSyl)BQ.

The dichlorohydroquinone glutathione conjugate (DC-(GSyl)HQ) was prepared by dissolving DC-(GSyl)BQ with a threefold molar excess of ascorbic acid in PBS immediately prior to administration to rats. The HPLC peak at retention time 22.4 min which corresponded to authentic DC-(GSyl)BQ, completely disappeared with the concomitant appearance of a new peak at 21.8 min. The UV spectrum of this product was also consistent with the conversion of a quinone to a hydroquinone.

Determination of oxidation potentials

DC-(GSyl)BQ and TC-(GSyl)BQ (0.5 mM) were dissolved in a mixture of DMSO: methanol (1:9; v:v) and diluted to a concentration of 5 µM with the HPLC mobile phase (see below). Oxidation potentials were determined by HPLC (Shimadzu LC-6A) with electrochemical (coulometric respons) detection (ESA Coulochem Model 5100A). The detector was equipped with two porous graphite test electrodes in series. An aliquot of each sample (10 µl; 50 pmol) was injected onto a Partisil 5 ODS-3 reversed phase analytical column (Whatman) and eluted with a mixture of 60% citric acid (12.5 mM)/ ammonium acetate (25 mM) and 40% methanol (pH 4.0) containing 20 mg/L EDTA, at a flow rate of 1 ml/min. A potential of -0.6 volts was applied to the first test electrode to effect reduction of the injected quinones. The applied potential at the second test electrode was varied from -0.2 to +0.3 volts. Peak areas were measured at each potential and expressed as a % of the maximum response obtained.

Animals

Male Sprague-Dawley rats (Harlan Sprague-Dawley, Houston, TX; 115-140 g) were used for all experiments and were allowed food and water ad libitum.

Toxicity studies

Each rat received a freshly prepared solution of the GSH-benzoquinones dissolved in DMSO. DC-(GSyl)HQ and TC-(GSyl)HQ were dissolved in PBS and DMSO/PBS (1/1) respectively. Conjugates (100 µl/100 g) were injected intravenously (i.v.) in the tail.

Inhibition of γGT was achieved by an intraperitoneal (i.p.) injection of acivicin in PBS (10 mg/kg; 200 µl/100 g) one hour before the i.v. injection with the conjugates (Monks et al, 1988a). Rats were housed in metabolism cages and urine was collected and kept in the dark at 4°C individual. After 19 h the volume of the urine was determined and urine was filtered using PD-10 columns (Sephadex, G25M, Pharmacia). A sample of 2.5 ml of urine was brought on the column and eluted with 3.5 ml of saline. The filtered urine was used for determination of lactate dehydrogenase (LDH) activity by measuring the decrease in NADH (0.23 mM) at 340 nm in the presence of pyruvate (0.6 mM). Urinary glucose and

γGT-activity were determined with Sigma Kit Glucose 15-UV, and a Sigma Kit 545, respectively. By orbital puncture 400 µl of blood were collected. Blood urea nitrogen (BUN) was measured with Sigma Kit 535A . Liver damage was assessed by measuring serum glutamate pyruvate transaminase (SGPT) in plasma according to Sigma Kit 505. Rats were euthanized by cervical dislocation, liver and kidneys were removed, and thin slices were placed in phosphate buffered formalin. Sections for histological study of liver and kidney were made and stained with eosin and hematoxylin .

Statistical analysis

All data are expressed as the mean ± standard error. Significance in differences was determined by the Wilcoxon-Mann-Whitney test.

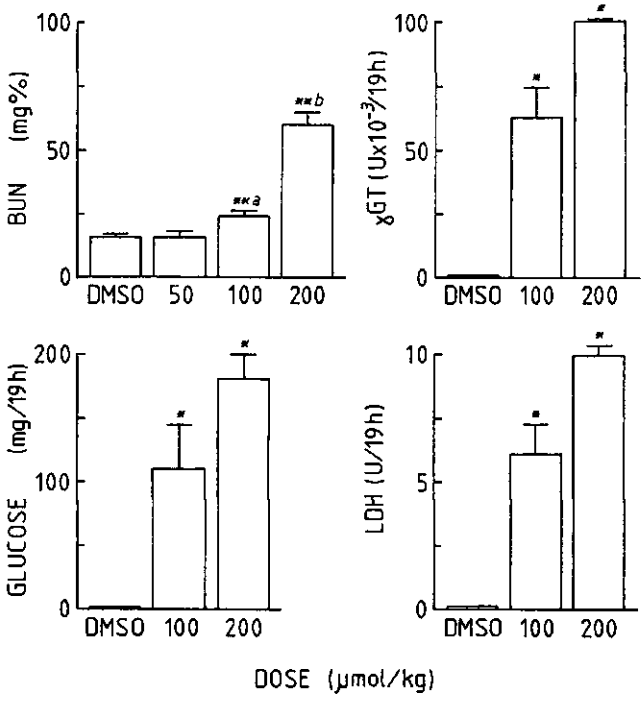


Figure 6.1 Dose-dependent nephrotoxicity of TC-(GSyl)BQ in male Sprague-Dawley rats. Rats were injected (100 µl/100 g) i.v. with TC-(GSyl)BQ dissolved in DMSO. Control animals received only DMSO. Data of BUN and urinary γGT, LDH, and glucose are given as the average ± S.E. (n=2-13). Values are significantly different from control values at: * $p<0.025$ or ** $p<0.005$. Values marked [b] are significantly different from those marked [a] at $p<0.005$ (Wilcoxon-Mann-Whitney).

RESULTS

Nephrotoxicity of DC-(GSyl)BQ and TC-(GSyl)BQ

Administration of either DC-(GSyl)BQ or TC-(GSyl)BQ to male Sprague-Dawley rats caused a dose-dependant increase in BUN concentrations and increases in the urinary excretion of γGT, LDH and glucose (Figs. 6.1 and 6.2). Of the two conjugates studied, TC-(GSyl)BQ appeared to be more toxic than DC-(GSyl)BQ since the former caused enzymuria and glucosuria at a dose of 100 µmol/kg, whereas doses of 150-200 µmol/kg were required to cause similar increases in these parameters by DC-(GSyl)BQ ($p<0.1$).

Histological examination of kidney slices from TC-(GSyl)BQ and DC-(GSyl)BQ treated animals also support the contention that the former conjugate is the more potent nephrotoxicant. For example, animals treated with either 50 or 100 µmol/kg DC-(GSyl)BQ showed little evidence of injury. Injury at these doses was limited to

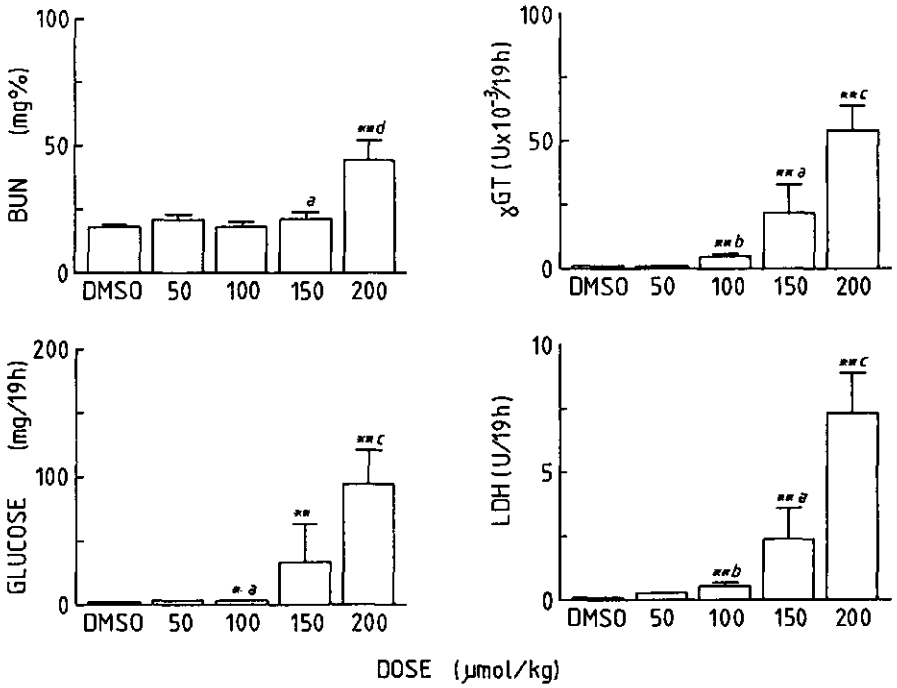


Figure 6.2 Dose-dependent nephrotoxicity of DC-(GSyl)BQ in male Sprague-Dawley rats. Rats were injected (100 µl/100 g) i.v. with DC-(GSyl)BQ dissolved in DMSO. Control animals received only DMSO. Data of BUN and urinary γGT, LDH, and glucose are given as the average ± S.E. (n=4-8). Values are significantly different from control values at: * $p<0.025$ or ** $p<0.005$. Values are significantly different from those marked [a] at: [b] $p<0.1$; [c] $p<0.025$ or [d] $p<0.005$ (Wilcoxon-Mann- Whitney).

individual cell death and necrosis, apparant as eosinophilic cells with pyknotic nuclei, scattered throughout the outer stripe of the outer medulla (OSOM) (Figs. 6.3 and 6.4). In contrast, animals treated with 100 μmol/kg TC-(GSyl)BQ

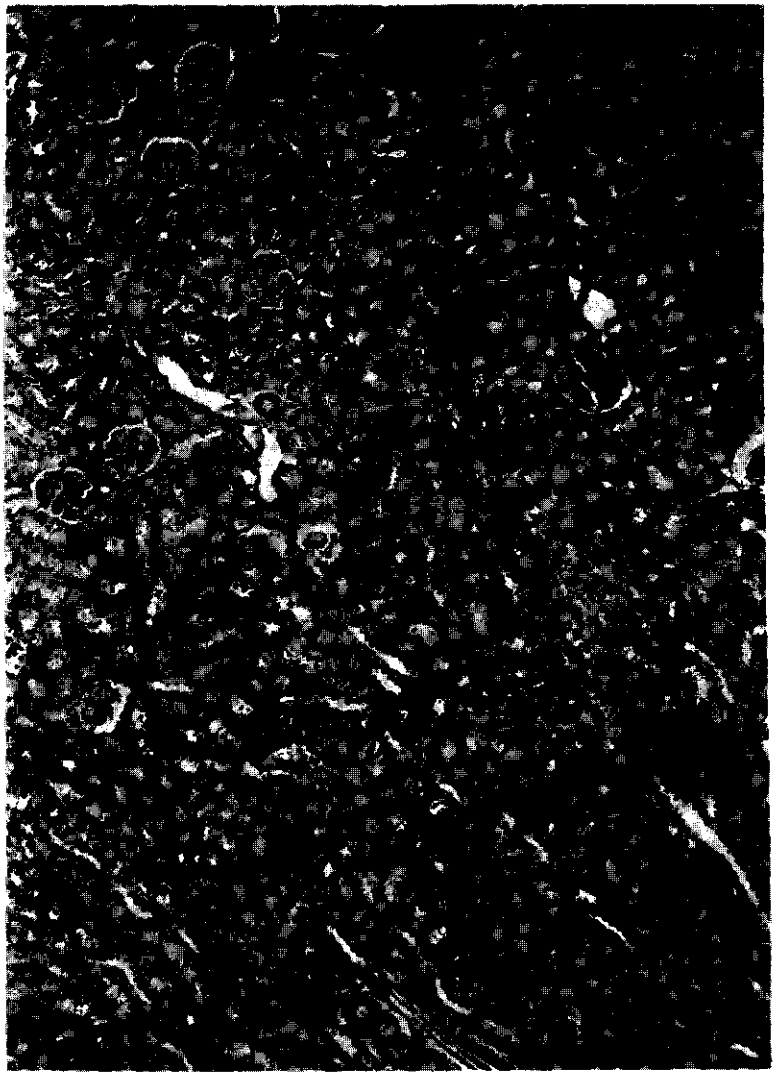


Figure 6.3 The renal cortex (C) and OSOM (O) 19 hours following the administration of 100 μmol/kg DC-(GSyl)BQ. Tubular injury is limited to single cell death and necrosis, as indicated by the dark staining (eosinophilic) cells with pyknotic nuclei, throughout the OSOM (arrowheads). Paraffin section stained with hematoxylin and eosin (x72).

displayed injury ranging from focal necrosis at the junction of the cortical S_3 (S_3 C) and the medullary S_3 (S_3 M) segment at the base of the medullary rays (MR), to more extensive injury forming a rim of necrosis at the junction of the cortex and the OSOM (Fig. 6.5). Single cell death and necrosis was also evident throughout the OSOM. Sublethally injured cells of the S_3 segment appeared highly vesiculated. At 150 µmol/kg DC-(GSyl)BQ proximal tubular injury was limited to the S_3 M segment. The injury ranged from individual cell death and necrosis involving the proximal portion of the S_3 M at the top of the OSOM, to a somewhat more severe injury which included focal tubular necrosis at the junction of S_3 C and S_3 M at the base of the MR. At 200 µmol/kg DC-(GSyl)BQ, individual cell death and necrosis progressed to involve much of the OSOM, with small foci of tubular necrosis evident at the junction of the S_3 C and S_3 M at the base of the MR (Fig. 6.6). Sublethally injured cells in the OSOM showed evidence of cytosolic vesiculation. Treatment of the animals with 200 µmol/kg TC-(GSyl)BQ caused a total loss of the OSOM with involvement of the lower portion of the MR

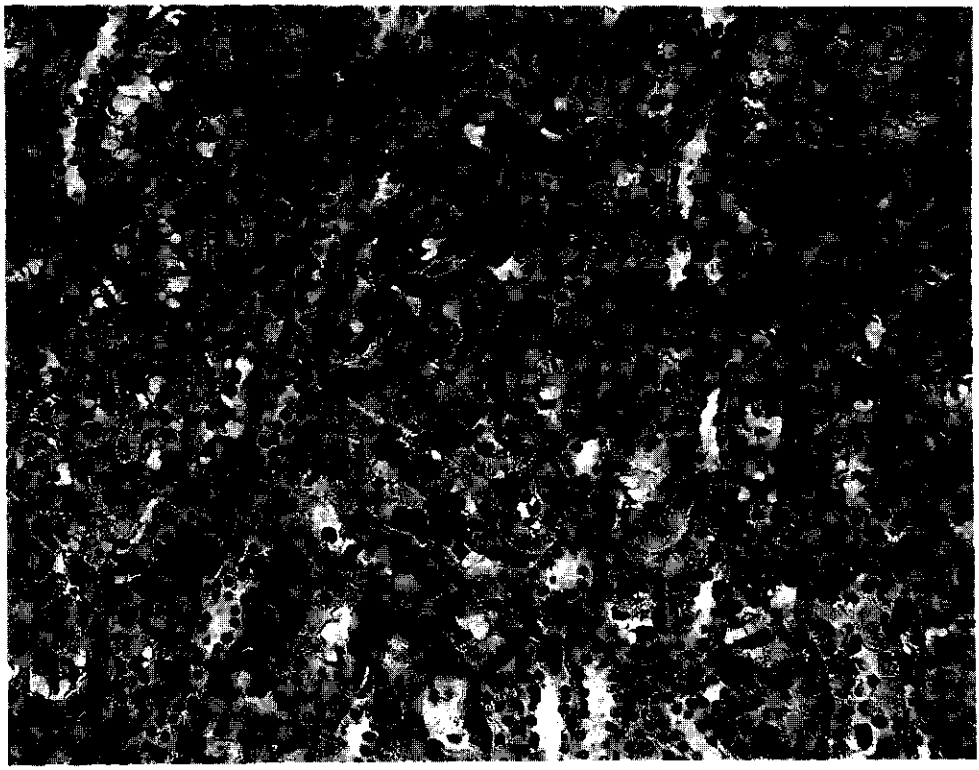
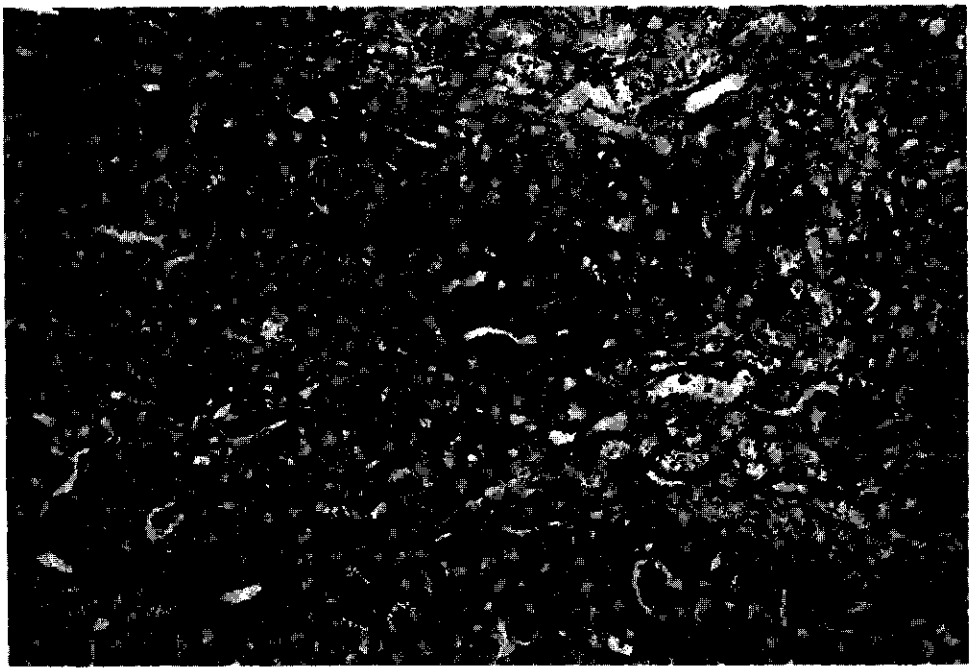


Figure 6.4 Higher magnification of the OSOM following the administration of 100 µmol/kg DC-(GSyl)BQ. Showing evidence of single cell death and necrosis affecting cells of the proximal tubule (arrowheads). Paraffin section stained with hematoxylin and eosin (x184).



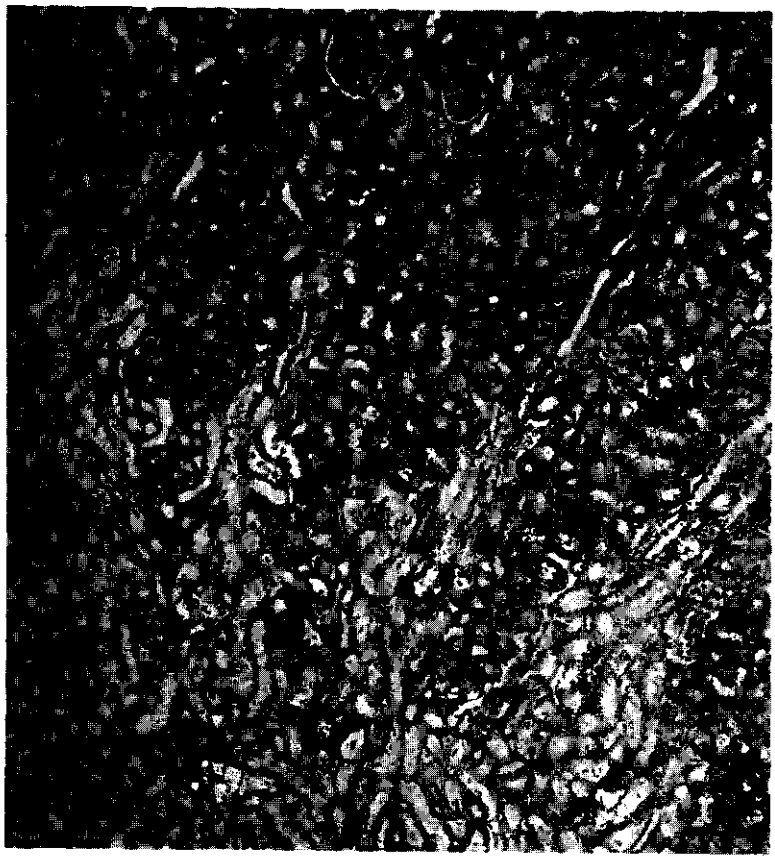


Figure 6.7 The renal cortex (C) and OSOM (O) 19 hours following the administration of 200 µmol/kg TC-(GSyl)BQ. Widespread proximal tubular necrosis is evident throughout the S_3 M segment in the OSOM (*). Paraffin section stained with hematoxylin and eosin (x64).

Figure 6.5 The renal cortex (C) and OSOM (O) 19 hours following the administration of 100 µmol/kg TC-(GSyl)BQ. Proximal tubular necrosis can be observed involving the S_3 segment at the junction of the medullary ray and the OSOM (*). Paraffin section stained with hematoxylin and eosin (x64).

Figure 6.6 The renal cortex (C) and OSOM (O) 19 hours following the administration of 200 µmol/kg DC-(GSyl)BQ. Necrosis is apparent primarily affecting the S_3 segment of the proximal tubule at the junction of the medullary ray and the OSOM (*). Paraffin section stained with hematoxylin and eosin (x64).

(S_3 C segment)(Fig. 6.7). The cortical proximal tubules (S_1 and S_2) and lower nephron segments appeared unaffected in both DC-(GSyl)BQ and TC-(GSyl)BQ treated rats. The morphology observed in all DMSO treated control animals was

Figure 6.8 Normal histology of the renal cortex (C) and OSOM (O) from a control animal. Paraffin section stained with hematoxylin and eosin (x64).

normal for immersion fixed rat kidney (Fig. 6.8). Thus based upon both biochemical and histological criteria, TC-(GSyl)BQ is a more potent nephrotoxicant than DC-(GSyl)BQ.

Effects of ascorbic acid on DC-(GSyl)BQ and TC-(GSyl)BQ-mediated nephrotoxicity

Coadministration of ascorbic acid with either DC-(GSyl)BQ (200 µmol/kg) or TC-(GSyl)BQ (100 and 200 µmol/kg) clearly potentiated the nephrotoxicity of both compounds. Thus when the conjugates were administered in the reduced, hydroquinone form, BUN concentrations were more than double those observed in animals treated with the quinone-GSH conjugates (Figs. 6.9 and 6.10). This protocol also amplified the urinary excretion of glucose (DC-(GSyl)HQ, 200 µmol/kg, ; Fig. 6.9). However, with TC-(GSyl)HQ, urinary glucose excretion

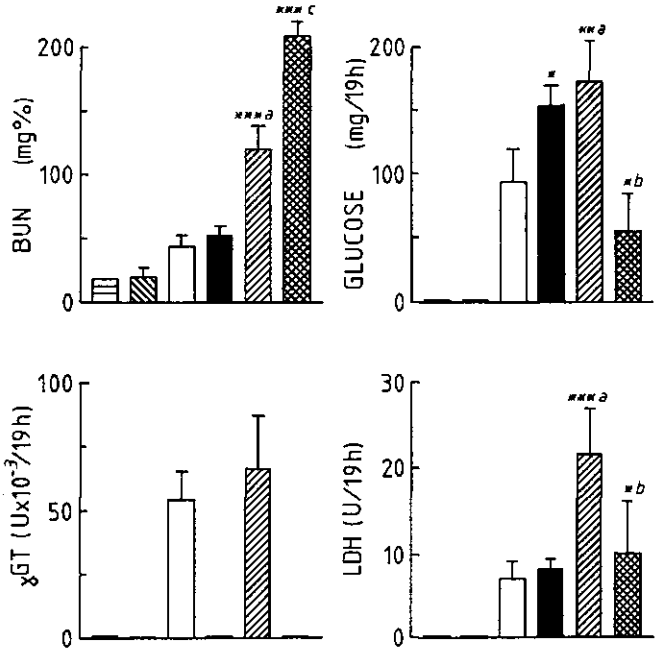


Figure 6.9 The effect of ascorbic acid and acivicin on the nephrotoxicity of DC-(GSyl)BQ. Male Sprague-Dawley rats were injected i.p. with acivicin in PBS (10 mg/kg; 200 µl/100 g) 1 h prior to DC-(GSyl)BQ dosing. DC-(GSyl)BQ (200 µmol/kg) was injected i.v. either alone (□) or after reduction with ascorbic acid (0.6 mmol/kg) (▨). Control animals received DMSO (▤) or acivicin and ascorbic acid dissolved in PBS (▧). Further experimental groups received DC-(GSyl)BQ plus acivicin (■) or plus a combination of acivicin and ascorbic acid (▩). Data of BUN and urinary glucose, γGT, and LDH are given as the average ± S.E. (n=3-8). Values are significantly different when compared to rats which received DC-(GSyl)BQ alone at: * $p<0.1$, ** $p<0.05$ or *** $p<0.025$. Values are significantly different from [a] at: [b] $p<0.1$ or [c] $p<0.025$ (Wilcoxon-Mann-Whitney).

was equal (100 µmol/kg) or even lower (200 µmol/kg) than in the quinone treated animals, which probably reflects a decrease in glomerular filtration rates concomitant with the severe toxicity. As noted in previous studies (Lau and Monks, 1990) the urinary excretion of γGT appears to be the most sensitive indicator of proximal tubular necrosis caused by quinol/quinone-thioethers. Thus the urinary excretion of γGT was not increased by coadministration of the quinone conjugates with ascorbic acid (Figs. 6.9 and 6.10). This is probably because the doses of DC-(GSyl)HQ and TC-(GSyl)HQ used in this study cause a maximal excretion of this brush border enzyme into urine. Indeed, when TC-(GSyl)HQ, at a dose of 200 µmol/kg, was administered, the appearance of γGT in urine actually decreased. This occurred despite the severe necrosis observed histologically (see below). The decrease in γGT activity may be a consequence of the ability of quinone-thioethers to apparently inhibit this enzyme (Hill et al, 1990). Administration of DC-(GSyl)HQ and TC-(GSylHQ did also cause statistically significant potentiation of the urinary excretion of LDH (Figs. 6.9 and 6.10).

Histological examination of kidney slices from animals coadministered ascorbic acid confirmed the potentiating effects of this protocol. In DC-(GSyl)HQ (200 µmol/kg) and TC-(GSyl)HQ (100 µmol/kg) treated animals extensive proximal tubular cell necrosis was observed, involving all of the OSOM and the MR (Figs. 6.11 and 6.13). Tubular necrosis was also found to involve the cortical proximal tubules. This injury appeared to primarily involve the S_2 segment in that all of the neck segments (S_1) were normal (DC-(GSyl)HQ (Fig. 6.12), TC-(GSyl)HQ)(Fig. 6.14) or at worse sublethally (TC-(GSyl)HQ) injured. Tubular casts were observed in the cortex and IM. At 200 µmol/kg TC-(GSyl)HQ, the

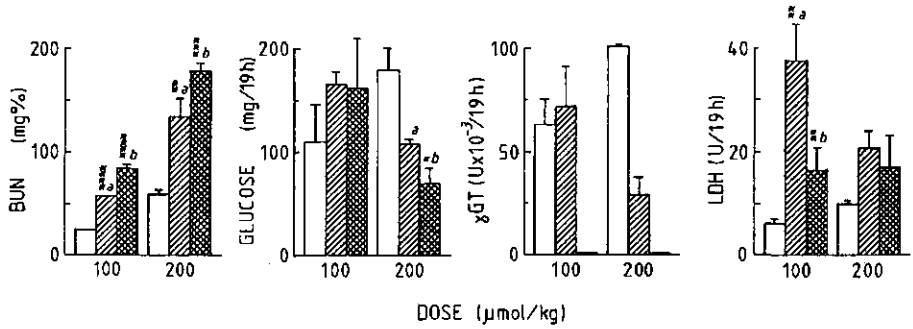


Figure 6.10 The effect of ascorbic acid and acivicin on the nephrotoxicity of TC-(GSyl)BQ. Male Sprague-Dawley rats were injected i.p. with acivicin in PBS (10 mg/kg; 200 µl/100 g) 1 h prior to TC-(GSyl)BQ dosing. TC-(GSyl)BQ was injected i.v. either alone (□) or after reduction with ascorbic acid (0.6 mmol/kg) (▨). Animals which received TC-(GSyl)BQ in combination with ascorbic acid and acivicin are indicated as ▩. Data of BUN and urinary glucose, γGT, and LDH are given as the average ± S.E. (n=2-13). Values are significantly different when compared to rats which received TC-(GSyl)BQ alone at: * $p<0.1$, ** $p<0.05$, *** $p<0.025$ or **** $p<0.005$. Values are significantly different from [a] at: [b] $p<0.1$ (Wilcoxon-Mann-Whitney).

extensive necrosis involved nearly the entire proximal tubule. Large casts and cystic spaces were observed in the cortex. The tubular casts containing necrotic debris were found to extend down into the IM. The lower nephron segments still

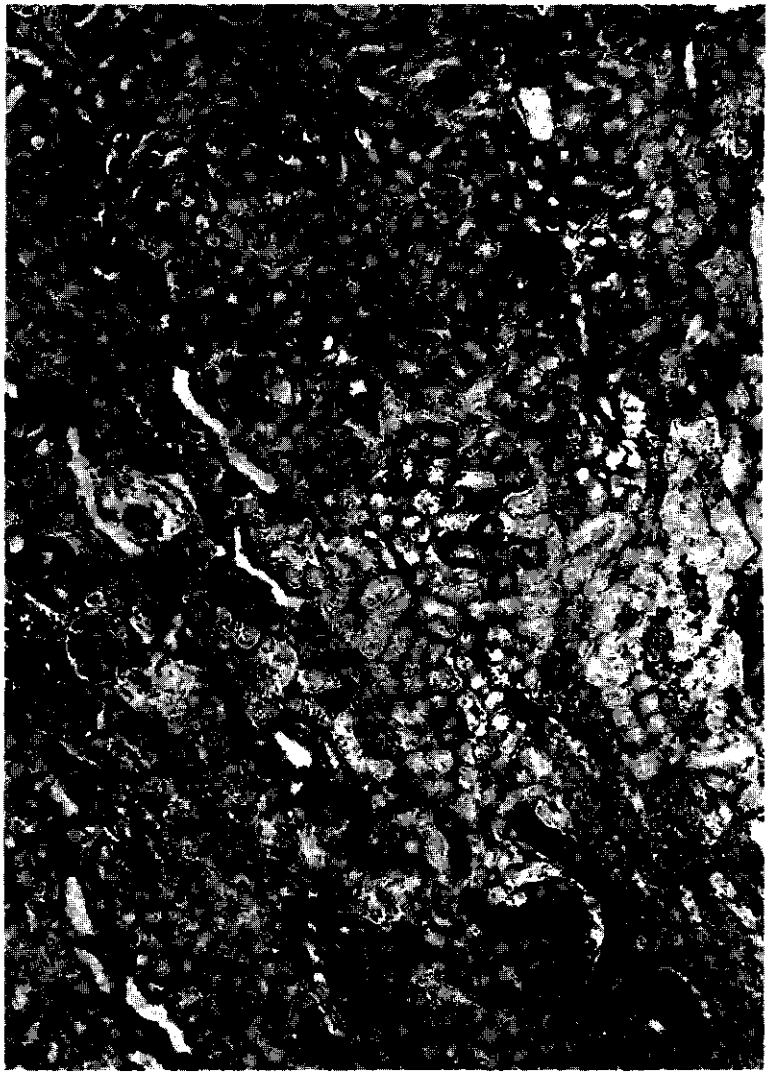


Figure 6.11 The renal cortex (C) and OSOM (O) 19 hours following the administration of 200 µmol/kg DC-(GSyl)BQ after reduction with 600 µmol/kg ascorbic acid. Widespread necrosis involving the proximal tubule throughout the cortex and the OSOM. Hyaline casts, frequently containing calcified debris, are evident (arrowheads). Cells of the lower uriniferous tubule (including ascending thick limb, distal tubule and collecting duct) appear unaffected. Paraffin section stained with hematoxylin and eosin (x64).

appeared unaffected.

Figure 6.12 Higher magnification of the renal cortex following the administration of 200 µmol/kg DC-(GSyl)BQ after reduction with 600 µmol/kg ascorbic acid. Note the necrotic proximal tubules surrounding the glomeruli (G). The cortical injury appears to primarily affect the cells of the S_2 segment; all of the neck (S_1) segments (arrowhead) appear normal. Hyaline casts containing calcified debris are also present (*). Paraffin section stained with hematoxylin and eosin (x180).

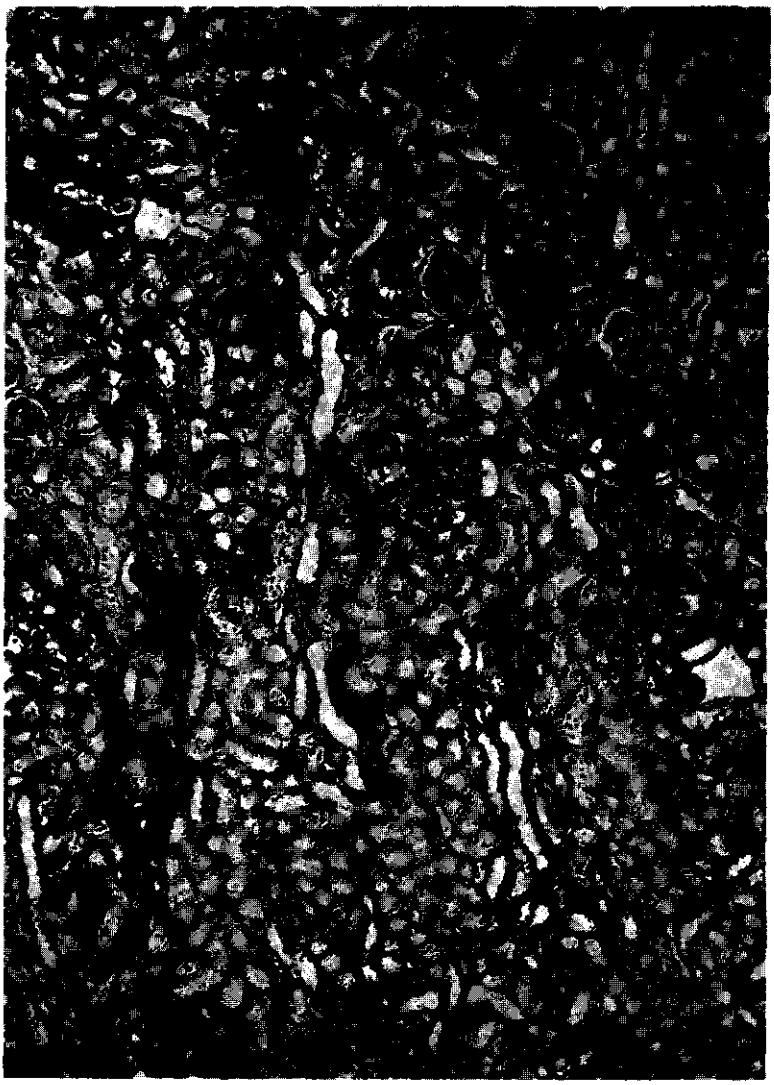


Figure 6.13 The renal cortex (C) and OSOM (O) 19 hours following the administration of 100 µmol/kg TC-(GSyl)BQ after reduction with 300 µmol/kg ascorbic acid. Proximal tubular necrosis is evident involving the S_3 segment throughout the OSOM and medullary ray. Many of the cortical tubules surrounding the glomeruli are also affected. Hyaline casts (arrowheads) are also evident in this animal. Paraffin section stained with hematoxylin and eosin (x64).

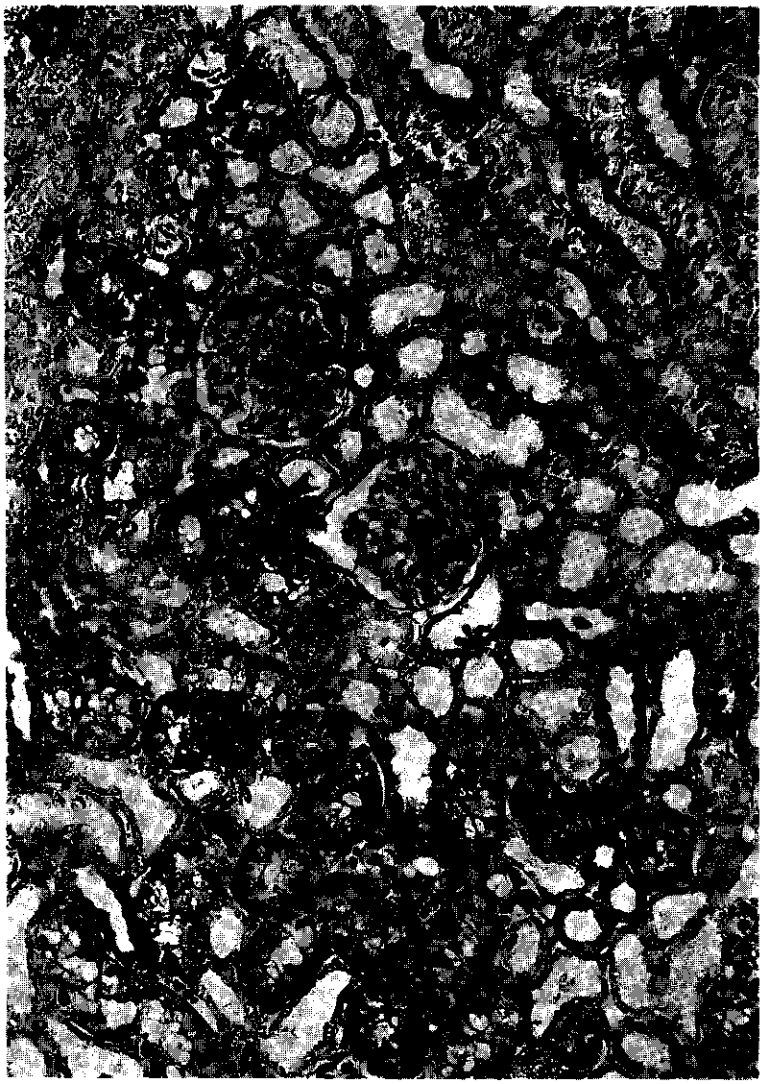


Figure 6.14 Higher magnification of the renal cortex following the administration of 100 µmol/kg TC-(GSyl)BQ after reduction with 300 µmol/kg ascorbic acid. Many of the proximal tubules around the glomeruli (G) are necrotic (*). The cortical injury appears to primarily affect the cells of the S_2 segment; all of the neck (S_1) segments (arrowhead) appear normal. Paraffin section stained with hematoxylin and eosin (x180).

Effects of acivicin on DC-(GSyl)BQ and TC-(GSyl)BQ-mediated nephrotoxicity

Urinary glucose excretion was greater in animals pretreated with acivicin, an irreversible inhibitor of γGT, prior to DC-(GSyl)BQ (200 µmol/kg) administration

than in animals treated with DC-(GSyl)BQ alone (Fig. 6.9). BUN concentrations and urinary LDH values were unaffected and γGT could not be measured due to inhibition by acivicin. Interestingly, the pattern of injury seen in these animals was different to that seen in animals treated with DC-(GSyl)BQ alone. Tubular necrosis with calcification was observed, involving the terminal portion of the S_3 M segment at the junction of the OSOM and the inner stripe of the outer medulla. The more proximal S_3 M and S_3 C segment in the MR appeared less severely injured, with individual cell necrosis and cytosolic vesiculation in the sublethally injured cells. Some casts were evident in the cortex, although the cortical proximal tubule cells appeared to be unaffected. The cells of the lower nephron remained unaffected. Thus, acivicin pretreatment clearly did not protect these animals from DC-(GSyl)BQ mediated nephrotoxicity.

Pretreatment of DC-(GSyl)BQ/ascorbate treated animals with acivicin actually potentiated the nephrotoxicity of the reduced conjugate, as evidenced by further increases in BUN concentrations (Fig. 6.9) and by macroscopic and histological examination of kidney slices. In addition, the decrease in glucose excretion previously observed with severely toxic doses of TC-(GSyl)HQ, was now seen in DC-(GSyl)HQ treated animals (Fig. 6.9). Histological examination revealed an extensive proximal tubule cell injury, similar to that described for the DC-(GSyl)HQ treated group. Moreover, calcification of necrotic tubule cells and cast material was widespread. Even the S_1 segment showed severe injury with massive cytosolic vesiculation. Perhaps surprisingly, the lower nephron segments still appeared unaffected. There was clearly no evidence for protection from DC-(GSyl)HQ mediated nephrotoxicity with acivicin , rather, acivicin treated animals displayed a greater degree of overall injury.

Pretreatment of TC-(GSyl)HQ treated animals with acivicin resulted in higher BUN concentrations than in either TC-(GSyl)BQ or TC-(GSyl)HQ treated animals without acivicin, at both the 100 µmol/kg and 200 µmol/kg dose levels (Fig.6.10). Moreover, at the higher dose, the glucosuria induced by TC-(GSyl)HQ was further reduced by pretreatment with acivicin. The effects of acivicin on TC-(GSyl)HQ induced urinary excretion of LDH were equivocal. At 100 µmol/kg TC-(GSyl)HQ, the injury ranged from involvement of the proximal tubule in the OSOM and MR (S_3 M and S_3 C), with no apparent involvement of the cortical tubules, to more extensive injury including focal to extensive necrosis of the cortex. The pattern of injury seen at 200 µmol/kg TC-(GSyl)HQ was similar in animals with or without acivicin pretreatment (Fig. 6.15). Injury appears maximal at this dose. Again, therefore, there was no evidence for protection with acivicin. Conversely, the increases in BUN and decrease in urinary glucose, and the pathological observations at 100 µmol/kg TC-(GSyl)HQ, are indicative of potentiation by acivicin.

Neither treatment with DC-(GSyl)BQ/HQ nor TC-(GSyl)BQ/HQ caused any hepatocellular alterations as evidenced by SGPT values (control, 46.9 ± 12.7 units/liter; 200 µmol/kg DC-(GSyl)BQ/ascorbic acid/acivicin, 54.9 ± 8.6 units/liter; 200 µmol/kg TC-(GSyl)BQ/ascorbic acid/acivicin, 34.8 ± 4.8 units/liter) or histological examination (not shown).

Figure 6.15 The renal cortex (C) and OSOM (O) 19 hours following the administration of 200 µmol/kg TC-(GSyl)BQ after reduction with 600 µmol/kg acorbic acid and pretreatment of the animals with 10 mg/kg acivicin. Cell injury and necrosis are apparent involving all segments of the proximal tubule. Hyaline casts, often containing calcified debris, are evident throughout the cortex and OSOM (arrowheads). Cells of the lower uriniferous tubule (including ascending thick limb, distal tubule and collecting duct) appear unaffected. Paraffin section stained with hematoxylin and eosin (x72).

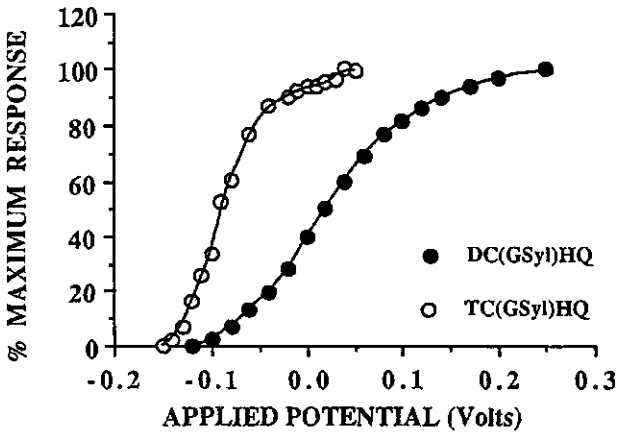


Figure 6.16 Hydrodynamic voltammograms of DC-(GSyl)HQ and TC-(GSyl)HQ. Oxidation potentials were determined by HPLC with electrochemical detection equipped with two porous graphite electrodes in series. A potential of -0.6 V was applied to the first electrode to affect reduction of the injected quinones. The applied potential at the second electrode was varied from -0.2 to +0.3 V. Peak areas were measured at each potential and expressed as % of the maximum response obtained. Under the used conditions (see methods), DC-(GSyl)BQ and TC-(GSyl)BQ were eluted with retention times of 4.8 and 8.4 min and the corresponding hydroquinones exhibited half-wave potentials ($E_{1/2}$) of +19 mV and -89 mV respectively.

Electrochemistry of DC-(GSyl)BQ and TC-(GSyl)BQ

Hydrodynamic voltammograms of the reduced, hydroquinone forms of DC-(GSyl)BQ and TC-(GSyl)BQ are illustrated in figure 6.16 . Reduction of DC-(GSyl)BQ and TC-(GSyl)BQ was achieved electrochemically, subsequent to their separation by HPLC, by applying a potential of -0.6 V to the first of two porous graphite electrodes connected in series. The half-wave oxidation potentials of the reduced conjugates, in protonic media at pH 4.0, were +19 mV and -89 mV respectively. Consequently, the reduced thrichloro- conjugate will be more readily oxidized than the dichloro-analog.

DISCUSSION

In the present study we have shown that both DC-(GSyl)BQ and TC-(GSyl)BQ are renal proximal tubule cell toxicants. Coadministration of these compounds with ascorbic acid, which effectively produces the corresponding hydroquinone-GSH conjugates increased the nephrotoxicity. Quinones can readily react with a variety

of cellular and extracellular nucleophiles. It is possible therefore, that a significant fraction of the DC-(GSyl)BQ and TC-(GSyl)BQ administered to rats reacts with nucleophilic sites on plasma proteins or other extrarenal macromolecules, substantially reducing the effective dose delivered to the kidneys. Reducing the quinone with ascorbic acid decreases the extra-renal removal of the conjugates and increases delivery to the kidney, thus a greater toxicity ensues.

Based upon both biochemical and histological criteria, TC-(GSyl)BQ and its corresponding hydroquinone, were more potent nephrotoxicants than either DC-(GSyl)BQ or its hydroquinone analog. Consistent with these findings, the TC-(GSyl)HQ conjugate exhibited a lower oxidation potential than the DC-(GSyl)HQ conjugate (Fig. 6.16). The additional chlorine atom in TC-(GSyl)HQ clearly facilitates its ready oxidation. We have previously shown that oxidation, rather than metabolism to reactive thiols catalysed by cysteine conjugate β-lyase, plays an important role in the nephrotoxicity of several benzoquinol-thiol conjugates (Monks et al, 1988 a,b; Lau et al, 1988a). The present data are consistent with these findings. The site and mechanism of oxidation of the DC-(GSyl)HQ and TC-(GSyl)HQ conjugates are unknown but warrant further investigation.

The renal specific toxicity of both 2-Br-(diGSyl)HQ and 2,3,5-(triGSyl)HQ appears to be a consequence of the high activity of γGT within the membrane of proximal tubule cells. The activity of γGT appears to be necessary for the accumulation of these conjugates into renal cells (Lau et al, 1988b) and also, perhaps for the activation of the conjugates by facilitating oxidation (Monks and Lau, 1990). Thus, pretreatment of rats with acivicin to inhibit γGT, protected the animals from both 2-Br-(diGSyl)HQ- and 2,3,5-(triGSyl)HQ-mediated nephrotoxicity (Monks et al, 1988a; Lau et al, 1988a). Interestingly however, acivicin pretreatment failed to protect rats from either DC-(GSyl)HQ- or TC-(GSyl)HQ-mediated nephrotoxicity. Rather, acivicin actually potentiated the toxic effects of these compounds. This observation suggests that, at least for the present conjugates, metabolism via γGT may lead to detoxication. Support for this interpretation is provided by recent studies on the γGT catalyzed metabolism of 2-Br-3-(GSyl)HQ (Monks et al, 1990). The products of this reaction, the cystein-S-ylglycine and cystein-S-yl conjugates, undergo an oxidative cyclization reaction that results in 1,4-benzothiazine formation. This is, in effect, an intramolecular detoxication reaction, since it results in removal of the reactive quinone function from the molecule. This same reaction appears to be occurring with both DC-(GSyl)HQ and TC-(GSyl)HQ. We observed the appearance of a characteristic green precipitate in urine of rats treated with both these conjugates, indicative of benzothiazine formation. Interestingly, pretreatment of animals with acivicin, and thus inhibition of γGT, prevented the appearance of the green precipitate in urine.

The reasons for the differential effects of acivicin on quinol/quinone-GSH conjugate-mediated nephrotoxicity are unclear, but are probably related to the relative rates at which the products of the γGT catalyzed reaction undergo cyclization (detoxication) and macromolecular alkylation (toxication). The ability of

both DC-(GSyl)HQ and TC-(GSyl)HQ to cause severe proximal tubular necrosis, under conditions where γGT activity is almost completely inhibited, also raises several questions. For example, since it is generally accepted that the activity of brush border γGT is required for the transport of GSH (as its corresponding constituent amino acids) and its S-conjugates, from the tubular lumen into proximal tubule cells, how do DC-(GSyl)HQ and TC-(GSyl)HQ gain access to these cells? In addition, the peritubular extraction of GSH and S-conjugates via the basolateral membrane is also a significant physiological process (Curthoys, 1990), and might also contribute to the renal uptake of quinol-GSH conjugates. Such uptake may be mediated either by γGT which is associated with the basolateral membrane (Abbott et al, 1984; Rankin et al, 1985) or via an electrogenic, Na^+-dependent transport system. Indeed it has been suggested that the Na^+-dependent, probenecid sensitive, transport system exists for the transport of GSH-conjugates (Lash and Jones, 1985). Although probenecid offered only slight protection against 2-Br-(diGSyl)HQ (Monks et al, 1988a) and failed to protect rats from 2,3,5-(triGSyl)HQ-mediated nephrotoxicity (Lau et al, 1988a), it is possible that this system contributes significantly to the transport of DC-(GSyl)HQ and TC-(GSyl)HQ. The relative importance of each of these pathways to the renal uptake of quinol-GSH conjugates is unclear and requires further study.

Alternatively, it is possible that quinone-GSH conjugates express their toxicity extracellularly. In this case, transport across a membrane would be unneccessary. This scenario would also implicate the membrane as a principal target of these compounds. The observation that the presence of γGT in urine is a sensitive indicator of the toxicity of these compounds supports the brush border membrane as a potential target. The extremely high reactivity of the quinone conjugates with protein thiols would be in line with this suggestion (van Ommen et al, 1988). An extracellular mode of action of these compounds would also have to explain the tissue selectivity of these conjugates. The physiological function of the kidney may provide the rationale behind the specificity of these compounds. The cells of the kidney are constantly exposed to higher concentrations of chemicals than cells of most other organs, as a consequence of its normal function of filtration, reabsorption and excretion.

In conclusion we have demonstrated that the nephrotoxicity of DC-(GSyl)BQ and TC-(GSyl)BQ can be potentiated by reduction of these conjugates with ascorbic acid, presumably due to an increased delivery of the reduced conjugates to the kidney. The unexpected increase in nephrotoxicity after inhibition of γGT activity suggests that different renal transport mechanism may be involved in the proximal tubular accumulation of GSH conjugated hydroquinones, and/or that metabolism by γGT can constitute a detoxication reaction for some of these conjugates.

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CHAPTER 7

In vitro cytotoxicity of nephrotoxic glutathione conjugated halohydroquinones. Influence of aqueous stability, and γ-glutamyltranspeptidase

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In vitro cytotoxicity of nephrotoxic glutathione conjugated halohydroquinones. Influence of aqueous stability, and γ-glutamyltranspeptidase

SUMMARY

The effects of several nephrotoxic glutathione(GSH) conjugated halogenated hydroquinones were studied in monolayer cultures of LLC-PK1 cells. Half-lives of the halogenated glutathion-S-yl hydroquinone ((GSyl)HQ) conjugates in aqueous solutions ranged from 19 to 88 min at pH 7.4. When oxygen was excluded however, 2-bromo-6-(glutathion-S-yl)hydroquinone (2-Br-6-(GSyl)HQ) was stable. At pH 5.8, the (GSyl)HQ conjugates were all relatively stable even in the presence of oxygen. The toxicities of 2-chloro-(diglutathion-S-yl)hydroquinone (2-Cl-(diGSyl)HQ), 2-Br-6-(GSyl)HQ and 2-bromo-(diglutathion-S-yl)-hydroquinone (2-Br-(diGSyl)HQ) to LLC-PK1 cells were all decreased at pH 5.8 as compared to pH 7.4. In contrast, 2,5-dichloro-3-(glutathion-S-yl)hydroquinone (DC-(GSyl)HQ) and 2,5,6-trichloro-3-(glutathion-S-yl)-hydroquinone (TC-(GSyl)HQ) were equally toxic at either pH.

Acivicin, an irreversible inhibitor of γ-glutamyltranspeptidase (γGT), had no effect on the cytotoxicity of any of the compounds studied. However, addition of exogenous γGT to the medium caused a decrease in the toxicitiy of 2-Br-6-(GSyl)HQ, DC-(GSyl)HQ, and TC-(GSyl)HQ, possibly as a consequence of either 1,4-benzothiazine formation and/or due to the formation of extracellular quinone-GSH-protein adducts. In the absence of γGT activity, breakdown products arising from 2-Br-(GSyl)HQ were toxic to LLC-PK1 cells, whereas those arising from DC-(GSyl)HQ or TC-(GSyl)HQ were without toxic effect.

The cytotoxicity of halohydroquinone-GSH conjugates is thus caused by a process which also results in the breakdown of the conjugate, presumably via oxidation. The most likely explanation for the contrasting effects of γGT is that, on the one hand, γGT initiates detoxication via 1,4-benzothiazine formation and/or polymerization, and on the other hand , targets the toxic glutathione conjugate to the membrane, thus enhancing the toxic effect.

ABBREVIATIONS

2-Br-6-(GSyl)HQ , 2-Bromo-6-(glutathion-S-yl)hydroquinone ; 2-Br-(diGSyl)HQ , 2-Bromo-(diglutathion-S-yl)hydroquinone; 2-Cl-(diGSyl)HQ , 2-Chloro(diglutathion-S-yl)hydroquinone; CYS(GLY), cysteinyl(glycine); DC-(GSyl)BQ , 2,5-Dichloro-3-(glutathion-S-yl)benzoquinone; DC-(GSyl)HQ , 2,5-Dichloro-3-(glutathion-S-yl)hydroquinone; EBSS, Earle's Balanced Salt Solution; γGT, γ-glutamyltranspeptidase; GSH, glutathione; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid; HPLC, high performance liquid chromatography; LDH, lactate dehydrogenase; PCBD-GSH, 1,2,3,4,4-pentachlorobutadienyl-glutathione; TC-(GSyl)BQ , 2,5,6-Trichloro-3-(glutathion-S-yl)benzoquinone; TC-(GSyl)HQ , 2,5,6-Trichloro-3-(glutathion-S-yl)hydroquinone; (triGSyl)HQ , (triglutathion-S-yl)hydroquinone; WE, Williams Medium E.

INTRODUCTION

Glutathione (GSH) conjugates are dualistic compounds. Their formation often results in detoxication and excretion of the parent compound. However, for an increasing number of xenobiotics, GSH conjugation can result in the formation of reactive metabolites and ensuing toxicity (van Bladeren, 1988). Although GSH conjugation of haloalkenes occurs in the liver, the target of these toxic conjugates very often is the proximal tubular epithelium of the kidney. Once formed within the liver, GSH conjugates can be released both into bile and plasma (Wahlländer and Sies, 1979). Biliary and/or intestinal metabolism results in the formation of cysteine conjugates which can be transferred to the kidney, either directly or following acetylation in the intestine or liver. Cysteine conjugates can also originate from the GSH conjugates at the proximal tubular epithelium through the activity of the brushborder enzymes , γ-glutamyltranspeptidase (γGT) and dipeptidases, and intracellularly after deacetylation of mercapturic acids. Further metabolism by cysteine conjugate β-lyase can result in the formation of a reactive thiol, and subsequent nephrotoxicity (Lock, 1988 ; Anders et al, 1988).

Several GSH-conjugated hydroquinones are potent nephrotoxicants. For example, 2-bromo-(glutathion-S-yl)hydroquinone (2-Br-(GSyl)HQ; 50 μmol/kg), and to a greater extent, 2-bromo-(diglutathion-S-yl)hydroquinone (2-Br-(diGSyl)HQ ; 10-30 μmol/kg) caused a severe necrosis of renal proximal tubules (Monks et al, 1985, and 1988a). The same effect was also caused by GSH-conjugated hydroquinones lacking the halogen atom, albeit at slightly higher doses (Lau et al, 1988) . In contrast to the pathway of haloalkene mediated nephrotoxicity, metabolism by β-lyase does not appear to play a role in the nephrotoxicity of hydroquinone-GSH conjugates, since pretreatment of animals with aminooxyacetic acid, an inhibitor of β-lyase, had only a negligible effect on the nephrotoxicity of both 2-Br-(diGSyl)HQ (Monks et al, 1988a) and tri(GSyl)HQ (Lau et al, 1988). However, inhibition of γGT by acivicin , an irreversible inhibitor, caused a decrease in the nephrotoxicity of 2-Br-(diGSyl)HQ (Monks et al, 1988a) and (triglutathion-S-yl)hydroquinone ((triGSyl)HQ) (Lau et al, 1988). We have also recently shown that 2,5-dichloro-3-(glutathion-S-yl)benzoquinone (DC-(GSyl)BQ), 2,5,6-trichloro-3-(glutathion-S-yl)benzoquinone (TC-(GSyl)BQ), and the corresponding hydroquinones cause renal proximal tubular necrosis in rats (Monks et al, 1990a; Mertens et al, 1990). In contrast to 2-Br-di(Gsyl)HQ and tri(GSyl)HQ however, inhibition of γGT resulted in an increase in nephrotoxicity . In view of the paradoxical effects of acivicin on the in vivo nephrotoxicity of halohydroquinone-GSH conjugates we therefore investigated the role of γGT on the cytotoxicity of these conjugates, in the LLC-PK1 cell line. The LLC-PK1 cell line maintains several characteristics of the proximal kidney tubule epithelia, including a brushborder and substantial γGT activity. Moreover, this cell line has been shown to respond to other nephrotoxic GSH and cysteine conjugates (Stevens et al, 1986; Mertens et al, 1988). Preliminary experiments with this cell model also indicated that several of the (GSyl)HQ conjugates were unstable under the conditions employed. The

study was therefore extended to investigate the effects of aqueous stability on the cytotoxicity of the halohydroquinone-GSH conjugates.

MATERIALS AND METHODS

Chemicals

Glutathione was purchased from Janssen (Beerse, Belgium). 2-Chlorohydroquinone (90% pure) was from Aldrich Chemie (Brussel, Belgium). Acivicin was a product from the Upjohn Company (Kalamazoo, MI), and γGT was obtained from Sigma (St. Louis, MO).

Synthesis of GSH-conjugates

2-Br-6-(GSyl)HQ and 2-Br-(diGSyl)HQ were synthesized as previously described (Monks et al, 1985). DC-(GSyl)BQ and TC-(GSyl)BQ were synthesized according to Mertens et al (1990) and the corresponding hydroquinones obtained by reducing the quinones in the presence of a threefold molar excess of ascorbic acid (Mertens et al,1990). 1,2,3,4,4-pentachlorobutadienylglutathione (PCBD-GSH) was synthesized according to Mertens et al (1988).

For the synthesis of 2-chloro-(diglutathion-S-yl)hydroquinone (2-Cl-(diGSyl)-HQ) , 2-chlorohydroquinone was twice recrystallized from chloroform after treatment with activated carbon. The resulting white/beige crystals had a melting point of 101-103°C. To a solution of 2.5 g of the 2-chlorohydroquinone in 15 ml of distilled water, 1.25 ml of 1 N sulfuric acid and 1.05 g of $KBrO_3$ in 10 ml of distilled water was added. The mixture was stirred at 60-65°C for 20 min . After 15 min a red oil and a yellow liquid had appeared. The yellow liquid was transferred to ice water and yellow crystals (2-chlorobenzoquinone) formed. The latter were dried with NaOH under vacuum (m.pt. 53.5 -55.5°C).

2-Chlorobenzoquinone (1.3 mmol) was dissolved in 100 ml of methanol and a solution of 0.13 mmol GSH in 5 ml of water was added dropwise with constant stirring. The reaction mixture was evaporated under vacuum and redissolved in water and ethyl acetate. The aqueous phase was extracted repeatedly with ethyl acetate, and evaporated until approximately 30 ml of solution remained. An additional 0.13 mmol GSH in 5 ml of water was added dropwise. The solvent was evaporated and the residue stored under nitrogen at 4°C.

For purification , the product was dissolved in water and an aliquot injected onto a Zorbax ODS 9.4 mm i.d. x 25 cm reversed phase semipreparative column, eluted with methanol/1% acetic acid in water (10:90) at a flow rate of 4 ml/min, and the eluate monitored at 254 nm. Two major peaks were collected from several injections of the crude mixture. The one eluting first was not further identified, the second one contained the required conjugate. After removing the methanol by evaporation the aqueous solution was frozen in dry ice/acetone and lyophilized. Purity exceeded 95% as determined by HPLC using a reversed phase analytical column (LiChrosorb 5RP18, 150x4.6 mm, Chrompack, Middelburg, The Netherlands)

and a linear gradient of methanol/1% acetic acid in water (10:90 to 100:0) at a flow rate of 1 ml/min , over 60 min. The eluate was monitored at 310 nm. The peak had a k' of 3.1 .

The identity of the conjugate was established by UV- and NMR-spectroscopy. The UV-spectrum (Beckman DU-8) showed a maximum at 336 nm and was similar to that reported for 2-Br-(diGSyl)HQ (Monks et al, 1985). The ^{1}H-NMR spectrum (Bruker CXP-300 in D_2O) showed the appropriate signals for a diglutathion-S-yl substituted monohalogenated hydroquinone (i.e., the aromatic proton at δ, 7.05 ppm; Cys α, 4.52-4.45 and 4.29-4.26 ppm; Gly α and Glu α, 3.80 and 3.76-3.73 ppm; Cys β, 3.40-3.33 and 3.24 -3.20 ppm; Glu γ, 2.49-2.45 ppm and Glu β, 2.15-2.07 ppm (Monks et al, 1985)).

Stability experiments

The aqueous stability of the conjugates was determined by analytical HPLC using the conditions described above. Conjugates (0.5 mM) were dissolved in water and kept in the dark under argon . At time zero, an aliquot was diluted (1:1) with a modified Earle's Balanced Salt Solution (containing twice the concentrations of the ingredients plus 20 mM $NaHCO_3$, 40 mM HEPES) (EBSSx2) pH 7.4 or pH 5.8. Ascorbic acid (1.5 mM) was dissolved in EBSS x2 . The solutions were incubated at 37 ^{o}C and 5% CO_2 in a humidified atmosphere. At different times a sample (20 µl) was taken and injected on the HPLC. Peak heights were related to the peak height of a sample diluted 1:1 with water at time zero .

The influence of oxygen on the stability was also investigated for 2-Br-6-(GSyl)HQ (0.25 mM). Argon or air was bubbled through the solution, after thirty minutes a sample was taken and injected on the HPLC.

Cell strain and culture

Epithelial pig kidney cells (LLC-PK1) (Flow, Irvine, Scotland) were cultured as described previously (Mertens et al, 1988). Cells were used in experiments between passage 193 and 230.

Cytotoxicity assay

Compounds were dissolved as described above. LLC-PK1 monolayers cultured in 24-well tissue culture plates (Costar, Mark II; $3.5x10^5$ cells/well) were exposed on day 3 to the compounds in 1 ml of EBSS at pH 5.8 or pH 7.4 for 2 h. After the exposure period EBSS was removed and the monolayers were cultured in Williams Medium E (WE) for 16 h. Lactate dehydrogenase (LDH) leakage was used as a parameter for cytotoxicity (Mertens et al, 1988) by measuring both the intra- and extracellular enzyme activity.

Statistical analyses

Statistical analyses were performed by the Wilcoxon-Mann- Whitney test.

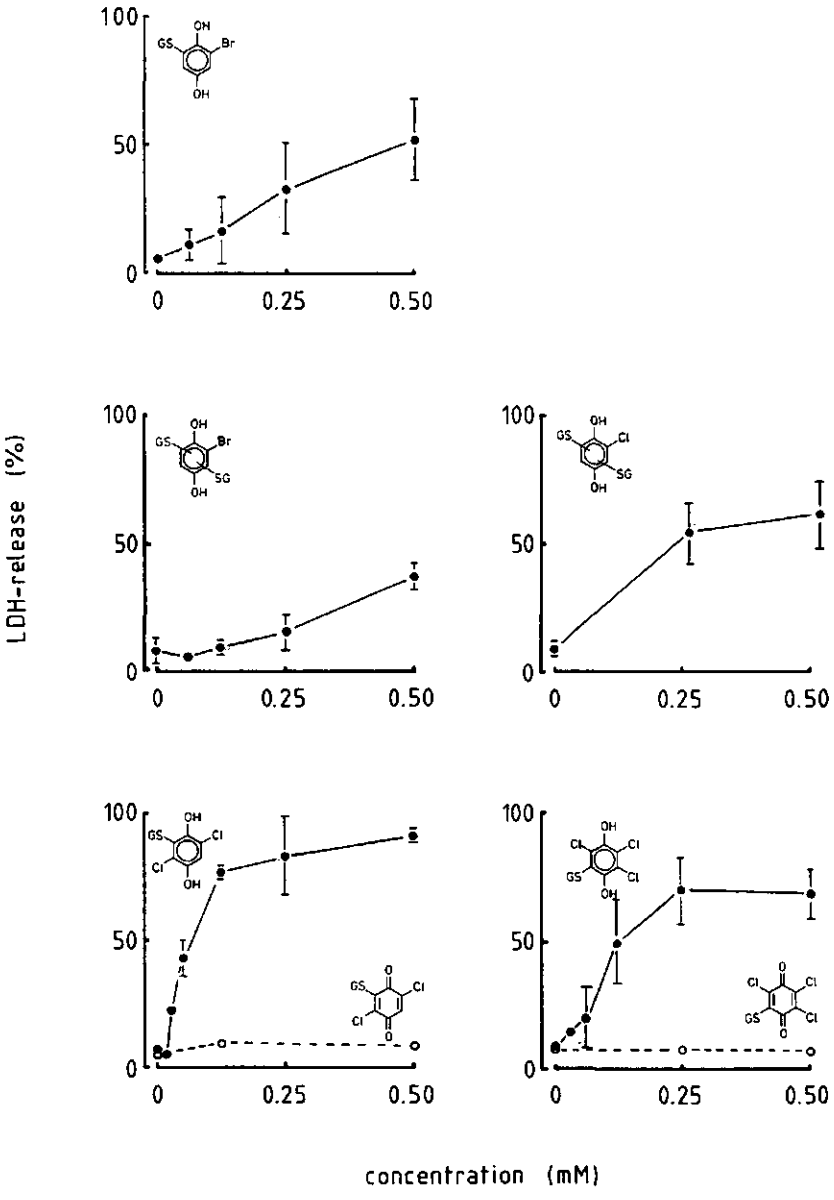

Figure 7.1 Cytotoxicity of halogenated quinoid GSH conjugates. LLC-PK1 monolayers were exposed for 2 h to GSH conjugates in EBSS (pH 7.4) (See Methods) . After a subsequent overnight incubation in WE, LDH-leakage was determined. Values represent the mean ±S.D. of at least three experiments in triplicate.

RESULTS

Cytotoxicity of (GSyl)hydroquinones at pH 7.4

Exposure of LLC-PK1 monolayers to GSH conjugated hydroquinones resulted in cytotoxicity as presented in figure 7.1 . In contrast to in vivo data, 2-Br-6-(GSyl)-HQ was more toxic than 2-Br-(diGSyl)HQ (at 0.5 mM, $p<0.1$). Of the (diGSyl)monohalohydroquinones 2-Cl-(diGSyl)HQ was the most toxic (at 0.25 mM, $p<0.05$). The cytotoxicity of dichloro and trichloro GSH conjugates were tested both in the oxidized (quinone) and reduced (hydroquinone) forms. As quinones they were not toxic at concentrations up to 0.5 mM; however, as hydroquinones, toxicity was observed at concentrations as low as 60 µM. The LLC-PK1 monolayers were slightly more sensitive to DC-(GSyl)HQ than to TC-(GSyl)HQ (at 0.125 mM, $p<0.025$), again, in contrast to observations in vivo. Pretreatment of LLC-PK1 monolayers for 30 min with acivicin (0.5 mM) caused a 70 % inhibition of γGT activity. However, such a treatment did not protect the cells from halohydroquinone-GSH conjugate-mediated cytotoxicity (data not shown).

Stability of the (GSyl) hydroquinones

From pilot studies it was clear that GSH-conjugated hydroquinones were unstable under standard in vitro incubation conditions. We therefore studied the stability of the compounds in more detail. In EBSS at pH 7.4 the half-life of the GSH conjugates varied between 4 and 80 min (Table 7.1). Of the mono-halogenated (GSyl)hydroquinones, 2-Br-(diGSyl)HQ ($t_{1/2}$ 72 min) was more stable than 2-Cl-(diGSyl)HQ ($t_{1/2}$ 52 min), while 2-Br-6-(GSyl)HQ was the least stable ($t_{1/2}$ 19 min). The difference in stability was also apparent at pH 5.8. Both brominated (GSyl)hydroquinones were stable throughout the 120 min testing period, whereas

Table 7.1 Stability of halogenated quinoid GSH conjugates. Conjugates dissolved in water (0.5 mM) were diluted (1:1) with EBSSx2 (pH 5.8 or 7.4), and incubated at 37°C in a humidified atmosphere with 5% CO_2 . At different times samples were injected on HPLC (see Methods).

Conjugate	$T_{1/2}$ (min)	
	pH 7.4	pH 5.8
2-Br-6-(GSyl)HQ	19	∞[a]
2-Br-(diGSyl)HQ	72	∞
2-Cl-(diGSyl)HQ	52	620
DC-(GSyl)HQ*	47	∞
DC-(GSyl)BQ	5	
TC-(GSyl)HQ*	77	∞
TC-(GSyl)BQ	4	

*in the presence of ascorbic acid

[a]no decrease in concentration throughout the 120 min testing period.

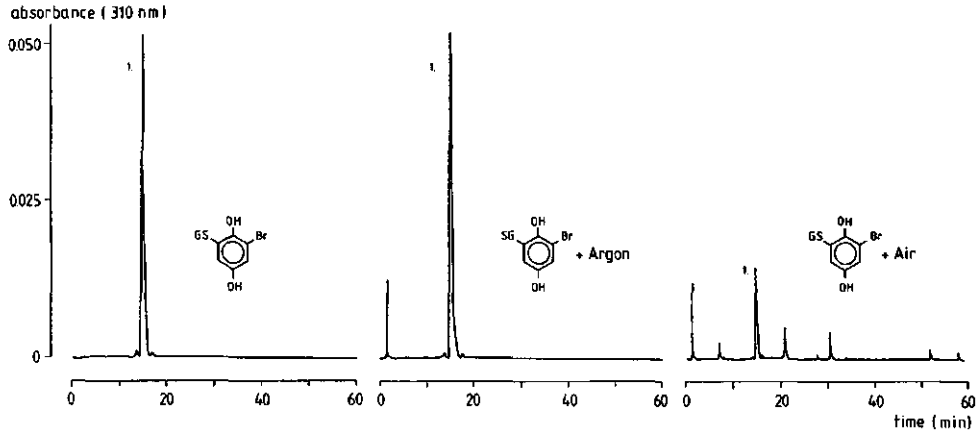

Figure 7.2 Oxygen dependence of the stability of 2-Br-6-(GSyl)HQ. 2-Br-6-(GSyl)HQ dissolved in water (0.5 mM) was diluted (1:1) with EBSS x2 (pH 7.4). After the solution was bubbled with air or argon during 30 min a sample was injected on HPLC (see Methods).

2-Cl-(diGSyl)HQ showed a slight decrease in concentration resulting in a half-life of 620 min. Stabilities of the di- and trihalogenated (GSyl)hydroquinones were similar: t1/2 47 and 77 min for DC-(GSyl)HQ and TC-(GSyl)HQ respectively at pH 7.4, and an absence of a decrease in concentration at pH 5.8 (t1/2 00). In contrast to the hydroquinones, the concentrations of the di- and trihalogenated (GSyl)quinone conjugates decreased very rapidly at pH 7.4 . Both DC-(GSyl)BQ and TC-(GSyl)BQ had a half-life of approximately 5 min.

The instability of the reduced conjugates is probably a consequence of the ease with which they autoxidize, since the exclusion of oxygen from the media completely prevented the alteration in the HPLC-pattern for 2-Br-6-(GSyl)HQ due to thirty minutes of aerobic incubation (Fig.7.2).

Cytotoxicity at pH 5.8

Because of the relative stability of the hydroquinone GSH conjugates at pH 5.8 we exposed LLC-PK1 monolayers at this pH. Preliminary experiments showed that LLC-PK1 cells were not adversely affected when incubated for short periods of time, even at pH 5.5. As a positive control, PCBD-GSH was used. As shown in figure 7.3 , the metabolic and transport systems needed for PCBD-GSH to cause toxicity were still present and effective at pH 5.8. In fact, the toxicity was slightly higher at pH 5.8 than at pH 7.4. Inhibition of γGT by 0.5 mM acivicin protected the monolayers from the effects of PCBD-GSH .

Exposure of LLC-PK1 monolayers at pH 5.8 to (GSyl)hydroquinones (0.25 mM),

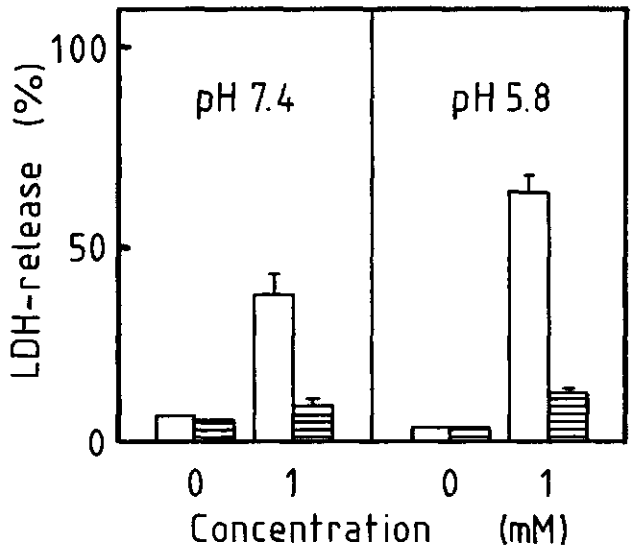

Figure 7.3 Cytotoxicity of PCBD-GSH at pH 5.8 and pH 7.4. LLC-PK1 monolayers were exposed to PCBD-GSH in EBSS (pH 7.4 or 5.8) with (▤) or without (□) 0.5 mM acivicin for 2 h . LDH-leakage was determined after a subsequent overnight incubation in WE. Data represent the mean ±S.D. of two experiments in triplicate.

which are stable under these conditions, resulted in cytotoxicity for the chlorinated (GSyl)hydroquinones only (Fig. 7.4). 2-Br-6-(GSyl)HQ and 2-Br-(diGSyl)-HQ were not cytotoxic under these conditions. Again addition of acivicin, to inhibit γGT, did not prevent the cytotoxic effects of the chlorinated (GSyl)hydroquinones (data not shown).

Role of γGT

As described above, acivicin did not affect the cytotoxicity of the (GSyl)hydroquinones, suggesting that γGT activity was not necessary for the expression of cytotoxicity. Addition of commercially obtained, exogenous γGT during the exposure to (GSyl)hydroquinones resulted in a dose related decrease in toxicity (Fig.7.5). However, preincubation of exogenous γGT with 1 mM acivicin for 45 min (which caused an 87% inhibition of the γGT activity as determined with L-γ-glutamyl-p-nitroanilide as the substrate) did not prevent the inhibitory effect of γGT on the cytotoxicity of DC-(GSyl)HQ and TC-(GSyl)HQ , and slightly increased the toxicity of 2-Br-6-(GSyl)HQ . This suggests that for 2-Br-6-(GSyl)-HQ, the catalytic activity of the exogenous γGT plays a more important role in its inhibitory effect than it does for the chlorinated conjugates , where γGT may be providing a source of alternative nucleophiles.

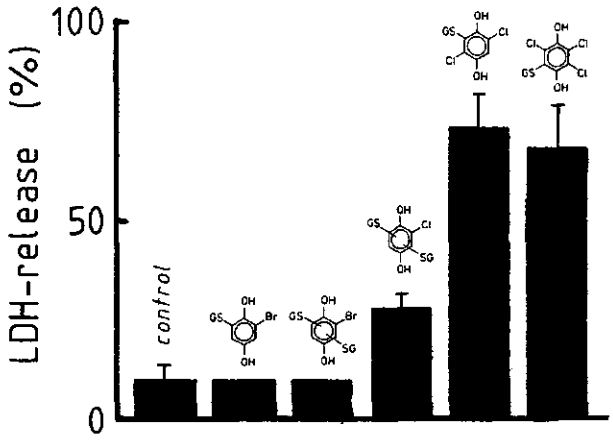

Figure 7.4 Cytotoxicity of halogenated (GSyl)hydroquinones at pH5.8. LLC-PK1 monolayers were exposed to 0.25 mM GSH conjugated chlorohydroquinones or 0.5 mM GSH conjugated bromohydroquinones in EBSS at pH 5.8 for 2 h . After a subsequent overnight incubation in WE, LDH-leakage was determined. Data represent the mean ±S.D. of at least two experiments in triplicate.

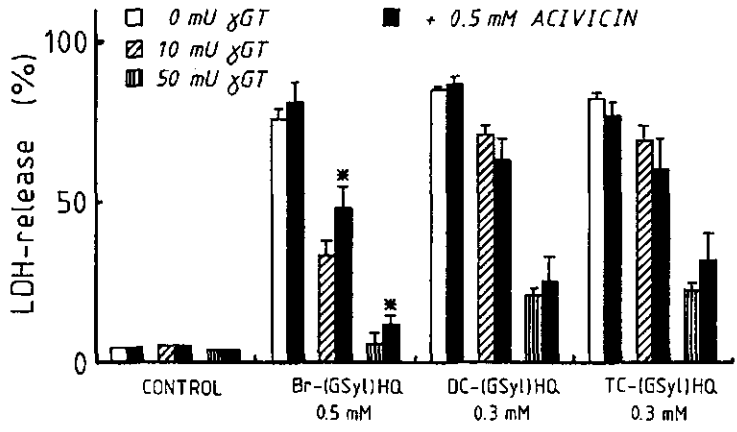

Figure 7.5 Cytotoxicity of DC-(GSyl)HQ, TC-(GSyl)HQ, and 2-Br-6-(GSyl)HQ in the presence of additional exogenous γGT. LLC-PK1 monolayers were exposed to DC-(GSyl)HQ, TC-(GSyl)HQ, or 2-Br-6-(GSyl)HQ in EBSS at pH 7.4 for 2 h in the presence of either active or inactive exogenous γGT. Exogenous γGT was inactivated by preincubating with acivicin for 45 min. After preincubation , 0.5 ml of the γGT solution was added to the cells , followed by 0.5 ml of medium containing the conjugate. The concentrations shown are the final concentrations. After a subsequent overnight incubation in WE, LDH-leakage was determined. Data represent the mean ±S.D. of two experiments in triplicate. Asteriks indicate when the effect of acivicin was statistically significant ($p<0.05$).

Stability vs toxicity

For the mono-halogenated (GSyl)hydroquinones there was an inverse correlation between stability and toxicity. To investigate whether toxicity was caused by the parent compound or a breakdown product(s), LLC-PK1 monolayers were exposed for two hours to either the parent conjugates or their breakdown products. The latter were obtained by incubating the parent compounds in EBSS pH 7.4 at 37 °C for 3 h before adding them to the cells. Such a time period was sufficient to obtain a solution in which no parent compound remained (Table 7.1). Exposure of LLC-PK1 monolayers to the breakdown product(s) derived from 2-Br-6-(GSyl)HQ caused toxicity, at least equal to that caused by incubation with the parent compound (Fig. 7.6) indicating that the derivatives formed remain toxic. In contrast, no cytotoxicity was observed after exposure of the cells to the breakdown product(s) of the poly-halogenated GSH-conjugates DC-(GSyl)HQ and TC-(GSyl)HQ.

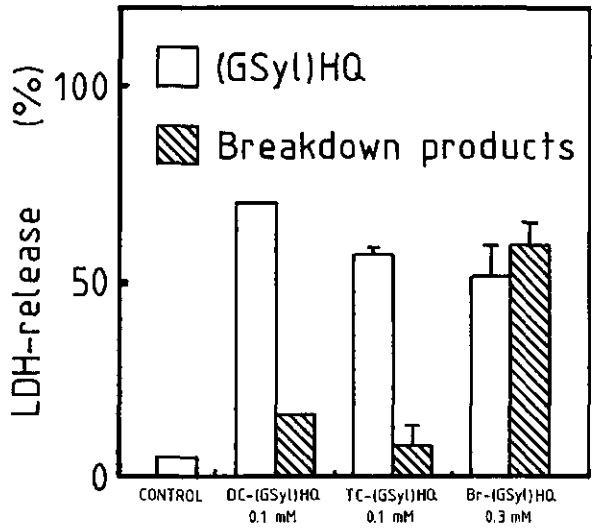

Figure 7.6 Cytotoxicity after exposure to 2-Br-6-(GSyl)HQ, DC-(GSyl)HQ, TC-(GSyl)HQ, or their breakdown products. LLC-PK1 monolayers were exposed for 2 h to DC-(GSyl)HQ, TC-(GSyl)HQ, 2-Br-6-(GSyl)HQ, or their breakdown products. The latter were obtained by incubation of the compounds for three hour at 37 °C in a humidified atmosphere containing 5% CO_2. After a subsequent incubation in WE, LDH-leakage was determined. Data represent the mean (±S.D.) of two experiments in triplicate.

DISCUSSION

The nephrotoxicity of haloalkene GSH conjugates appears to be mediated by the combined activities of γGT and cysteine conjugate β-lyase, which results in the formation of a reactive thiol(s) (Anders et al, 1988; Lock, 1988). The reactive thiol can rearrange to form a thioacylating intermediate, probably a thioketene and/or a thionoacyl halide (Dekant et al, 1987; 1988; Commandeur et al, 1989). However, for the nephrotoxicity of GSH conjugated hydroquinones, β-lyase mediated metabolism appears to be of lesser importance. Oxidation resulting in the formation of the corresponding quinone conjugates, seems to play a major role in the nephrotoxicity of these compounds (Monks et al, 1988a, and 1988b). However, the exact mechanism of toxicity is unknown: Nephrotoxicity might be initiated by either quinone-mediated macromolecule arylation and/or via the formation of reactive oxygen species during the oxidation process(Fig. 7.7).

Thus, although β-lyase appears to play a minor, if any, role in quinol-GSH conjugate-mediated nephrotoxicity, γGT activity can effectively modulate the toxicity of these compounds. For example, the toxicity of 2-Br-(diGSyl)HQ and (triGSyl)HQ can be attenuated by inhibition of γGT by acivicin (Monks et al, 1988a; Lau et al, 1988). Conversely, acivicin potentiated the nephrotoxicity of DC-(GSyl)-

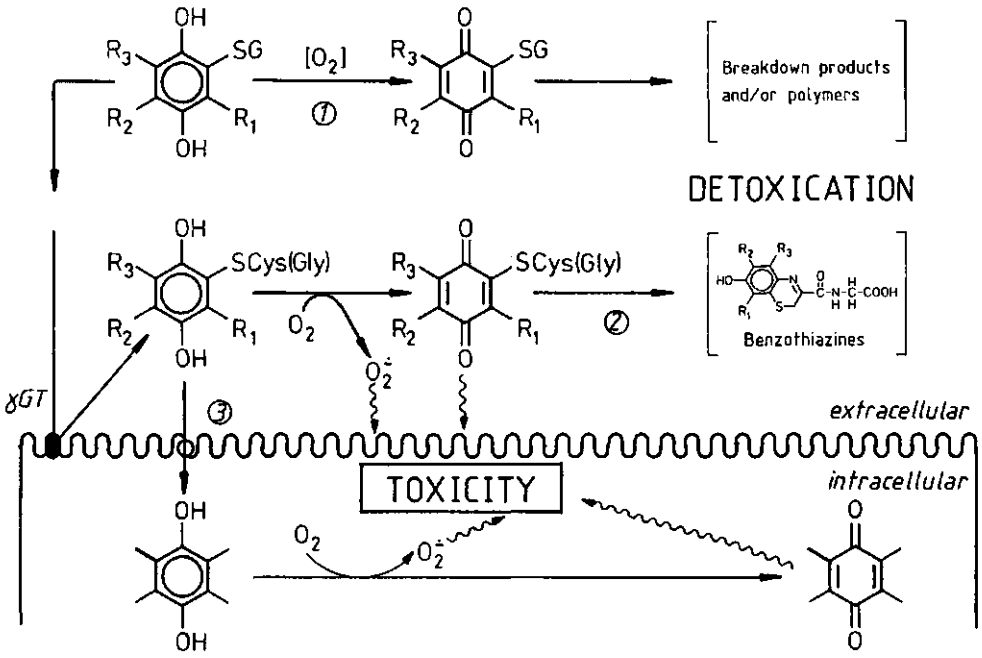

Figure 7.7 Postulated reaction pathways for (GSyl)HQ conjugates resulting in toxicity or detoxication (See text).

HQ and TC-(GSyl)HQ (Monks et al, 1990a ; Mertens et al, 1990). The reason(s) for this paradoxical behavior is unclear but may be related to events subsequent to the γGT mediated removal of the γ-glutamyl moiety. Thus, after the γ-glutamyl-moiety is removed by γGT, subsequent oxidative cyclization of the cysteinylglycine-conjugate can result in 1,4-benzothiazine formation (Monks et al, 1990b). This reaction sequence seems to constitute a detoxication mechanism. Consistent with this view, the L-homocysteine-conjugates of 2-bromohydroquinone, which, as a consequence of the additional methylene group, should not undergo the cyclization reaction, are as nephrotoxic as the corresponding cysteine conjugates (Lau and Monks, 1990). Thus the γGT catalysed removal of the γ-glutamyl moiety can have the ultimate effect of removing the reactive quinone function from the molecule (Fig.7.7 (2)).

GSH-conjugated hydroquinones are sensitive to air-oxidation (Table 7.1; Fig. 7.2), and this is influenced by the pH and the nature of the ring substituents (Musso and Döpp, 1967). Upon oxidation the hydroquinones (Fig.7.7 (1)) may form several breakdown products, among them, presumably, polymers with an intact GSH-moiety similar to those reported for GSH conjugates of acetaminophen (Potter et al, 1986). In addition, γGT may facilitate the oxidation process. Removal of the γ-glutamyl and glycine-moiety decreases the oxidation potential drastically: for example 2-Bromo-3-(cystein-S-yl)hydroquinone is more readily oxidized than 2-Br-3-(GSyl)HQ (Lau and Monks, 1990; Monks and Lau, 1990). This decrease may be sufficient to permit oxidation of the cysteinyl(glycine) (CYS(GLY)) conjugates of dichlorohydroquinone and trichlorohydroquinone but, due to the differences in halogenation, insufficient to permit oxidation of 2-bromo-(cystein-S-ylglycine)HQ and 2-bromo-(dicystein-S-ylglycine)HQ at pH 5.8. Thus, the chlorohydroquinones remain toxic at pH 5.8, whereas the bromohydroquinones are not (Fig.7.4).

Does the oxidation of these conjugates occur intracellularly and/or extracellularly? Will oxidation in either compartment lead to cytotoxicity or is toxicity initiated by preferential oxidation in a specific compartment? If extracellular oxidation was responsible for the in vitro toxicity of these conjugates then one might expect to see a decrease in toxicity after inhibition of γGT. However, pretreatment of LLC-PK1 cells with acivicin, at concentrations sufficient to cause an 70% inhibition of γGT activity, failed to protect the cells from halohydroquinone-GSH conjugate mediated cytotoxicity. It should be noted that the residual γGT activity may be sufficient to permit efficient processing of the GSH-conjugates. In addition, the lack of toxicity of DC-(GSyl)BQ and TC-(GSyl)BQ when added directly to LLC-PK1 cells (Fig. 7.1) further suggests that the presence of the quinones extracellularly does not, per se, cause toxicity. This suggests an intracellular mechanism of toxicity, with the rate-limiting step distal to metabolism by γGT. Of the CYS(GLY) conjugate formed, á fraction may be accumulated intracellularly (Fig.7.7(3)) with the remaining fraction detoxified via extracellular autoxidation and subsequent 1,4-benzothiazine formation. Inhibition of γGT should cause a decrease in the formation of CYS(GLY) conjugates , and an increase in oxidation of the intact GSH conjugates (Fig.7.7 (1)). The relative rates of these two

reactions will determine the consequences of γGT inhibition for each compound.

The addition of exogeneous γGT to the LLC-PK1 cells decreased the toxicity of 2-Br-(GSyl)HQ , TC-(GSyl)HQ and DC-(GSyl)HQ (Fig.7.5). When the activity of exogenously added γGT was decreased 87% by pretreatment with acivicin prior to its addition to LLC-PK1 monolayers, the cytotoxicity of DC-(GSyl)HQ and TC-(GSyl)-HQ was the same as in the presence of exogenously added active γGT. In contrast, this protocol partially restored the cytotoxic effects of 2-Br-6-(GSyl)HQ (Fig.7.5). This suggests that the effect of exogenously added γGT on TC-(GSyl)HQ and DC-(GSyl)HQ may be a consequence of the availability of additional nucleophilic sites on the protein which scavenge any reactive quinone formed and thereby protects critical sites on cellular membranes from electrophilic attack.The extremely high reactivity of the quinone conjugates with protein thiols would be in line with this suggestion (van Ommen et al, 1988).In contrast it is clear that the enzymatic activity of exogenously added γGT is at least partially responsible for its ability to protect LLC-PK1 cells from 2-Br-6-(GSyl)HQ cytotoxicity in vitro.

Data from in vivo studies also support the contention that γGT can serve both a toxication and detoxication function with respect to the metabolism of halohydroquinone-GSH conjugates. Inhibition of γGT in vivo caused an increase in nephrotoxicity after administration of DC-(GSyl)HQ or TC-(GSyl)HQ (Monks et al, 1990a; Mertens et al, 1990), and a decrease of the nephrotoxicity of 2-Br-(diGSyl)HQ and (triGSyl)HQ (Monks et al, 1988a; Lau et al, 1988). Apparently, for the toxicity of these latter two conjugates, γGT is the rate-limiting factor in vivo, whereas for DC-(GSylHQ and TC-(GSyl)HQ the detoxication reaction is predominant, possibly due to the lower oxidation potential compared to 2-Br-(diGSyl)HQ and (triGSyl)HQ (Monks et al, 1988b; Lau et al, 1988; Mertens et al, 1990). Finally, Lau et al (1990) have shown that differences in species susceptibility to 2-Br-di(GSyl)HQ do not correlate with variability in γGT-activity. These data indicate that factors other than γGT are important determinants of quinol-GSH mediated nephrotoxicity. For example, difference in the relative rates of cysteine conjugate N-acetylation and deacetylation may contribute to the observed difference in species susceptibility to 2-Br-di(GSyl)HQ nephrotoxicity. In addition, differences in the direct alkylating ability of the mono- versus the di- and trihalogenated quinones might contribute to substrate-specific differences in toxicity and are areas worthy of future investigation.

In conclusion, the present study indicates that : 1) Oxidation of halogenated (GSyl)hydroquinones appears necessary for toxicity. 2) The dualistic effects of γGT may be a consequence of its ability to initiate a detoxication sequence via 1,4-benzothiazine formation and/or polymerization , and on the other hand by targetting the toxic glutathione conjugate to the membrane, thus enhancing the toxic effect. 3) The role of breakdown products in the toxicity of these compounds deserves further attention especially in the case of 2-Br-6-(GSyl)HQ. Further studies are necessary to elucidate the mechanism and site of oxidation of these compounds.

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PART IV

SUMMARY AND CONCLUDING REMARKS

CHAPTER 8

Summary and concluding remarks

Summary and concluding remarks

8.1 SUMMARY

Presently in our society animal tests still form the main starting point for the assessment of the possible risks of chemicals with regard to human and animal health. For scientific, economic, and ethical reasons, attempts are undertaken continuously to develop cell models as alternatives to animal testing. However, the predictive value of in vitro test systems is often limited due to the unawareness about the mechanisms of toxicity and the complexity of organisms. As a consequence, a strategy increasingly used is one of studying mechanisms of toxicity evolving in rational cell models. Following this strategy, this thesis aims at contributing to the development of cell models as an alternative to animal testing.

A usefull cell model for studying mechanisms of toxicity requires the presence of characteristics which may play a role in this toxicity in vivo, and the possibility to demonstrate their involvement. The organ of our choice was the kidney. Since very often the cortex, more in particular the proximal tubule, is affected by chemicals, cortical cells of different origin have been used: A renal cell line derived from a Hamshire pig (LLC-PK1) and primary cultures of rat renal cortical cells (Chapter 2). The applicability of these cells in a cell model was tested considering two major aspects of importance for nephrotoxicity: Firstly, the functional polarity of the proximal tubular cell, and secondly the presence of specific biotransformation processes and transport systems (Chapter 1).

In vivo luminal (apical) as well as serosal (basolateral) exposure of the proximal tubular cell can occur. However, when monolayers are cultured in a conventional way, i.e. on a solid support, only the apical side is accessible for the compounds to be investigated. To overcome this problem a new system for nephrotoxicological in vitro studies has been developed. Cells were cultured on porous substrates, which enables exposure of monolayers on either side (Chapter 2).

The nephrotoxic effects of haloalkenes and halobenzenes seem to be mediated via the formation of glutathione conjugates (Chapter 1). In part II of this thesis a glutathione conjugate of hexachlorobutadiene and its derivatives are used as model compounds to investigate the possibilities of the cell systems described in chapter 2. LLC-PK1 monolayers possess the most important characteristics necessary for cytotoxicity of S-(1,2,3,4,4-pentachlorobutadienyl)glutathione (PCBD-GSH) to occur (Chapter 3). Via the use of acivicin, an irreversible inhibitor of γ-glutamyltranspeptidase (γGT), and aminooxyacetic acid, an inhibitor of β-lyase, the importance of these enzymes in the activation of PCBD—GSH was demonstrated. After metabolism by γGT and dipeptidase S-(1,2,3,4,4-pentachlorobutadienyl)-L-cysteine (PCBD-CYS) is formed extracellularly. This is taken up and converted by β-lyase into a reactive thiol resulting in toxicity. In vivo and in the LLC-PK1 cell line γGT is predominantly present on the luminal and apical membrane respectively. In agreement with this, apical exposure of LLC-PK1 monolayers to

PCBD-GSH caused a stronger toxicity than basolateral exposure . In the nephrotoxicity of the corresponding mercapturate N-acetyl-S-(1,2,3,4,4-pentachlorobutadienyl)-L-cysteine (PCBD-NAC), which is deacetylated intracellularly into PCBD-CYS, a serosally located probenecid sensitive organic anion transporter has been suggested to be of importance (Chapter 1). Apical exposure of LLC-PK1 monolayers to PCBD-NAC caused hardly any toxicity; only at relatively high concentrations a toxic effect was detected. Surprisingly, basolateral exposure and consequently administration of the compound to the possible transporter, did not cause substantial toxicity either. Further investigations revealed that this is probably caused by the absence of an organic anion transporter in the LLC-PK1 cell line: The model organic anion para-aminohippurate (PAH) was not transported by LLC-PK1 cells. In contrast, LLC-PK1 monolayers are in the possession of an organic cation transporter. A quinine and ouabaine inhibitable transcellular transport of the cation tetraethylammonium (TEA) was demonstrated (Chapter 3).

The organic anion transporter was also postulated (Lash and Anders, 1986) to play a role in the cytotoxicity of cysteine conjugates . However, in spite of the demonstrated absence of an organic anion transporter , PCBD-CYS causes toxicity in the LLC-PK1 monolayer. In contrast to PCBD-GSH, basolateral exposure to PCBD-CYS resulted in a stronger toxic effect than apical exposure (Chapter 4). A possible candidate for the transport of PCBD-CYS in LLC-PK1 cells is an amino acid transporter. The inhibitory effect of D-tryptophan and histidine on the toxicity of PCBD-CYS strongly suggests the involvement of the amino acid transport System T (Chapter 4).

Culturing primary cultures of rat renal cortical cells resulted in confluent monolayers (Chapter 2 and 5). However, investigating transcellular transport of organic ions in a similar way as with the LLC-PK1 monolayers was not possible, because a non-leaking monolayer on a porous support was not obtained. This may be due to the diversity of the cell population in the culture, and the concomitant differences in junctional complexes necessary for complete sealing of the monolayer. The presence of both PAH and TEA transporters was demonstrated by measuring intracellular accumulation, which could be inhibited by probenecid and quinine respectively. Inhibition of γGT and/or β-lyase decreased the toxic effect of PCBD-GSH, PCBD-CYS, and PCBD-NAC. In contrast to the experiments with LLC-PK1 monolayers, no effect of amino acid transporter substrates or probenecid on the toxicity was observed. Apparently uptake of PCBD-CYS or PCBD-NAC in primary cultures of rat renal cortical cells is not the rate-limiting step in the toxicity. The probenecid sensitivity of toxicity observed by others thus must be caused by other mechanisms.

In part III, glutathione conjugated halohydroquinones are the subject of investigation. These conjugates resemble the bromohydroquinone glutathione conjugates which are supposed to be responsible for the nephrotoxic effects of bromobenzene. The exact mechanism of nephrotoxicity is not known. In vivo experiments indicate that γGT is an important factor, as in the case of PCBD-GSH. However, β-lyase mediated metabolism appears to be of lesser

importance than oxidative metabolism resulting in quinone conjugates (Chapter 1). In a first attempt to validate the in vitro test system and to see whether the mechanism of nephrotoxicity of glutathione conjugated bromohydroquinones is more generally valid, dichloro(glutathion-S-yl)hydroquinone (DC-(GSyl)HQ) and trichloro(glutathion-S-yl)hydroquinone (TC-(GSyl)HQ) were studied in vivo (Chapter 6). Administration (i.v.) of these compounds in the quinone form caused nephrotoxicity evidenced by elevations in blood urea nitrogen (BUN) , an increase in the urinary excretion of glucose, lactate dehydrogenase and γGT, and by the pathological changes in the kidney slices. Reducing the glutathione conjugated quinones with ascorbic acid caused a drastic increase in nephrotoxicity . This protocol may result in an increased delivery of DC-(GSyl)HQ and TC-(GSyl)HQ to the kidney , by preventing their interaction with nucleophilic sites on plasma proteins and/or with other extra-renal macromolecules. Surprisingly, inhibition of γGT increased the nephrotoxicity.

Apparently γGT has a dual role in the nephrotoxicity of halogenated hydroquinone glutathione conjugates. This was further investigated in vitro in chapter 7. The LLC-PK1 cell line was chosen, since the apical presence of γGT makes this cell line a very suitable model for this purpose. However, in contrast to the experiments with PCBD-GSH and its derivatives, the in vitro experiments did not completely clarify the mechanism of activation of halogenated hydroquinone-glutathione conjugates. In a postulated scheme (Fig.7.7) the reactions that can occur with halogenated GSyl-HQ conjugates are summarized , and an explanation for the results obtained in vitro as well as in vivo is offered. The results indicate that γGT is not the rate-limiting step in the toxicity. They suggest that γGT on the one hand initiates a detoxication by 1,4-benzothiazine formation and/or polymerization, and on the other hand activates by targetting the oxidation that causes the toxicity. In addition, without metabolism by γGT these compounds are not stable and can be detoxified via air-oxidation and presumably polymerization. Further studies will be necessary to elucidate the exact site and mechanism of oxidation.

8.2. CONCLUDING REMARKS

The studies described in this thesis demonstrate that exposing of monolayers on porous substrates closely resembles the in vivo luminal and serosal exposure of a renal proximal tubular cell. In the future this system offers the possibility to investigate and compare the relative importance of polarized functions of epithelial cells of different organs and origin.

By using model compounds it could be demonstrated that the LLC-PK1 cell may be a relatively simple model for determining a specific nephrotoxic potential of glutathione and cysteine conjugates. For this purpose, the absence of an organic anion transporter in the LLC-PK1 cell line proved not to be a handicap. However, for investigating the nephrotoxic potential of other chemicals, the presence of an

organic anion transporter may be a prerequisite. Consequently, further characterization of other cell lines as the Opussum Kidney cell line (Koyama et al, 1978) or the recently established human renal cell line (KRC/Y) (Yano et al, 1988) should focus on the presence of this transporter.

Finally, the experiments on the role of γGT in the nephrotoxicity of glutathione conjugated halogenated hydroquinones underline once more the importance of in vivo validation. Differences of a more physical natures between in vivo and in vitro, as e.g. oxygen pressure and pH, may cause different or additional effects of compounds in vivo, which may be missed in vitro when these differences are not taken into account.

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CHAPTER 9

Samenvatting en slotbeschouwingen

Samenvatting en slotbeschouwingen

9.1. SAMENVATTING

In onze huidige maatschappij vormen dierexperimenten nog steeds het belangrijkste uitgangspunt voor de vaststelling van mogelijke risico's van chemicaliën t.a.v. de gezondheid van mens en dier. Vanwege wetenschappelijke, economische en ethische redenen wordt er doorlopend getracht celmodellen te ontwikkelen ter vervanging van dierproeven. De voorspellende waarde echter van in vitro testsystemen is vaak beperkt door de onbekendheid van toxiciteits-mechanismen en het complexe functioneren van organismen. Dientengevolge wordt steeds meer de strategie gevolgd: bestuderen van toxiciteitsmechanismen evoluerend in rationele celmodellen. Middels deze benadering probeert dit proefschrift een bijdrage te leveren in de ontwikkeling van celmodellen als een alternatief voor proefdierexperimenten.

Een bruikbaar celmodel ter bestudering van toxiciteitsmechanismen dient te beschikken over eigenschappen die een rol kunnen spelen in de toxiciteit in vivo en over de mogelijkheid hun betrokkenheid te demonstreren. Het orgaan van onze keuze was de nier. Aangezien chemicaliën vooral de cortex, meer in het bijzonder de proximale tubulus beïnvloeden is er gebruik gemaakt van corticale niercellen van verschillende afkomst: Een niercellijn afkomstig van een Hampshire varken (LLC-PK1) en primaire kweken van corticale ratteniercellen (Hoofdstuk 2). De toepasbaarheid van deze cellen is in een celmodel getest waarbij rekening werd gehouden met twee aspecten van belang voor niertoxiciteit: Ten eerste, de functionele polariteit van de proximale tubuluscel en ten tweede, de aanwezigheid van specifieke biotransformatieprocessen en transportsystemen (Hoofdstuk 1).

In vivo kan er zowel luminale (apicale) als serosale (basolaterale) blootstelling plaatsvinden. Wanneer echter cellen op conventionele wijze gekweekt worden, d.w.z. op een vaste ondergrond, is alleen de apicale zijde toegankelijk voor de te bestuderen stoffen. Om dit probleem op te lossen is een nieuw systeem voor niertoxicologische in vitro studies ontwikkeld. Cellen zijn gekweekt op een poreuze ondergrond , waardoor monolayers aan beide zijden kunnen worden blootgesteld (Hoofdstuk 2).

De niertoxische effecten van haloalkenen en halobenzenen lijken veroorzaakt te worden via de vorming van glutathion-conjugaten (Hoofdstuk 1). In deel II van dit proefschrift zijn een glutathion-conjugaat van hexachloorbutadieen en zijn derivaten gebruikt als modelstoffen ter bestudering van de mogelijkheden van de in hoofdstuk 2 beschreven celsystemen. LLC-PK1 monolayers bezitten de meest essentiële eigenschappen noodzakelijk voor het optreden van cytotoxiciteit van S-(1,2,3,4,4-pentachloorbutadiënyl)glutathion (PCBD-GSH) (Hoofdtuk 3). Door gebruik te maken van acivicin, een irreversibele remmer van γ-glutamyl-transpeptidase (γGT), en van aminooxyazijnzuur, een remmer van β-lyase, kon het belang van deze enzymen voor de activatie van PCBD-GSH worden aangetoond. Na

omzetting door γGT en dipeptidase ontstaat er extracellulair het S-(1,2,3,4,4-pentachloorbutadiënyl)-L-cysteïne (PCBD-CYS). Dit wordt opgenomen en omgezet in een reactief thiol, resulterend in toxiciteit. In vivo en in de LLC-PK1 cellijn is γGT voornamelijk aanwezig op het luminale/apicale membraan. In overeenstemming hiermee veroorzaakte apicale blootstelling van LLC-PK1 monolayers aan PCBD-GSH een sterkere toxiciteit dan basolaterale blootstelling. Men neemt aan dat in de nephrotoxiciteit van het corresponderende mercaptuurzuur, het N-acetyl-S-(1,2,3,4,4-pentachloorbutadiënyl)-L-cysteïne (PCBD-NAC) dat intracellulair gedeacetyleerd wordt tot PCBD-CYS, een serosaal gelocaliseerde probenecide gevoelige organisch-aniontransporter van belang is (Hoofdstuk 1). Apicale blootstelling van LLC-PK1 monolayers aan PCBD-NAC veroorzaakte nauwelijks toxiciteit; alleen t.g.v. relatief hoge concentraties werd er een toxisch effect waargenomen. Verwonderlijk was dat basolaterale blootstelling en dientengevolge toediening van de stof aan de mogelijke transporter, evenmin toxiciteit veroorzaakte. Verdere experimenten toonden aan dat dit waarschijnlijk veroorzaakt wordt door de afwezigheid van een organisch-aniontransporter in de LLC-PK1 cellijn; het model organisch-anion para-aminohippuurzuur (PAH) werd niet getransporteerd door LLC-PK1 cellen. Daarentegen bleken LLC-PK1 monolayers wel in het bezit te zijn van een organisch-cationtransport; een door quinine en ouabaine rembaar transcellulair transport van het cation tetraethylammonium (TEA) kon worden aangetoond (Hoofdstuk 3).

De organisch-aniontransporter zou ook een rol spelen in de cytotoxiciteit van cysteïne-conjugaten (Lash en Anders, 1986). Echter, ondanks de aangetoonde afwezigheid van een organisch-aniontransporter bleek PCBD-CYS cytotoxisch te zijn voor de LLC-PK1 monolayer. In tegenstelling tot PCBD-GSH, veroorzaakte basolaterale blootstelling aan PCBD-CYS een sterker toxisch effect dan apicale blootstelling (Hoofdstuk 4). Een mogelijke kandidaat voor het transport van PCBD-CYS door LLC-PK1 cellen is een aminozuurtransporter. Het remmende effect van D-tryptofaan en histidine op de toxiciteit van PCBD-CYS wijst sterk in de richting van een betrokkenheid van de aminozuurtransporter System T (Hoofdstuk 4).

De kweek van corticale ratteniercellen resulteerde in confluente monolayers (Hoofdstuk 2 en 5). Echter, transcellulair transport van organische ionen onderzoeken op eenzelfde wijze als bij de LLC-PK1 monolayers bleek niet mogelijk, omdat een niet-lekkende monolayer op een poreuze ondergrond niet verkregen werd. De oorzaak daarvan ligt waarschijnlijk in het diverse karakter van de celpopulatie in de kweek en de daarmee samenhangende verschillen in junctionele complexen die noodzakelijk zijn voor een complete dichting van de confluente monolayer. De aanwezigheid van zowel PAH- als TEA-transporters kon aangetoond worden door meting van intracellulaire accumulatie, welke geremd kon worden respectievelijk door probenecide en quinine. Remming van γGT en/of β-lyase verminderde het toxische effect van PCBD-GSH, PCBD-CYS en PCBD-NAC. In tegenstelling tot de experimenten met de LLC-PK1 monolayers hadden aminozuur-transportsubstraten en probenecide geen effect op de toxiciteit. Blijkbaar is de

opname van PCBD-CYS en PCBD-NAC niet de snelheidsbepalende stap in de toxiciteit in de primaire kweek van corticale niercellen. De probenecide gevoeligheid van de toxiciteit die door anderen is waargenomen moet dus veroorzaakt worden door andere mechanismen.

In deel III worden glutathion-geconjugeerde halohydrochinonen onderzocht. Deze conjugaten lijken op broomhydrochinon-glutathion-conjugaten die verondersteld worden verantwoordelijk te zijn voor de niertoxische effecten van broombenzeen. Het exacte mechanisme van niertoxiciteit is niet bekend. In vivo experimenten geven aan dat γGT een belangrijke factor is, zoals in het geval van PCBD-GSH. β-Lyase gemedieerd metabolisme lijkt hier van minder belang te zijn dan oxydatief metabolisme resulterend in de vorming van chinonen (Hoofdstuk 1). In een eerste poging het in vitro testsysteem te valideren en om te zien of het mechanisme van niertoxiciteit van glutathion-geconjugeerd broomhydrochinon algemeen geldig is, werden dichloor(glutathion-S-yl)hydrochinon (DC-(GSyl)HQ) en trichloor(glutathion-S-yl)hydrochinon (TC-(GSyl)HQ) in vivo bestudeerd (Hoofdstuk 6). Toediening (i.v.) van deze stoffen in de chinon vorm veroorzaakte niertoxiciteit aangetoond door verhoogde bloed ureum stikstof (BUN) gehaltes, een toename in de urinaire excretie van glucose, lactaatdehydrogenase en γGT, en pathologische afwijkingen in de nier. Reduceren van de glutathion-geconjugeerde chinonen d.m.v. vitamine C veroorzaakte een drastische toename in de niertoxiciteit. Deze werkwijze veroorzaakt waarschijnlijk een verhoogd aanbod van DC-(GSyl)HQ en TC-(GSyl)HQ aan de nier, doordat hun interactie met nucleofiele plaatsen op plasma eiwitten en/of met andere extrarenale macromoleculen wordt verhinderd. Verbazingwekkend was dat de remming van γGT een verdere toename in de niertoxiciteit veroorzaakte.

Blijkbaar speelt γGT een tweeledige rol in de niertoxiciteit van gehalogeneerde hydrochinon-glutathion-conjugaten. Dit is verder onderzocht in vitro in hoofdstuk 7. Hiervoor is de LLC-PK1 cellijn gekozen, aangezien de apicale aanwezigheid van γGT deze cellijn tot een goed bruikbaar model maakt voor dit doel. In tegenstelling echter tot de experimenten met PCBD-GSH en zijn derivaten, konden de in vitro experimenten geen complete opheldering verschaffen omtrent het mechanisme van activatie van gehalogeneerde hydrochinon-glutathion-conjugaten. In een gepostuleerd schema (Fig 7.7) zijn de mogelijke reacties die kunnen plaatsvinden met gehalogeneerde GSyl-HQ-conjugaten samengevat en wordt een verklaring gegeven voor de verkregen in vitro en in vivo resultaten. De resultaten duiden erop dat γGT niet de snelheidsbepalende stap in de toxiciteit is en ze geven aan dat γGT enerzijds een detoxificatie initieerd door 1,4-benzothiazine vorming en/of polymerisatie en anderzijds een activatie teweegbrengt door "targetting" van de oxydatie die verantwoordelijk is voor de toxiciteit. Daarnaast zijn deze conjugaten zonder metabolisme door γGT niet stabiel en kunnen ze gedetoxificeerd worden via oxydatie aan de lucht en vermoedelijk polymerisatie. Verder onderzoek is noodzakelijk om de exacte plaats en het exacte mechanisme van oxydatie op te helderen.

9.2. SLOTBESCHOUWINGEN

De studies beschreven in dit proefschrift laten zien dat het mogelijk is de in vivo luminale en serosale blootstelling van een proximale tubuluscel te imiteren middels blootstelling van een monolayer op een poreuze ondergrond. In de toekomst biedt dit systeem de mogelijkheid het relatieve belang van gepolariseerde functies van epitheliale cellen van diverse organen en afkomst te bestuderen en te vergelijken.

Door gebruik van modelstoffen kon worden aangetoond dat de LLC-PK1 cellijn een relatief eenvoudig model kan zijn voor het bepalen van een specifiek niertoxisch potentieel van glutathion- en cysteïne-conjugaten. Voor dit doel bleek de afwezigheid van een organisch-aniontransport in de LLC-PK1 cellijn geen belemmering. Wanneer echter andere chemicaliën op hun mogelijke niertoxiciteit onderzocht dienen te worden, kan de aanwezigheid van een organisch-aniontransport een voorwaarde zijn. Dientengevolge zou verdere karakterisering van andere cellijnen zoals de opossum niercellijn (Koyama et al, 1978) of een recent ontwikkelde humane niercellijn (KRC/Y) (Yano et al, 1988) zich moeten richten op de aanwezigheid van deze transporter.

Ten slotte onderstrepen de experimenten waarin de rol van γGT in de niertoxiciteit van glutathion-geconjugeerde gehalogeneerde hydrochinonen bestudeerd werd, nogmaals het belang van in vivo validering . Verschillen tussen in vivo en in vitro van meer fysische aard, zoals bijv. zuurstofdruk en pH, kunnen verschillende of additionele effecten van stoffen veroorzaken, die in vitro kunnen worden gemist wanneer geen rekening wordt gehouden met deze verschillen.

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Jos Mertens werd op 18 mei 1958 geboren te Oirsbeek. In 1976 behaalde hij het Atheneum B diploma aan het St. Janscollege te Hoensbroek. In hetzelfde jaar begon hij met de studie Biologie aan de Landbouwhogeschool (nu Landbouwuniversiteit) te Wageningen. Zijn stage vervulde hij bij het Rijksinstituut voor Volksgezondheid en Milieuhygiëne, Laboratorium voor Pathologie. Het doctoraalexamen, met als hoofdvakken Dierfysiologie en Toxicologie, behaalde hij in september 1984. Na het vervullen van zijn militaire dienstplicht, startte hij, in december 1985, het in dit proefschrift beschreven onderzoek op de vakgroep Toxicologie van de Landbouwuniversiteit. Vanaf 15 januari 1991 is hij werkzaam aan de University of Texas at Austin, College of Pharmacy, Division of Pharmacology and Toxicology.