

Paracetamol (Acetaminophen)-Induced Toxicity: Molecular and Biochemical Mechanisms, Analogues and Protective Approaches

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ABBREVIATIONS: **3'-HAA**, 3'-hydroxyacetanilide; **4'-HAA**, 4'-hydroxyacetanilide = paracetamol (PAR); **3',4'-diHAA**, 3',4'-dihydroxyacetanilide = 3-hydroxyparacetamol; **3-OH-PAR**, 3-hydroxyparacetamol; **ALT**, alanine aminotransferase (ALAT) = an indicator of hepatotoxicity; **AST**, aspartate aminotransferase (ASAT) = an indicator of hepatotoxicity; **BNF**, β -naphthoflavone; **CYP**, microsomal cytochrome P450 (used for isoform designation); **Cys**, cysteine***; **γ GT**, γ -glutamyltranspeptidase = γ -glutamyltransferase; **GSH**, glutathione = γ -glutamylcysteinylglycine; **GSSG**, oxidized glutathione; **LPO** = lipid peroxidation; **NAC**, *N*-acetylcysteine; **NAPQI**, *N*-acetyl-*p*-benzoquinone imine; **NAPSQI**, *N*-acetyl-*p*-benzosemiquinone imine; **P450** = microsomal cytochrome P450; **PAP** = *p*-aminophenol; **PAR**, paracetamol = acetaminophen = 4'-hydroxyacetanilide (4'-HAA); **PAR-CG**, cysteinylglycine conjugate of PAR; **PAR-Cys**, cysteine conjugate of PAR; **PAR-GLUC**, glucuronide conjugate of PAR; **PAR-NAC**, mercapturic acid of PAR; **PAR-SG**, glutathione conjugate of PAR; **PAR-SULP**, sulfate conjugate of PAR; **PGES**, prostaglandin endoperoxide synthetase; **UDPGA** = uridine diphosphoglucuronic acid (cofactor UDPGT); **UDPGT** = uridine diphosphoglucuronyl transferase.

*** Although mostly not indicated for the sake of readability, in the case of amino acids or amino acid-like compounds, the stereoisomer with the L-configuration is always meant, unless stated otherwise.

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ABSTRACT: An overview is presented on the molecular aspects of toxicity due to paracetamol (acetaminophen) and structural analogues. The emphasis is on four main topics, that is, bioactivation, detoxication, chemoprevention, and chemoprotection. In addition, some pharmacological and clinical aspects are discussed briefly. A general introduction is presented on the biokinetics, biotransformation, and structural modification of paracetamol. Phase II biotransformation in relation to marked species differences and interorgan transport of metabolites are described in detail, as are bioactivation by cytochrome P450 and peroxidases, two important phase I enzyme families. Hepatotoxicity is described in depth, as it is the most frequent clinical observation after paracetamol-intoxication. In this context, covalent protein binding and oxidative stress are two important initial (Stage I) events highlighted. In addition, the more recently reported nuclear effects are discussed as well as secondary events (Stage II) that spread over the whole liver and may be relevant targets for clinical treatment. The second most frequent clinical observation, renal toxicity, is described with respect to the involvement of prostaglandin synthase, *N*-deacetylase, cytochrome P450 and glutathione *S*-transferase. Lastly, mechanism-based developments of chemoprotective agents and progress in the development of structural analogues with an improved therapeutic index are outlined.

KEY WORDS: paracetamol (acetaminophen), structural analogues, hepatotoxicity, bioactivation, interspecies differences, chemoprevention, chemoprotection.

I. INTRODUCTION

A. Kinetics and Biotransformation

Paracetamol (4'-hydroxyacetanilide, *N*-acetyl-*p*-aminophenol, acetaminophen, PAR) is a widely used over-the-counter analgesic and antipyretic drug. In the UK, approximately 3.2×10^9 tablets of PAR are consumed every year, which is an average of 55 tablets/person (Jones, 1998). The pharmacological effects of PAR are generally considered to be based on inhibition of prostaglandin synthesis (Flower and Vane, 1972; Mattamal *et al.*, 1979; Malmberg and Yaksh, 1982). At therapeutic levels, PAR is considered to be safe for humans upon normal drug use (Thomas, 1993). The absorption of low therapeutic doses of PAR is usually rapid and complete, the systemic bioavailability and the plasma half-life being about 75% and 1.5 to 2.5 h, respectively. The toxicokinetics of PAR were reviewed in the past (Prescott, 1980) and investigated recently using a pharmacokinetic model (Tone *et al.*, 1990). As delineated from urinary metabolites and discussed in various review articles (Thomas, 1993), PAR is metabolized primarily by glucuronidation and sulfation (Figure 1). These major conjugates PAR-sulfate (PAR-SULP) and PAR-glucuronide (PAR-GLUC), being more water-soluble than the parent compound, are eliminated from the liver and blood mainly via urine (both) and a little via bile (PAR-GLUC). About 30 and 55% of administered PAR is excreted in urine as PAR-SULP and PAR-GLUC, respectively (Howie *et al.*, 1977; Tone *et al.*, 1990). Like in various laboratory animals, a small amount of PAR is probably metabolised via

a third metabolic pathway, that is, oxidation by the microsomal cytochrome P450 (CYP)-containing mixed-function oxidase system (MFO) to NAPQI. A glutathione 1,4-Michael adduct of NAPQI and the corresponding cysteine conjugate and mercapturic acid breakdown products were found in human urine after ingestion of PAR (Howie *et al.*, 1977; Knox and Jurand, 1977; Prescott, 1980). Although a minor oxidation reaction, hydroxylation of PAR to 3-hydroxyparacetamol (3-OH-PAR) is probably also occurring in man as methylated 3-hydroxy-paracetamol has been found in urine of patients who had taken an overdose of PAR (Knox and Jurand, 1977). In addition, as shown for various laboratory animals, probably also human enzymes with peroxidase activity, like prostaglandin synthase and myeloperoxidase, have the ability to catalyze the metabolism of PAR. The enzyme-catalyzed bioactivation by cytochrome P450 as well as peroxidase-like enzymes is discussed in Section III.

Most of the large amount of investigations regarding PAR-induced toxicity have of course been performed in (laboratory) animals. In either one or more of the species rat, mouse, hamster, and dog, besides the human metabolites, several other metabolites were found in urine, that is, 3-thiomethylparacetamol (including its sulfate, its glucuronide and its sulfone derivative), *p*-aminophenol and the (pre)mercapturic acid conjugates of *p*-benzoquinone (Wong *et al.*, 1976; Howie *et al.*, 1977; Knox and Jurand, 1977; Gemborys and Mudge, 1981; Hart *et al.*, 1982; To and Wells, 1985; Betowski *et al.*, 1987; Lubek *et al.*, 1988a). A composition of the metabolic pathways of PAR in the various species is depicted in Figure 1.

B. Toxicodynamics

After intake of a toxic dose, PAR mainly causes P450-dependent centrilobular hepatotoxicity in man and various laboratory animals (Mitchell *et al.*, 1973a; Hinson, 1980), as observed by the release of serum alanine aminotransferase (ALT) into the serum. Serum ALT is often used as monitoring parameter for hepatic damage. Much evidence has been presented for *N*-acetyl-*p*-benzoquinone imine (NAPQI) to be the reactive electrophilic intermediate responsible for the observed toxicity (Albano *et al.*, 1985; Van de Straat *et al.*, 1988b), although an as yet elusive *N*-acetyl-*p*-benzosemiquinone imine (NAPSQI) was proposed as well (De Vries, 1981; Potter and Hinson, 1989; Bessems *et al.*, 1998). In addition, the nontoxic catechol 3-hydroxy-*p*-paracetamol (3-OH-PAR) is formed by it as a minor metabolite (Hinson *et al.*, 1980; Forte *et al.*, 1984; Harvison *et al.*, 1988b). At normal doses of PAR, in most species including man, only a trace amount of the reactive intermediate NAPQI is formed. In the presence of reduced glutathione (GSH), for example, in isolated rat hepatocytes, NAPQI can either be reduced back to PAR or covalently linked to GSH to form a 3-glutathione-S-yl-paracetamol conjugate (PAR-SG) (Moldéus, 1978; Van de Straat *et al.*, 1986) without displaying significant adverse effects. After an overdose, however, or when specific microsomal P450s are increased, hepatic GSH is depleted more extensively and can no longer compensate for a massive production of NAPQI. Especially, depletion of mitochondrial GSH is correlated with hepatic toxicity (Vendemiale *et al.*, 1996). Paracetamol, most likely via NAPQI, can form adducts with proteins (Jollow *et al.*, 1973; Hinson *et al.*, 1995; Holtzman, 1995; Nelson, 1995), oxidise protein sulfhydryls (Birge *et al.*, 1988; Tirmenstein and Nelson, 1990), covalently bind to liver (as well as renal) DNA (Hongslo *et al.*, 1994) and eventually disrupt cellular homeostasis. A short overview mainly on the most common liver effects but also some other effects of acute and high ingestion of PAR is depicted in Table 1.

Thus, as described more in detail in Section IV, in mice, rats, and humans, an overdose

of PAR may result in severe centrilobular hepatic necrosis (Hinson *et al.*, 1981; Prescott, 1983; Hinson *et al.*, 1990; Vermeulen *et al.*, 1992). However, also renal tubular necrosis (Section V) (Cobden *et al.*, 1982; Newton *et al.*, 1985b; Björck *et al.*, 1988; Emeigh Hart *et al.*, 1991a; Emeigh Hart *et al.*, 1991b; Möller-Hartmann and Siegers, 1991; Hoivik *et al.*, 1995; Emeigh Hart *et al.*, 1996) may develop. Although uncommon, acute renal failure due to a large PAR overdose may occur in the absence of fulminant hepatic failure (Eguia and Materson, 1997). Moreover, in mice pulmonary nonciliated bronchiolar epithelial (Clara cell) necrosis (Jeffery and Haschek, 1988), covalent protein adduct formation in lungs (Bartolone *et al.*, 1989) and strain specifically, cataractogenesis (Lubek *et al.*, 1988a; Wells *et al.*, 1995) were reported to occur. In man, deleterious effects on blood platelets were found upon acute ingestion of large amounts of PAR (Fischereder and Jaffe, 1994). Furthermore, it is indicated that long-term exposure of humans to high but still therapeutic doses of PAR is correlated with increased risk of chronic renal disease (Sandler *et al.*, 1989). PAR-induced liver tumours in mice (Flaks and Flaks, 1983) and bladder carcinomas in rats (Flaks *et al.*, 1985) have been reported as well. Genotoxic effects of PAR have been found *in vitro* (Brunborg *et al.*, 1995), in various laboratory animals (Hongslo *et al.*, 1994), and in man (Rannug *et al.*, 1995). Recently, even adverse as well as positive effects on estrogen-related physiologic processes, such as proliferation of cultured breast cancer cells but, although preliminary, an epidemiological finding of a decrease of ovarian cancer, were reported (Cramer *et al.*, 1998; Harnagea-Theophilus *et al.*, 1999; Miller *et al.*, 1999).

Significant differences exist with respect to the susceptibility of various species and even the various strains of laboratory animals to the deleterious effect of PAR on a toxic dose (Hinson, 1980; Ioannides *et al.*, 1983; Gregus *et al.*, 1988; Lubek *et al.*, 1988a). In general, hamsters and mice are sensitive whereas rats, rabbits, and guinea pigs are relatively resistant to PAR-induced liver injury. These species differences (toxicodynamic and/or toxicokinetic) should be kept in mind, es-

TABLE 1
Subcellular Effects and Physiological Changes Caused by Paracetamol

Parameters/mechanism	Species/Test system	Reference
GSH depletion	many species	(Mitchell <i>et al.</i> , 1973b; Vendemiale <i>et al.</i> , 1996)
GSH oxidation	many species	(Albano <i>et al.</i> , 1985)
protein thiol depletion:	many species	
-liver proteins	-various species	(Roberts <i>et al.</i> , 1987a)-
-plasma membrane proteins	-rat	(Tsokos-Kuhn <i>et al.</i> , 1988)
-hemoglobin	-mouse	(Axworthy <i>et al.</i> , 1988)
protein thiol oxidation	many species	(Albano <i>et al.</i> , 1985; Kyle <i>et al.</i> , 1990)
lipid peroxidation (LPO)	many species	(Wendel <i>et al.</i> , 1979)
production hydroperoxides	mouse, hepatocytes	(Adamsen and Harman, 1989)
incr. malondialdehyde	rat liver, <i>in vivo</i>	(Vendemiale <i>et al.</i> , 1996)
mitochondrial membrane potential; change within 30 min	rat, liver slices	(Nazareth <i>et al.</i> , 1991)
plasma membrane potential; change after 4 hours	rat, liver slices	(Nazareth <i>et al.</i> , 1991)
decr. Na ⁺ /K ⁺ -ATPase activity; protein arylation	mouse / rat, liver plasma-membrane	(Corcoran <i>et al.</i> , 1987a; Corcoran <i>et al.</i> , 1987b; Corcoran <i>et al.</i> , 1988)
decr. Ca ²⁺ /Mg ²⁺ -ATPase activity; protein arylation; no support ox stress hypothesis	rat, liver plasma membrane	(Tsokos-Kuhn <i>et al.</i> , 1988)
membrane blebbing	rat, hepatocytes	(Moore <i>et al.</i> , 1985)
inhibition carbamyl phosphate synthetase-I	mice, <i>in vivo</i>	(Gupta <i>et al.</i> , 1997)
inhibition glutamine synthetase	mice, <i>in vivo</i>	(Gupta <i>et al.</i> , 1997)
incr. mitochondrial respiration	rat liver, <i>ex vivo</i>	(Vendemiale <i>et al.</i> , 1996)
liver necrosis	many species	many references (see text)
renal tubular necrosis	many species	many references (see text)
cataracts	susceptible mice strains	(Lubek <i>et al.</i> , 1988a; Lubek <i>et al.</i> , 1988b; Wells <i>et al.</i> , 1995)
incr. nuclear Ca ²⁺ ; within 2-6 h	mouse, <i>in vivo</i>	(Ray <i>et al.</i> , 1990; Ray <i>et al.</i> , 1991)
inhibition of replicative DNA synthesis	Chinese hamster V79 cells	(Richard <i>et al.</i> , 1991)
inhibition of DNA synthesis	rat, <i>in vivo</i>	(Lister and McLean, 1997)
loss of large genomic DNA; within 2-6 h	mouse, <i>in vivo</i>	(Ray <i>et al.</i> , 1990; Ray <i>et al.</i> , 1991)
inhibition of cell cycling	HL-60 cells	(Wiger <i>et al.</i> , 1997)
induction of apoptosis	HL-60 cells	(Wiger <i>et al.</i> , 1997)

pecially when one realises that the choice of the species is not always a scientific choice but an economical or a practical one. Many *in vivo* investigations are performed with mice whereas for many *in vitro* experiments the rat is chosen. Important differences in conjugation and deconjugation pathways between species and between organs (which is further discussed in Section II) may be involved in this differential sensitivity to PAR toxicity (Miller *et al.*, 1993). In Table 2 a short overview is presented of interspecies and intraspecies/interindividual differences — genotype (inbred strains) as well as phenotype (including environmental factors) — in the susceptibility to PAR toxicity.

C. Chemoprotection and Structural Modification

1. A Tool in Biomedical and Toxicological Research

In the last 10 to 15 years, remarkably, PAR has become a model toxin and a tool in biochemical and clinical toxicological research, that is, PAR has developed into a model compound for examination of the similarities and differences in toxicity mechanism between PAR and other xenobiotics, either with a similar structure (regioisomers and analogues or derivatives) or with similar toxicological features, such as pulegone and bromobenzene (Manautou *et al.*, 1995). Also, PAR is used as model toxin for establishing the usefulness of *in vitro* models such as liver slices in investigation of, for example, species differences in centrilobular damage (Miller *et al.*, 1993). With the elucidation of the many stages in the toxicity mechanism of PAR, an ever increasing number of chemical substances has been examined for potential chemoprotective properties (mechanism-based testing of chemoprotective properties of many substances [Mourelle *et al.*, 1990; Chanda *et al.*, 1995]), all targeted at a relatively specific biochemical action/lesion (Section VI) and investigated in various test systems, from reconstituted enzyme systems, microsomal incubations, via isolated hepatocytes, liver slices and perfused livers to whole animals, for example, on coincubation of PAR in rat liver

slices with a suitable inhibitor of cytochrome P450, cell injury is prevented whereas addition after 2 h of incubation with PAR is ineffective. Then, however, treatment with the antioxidant dichlorophenol indophenol (DCPIP) is effective (Mourelle *et al.*, 1990). Some more important compounds examined and biochemical features studied as possible target site in chemoprotection are mentioned here: addition of GSH status recovering compounds like cysteine, clofibrate (Manautou *et al.*, 1996), addition of reducing compounds like ascorbic acid (De Vries, 1981), addition of antioxidant drugs like lobenzarit (anti-rheumatic) and curcumin (Donatus *et al.*, 1990; Ramirez *et al.*, 1995), modulation of the conjugation with uridine-diphosphoglucuronic acid (UDPGA) and 3'-phosphoadenosine-5'-phosphosulfate (PAPS) (Fayz *et al.*, 1984), administration of garlic and related organosulfur compounds (Wang *et al.*, 1996), the antidotal effect of *N*-acetylcysteine (Prescott, 1983; Corcoran *et al.*, 1985), agents that protect against oxidative stress and lipid peroxidation (LPO) (Harman and Fischer, 1983; Harman, 1985; Dai and Cederbaum, 1995), compounds with glutathione peroxidase-like activity (Li *et al.*, 1994a), a compound with possible GSH reductase stimulating effect (Ramirez *et al.*, 1995), inhibitors of P450 (Snawder *et al.*, 1993; Li *et al.*, 1994b; Alexidis *et al.*, 1996). The result of this type of research is also an increased understanding of the mechanisms of cytoprotective effects as such, that is, independent of the primary damaging agent.

2. Structural Modification

Besides efforts to modulate the PAR toxicity or to understand the toxicity mechanism(s), important progress has been made as well by modifying the molecular structure of PAR itself. Regioisomers (congeners, e.g., 2'- and 3'-hydroxyacetanilide) and substituted analogues (derivatives) of PAR were synthesized and used in elucidating the relevance and the molecular mechanism of oxidative biotransformation in the observed toxicity of PAR (Van de Straat *et al.*, 1986; Van de Straat *et al.*, 1987b; Holme *et al.*, 1991; Barnard *et al.*, 1993a; Bessems *et al.*, 1996; Bessems *et al.*, 1997). Also, regioisomers and

TABLE 2
Interspecies, Interstrain and Interindividual Differences with Respect to Paracetamol-Induced Toxicity or Biotransformation of Paracetamol

Factor	Mechanism of action	Toxic effect	Reference
<u>Interspecies differences</u>			
hamster = mouse > guinea pig > rat	incr. bioactivation of PAR	incr. protein arylation	(Ioannides <i>et al.</i> , 1983)
hamster = mouse > guinea pig > rat	ratio toxication/detoxication pathways	incr. susceptibility	(Gregus <i>et al.</i> , 1988)
hamster > rat	ratio toxication/detoxication pathways	incr. susceptibility	(Miller <i>et al.</i> , 1993)
<u>Interstrain/ethnic differences</u>			
rat, Sprague-Dawley vs Long Evans Hooded	incr. glucuronidation	decr. hepatotoxicity	(Price and Jollow, 1986)
rat, Fisher 344 vs Sprague-Dawley	yes and no deacetylation to p-aminophenol	Fisher 344 rats are susceptible to renal toxicity	(Newton <i>et al.</i> , 1985b)
rat, hepatocytes (Sprague-Dawley vs Fischer344)	[GSH] in Fisher 344 higher than Sprague-Dawley rats	still, Fisher 344 rats are more susceptible to hepatotoxicity	(Wilson <i>et al.</i> , 1991)
mouse, C57BL/6 vs DBA/2 strain	inducibility vs non-inducibility of CYP1A	susceptible vs resistant to cataractogenesis	(Lubek <i>et al.</i> , 1988a; Lubek <i>et al.</i> , 1988b; Wells <i>et al.</i> , 1995)
man, in vivo	genetic or environmental factors	2-fold inter-ethnic and 60-fold inter-subject variation in mercapturic acid excretion	(Critchley <i>et al.</i> , 1986)
<u>Genetic deficiencies</u>			
man, hepatoma line	lack of CYP2E1 expression	no cytotoxicity	(Dai and Cederbaum, 1995)
man, 'Gilbert's syndrome'	lack of bilirubin UDPGT ¹ ; decreased glucuronidation	incr. ratio urinary toxication/detoxication metabolites	(De Morais <i>et al.</i> , 1992)
<u>Interindividual differences/lifestyle factors</u>			
ageing as shown in mouse kidney, in vivo	incr. GSH and cysteine depletion	incr. susceptibility with age	(Richie <i>et al.</i> , 1992)
certain medicinal drugs	incr. glucuronidation	incr. excretion of glucuronide	references in (Burchell and Coughtrie, 1997)
diabetes (rat)	incr. UDPGA ² , incr. glucuronidation	decr. hepatotoxicity	(Price and Jollow, 1986)
diet (cruciferous vegetables) in man	incr. glucuronidation due to indoles	incr. excretion of glucuronide	referenced in (Burchell and Coughtrie, 1997)
fasting (rat)	altered carbohydrate metabolism	decr. glucuronidation (UDPGA ²)	(Price and Jollow, 1988)
obesity (rat)	incr. glucuronidation / decr. sulphation	incr. hepatic and renal toxicity	(Corcoran and Wong, 1987)
5-oxoprolinuria shown in human lymphocytes + activating system	GSH-synthetase deficiency	incr. cytotoxicity	(Spielberg and Gordon, 1981)
pregnancy in man	incr. glucuronidation and oxidation	incr. oral clearance / decr. elimination half-life	(Miners <i>et al.</i> , 1986)
sulfur deficiency (rat)	decr. sulphation	incr. hepatic necrosis	(Price and Jollow, 1989)

¹ UDPGT = uridine diphosphoglucuronosyltransferase

² UDPGA = uridine diphosphoglucuronic acid

substituted analogues were studied for possible improvement of the analgesic properties and diminution of the toxic properties of PAR (Nelson *et al.*, 1978; Harvison *et al.*, 1986b; Van de Straat *et al.*, 1986; Van de Straat *et al.*, 1987b; Harvison *et al.*, 1988a; Rundgren *et al.*, 1988; Ramsay *et al.*, 1989; Holme *et al.*, 1991; Barnard *et al.*, 1993a; Barnard *et al.*, 1993b; Bessems *et al.*, 1995; Weis *et al.*, 1996). These aspects are discussed in detail in Section VII. Notwithstanding the advantages of these more fundamental aspects of this research, the clinical relevance remains high as in 1992, in England and Wales, out of 1951 deaths due to overdoses of medicines, still 144 deaths were due to poisoning with PAR (Spooner, 1995).

D. Summary

Improvement of the therapeutic index of PAR could result from increase of the intrinsic analgesic activity (thereby lowering the therapeutic dose and possibly the size of a package), from decreased production of the toxic intermediate(s), from increased capacity to detoxify the toxic intermediate(s), from increased ability of tissue to withstand or even repair the molecular damage caused by toxic species and from modification of the chemical structure of PAR. This article aims at reviewing studies that deal with the molecular, biochemical, and cellular aspects of the analgesic and, more importantly in this context, the toxic properties of PAR. Furthermore, the role of biotransformation in the activation and detoxification of PAR and some of the tools available for the protection against PAR toxicity will be discussed (chemoprevention and/or -protection). Special emphasis is laid on the recent findings on arylation by PAR of a number of cytosolic, mitochondrial, microsomal and nuclear proteins and the consequences of modification of its molecular structure.

II. BIOTRANSFORMATION — PHASE II

A. Bioinactivation and Species Differences

As indicated in the introduction, a large portion of ingested PAR is directly conjugated in

detoxifying phase II reactions by sulfation and glucuronidation in most laboratory species. In humans, about 30 and 55% of administered PAR is excreted in urine as PAR-SULP and PAR-GLUC, respectively, whereas PAR-Cys and PAR-NAC each account for some 4% of the dose. At elevated although still therapeutic doses (1.5 g), sulfate conjugation becomes saturated with less than 20% and more than 75% being excreted as PAR-SULP and PAR-GLUC, respectively (Howie *et al.*, 1977; Tone *et al.*, 1990).

These findings are in line with general findings regarding sulfotransferases and UDP-glucuronosyltransferases (Mulder, 1990) and substantiated by data from rat. In liver perfusion experiments (via the portal vein), in contrast to PAR-SULP, recovery of PAR-GLUC in the perfusate and bile increased more than threefold upon increase of the dose, indicating a higher capacity for glucuronidation than for sulfation, but a higher affinity for sulfation (Fayz *et al.*, 1984; Mitchell *et al.*, 1989). Similar results were obtained in rat hepatocyte incubations, where V_{\max} values for sulfation and glucuronidation were comparable (1.1 and 1.3 nmol/10⁶ cells/min, respectively), whereas K_M values were significantly different (0.03 and 2.1 mM, respectively) (Mizuma *et al.*, 1985).

As mentioned in Section I, significant differences exist with respect to the susceptibility of various species to the hepatotoxic effect of PAR (Hinson, 1980; Ioannides *et al.*, 1983; Gregus *et al.*, 1988). Although even strain specificities exist, in general hamsters and mice are most sensitive, whereas rats, rabbits, and guinea pigs are relatively resistant to PAR-induced liver injury. After i.v. administration of 1 mmol/kg to the susceptible animal species, 27 to 42% of the dose is excreted as toxication pathway-related metabolites (PAR-SG and its hydrolysis breakdown products) in contrast to only 5 to 7% for the less sensitive species. In the sensitive species hamsters and mice, only 12 and 41%, respectively, of the dose are excreted as metabolites of the detoxifying route (PAR-GLUC and PAR-SULP) whereas in rats, rabbits, and guinea pigs these percentages are 62%, 27%, and 74%, respectively. Furthermore, hamsters and mice excreted mainly PAR-SG as such via bile (low γ -glutamyltranspeptidase (γ GT) activity), whereas rabbits

and guinea pigs excreted significant amounts of PAR-SG hydrolysis products via bile (high γ GT activity) (Gregus *et al.*, 1988). These *in vivo* differences with respect to the ratio of toxication/inactivation in relation to centrilobular damage have been substantiated for rat and hamster in liver slices (Miller *et al.*, 1993). Therefore, it seems conceivable that the ratio between excretion (via bile) of PAR-SG (including breakdown products) and the combined excretion of PAR-GLUC and PAR-SULP, to some extent predict the toxicity of PAR in a specific species or even strain. The tendency of mice and hamsters to excrete more toxication pathway-related metabolites compared with rats and rabbits appears from Table 3.

B. Interorgan Transport of Metabolites

Two major organs, the kidneys and intestine, have been implicated in the metabolism of sulfur-containing metabolites of PAR. Both organs contain γ GT and dipeptidase for the breakdown of PAR-SG to PAR-CG and PAR-Cys. The kidneys play a major role in the disposition of sulfur-containing metabolites of PAR, either by direct excretion (glomerular filtration of PAR-Cys and probenecid-sensitive active transport of PAR-NAC) or by further biotransformation with subsequent renal excretion. The hamster urinary metabolites PAR-SCH₃ and PAR-SOCH₃ are derived from PAR-SG breakdown products within the enterohepatic circulation (Gemborys and

TABLE 3
Metabolites of Paracetamol Found in Excreta of Species More and Less Sensitive to Paracetamol Poisoning¹

	Mouse		Hamster		Rat		Rabbit	1.5 g Man >20 g	
	Plasma	Urine	Bile	Urine	Bile	Urine	Urine	Urine	Urine
PAR	++	++		+			+	+	+
PAR-SULP	++	+	+-	+	+	+++	+	++	+++
PAR-GLUC	++	++	+-	++	+++	++	+++++	+++++	++++
3-OH-PAR ⁽²⁾		+		-				+	
3-OCH ₃ -PAR ⁽²⁾		+		+				+	+
PAR-SG		+	+++++		++		+		
PAR-Cys	+	++	+-	+		-	+	+-	++
PAR-NAC	+	++		++++		+		+	+
PAR-SCH ₃		++		+++		+			
PAR-SOCH ₃		++		++		+			
HQ-Cys		+							
HQ-NAC		+							
PAP				+					
References	a	b	c	d	e	f	g	h	i

⁽¹⁾ Most abbreviations are self-explanatory (see also text, mostly Part I), except maybe the following: HQ-Cys and HQ-NAC for the cysteine conjugate and mercapturic acid of 1,4-hydroquinone, respectively, and PAP for *p*-aminophenol. Furthermore, a (-)-sign means that analysis was amongst others aimed at that specific metabolite. A blank cell in the table means that the specific metabolite was just not encountered.

⁽²⁾ Only data for the conjugated (GLUC and SULP) of PAR are indicated separately. Data for those conjugates of primary and secondary metabolites of PAR are included in the quantities of the corresponding aglycone.

References: (a) (To and Wells, 1965), (b) (Hart *et al.*, 1982; Forte *et al.*, 1984; To and Wells, 1985; Pascoe *et al.*, 1988; Wang *et al.*, 1996), (c) (Madhu and Klaassen, 1991), (d) (Gemborys and Mudge, 1981; Warrander *et al.*, 1985), (e) (Mitchell *et al.*, 1989), (f) (Hart *et al.*, 1982; Price and Jollow, 1982; Corcoran and Wong, 1987), (g) (Lubek *et al.*, 1988b), (h) (Howie *et al.*, 1977), (i) (Mrochek *et al.*, 1974; Knox and Jurand, 1977; Slattery *et al.*, 1987)

Mudge, 1981; Newton *et al.*, 1986). The fate and behavior of glutathione- and cysteine-conjugates in general were reviewed (Commandeur *et al.*, 1995).

Collection of bile from PAR-dosed mice, containing mainly the GSH conjugate of PAR, reduced the urinary excretion of the premercapturate (cysteine conjugate) and the mercapturate by >70%, indicating that these urinary metabolites originated from the biliary GSH-conjugate. However, ligation of the common bile duct did not alter this urinary excretion, indicating that enterohepatic circulation is not obligatory for the appearance of the (pre)mercapturates of PAR in urine. Intravenous administration of purified PAR-SG conjugate did not result in biliary excretion of the parent conjugate but in urinary excretion of primarily the PAR-Cys conjugate. Together with other results, these findings suggest that if the PAR-SG leaves the liver (via the blood), it can rapidly be converted to the PAR-Cys conjugate by γ GT and dipeptidase, which appear in the intestine as well as the kidneys (Fischer *et al.*, 1985a). However, γ GT-dependent breakdown may also occur in the bile duct and gallbladder.

It was suggested that in species such as guinea pig and perhaps also in humans, the liver and the bile duct play a more important role in breakdown of GSH-conjugates than in rat and mouse, two species that have been used extensively in research on PAR-dependent toxicity (Hinchman and Ballatori, 1990). As in liver of rat and mouse γ GT-activity is very low relative to that of kidneys (Hinchman and Ballatori, 1990), the importance of the extrarenal breakdown of PAR-SG in humans may have been widely underestimated. Moreover, also the absence of a gallbladder in rat, a site where significant γ GT activity is localized in other species, may have contributed to this underestimation. In macaque relative to rat, the γ GT activity (per mg protein) in kidneys and liver is about eightfold lower and two- to threefold higher, respectively, making the ratio of γ GT activities between liver and kidneys almost 20-fold higher in macaques relative to rats. In addition, the γ GT activity in gallbladder (which is absent in rats) in macaque is even a little higher than in liver (Hinchman and Ballatori, 1990).

C. Summary

The species-specific susceptibility to PAR-dependent toxicity seems to be quite accurately reflected by the urinary metabolites. The susceptible species mice and hamster mainly excrete detoxification pathway-related metabolites (PAR-SG and breakdown products), whereas the relatively insensitive species guinea pig, rat, and rabbit excrete much more detoxification pathway-related products, such as PAR-SULP and PAR-GLUC. The site of breakdown of PAR-SG starting with activity of γ GT may be also important as PAR-Cys, although a breakdown product of PAR-SG, may still possess hazardous properties. Some species dispose mainly PAR-SG via bile and/or hepatic vein, whereas others dispose mainly hydrolysis products of PAR-SG. It should be noted that these differences are caused not solely by species variance in phase II biotransformation enzymes but also by differences in the activity of phase I enzymes (see Section III).

The complex pattern of the primary as well as secondary biotransformation of PAR in man, including the distribution of PAR and metabolites, is presented in Figure 2. The most striking interspecies differences with respect to metabolite formation, especially for detoxification vs. toxification pathway-related metabolites, are presented in Table 3.

III. BIOACTIVATION — PHASE I

A. Cytochrome P450

1. Mechanism of Oxidation

P450 enzymes are the most predominant drug-metabolizing enzymes in the liver and are also present in most other tissues of the body. Thus, it is not unexpected that the cytochrome P450-mediated oxidative bioactivation of PAR was the subject of several extensive reviews (Vermeulen *et al.*, 1992; Hinson *et al.*, 1995; Nelson, 1995). There is general consensus now that *N*-acetyl-*p*-benzoquinone imine is the main electrophilic reactive metabolite formed in the oxidative biotransformation of PAR *in vitro* and *in vivo* (Miner and Kissinger, 1979; Hinson *et al.*, 1981). Although

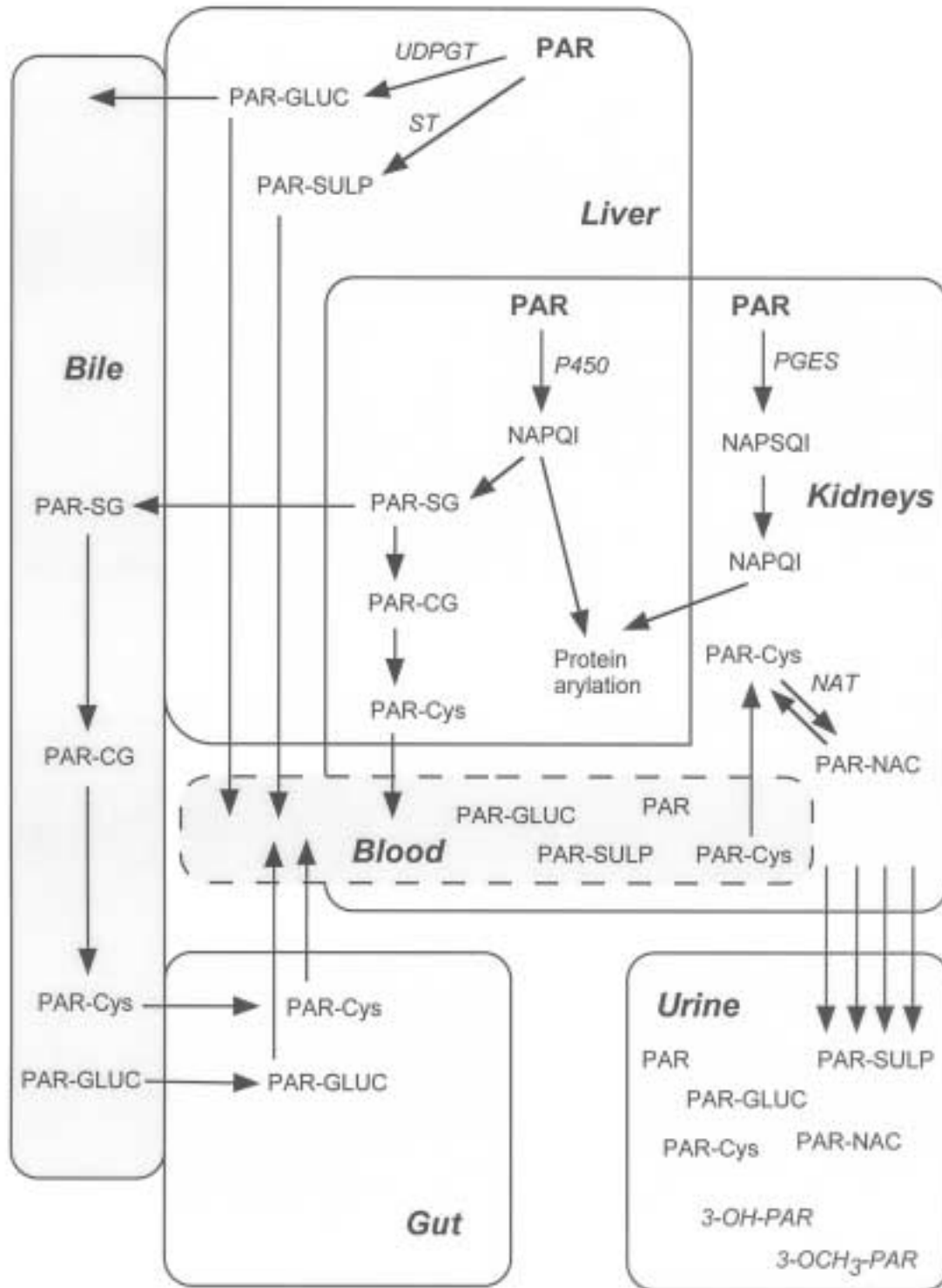


FIGURE 2. Disposition of paracetamol in humans. Paracetamol disposition (distribution and metabolism) in human serum, urine and feces (see text for references). The disposition of the minor urinary metabolites 3-OH-PAR and 3-OCH₃-PAR is not indicated but suggested to occur via hepatic formation and renal excretion.

its direct detection in *in vitro* systems is very difficult (Dahlin *et al.*, 1984), and the exact mechanism of formation is still not unequivocally identified (Koymans *et al.*, 1989; Myers *et al.*, 1994; Bessems *et al.*, 1998), NAPQI has been detected as its glutathione conjugate in numerous *in vitro* and *in vivo* systems (Hinson *et al.*, 1982; Newton *et al.*, 1986; Van de Straat *et al.*, 1986; Harvison *et al.*, 1988b; Bessems *et al.*, 1997).

Originally, *N*-hydroxylation or 3,4-epoxidation was postulated to be the first step in P450 catalyzed oxidation of PAR followed by dehydration to the electrophile NAPQI. However, after synthesis, the hydroxamic acid (hydroxylamine derivative) that would be formed on *N*-hydroxylation, exhibited a reported half-life of about 15 to 80 minutes, but was never detected in oxidative microsomal systems. Therefore, *N*-hydroxylation was ruled out as the mechanism of bioactivation of PAR by P450 (Hinson *et al.*, 1979; Hinson *et al.*, 1980; Calder *et al.*, 1981; Gemborys and Mudge, 1981). A second postulated mechanism, that is, the 3,4-epoxidation in the aromatic ring, was rejected by using $^{18}\text{O}_2$ and epoxide hydrolase in a rat liver microsomal incubation (Hinson *et al.*, 1980).

As a third mechanism, oxidation of PAR to NAPQI via the free radical species *N*-acetyl-*p*-benzosemiquinone imine (NAPSQI) was proposed (De Vries, 1981). Shortly thereafter computational data were presented for two sequential hydrogen abstractions leading to NAPQI (designated as the peroxidase pathway), being thermodynamically favoured over oxygenation with hydroxylamine formation (*N*-hydroxy metabolite) (Loew and Goldblum, 1985). However, no important differences were predicted by the semi-empirical model of Loew and Goldblum for the first hydrogen abstraction being abstracted from the phenolic oxygen of PAR or from the acetylamino-nitrogen (Loew and Goldblum, 1985). By using an improved computational method (*ab initio*), Koymans *et al.* argued that primary hydrogen abstraction from the phenolic oxygen was energetically favoured over an abstraction from the acetylamino-nitrogen. In their model, this was predicted to be followed by either a second hydrogen abstraction, leading to NAPQI, or hydroxyl radical recombinations, leading to 3-hydroxy-*paracetamol* (3-OH-PAR) or an *ipso*-adduct in

intermediate, the latter giving rise to *p*-benzoquinone (PBQ) and acetamide. Also NAPQI was proposed to be able to hydrolyze to pBQ and acetamide (Koymans *et al.*, 1989). This mechanism is depicted in Figure 3.

Moreover, the experimental finding of 3-OH-PAR, PBQ, and acetamide, next to NAPQI as a P450-dependent metabolites of PAR in the past (Miner and Kissinger, 1979; Corcoran *et al.*, 1980; Hinson *et al.*, 1980; Hinson *et al.*, 1982; Forte *et al.*, 1984), combined with NMR-relaxation studies on the binding of PAR in the active site of different P450 enzymes (Van de Straat *et al.*, 1987a; Myers *et al.*, 1994), prompted several authors to support this mechanistic interpretation (Hoffmann *et al.*, 1990; Myers *et al.*, 1994). Interestingly, NAPSQI was detected indirectly (a melanine-like signal was actually observed) with ESR in a reductive mixed-function oxidase-catalyzed reaction (Van de Straat *et al.*, 1987d) after the addition of NAPQI to an anaerobic, reconstituted system containing P450 and P450-RED. The absence of oxygen, as indicated by the authors, might imply that the reduction of NAPQI was due to the electron-donating activity of P450-RED only or of P450 + P450-RED in combination, but without involvement of oxygen as shown also under anaerobic conditions for 2,3,5,6-tetramethylbenzoquinone (Goeptar *et al.*, 1992).

However, it cannot be fully excluded that reduction of NAPQI was due to the oxygen reductase activity of P450 in the presence of small quantities of oxygen, as reviewed recently by Goeptar *et al.* (Goeptar *et al.*, 1995). As suggested here this could imply that the thus formed superoxide anion radical could be responsible for the reduction of NAPQI to NAPSQI with concomitant oxidation of the superoxide anion radical to molecular oxygen. Also, the NAPSQI could oxidise NADPH to NADP \cdot . The NADP \cdot thus formed might react with O_2 to produce the superoxide anion $\text{O}_2\cdot^-$ again as was suggested earlier (Keller and Hinson, 1991). An 'all in mechanism' as proposed here is depicted in Figure 4. Moreover, so far still no direct evidence has been found for the existence of such semiquinone imine radical intermediates during their P450 catalyzed oxidation of PAR and substituted analogues beyond the active site of the P450 enzymes (Bessems *et al.*, 1998). Therefore, as yet any discussion with respect to the relevance of

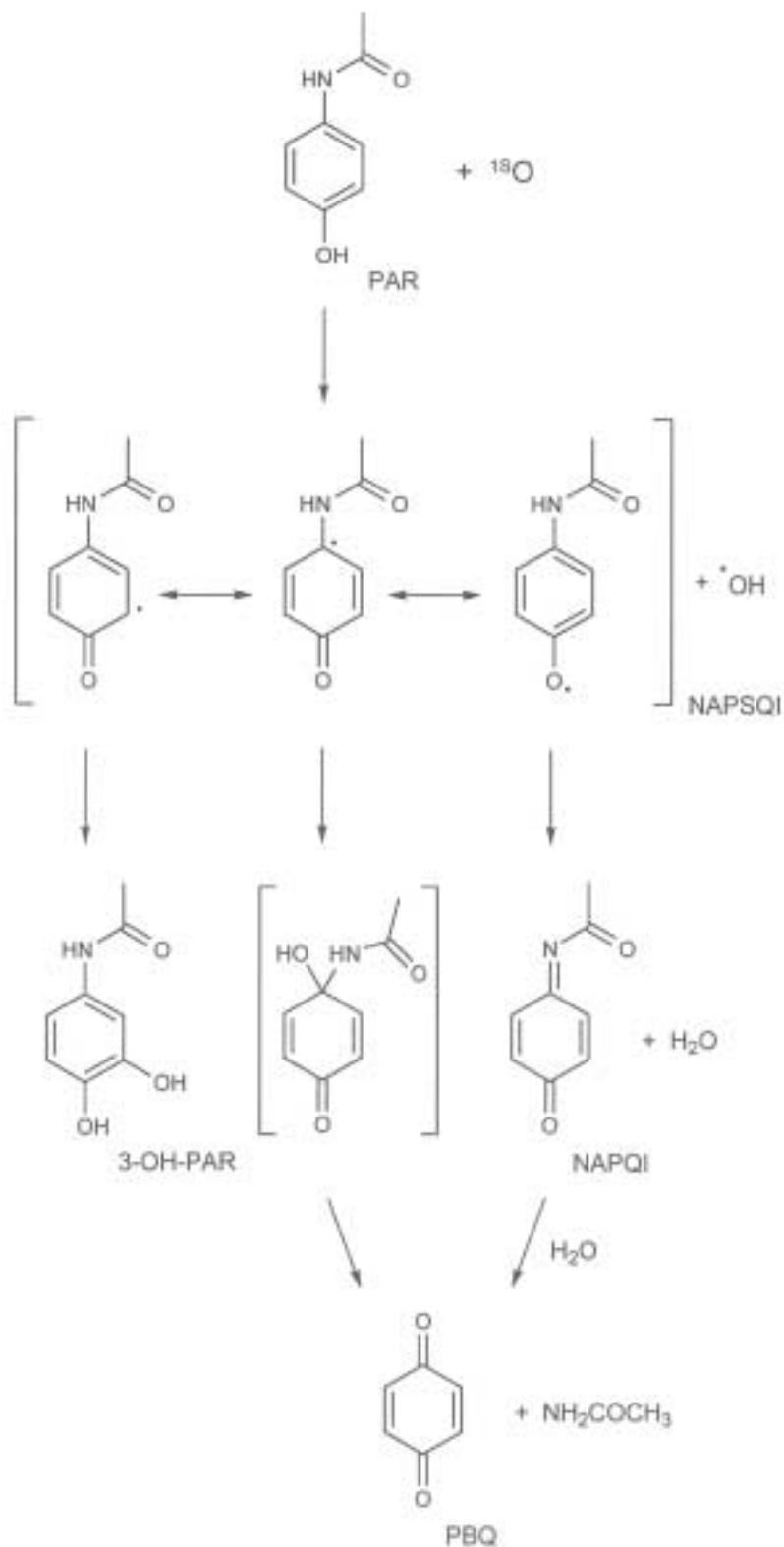


FIGURE 3. Hypothesized phenoxy radical pathway. Hypothesized oxidation of PAR by singlet oxygen, a substitute for cytochrome P450. An initial hydrogen abstraction occurs at the phenolic hydroxyl group of PAR and is followed by delocalisation of the radical. Recombination of the hydroxyl radical, formed in the active site of P450 from singlet oxygen, can give rise to 3-hydroxy-paracetamol (3-OH-PAR), *p*-benzoquinone (PBQ) plus acetamide and NAPQI, which have been found as three minor and one major P450-dependent metabolites of PAR, respectively. (Modified from Koymans *et al.* [1989].)

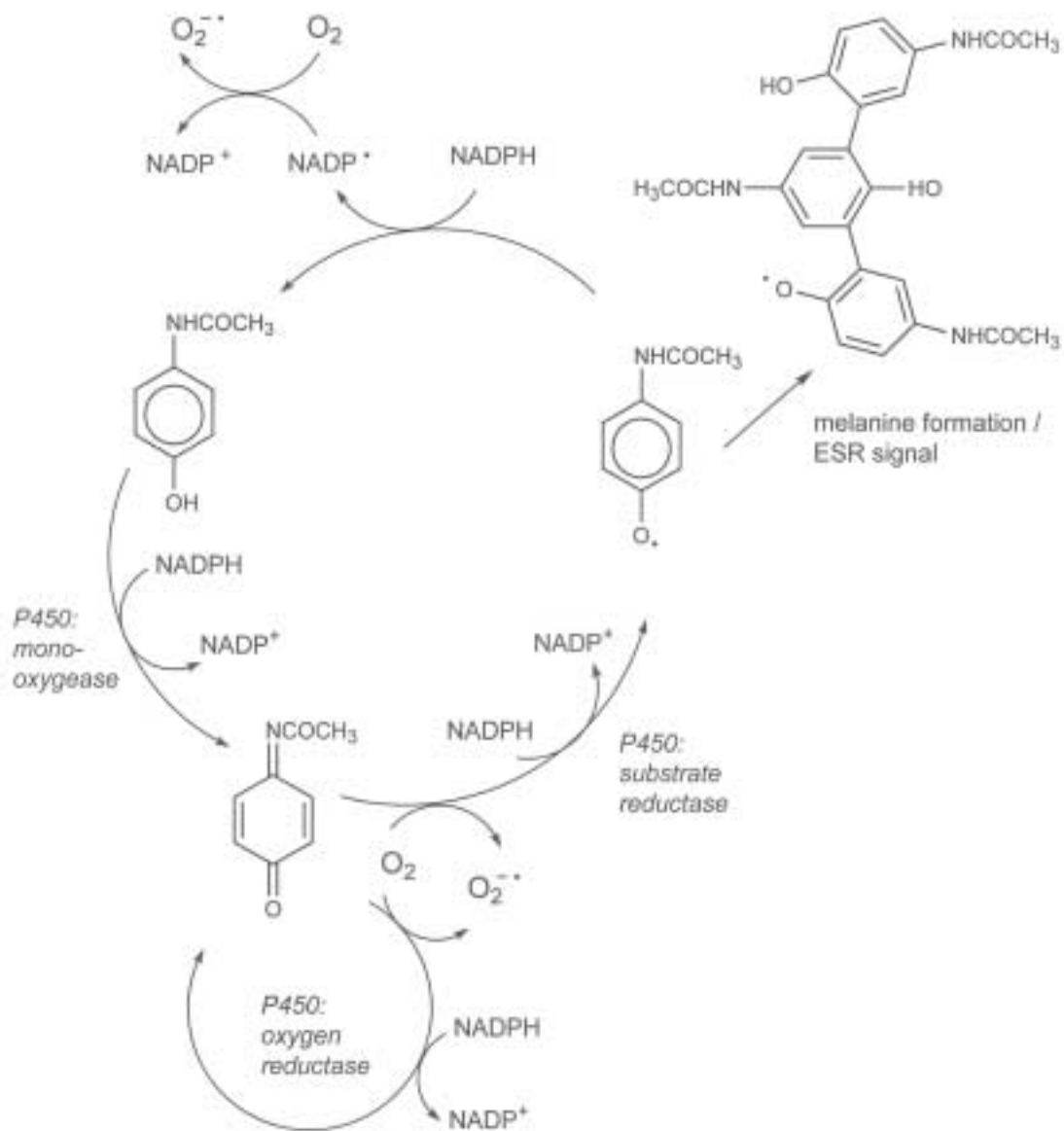


FIGURE 4. Proposed 'all in mechanism' for P450 incubations with NAPQI. Composed from observations reported in Van de Straat *et al.* (1987d) and Keller and Hinson (1991). (See text for further explanations.)

NAPSQI in the mechanism of P450-dependent toxicity of PAR remains speculative.

It cannot be excluded that recently found differences in kinetics between cysteine conjugation and liver microsomal protein binding of PAR should be seen in the context of the P450-based mechanism of formation of NAPQI from PAR. After an initial linear increase in both reactions with increase of NADPH concentrations, further increase of this cofactor significantly decreased cysteine conjugation while the rate of protein binding plateaued. Furthermore, NADH and NADPH reacted fairly different after the addition to incu-

bations with PAR concerning modulation of cysteine conjugation and protein binding. Also, ethanol feeding of mice (with probably CYP2E1 induction) before isolation of the microsomes, increased protein binding by about 97%, but cysteine conjugation by only 33% (Zhou *et al.*, 1997b). Although the absolute rates of formation were in the range of pmol/mg and nmol/mg microsomal protein for protein binding and cysteine conjugation, respectively, these differences might be due to some formation of NAPSQI by CYP2E1. Protein binding of NAPSQI could be favored over cysteine conjugation.

2. P450s Involved

Incubations with purified and reconstituted rat liver microsomal cytochrome P450 and PAR showed that, from 9 purified P450 enzymes studied, the constitutive and male-specific CYP2C11 (cytochrome P450_{UT-A}) exhibited the highest rate of formation of NAPQI (Table 4). The next highest was the β NF-inducible CYP1A1 (cytochrome P450_{BNF-B}). In addition, almost all of these rat P450s exhibited significant formation of 3-hydroxyacetamol (3-OH-PAR). Moreover, the phenobarbital (PB)-inducible CYP2B1 (cytochrome P450_{PB-B}) primarily formed (3-OH-PAR) (Harvison *et al.*, 1988b). Except that species differences exist with respect to the expression of specific P450s, for example, humans do not constitutively express CYP1A1 (Berthou *et al.*, 1992), it must be kept in mind, however, that results from reconstituted enzyme incubations may be less predictive for the *in vivo* situation compared with microsomal incubations. In microsomal incubations, rat liver CYP1A2, CYP2E1, CYP3A1, CYP3A2 and the human liver CYP3A4 catalyze the oxidation of PAR to NAPQI (Patten *et al.*, 1993; Thummel *et al.*, 1993). In contrast to recent studies where PAR metabolites were not detected in human Hep G2 and lymphoblast cell lines transfected with P450 2A6 DNA (Patten *et al.*, 1993), baculovirus-expressed and purified human CYP2A6 (as well as CYP2E1) was shown to oxidize PAR to NAPQI as well as the nontoxic 3-OH-PAR (Chen *et al.*, 1998), and, albeit at toxic doses of PAR, CYP2E1 was found to be the more efficient catalyst for the bioactivation to NAPQI (relative ratio NAPQI:3-OH-PAR formation was approximately 6:1), CYP2A6 also can contribute significantly to NAPQI formation (relative ratio 1:3) (Chen *et al.*, 1998). In addition, strong *in vitro* evidence was obtained from microsomal incubations retrieved from a transfected human lymphoblast cell line that also human CYP2D6 is involved in oxidation of PAR (Zhou *et al.*, 1997a). For an overview see Table 4.

In mice, CYP2E1 is probably the most important hepatic P450 enzyme involved in the bioactivation of PAR at low doses with little additional contribution at the high dose, whereas CYP1A2, probably exhibiting a higher K_M , contributes more to the bioactivation and toxicity of

PAR at high doses (Hu *et al.*, 1993; Snawder *et al.*, 1994). Findings of resistance, only slight resistance and high resistance against PAR-mediated toxicity with *cyp2e1* knock-out mice (CYP2E1 null phenotype), CYP1A2 null mice and mice being double-null for CYP1A2 and CYP2E1, respectively, provided definite proof for the dose-dependent involvement of CYP2E1 and CYP1A2 in bioactivation of PAR (Lee *et al.*, 1996b; Tonge *et al.*, 1998; Zaher *et al.*, 1998). In human *in vitro* systems, CYP1A2 also exhibits high K_M kinetics (low affinity; only effective at high concentrations) whereas CYP2E1 — and actually CYP3A4 even more — displays low K_M kinetics (high affinity; already effective at low concentrations) (Raucy *et al.*, 1989; Patten *et al.*, 1993; Thummel *et al.*, 1993). The human CYP2C8 and CYP2C9 exhibited only negligible activity (Raucy *et al.*, 1989). The major P450 enzymes in the Caucasian as well as the Japanese population are those of the CYP3A (about 30% of total P450) and CYP2C (about 20%) subfamily, followed by CYP1A2 (about 13%), CYP2E1 (about 7%), CYP2A6 (about 4%), CYP2D6 (about 2%) and CYP2B6 (< 1%) (Shimada *et al.*, 1994). Thus, CYP3A4 is probably the most important P450 at therapeutic concentrations whereas CYP2E1 and CYP1A2 becoming significantly involved at high plasma levels and at serious intoxication, respectively.

However, even incubations using human liver microsomes are a flattered mirror imaging of actual situations in which humans may be exposed to PAR overdose situations. Scaling to whole organ situations by using Michaelis Menten kinetic parameters like K_M (Michaelis constant) and V_{max} (as determined in microsomal incubations or reconstituted enzyme systems) might therefore be an important tool in finding out the practical relevance of the various P450 enzymes. Another tool for *in vivo* investigations of agents that are known to specifically modify one or more enzymatic biotransformation steps *in vitro*, is pharmacokinetic analysis of human plasma and urine levels of PAR and various metabolites. Using a simple descriptive combined one compartment pharmacokinetic model (first-order absorption and elimination of PAR combined with first-order formation and elimination of its metabolites), the human pharmacokinetics of PAR were studied. In addition, fits of plasma and urine data clearly

TABLE 4
Role of Different P450 Enzymes (CYP) from Liver in Deactivation and Activation of Paracetamol

CYP	Mouse		Hamster		Rat ^(*)		Rabbit	Man		
	(1)	(2)	(3) [*]	<i>in vivo</i>	(4)	(5) [*]	(6)	(7) [*]	(8) [*]	(9)
1A1					++++ ^(*)		+			
1A2	++	+	++	++	++	++++	+	++++	+	
2A1					+ ^(*)					
2A6										+
2B1					±					
2B2					*					
2C3							-			
2C6					+					
2C8								±		
2C9								±		
2C11					+++++					
2C12					+ ^(*)					
2E1	++	++	+			++++	+	+++++	+++	++
2D6									++	
3A7				++						
3A1						+				
3A2					±	+				
3A4								++ ^(*)	+	
3A6							-			
Reference	a	b	c	d	e	f	g	h	i	

- (1) Reconstituted CYP2E1 at low concentrations; CYP1A2 at high concentrations
(2) CYP1A2 null mice were only slightly resistant to PAR-mediated toxicity at relatively low dose; CYP1A2 and CYP2E1 double-null mice were highly resistant to PAR-induced toxicity
(3) Microsomal incubations.
(4) Reconstituted systems. In the original reference, the following nomenclature of the CYP enzymes was used: BNF-B (CYP1A1), ISF-G (CYP1A2), UT-F (CYP2A1), PB-B (CYP2B1), PB-D (CYP2B2), PB-C (CYP2C6), UT-A (CYP2C11), UT-I (CYP2C12), PCN-E (CYP3A2). For a large part of the enzymes, also significant amounts of 3-OH-PAR were formed.
(5) Control male rat microsomes.
(6) Reconstituted systems. In the original reference, the following nomenclature of the P450's was used: 6 (CYP1A1), 4 (CYP1A2), 3b (CYP2C3), 3a (CYP2E1), 3c (CYP3A6)
(7) Levels from microsomal incubations using monoclonal antibodies are presented. However, also reconstituted systems were used. Activities correlated with the specific content of the P450's in human liver microsomal samples.
(8) Incubations using microsomes from human lymphoblasts that were transfected with human P450's.
(9) Reconstituted systems from baculovirus-expressed and purified P450's
(*) In rat, the formation of 3-OH-PAR was investigated and appeared to be substantial or even outreached the NAPQI formation, measured as the GSH-conjugate
(i) It should be noted that the levels presented are for microsomal incubations. However, the K_M observed corresponds to the K_M for CYP3A4 (0.15 mM), suggesting that at therapeutic concentrations in humans, CYP3A4 is the most important P450 involved in bioactivation.
* Microsomal incubations are to be considered more relevant for estimation of the relative contribution of the various P450 enzymes

References: (a) (Snawder *et al.*, 1994), (b) (Tonge *et al.*, 1998; Zaher *et al.*, 1998), (c) (Madhu *et al.*, 1989; Raucy *et al.*, 1989), (d) (Madhu *et al.*, 1989), (e) (Harvison *et al.*, 1988b), (f) (Patien *et al.*, 1993), (g) (Morgan *et al.*, 1983; Jeffery *et al.*, 1991), (h) (Raucy *et al.*, 1989; Thummel *et al.*, 1993), (i) (Zhou *et al.*, 1997a), (j) (Chen *et al.*, 1996)

demonstrated the inhibitory effect of intake of watercress (which probably contains a precursor of the CYP2E1 inhibitor phenethyl isothiocyanate) on *in vivo* oxidative metabolism of PAR (leading to PAR-Cys and PAR-NAC) (Chen *et al.*, 1996). Even more sophisticated is the integration of *in vitro* biotransformation data inhibitors in predictive physiologically based models that can be used to predict the effects of various modulators. Such PBPK (physiologically based pharmacokinetic) models have been set up for dibromoethane (Ploemen *et al.*, 1997) and offer the possibility to incorporate and modulate other relevant biotransformation (e.g., conjugating) enzymes and other relevant organs. Also, other process than biotransformation only, absorption, distribution and excretion, could be incorporated in this way (Tone *et al.*, 1990).

As mentioned above, in mice, rats, as well as humans exposed to ethanol, next to CYP2E1, CYP3A is an important subfamily of P450 enzymes involved in the bioactivation and consequently the hepatotoxicity of PAR. Interestingly, CYP2E1 as well as enzymes of the CYP3A subfamily are very likely suicidally inactivated by PAR (Kostrubsky *et al.*, 1997b). This supports the ambiguous results that have been presented in the past regarding the protecting or activating role of CYP2E1 ligands (Burk *et al.*, 1990; Anundi *et al.*, 1993). Many inducers act by stabilization of the CYP2E1 protein (Eliasson *et al.*, 1992) but at the same time, are competitive inhibitors. The balance between stabilization and competitive inhibition of PAR bioactivation will determine the observed effect (Dai and Cederbaum, 1995). In humans, isoniazid inhibited oxidation of PAR, measured as urinary excretion of the thioether of PAR and 3-OH-PAR, when both drugs were present at the same time, but 1 day after isoniazid was discontinued, bioactivation of PAR increased (Zand *et al.*, 1993). In addition to the importance in hepatic biotransformation, CYP2E1 is responsible for about 50% of renal bioactivation of PAR in mice. Interestingly, the oxidative metabolism of APAP in control male mouse kidney microsomes displayed an apparent low K_M of 43 to 45 μM and an apparent high K_M of 603 to 702 μM (Hu *et al.*, 1993).

The ratio between formation of NAPQI and 3-OH-PAR after P450-dependent oxidation of

PAR is not constant (Harvison *et al.*, 1988b; Zand *et al.*, 1993). The relative extent of NAPQI and 3-OH-PAR metabolite formation by different cytochrome P450 enzymes could be related to a P450 enzyme-specific orientation in the respective active sites (Van de Straat *et al.*, 1987a; Myers *et al.*, 1994). In humans, the relative contribution of the three main P450 enzymes involved (CYP1A2, CYP2E1, and CYP3A4) varies considerably and depends on various life style factors and the use of pharmaceutical drugs (Raucy *et al.*, 1989; Guengerich, 1995). An overview of the P450 enzymes involved is presented in Table 4.

Recently, strong indications were found that also 3'-hydroxyacetanilide (3'-HAA), a nontoxic regioisomer of PAR (4'-HAA), is a substrate of CYP2E1. Liver microsomal CYP2E1 enzyme activity was decreased and an anti-arylacetamide reactive protein adduct was detected in a protein that comigrated with CYP2E1 in mice treated with 3'-HAA. Also, incubation of 3'-HAA with hepatic microsomes resulted in a time-dependent decrease in CYP2E1 enzyme activity, whereas preincubation of microsomes with PAR did not result in covalent binding to or inhibition of CYP2E1 (Myers *et al.*, 1995; Matthews *et al.*, 1997; Halmes *et al.*, 1998; Salminen *et al.*, 1998).

B. Peroxidases

1. General

Although a wide range of xenobiotic substrates can be bioactivated by one or more P450 enzymes of the cytochrome P450 superfamily, also other enzymes may be relevant in the process of activation of PAR (phase I bioactivation). These are the peroxidase group of enzymes comprising myeloperoxidase, chloroperoxidase and lactoperoxidase (Nelson, 1981; Potter *et al.*, 1986; Potter and Hinson, 1989) but also prostaglandin *H* synthase (Harvison *et al.*, 1986a; Potter and Hinson, 1987; Harvison *et al.*, 1988a), which has been found in almost every mammalian tissue that has been investigated (Eling and Curtis, 1992). In white blood cells, for example, myeloperoxidase has been shown to bioactivate a wide range of drugs. In other tissues low in P450 activity, pros-

taglandin *H* synthase may also be responsible for bioactivation. For example in the kidneys, PAR toxicity is thought to result from activation via this enzyme (Pirmohamed *et al.*, 1996). Horseradish peroxidase, as it is an enzyme isolated from a plant, has no direct relevance for these processes in mammals, although it has been used widely as a model enzyme system in mechanistic investigations into the bioactivation processes of xenobiotics (Josephy *et al.*, 1983; Ross *et al.*, 1985; Potter *et al.*, 1986; Metodiewa *et al.*, 1992; Bessems *et al.*, 1998).

2. Catalytic Activities

It has to be noted that important differences exist between peroxidases in general and prostaglandin *H* synthase (PGHS) or prostaglandin synthase (PGS), which are both synonymous for prostaglandin-endoperoxide synthase (PGES, which is the official name, EC 1.14.99.1). PGES exhibits two distinct activities of which one is exhibited only by PGES: the cyclooxygenase activity (synonymous to prostaglandin cyclooxygenase and fatty acid oxygenase) that catalyzes the oxygenation of arachidonic acid (AA) to its hydroperoxy endoperoxide (ROOH; PGG₂) with concomitant formation of water and consumption of two molecules of oxygen. The other one, the peroxidase or hydroperoxidase activity, that catalyzes the reduction of the hydroperoxy endoperoxide of arachidonic acid (PGG₂) to the hydroxy endoperoxide (ROH; PGH₂), is exhibited by all peroxidases (Moldéus and Rahimtula, 1980; Moldéus *et al.*, 1982; Kulmacz *et al.*, 1991; Kulmacz *et al.*, 1994). The therapeutic action of PAR is almost undoubtedly based on inhibition of the cyclooxygenase activity of PGES, which prevents prostaglandins from being formed thereby lowering body temperature from fever to more normal levels (antipyretic activity) and tempering the pain sensation (analgesic activity) (Flower and Vane, 1972; Mattamal *et al.*, 1979; Malmberg and Yaksh, 1982; Harvison *et al.*, 1986a). Although peroxidase activity is only one aspect of PGES, various laboratories have been trying to investigate the activation mechanism of PAR by using specific peroxidases, for example, by studying possible structure activity relationships (oxi-

dation potentials, coplanarity of *N*-acetyl side chain, etc.) for various PAR analogues (Harvison *et al.*, 1986a; Harvison *et al.*, 1986b; Harvison *et al.*, 1988a; Barnard *et al.*, 1993b; Park and Kitteringham, 1994; Bessems *et al.*, 1995; Bessems *et al.*, 1998).

3. Pharmacological Cyclooxygenase Inhibition

The cyclooxygenase-inhibiting activity of PAR was suggested to be related to its capacity to quench the tyrosyl radical present in PGES (Kulmacz *et al.*, 1991). Even monomethylated analogues of PAR were found to be capable of inhibiting PGES (Harvison *et al.*, 1986b; Harvison *et al.*, 1988a). Also, PAR analogues with fluorine substitutions adjacent to the hydroxyl group, adjacent to the amide or in the acetamide group exhibited, although varying, *in vivo* analgesic and *in vitro* cyclooxygenase inhibiting capacities (Barnard *et al.*, 1993b; Park and Kitteringham, 1994; Bessems *et al.*, 1995). In general, coplanarity of the acetamide group with the phenyl ring is important in the cyclooxygenase-inhibiting capacity of PAR analogues (Barnard *et al.*, 1993b; Bessems *et al.*, 1995).

4. Bioactivating Activities

In contrast to PGES (see below), the peroxidase enzymes probably exhibit only one-electron oxidation activity toward PAR under physiological conditions (Potter and Hinson, 1989). PAR and several ring-alkylated and -halogenated analogues were shown to be liable to one-electron oxidative biotransformations by peroxidase enzymes to their respective alkylated and halogenated NAPSQI analogues (Fischer and Mason, 1984; Fischer *et al.*, 1985b; Mason and Fischer, 1986; Bessems *et al.*, 1998). PAR could also substitute catalytic amounts of the cosubstrate serotonin in myeloperoxidase-oxidase reactions with cysteine as substrate. Eosinophil, lacto- and horseradish peroxidase could catalyze these reactions as well (Svensson, 1989). As myeloperoxidase is mainly present in polymorphonuclear leukocytes (PMNs) in humans (Chamulitrat *et al.*,

1991), this enzyme might be involved in the later stages of hepatic damage as observed after PAR intoxication (see Section IV). A significant role for myeloperoxidase in the bioactivation process of PAR was suggested when protein binding and nucleic acid binding (DNA and RNA) of PAR was observed after stimulation of the respiratory burst in neutrophilic type differentiated leukemic HL-60 cells. A mechanism was proposed requiring one-electron oxidation of PAR (Corbett *et al.*, 1989; Corbett *et al.*, 1992). Furthermore, metabolic activation by myeloperoxidase in neutrophils or stem cells, leading to free radical metabolite formation was suggested to be the cause of agranulocytosis, as observed rarely following PAR intake (Mason and Fischer, 1992).

In contrast, PGES is suggested to exhibit two activities with respect to PAR, similar to the dual activity in the synthesis of prostaglandins. Strong indications for one-electron oxidation as well as two-electron oxidation to NAPSQI and NAPQI, respectively, have been obtained with *in vitro* experiments using microsomes from sheep seminal vesicles (Moldéus and Rahimtula, 1980; Moldéus *et al.*, 1982; Potter and Hinson, 1987; Potter and Hinson, 1989). Also, PGES from rabbit kidney inner medulla was suggested to exhibit metabolic oxidation of PAR, next to being inhibited by PAR (Mattamal *et al.*, 1979; Mohandas *et al.*, 1981). As mentioned above, the inhibition of PGES by PAR is the basis of the pharmacological properties of PAR which in its essence, are due to inhibition of the prostaglandin synthesis (Harvison *et al.*, 1986a). Unfortunately, however, it might be as well this PGES activity in the kidneys, as is hypothesized in this review, that is responsible for the increased risk of chronic renal disease that has been ascribed to long-term use of PAR (Sandler *et al.*, 1989).

C. Summary

With respect to PAR-dependent hepatotoxicity it is generally accepted that P450-dependent bioactivation of PAR is a main cause for ultimate potentially fulminant hepatic necrosis upon administration or intake of a lethal dose of PAR. *N*-acetyl-*p*-benzoquinone imine (NAPQI) is presumed to be the ultimate metabolite causing deple-

tion of GSH and protein thiols and arylation of the latter. The exact catalytical mechanism of formation of NAPQI is as of yet unknown, be it a direct two-electron oxidation (abstraction of two hydrogen atoms) or a sequential mechanism of two successive single hydrogen atom abstractions with a free radical intermediate, that is, *N*-acetyl-*p*-benzosemiquinone imine (NAPSQI). Another, nontoxic P450-dependent metabolite is 3-hydroxyacetamol (3-OH-PAR). In man, CYP3A4 is probably the most important P450 at therapeutic concentrations with CYP2E1 and CYP1A2 becoming significantly involved at higher concentrations and at serious intoxication, respectively. In mice and rats CYP2E1 and CYP1A2 are most important. Furthermore, the formation in significant amounts of the nontoxic 3-OH-PAR in rat by various of the P450 enzymes involved in the oxidation of PAR (Table 4) may be important in the low sensitivity of rat compared with mouse for PAR-dependent hepatotoxicity. In the less sensitive rabbit, P450 catalyzed biotransformation may be at all of minor importance, although few data were found on P450 activities with respect to PAR in this species.

The analgesic action of PAR is dependent on the inhibition of the cyclooxygenase activity of prostaglandin endoperoxide synthase (PGES), although the exact mechanism is not known. PGES is present in many tissues. For cyclooxygenase inhibitory activity, a *p*-hydroxyacetanilide structure is required, although also analogues substituted *ortho*- to the phenolic hydroxyl group and analogues substituted in the methyl group of the acetyl moiety, such as *N*-trifluoromethylacetyl-*p*-aminophenol, still possess this potential. Importantly, the *N*-acetyl group should be coplanar.

Next to activity of the general peroxidases such as myeloperoxidase, the PGES activity may also be relevant for toxicity of PAR, especially in the kidneys at chronic low intake levels. Protein, but also RNA and DNA adducts, may result from peroxidase-mediated bioactivation of PAR, maybe via formation of the radical *N*-acetyl-*p*-benzosemiquinone imine (NAPSQI). At high concentrations in the kidneys, P450 is probably more important for bioactivation (i.e., CYP2E1). Renal CYP2E1-dependent bioactivation displays species and even sex differences.

IV. MECHANISMS OF HEPATOTOXICITY

A. General

1. Activation and Inactivation

The hepatotoxicity of PAR is generally accepted to be primed by the formation of NAPQI, a metabolite formed during cytochrome P450 catalyzed oxidation of PAR (Albano *et al.*, 1985; Van de Straat *et al.*, 1988b). Hepatotoxic damage occurs mainly in the centrilobular (perivenous) zone (Anundi *et al.*, 1993). Recently, a model, filled with known published parameters on GSH synthesis, degradation, and transport, was developed to examine the bimolecular reaction of GSH conjugation with acceptor substrates. Simulations were performed to obtain the vascular and intracellular GSH concentrations in the absence and presence of PAR. The simulated results suggest that the average tissue GSH concentration as normally determined in liver homogenate and the formation of the PAR-SG conjugate are poor indices of the extent of toxic exposure. As the formation of NAPQI is regarded to be the rate-limiting step in the formation of the PAR-SG conjugate, the high concentration of cytochrome P450 enzymes in the perivenous region is probably more important for the observed zonal toxicity than the low GSH content (Chiba and Pang, 1995). It has to be noted, however, that also the zonal distribution of the primary phase II detoxication reactions, sulfation and glucuronidation, may be important in the observed zone-specific toxicity. Sulfation (which exhibits high affinity) is predominant in the periportal region (Pang, 1990), as shown specifically for PAR in the isolated perfused rat liver (Mitchell *et al.*, 1989). This finding was confirmed specifically for phenol sulfotransferase, which exhibited a slight predominance in (isolated) perivenous hepatocytes (Tosh *et al.*, 1996). Glucuronidation was found to be active in both zones, however, at high concentrations, the high capacity of the glucuronidation seemed to be most predominant in the periportal zone (Mitchell *et al.*, 1989). This is probably due to the high capacity properties of UDP-glucuronosyltransferase (higher K_M and higher V_{max} than sulfotransferase). See also Section II for conjugating reactions.

2. Toxicity and Defense Mechanisms

In an attempt to enhance survival from noxious injury, organisms have developed several lines of defense mechanisms. One is represented by biochemical mechanisms that enable the organism to prevent injury after noxious insults, such as the early hepatic damage events oxidative stress and covalent protein binding. In the case of PAR intoxication, the internal rescue mechanisms can be supported by increasing synthesis of GSH or possibly other sulfhydryl compounds by giving methionine or *N*-acetylcysteine (Mourelle *et al.*, 1990). The second class of defense mechanism is a biological response intended to overcome injury, by promoting tissue healing after the noxious insult (Mehendale, 1995). Establishing that the initial toxic or injurious events can be separated from the subsequent events that determine the ultimate outcome of injury offers promising opportunities for developing new avenues for therapeutic intervention, with the aim of restoring and boosting the hormetic tissue repair mechanisms. In the late stages after PAR has been metabolized, patients often present many hours after taking an overdose, *S*-amino acids are no longer effective (Mourelle *et al.*, 1990). The initial (bio)chemical reactions between the reactive metabolite(s) and macromolecular cell components (proteins, lipids, DNA), were grouped and called Stage I, whereas subsequent processes of adaptation or failure of response to modification of essential cellular processes (such as energy supply and the protein machinery) were grouped in Stage II of toxicity (Chanda and Mehendale, 1996b; Chanda and Mehendale, 1996a). These potential causative events described shortly above (Stage I) are reviewed in this Section (for a short overview of Stage I and Stage II see Figure 5). The Stage II processes will only be mentioned shortly, especially in the last paragraph of this section.

B. Stage I — Initial Events in Hepatocytes

Several hypotheses have been put forward in the last 20 years regarding the crucial early steps in the development of hepatic damage taking place

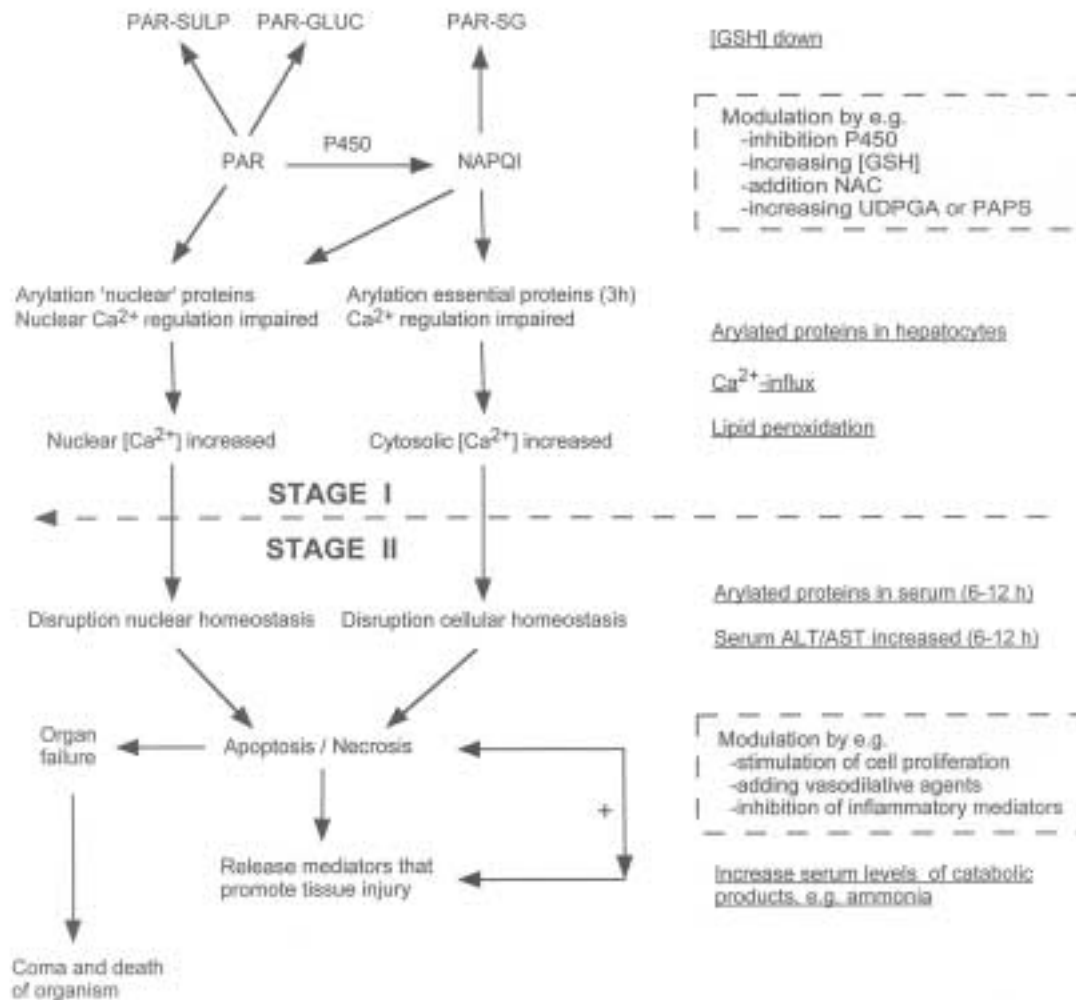


FIGURE 5. Stage I and II in paracetamol-induced hepatotoxicity. Model of stages I and II that are discerned in the paracetamol-induced development of hepatotoxicity as deduced from various references (see text).

directly after ingestion of PAR, once NAPQI is released in quantities that exhaust cellular GSH significantly. It must be stressed, however, that these hypotheses do not exclude each other, that is, several mechanisms could contribute more or less to early hepatic damage. As reviewed by several authors (Vermeulen *et al.*, 1992; Hinson *et al.*, 1995; Holtzman, 1995; Nelson, 1995), one hypothesis is that oxidative stress, i.e. thiol oxidation, mediated by the oxidative capacities of NAPQI, is the main cause of hepatotoxicity. NAPQI can oxidize GSH, thereby lowering the GSH/GSSG status, and it can oxidize protein SH groups, leading to the formation of interstrand disulfide bridges, to interprotein crosslinking, or to mixed disulfides (between protein and glutathione). Another hypothesis is that oxidative stress accompanied often by lipid peroxidation

(LPO) as caused by a redox cycling metabolite of PAR is the crucial step (Younes *et al.*, 1986). NAPQI was suggested to give rise to futile cycling of P450, using reducing equivalents of NADPH with concomitant reduction of molecular oxygen to the superoxide anion radical (O₂^{•-}). The superoxide anion radical is enzymatically reduced to hydrogen peroxide (H₂O₂), which in turn may lead to hydroxyl free radical (OH[•]) formation in the presence of traces of metal ions in the Fenton reaction (Goepfert *et al.*, 1995). When reacting with lipids, these very reactive hydroxyl free radicals may initiate LPO (Bast, 1986). The third hypothesis is that covalent protein binding of NAPQI as an electrophile is the most important event, leading to disrupted homeostases once critical proteins have been modified (Gibson *et al.*, 1996; Cohen *et al.*, 1997; Pumford and Halmes,

1997). Not regarded as a very important event in the development of toxicity, a fourth event, that is, covalent binding of NAPQI to lipids *in vitro* as well as *in vivo* has received some attention. With respect to hazard assessment of the use of PAR, more and more attention is paid to a fifth potential causative event for damage, that is, the nuclear effects that are observed experimentally as well as epidemiologically (Bergman *et al.*, 1996). Mostly, low but chronic levels of exposure are studied with respect to potential nuclear effects of PAR. However, DNA effects as a result of high hepatotoxic doses of PAR, leading to apoptosis, have been receiving attention as well (Ray *et al.*, 1991; Ray *et al.*, 1993; Ray *et al.*, 1996). As apoptosis with respect to PAR-induced toxicity is a relatively new phenomenon, it remains to be established where in the Stage I or Stage II processes, apoptosis should be positioned. As the direct nuclear effects seem to be early processes, taking place within 6 h (Ray *et al.*, 1996), apoptosis is described here (although only briefly) under the nuclear effects in Stage I as well.

1. Oxidative Stress and Thiol Oxidation

Several patho-physiological conditions may give rise to an imbalance between the production of and the protection against oxygen free radicals. This imbalance is called oxidative stress (Sies, 1986). This definition is often broadened to a condition of decreased reductive potential and an impaired capacity to cope with endogenous or exogenous oxidants. Oxidant stress mechanisms may be mediated either by reactive oxygen species or by the direct oxidant action of a reactive metabolite in PAR-induced hepatotoxicity. This

may be detectable as decreased ratios of NADPH/NADP⁺ (Keller and Hinson, 1991), GSH/GSSG (Subrahmanyam *et al.*, 1987), ProtSH/ProtSSProt (Albano *et al.*, 1985). The latter two phenomena result from the fact that NAPQI can oxidise cysteine thiols in GSH, leading to GSSG, and in proteins, giving rise to protein disulfides and GSH-protein mixed disulfides (Albano *et al.*, 1985; Kyle *et al.*, 1990; Birge *et al.*, 1991a). Increased oxidation of protein thiol groups has been reported in hepatocytes to play a causal role in the observed PAR-mediated toxicity (Adamson and Harman, 1993). Oxidation of GSH by NAPQI may occur via *ipso*-attack of GSH on the electrophilic C1-carbon (Figure 6), as proposed by several authors for NAPQI as well as 2,6-diCH₃-NAPQI and 3,5-diCH₃-NAPQI (Fernando *et al.*, 1980; Smith and Mitchell, 1985; Coles *et al.*, 1988; Ketterer *et al.*, 1988; Rundgren *et al.*, 1988; Nelson *et al.*, 1991). This occurs via the formation of a Meisenheimer complex like the formation of a carbinolamide after hydration of NAPQI (*ipso*-adduct) (Novak *et al.*, 1986; Novak *et al.*, 1989). A similar *ipso*-adduct of a thiol is formed after reaction of ethanethiol with NAPQI (Fernando *et al.*, 1980). In a similar bimolecular redox reaction, protein thiol oxidation may take place via the formation of unstable *ipso*-adducts, as proposed by Rundgren *et al.* after investigating the effects of dithiothreitol on the metabolism, covalent protein binding, and cytotoxic effects of the quinone imines (Rundgren *et al.*, 1988). Moreover, even protein *S*-thiolation of proteins that are sensitive for such inactivation like glyceraldehyde-3-phosphate dehydrogenase could start by formation of an *ipso*-adduct of NAPQI as indicated, followed by displacement by GSH (Dietze *et al.*, 1997). It has to be noted that some oxidant

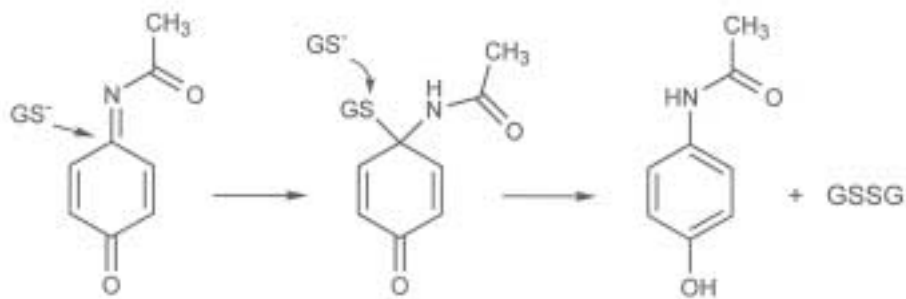


FIGURE 6. Proposed pathway for *ipso* attack of GSH with NAPQI. Proposed pathway of reduction of *N*-acetyl-*p*-benzoquinone imine via *ipso* attack. (Adapted from Ketterer *et al.* [1988].)

stress phenomena could be Stage II events initiated by an increased role of Kupffer cells (hepatic macrophages) as described at the end of this section (Hinson *et al.*, 1998).

Results from preliminary experiments with a PAR analogue in our laboratory suggesting oxidation followed by semipermanent protein binding support the *ipso*-adduct hypothesis. Briefly, in a buffered solution, the PAR analogue 3,5-diCH₃-PAR was oxidized with horseradish peroxidase and H₂O₂ with 3,5-diCH₃-NAPQI being formed as observed by UV spectrophotometry (Bessemers *et al.*, 1996). When microsomal protein was present during the oxidation, no formation of the 3,5-diCH₃-NAPQI was observed, although 3,5-diCH₃-PAR seemed to disappear. After subsequent addition of GSH or dithiothreitol, 3,5-diCH₃-PAR was observed again spectrophotometrically. This findings can be explained by the formation of a Meisenheimer-type complex between microsomal protein thiol groups and 3,5-diCH₃-NAPQI, being responsible for loss of π -conjugation and loss of absorbance at λ_{\max} . The addition of an excess of GSH or dithiothreitol removes the semipermanent adduct. The observed phenomena were much more significant when BSA was used instead of microsomal protein (unpublished observations). These findings are similar to those mentioned above (Rundgren *et al.*, 1988) and thus support the *ipso*-adduct hypothesis as formulated by the group of Nelson (Rundgren *et al.*, 1988; Nelson *et al.*, 1991).

Recently, the *in vivo* formation of hepatic protein aldehyde groups was used as a marker of oxidative damage on the treatment of mice with FeSO₄, while increased serum levels of alanine aminotransferase (ALT; used as a marker for hepatic damage) were not yet observed (Gibson *et al.*, 1996). Toxic doses of PAR did not result in protein aldehyde formation, while even the serum ALT levels were significantly increased. Moreover, combined treatment with FeSO₄ and PAR did not present protein aldehyde formation, while serum ALT was increased compared to control mice, thus indicating antioxidant properties of PAR (Gibson *et al.*, 1996). The antioxidant properties of PAR were previously reported and confirmed (Van de Straat *et al.*, 1988a). Together with other findings, these data are consistent with the theory that PAR covalent binding is the pri-

mary mechanism of toxicity and argue against a major role for a specific protein oxidation in PAR hepatotoxicity (Gibson *et al.*, 1996). This does not exclude, however, that thiol groups in some specific proteins become oxidised or glutathiolated (Birge *et al.*, 1991a), phenomena, which in general must be regarded as adverse effects, but not necessarily as significant toxic reactions. It may well be that protein thiol oxidation and glutathiolation are protective mechanisms after exposure to higher doses of PAR.

2. Oxidative Stress and Lipid Peroxidation

As mentioned above, one of the phenomena often observed in combination with oxidative stress is lipid peroxidation (LPO). Reactive oxygen species (hydrogen peroxide, superoxide anions, and hydroxyl radicals) are required for its initiation as NAPQI is expected to be incapable of initiating a radical hydrogen abstraction from lipid molecules. However, reduction of NAPQI, which could occur in the presence of flavoproteins, followed by reoxidation by oxygen could give rise to superoxide anions with a consequent formation of reactive reduced oxygen species. Even protein bound NAPQI was suggested to be liable to one-electron reduction (Mourelle *et al.*, 1990). LPO has been regarded to be an important initiation event in the toxicity mechanism of PAR in the 1970s and early 1980s (Wendel *et al.*, 1982; Thelen and Wendel, 1983). Some dispute has existed since the late 1980s, however. Hepatotoxic doses of PAR to Fisher 344 rats were not accompanied by increased biliary efflux of GSSG (Smith and Mitchell, 1985). Especially in isolated and cultured rat hepatocytes this phenomenon has been studied although results were not unambiguous (Albano *et al.*, 1983; Van de Straat *et al.*, 1987b; Donatus *et al.*, 1990; Harman *et al.*, 1992). A possible explanation for this ambiguity might be the increased sensitivity to oxidative stress in hepatocytes that were isolated from fasted compared with fed rats, as fastening may lower the ATP content and thus the normal physiology of the cell. Moreover, fastening increases CYP2E1 activity substantially in rat (Johansson *et al.*, 1988; Hu *et al.*, 1995). Associated with the induction of

CYP2E1 is an elevated production of reactive oxygen species (ROS) such as superoxide radicals and H_2O_2 in kidney and liver microsomes (Johansson *et al.*, 1988; Nordmann *et al.*, 1992; Liu *et al.*, 1993b; Rashba-Step *et al.*, 1993; Ueng *et al.*, 1993; Wu and Cederbaum, 1994). Furthermore, it has been concluded in various papers that LPO is not playing a causal role or only a minimal role in loss of cell viability induced by PAR (Mitchell *et al.*, 1985; Van de Straat *et al.*, 1988a; Younes *et al.*, 1988; Donatus *et al.*, 1990; Garrido *et al.*, 1991; Kamiyama *et al.*, 1993). The indigenous medicine curcumin for example was found to protect against PAR-induced LPO, without protecting against LDH leakage and GSH depletion (Donatus *et al.*, 1990). By showing that 3-mono-alkylation of PAR diminished LPO but not cytotoxicity in rat hepatocytes compared to unsubstituted PAR, Van de Straat *et al.* provided support for the hypothesis that LPO and cytotoxicity are not causally related in hepatocytes (Van de Straat *et al.*, 1988a).

The general question is whether PAR-induced LPO is a consequence of, for example, P450 oxidase activity or a consequence of other initial phenomena such as GSH depletion or oxidation of, and covalent adduct formation with proteins (Dai and Cederbaum, 1995). Also, it cannot be excluded that on a limited scale in other organs, in, for example, the kidneys, local peroxidase activity leads to some LPO as it was shown *in vitro* that peroxidase catalyzed oxidation of PAR in the presence of NADPH leading to, among others, polymers of PAR, very likely was accom-

panied by superoxide anion production (Figure 7) (Keller and Hinson, 1991). Furthermore, LPO may well be a Stage II phenomenon instead of a Stage I phenomenon as discerned by Mehendale (Mehendale, 1991; Mehendale, 1995; Chanda and Mehendale, 1996b). After release of chemotactic and activating factors (such as interleukines and tumour necrosis factors), possibly following changes in DNA binding activities in transcription factors in hepatocytes that are damaged by PAR in Stage I of toxicity, Kupffer cells, peritoneal macrophages, polymorphonuclear leukocytes and eosinophils may be activated with concomitant release of myeloperoxidases, H_2O_2 and other activated oxygen species (O'Brien *et al.*, 1990; Blazka *et al.*, 1995; Blazka *et al.*, 1996). Very likely, this may result in LPO as well.

3. Covalent Binding to Proteins ('Acetaminophen-Binding Proteins')

a. General

Just as NAPQI can oxidize GSH, it can covalently bind to GSH. Analogously, just as NAPQI can oxidize cysteine groups in proteins (Albano *et al.*, 1985; Kyle *et al.*, 1990; Birge *et al.*, 1991a) it can covalently bind to these amino acids in proteins leading to protein arylation *in vitro* and *in vivo* (Hoffmann *et al.*, 1985a; Hoffmann *et al.*, 1985b). Already in the 1970s, covalent binding of radiolabeled PAR to proteins was described and suggested to play an important role in the toxicity

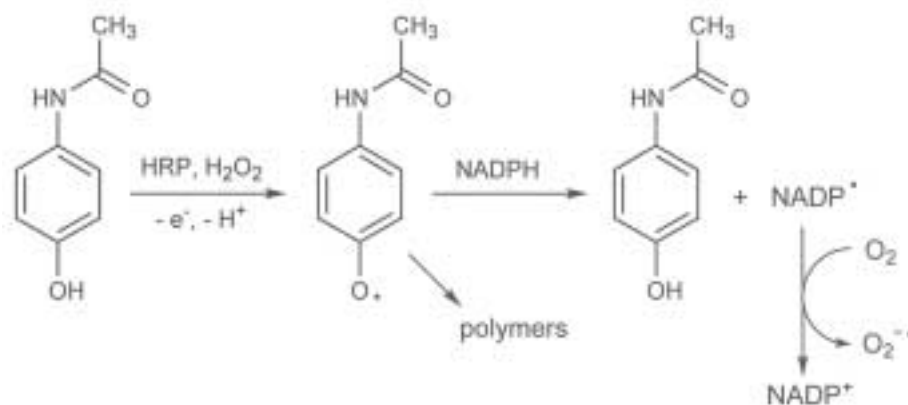


FIGURE 7. Paracetamol- and peroxidase-dependent NADPH oxidation. Proposed mechanism of PAR-stimulated NADPH oxidation catalyzed by the peroxidase/ H_2O_2 system. (Adapted from Keller and Hinson [1991].)

TABLE 5
(Covalent) Protein Modifications in Subcellular Compartments in Various Tissues Observed
after Incubation with Paracetamol

Protein	kDa	a.a.(*)	Time	Matrix	Effect (on)	Reference (s)
Microsomal (ER)						
calreticulin (a)		Lys		mouse liver in vitro	postranslational modifications	(Holtzman, 1995; Zhou et al., 1996)
glutamine synthetase subunit (b)	44	Cys	30 min	mouse liver in vivo	synthesis of Glu from Glu and ammonia	(Bartolone et al., 1989; Birge et al., 1991b; Bulera et al., 1995)
glutathione S-transferase (c)	17	?		rat liver in vivo + in vitro	increased glutathione S-transferase activity	(Wels et al., 1992b; Yonamine et al., 1996)
Q2 isoform of thioprotein disulfide oxidoreductase (d)		Lys		mouse liver in vitro	postranslational modifications	(Holtzman, 1995; Zhou et al., 1996)
Q5 isoform of thioprotein disulfide oxidoreductase (e)		Lys		mouse liver in vitro	postranslational modifications	(Holtzman, 1995; Zhou et al., 1996)
Mitochondrial						
aldehyde dehydrogenase (f)	54	Cys	1 h	mouse liver in vivo	oxidation of aldehydes; LPO	(Landin et al., 1996)
carbamyl phosphate synthetase (g)		Cys	6 h	mouse liver in vitro	consumption of ammonia	(Gupta et al., 1997)
glutamate dehydrogenase (h)	50	Cys	30 min	mouse liver in vivo	synthesis of Glu from α -ketoglutarate and ammonia	(Halmes et al., 1996)
unknown (i)	67		30 min	mouse liver in vivo	unknown	(Halmes et al., 1996)
Cytosolic						
acetoaminophen and/or selenium binding protein (j)	55-58	Cys	30-60 min	mouse liver in vivo	scavenging of electrophiles	(Bartolone et al., 1989; Pumford et al., 1992; Holvik et al., 1996b)
N-10-formyltetrahydrofolate dehydrogenase (k)	100	Cys	2 h	mouse liver in vivo	donation of one-carbon units in biosynthesis	(Pumford et al., 1997)
glutamine synthetase subunits released from ER (l)			30 min	mouse liver in vivo	see microsomal proteins	(Bartolone et al., 1989; Birge et al., 1991b; Bulera et al., 1995)

TABLE 5 (continued)

glyceraldehyde-3-phosphate dehydrogenase	3B	Cys	2 h	mouse liver <i>in vivo</i>	glycolytic pathway (ATP synthesis)	(Dietze <i>et al.</i> , 1997)
glutathione S-transferase (n)	2.5	?	?	mouse liver homogenate	not presented	(Wendel and Cikryt, 1981)
unknown (o)	3.3			mouse kidney <i>in vivo</i>	unknown	(Bartolone <i>et al.</i> , 1989)
Nucleolar						
lamin A (p)	7.5	Cys	< 2 h		disruption of nuclear lamina	(Hong <i>et al.</i> , 1994; Khairallah <i>et al.</i> , 1995)
ribonucleotide reductase (q) (3)		Tyr		human mononuclear blood cells; mouse mammary tumor cell line	quenching of tyrosyl; blocking replicative DNA synthesis	(Horigiso <i>et al.</i> , 1990; Richard <i>et al.</i> , 1991; Brumberg <i>et al.</i> , 1995)

- (1) Amino acid most likely covalently modified.
 (2) Specific enzymatic catalysis steps were retrieved from (Stryer, 1981)
 (3) Actually this is not protein binding but quenching of the tyrosyl radical in the active site of ribonucleotide reductase.
- (a) ε-Amino group of Lys-233 or Lys-23; possibly blocking of posttranslational modification of proteins
 (b) see 1.
 (c) ε-Amino group of Lys-103; possibly blocking of posttranslational modification of protein
 (d) ε-Amino group of Lys-202, Lys-209 or Lys-210 and Lys-354; possibly blocking of posttranslational modification of protein
 (e) Unknown
 (f) Probably cysteine; Cys-302 critical residue at active site; decreased oxidation of aldehydes, lipid peroxidation (LPO)
 (g) Catalyzes synthesis of carbamoyl phosphate (consumed in the urea production cycle) from ammonia and carbon dioxide
 (h) Probably cysteine; 6 free reactive cysteines in protein; important in carbon and nitrogen metabolism
 (i) Unknown
 (j) Probably cysteine; 8 cysteines in protein; evidence to function as common target protein for toxicants
 (k) Probably cysteine; Cys-707 essential cysteine at the active site; important enzyme in one carbon metabolism
 (l) Probably cysteine; 11 cysteines of which 3 not buried inside the native protein; catalyzes synthesis of glutamine from glutamate & ammonia
 (m) Probably Cys-149 in the active site; at least *in vitro* reaction of NADPH with this enzyme definitely caused Cys-149 adduct formation; probably also at lower rate other 2 cysteines in each subunit of glyceraldehyde-3-phosphate dehydrogenase
 (n) Unknown
 (o) Unknown
 (p) Unknown
 (q) Tyrosyl

mechanism of PAR (Potter *et al.*, 1973; Potter *et al.*, 1974). Since the identification of 3-cysteine-*S*-yl-4-hydroxyaniline (probably the *N*-acetyl moiety is lost during sample preparation) as the major covalent adduct formed *in vitro* and *in vivo* between PAR and mouse liver proteins (Hoffmann *et al.*, 1985a), numerous papers appeared that attempted to elucidate which specific proteins became arylated (Bartolone *et al.*, 1989; Bartolone *et al.*, 1992; Pumford *et al.*, 1992; Halmes *et al.*, 1996; Hoivik *et al.*, 1996b; Zhou *et al.*, 1996; Pumford *et al.*, 1997). These attempts were triggered by the fact that covalent binding of the radiolabeled regioisomer 3'-hydroxyacetanilide (3'-HAA) was occurring without toxicity, although PAR toxicity was never observed without covalent binding (Tirmenstein and Nelson, 1991; Halmes *et al.*, 1998; Salminen *et al.*, 1998). For example, recently strong indications were obtained for the covalent binding of 3'-HAA to liver microsomal CYP2E1 with concomitant loss of activity (Halmes *et al.*, 1998; Salminen *et al.*, 1998).

The search for critical proteins and the investigations into the time progression of adduct formation was accelerated when antibodies were raised against various epitopes of the 3'-(cystein-*S*-yl)-4'-hydroxyacetanilide adduct, in order to use them in ELISA and Western blot (immunoblot) analysis of protein adducts on PAR administration (Roberts *et al.*, 1987a; Bartolone *et al.*, 1988; Pumford *et al.*, 1989; Hinson *et al.*, 1996). Proteins arylated by PAR were found in hepatic fractions as well as in serum of B6C3F1 mice that were administered PAR in a dose range of non-toxic to toxic. The concentration- and time-dependent level of arylated proteins in serum closely paralleled serum ALT levels. Arylated proteins in liver of intoxicated mice peaked after 2 h whereas those in serum peaked 6 to 12 h after dosing (Pumford *et al.*, 1990b). The most intense immunostaining was found in the plasma membrane and the mitochondria, whereas the most intense arylated individual protein appeared to be a 55-kDa cytosolic protein (Pumford *et al.*, 1990a; Pumford *et al.*, 1990b). In addition, the presence of 3-(cystein-*S*-yl)paracetamol adducts in liver proteins prior to hepatotoxicity suggests a threshold for adduct formation in the development of toxicity (Pumford *et al.*, 1990a). More and more, specific proteins were found to be arylated in

mice and man exposed to PAR, the extent of which for some proteins did and for others did not correlate with cellular damage (Birge *et al.*, 1990; Hinson *et al.*, 1990; Hinson *et al.*, 1995). An antiserum raised against a 4-acetamidobenzoic acid (antiarylacetamide) protein adduct detected the same primary PAR-protein adducts as an antiserum against a 3'-(cystein-*S*-yl)-4'-hydroxyacetanilide protein adduct (3-(cystein-*S*-yl)paracetamol protein adduct). However, minor differences were observed, indicating additional covalent protein binding to amino acids other than cysteine (Matthews *et al.*, 1996). For an overview of hepatic proteins in the various sub-cellular compartment being covalently modified by PAR ('acetaminophen-binding proteins'), and the effects of this modification on biochemical processes, see Table 5. For a visual perception see Figure 8. For another review on these aspects, including, for example, the quantification of concomitant enzyme inhibition, see Pumford and Halmes (Pumford and Halmes, 1997).

A concise description of the relationship between covalent binding of PAR to hepatic proteins and the development of hepatotoxicity in mice treated with a toxic dose of PAR (400 mg/kg) was presented by Hinson *et al.* (Hinson *et al.*, 1996), based on previous experiments (Roberts *et al.*, 1991). Adducts of PAR were observed immunohistochemically in the innermost layers of cells surrounding the central hepatic vein as early as 15 min following a hepatotoxic dose. By 30 min there was a 90% depletion of hepatic glutathione and PAR-protein adducts were evident in the centrilobular area. By 1 h following this dose the protein adducts reached their maximum extent and were found exclusively in the centrilobular region of the liver. By 2 h, vacuolization and shrinking of hepatocytes were prominent. These events correlated with increases in serum ALT levels and PAR-protein adducts in serum that occurred as a result of hepatocyte lysis. By 6 h of treatment substantial loss of adducts from the necrotic cells was prominent (Hinson *et al.*, 1996). Moreover, in PAR overdose patients, a relation was found between plasma ALT and 3-(cystein-*S*-yl)paracetamol protein adducts in plasma. This highly suggests a dominant mechanistic role of this binding in humans and provides direct evidence of a similar mechanism of PAR-

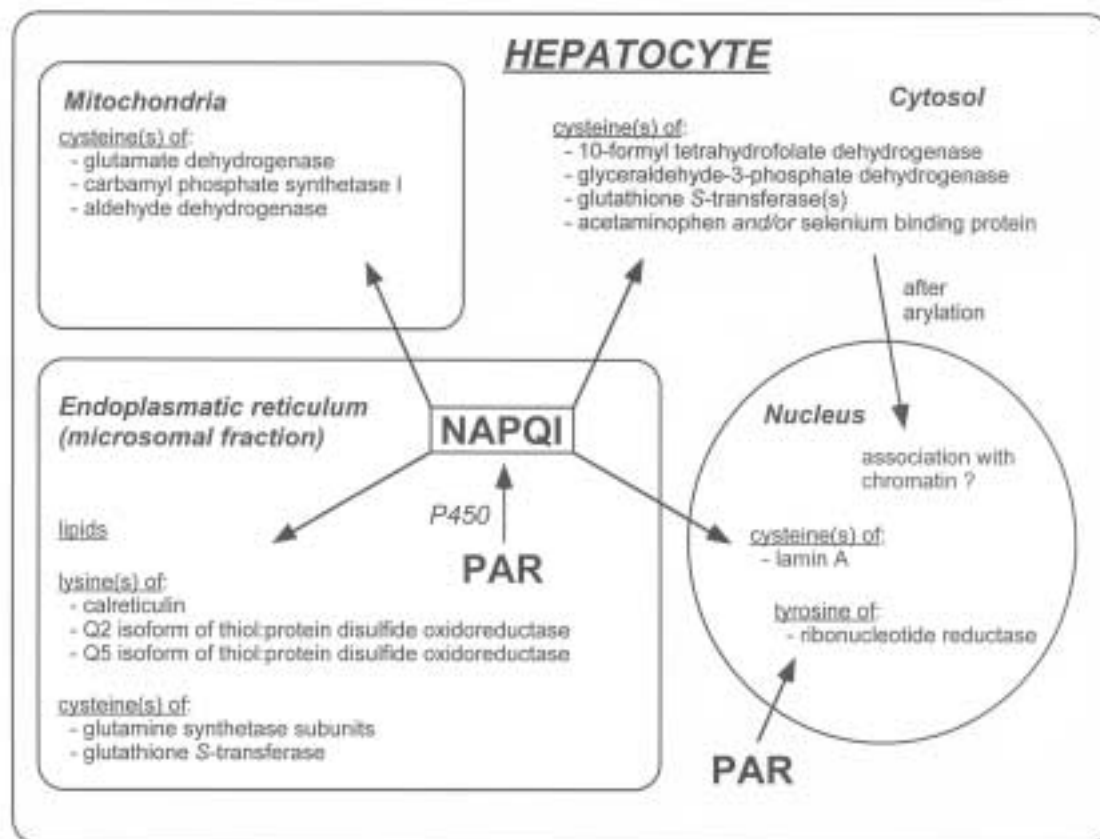


FIGURE 8. (Covalent) modification of macromolecules. Multiple proteins and other macromolecular structures covalently modified upon high doses of PAR (mostly investigated in mice). Retrieved from various references (see text).

induced hepatic necrosis in man and in laboratory animals (Hinson *et al.*, 1990).

a. Cytosolic Proteins

It has been known for quite some time that PAR becomes preferably covalently bound to a cytosolic GST after a mouse liver homogenate incubation (Wendel and Cikryt, 1981). The first reported specific hepatic proteins in cytosol becoming arylated on *in vivo* exposure of mice to hepatotoxic doses of PAR were a 44-kDa protein (Bartolone *et al.*, 1989; Birge *et al.*, 1991b), a 55-kDa protein (Pumford *et al.*, 1992), and a 58-kDa protein (Bartolone *et al.*, 1989; Bartolone *et al.*, 1992). The 58-kDa ‘acetaminophen-binding protein’ appeared to be native in many tissues, although adduct formation was only found in tissues that were prone to PAR-based damage (Bartolone *et al.*, 1989). Antibodies raised against the 58-kDa PAR-arylated protein revealed a simi-

lar protein in the cytosol of a human liver specimen (Bartolone *et al.*, 1992). The 44-kDa ‘acetaminophen-binding protein’ showed high homology with a subunit of liver microsomal glutamine synthetase, indicating that it might be ‘disrupted’ from the endoplasmatic reticulum and becoming cytosolic on arylation by PAR (Bulera *et al.*, 1995). Recently, two more cytosolic enzymes were shown to be modified on PAR intoxication in mice. *N*-10-formyltetrahydrofolate dehydrogenase was identified as a 100-kDa cytosolic target (Pumford *et al.*, 1997). A toxic dose of 400 mg/kg to mice resulted in a 25% decrease in cytosolic *N*-10-formyltetrahydrofolate dehydrogenase activity at 2 h already. Glyceraldehyde-3-phosphate dehydrogenase was identified as a 38-kDa subunit becoming covalently modified at the Cys-149 in the active site by NAPQI *in vitro*, as well as covalently modified and inhibited *in vivo* already within 2 h after PAR administration to mice (Dietze *et al.*, 1997). Recently, it was found that cytotoxic concentrations of PAR selectively

inhibited protein phosphatase activity and altered the phosphorylation state of several cytosolic proteins in cultured mouse hepatocytes. As phosphatases exhibit crucial roles in the physiology of each cell, these alterations may have an as yet unforeseen role in the toxicity mechanism of PAR (Bruno *et al.*, 1998).

In the last 10 years, numerous investigations were undertaken in order to elucidate the identity of the 55-kDa and the 58-kDa 'acetaminophen-binding proteins'. Partial internal peptide sequences of both the 55-kDa and the 58-kDa 'acetaminophen-binding proteins' that become arylated by PAR present a high homology (87 to 100%) with the cDNA-deduced amino acid sequence of a cytosolic 56-kDa 'selenium-binding protein' (SP56), discovered 2 years before (Bansal *et al.*, 1990; Bartolone *et al.*, 1992; Pumford *et al.*, 1992; Cohen *et al.*, 1997). The average 97% overall homology of the 55-kDa 'acetaminophen-binding protein' with SP56 is based on seven peptide fragments of 3 to 25 amino acids length (Pumford *et al.*, 1992; Cohen *et al.*, 1997). The 100% homology of the 58-kDa 'acetaminophen-binding protein' with SP56 is based on two peptide fragments of 9 and 18 amino acids, respectively (Bartolone *et al.*, 1992; Cohen *et al.*, 1997). By using a mouse genomic DNA library and a mouse liver cDNA library, one full-length cDNA, encoding the seven peptide fragments as found in the 55-kDa 'acetaminophen-binding protein' as well as the two fragments as found in the 58-kDa 'acetaminophen-binding protein' was picked up and cloned. Next, this full-length cDNA was used to deduce the full amino acid sequence of a virtual 56-kDa protein, designated as (deduced) AP56, and closely related to SP56, showing 100% homology in the total of nine peptide fragments used for screening. The cDNA deduced amino acid sequence of AP56 differs at only 14 amino acids from the deduced sequence of SP56 (the 56-kDa 'selenium binding protein') (Lanfear *et al.*, 1993). The amino acid composition (in percentage of the total amino acids) of the real 58-kDa 'acetaminophen-binding protein' 58 ABP as isolated from mouse liver, appeared to be fairly similar with the calculated amino acid composition of SP56 (Bartolone *et al.*, 1992).

Although strong support was present that the 55-kDa and the 58-kDa 'acetaminophen-binding

proteins' would be identical to deduced AP56, some findings deserve attention. Of all the fragments of the 55-kDa and 58-kDa PAR-arylated protein used for screening and homology analysis, not one contains a cysteine residue, expected to be the residue where PAR is bound. It is possible, however, that PAR binds covalently to other amino acids such as lysine or histidine instead of cysteine (Figure 9) (Streeter *et al.*, 1984b). This is not unlikely as a relatively low number of 10 cysteine residues is present in deduced AP56 as well as SP56. Arylation of lysine residues in microsomal proteins has been found recently on *in vitro* incubation (Holtzman, 1995). Sequencing of radioactive peptide fragments that had become ¹⁴C-labeled on incubation with ¹⁴C-PAR would aid in the verification of the identity of ¹⁴C-PAR-arylated proteins. Lastly, it has to be noted that the 58-kDa 'acetaminophen-binding protein' of Khairallah and Cohen's group is actually a mixture of 4 isoforms, as revealed by two-dimensional gel electrophoresis (Bartolone *et al.*, 1992). Definitive conclusions on the 55-kDa, 56-kDa, or 58-kDa 'acetaminophen-binding proteins' being equal to SP56 and/or deduced AP56 may need analysis of covalently modified proteins by liquid chromatography/mass spectrometry (LC/MS).

Interestingly, Lanfear *et al.* showed recently that SP56 and deduced AP56 are different as they are encoded by two different genes. In addition, SP56 mRNA is highly expressed in liver, kidneys and, to a lesser extent, lung, whereas deduced AP56 mRNA is mainly expressed in liver (Lanfear *et al.*, 1993). The 58-kDa 'acetaminophen-binding protein' was shown to be constitutive in many tissues, although it became covalently modified by PAR only in tissues sensitive to PAR toxicity (Bartolone *et al.*, 1989). In addition, the 58-kDa 'acetaminophen-binding protein' probably is not native in plasma, whereas PAR-bound 58-kDa 'acetaminophen-binding protein' was the most important modified protein found in plasma of PAR-intoxicated mice (Bartolone *et al.*, 1989). Although speculative, migration of the PAR-arylated 58-kDa 'acetaminophen-binding protein' to extrahepatic tissues on lysis of the hepatocyte is conceivable. NAPQI was postulated before to escape from hepatocytes and participate in the arylation of protein thiols in erythrocytes. This was suggested before to occur in mice that were

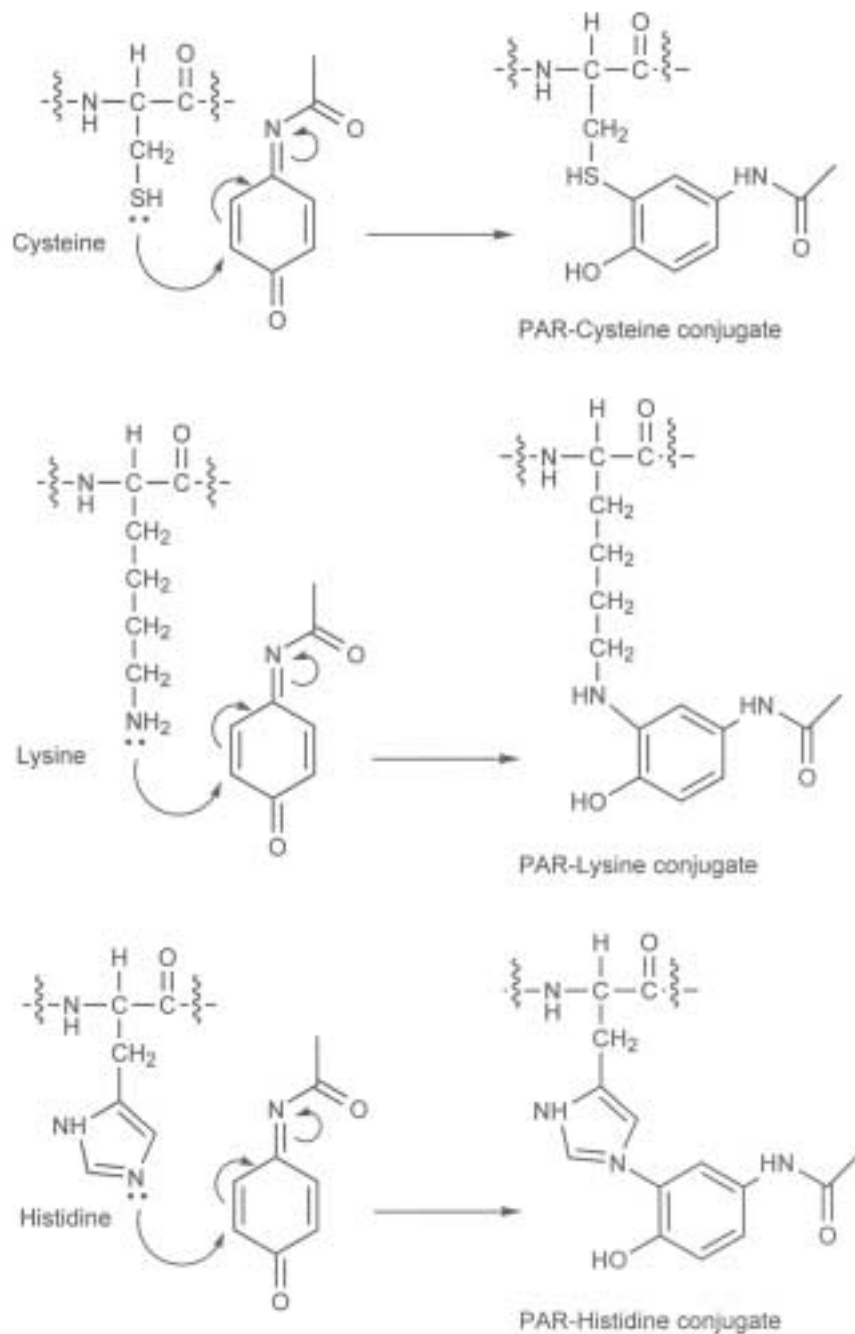


FIGURE 9. Reactions of NAPQI with amino acids. Reactions of NAPQI with various protein amino acid monomers as suggested in various references (see text).

administered PAR where arylation of hemoglobin by PAR was observed (Axworthy *et al.*, 1988).

Although the relevance of adduct formation of PAR to a specific target protein remains to be investigated, the recent reporting of a new mass spectrometry-based strategy, including two-dimensional gel electrophoresis (Qiu *et al.*, 1998) offers tremendous opportunities at least for identification of all target proteins. More than 20 new

PAR-adducted proteins were already identified in a liver tissue lysate of mice after administration of radiolabelled PAR. In-gel digestion of radiolabeled gel spots gave a set of tryptic peptides, which were analyzed by matrix-assisted laser desorption ionisation mass spectrometry (Qiu *et al.*, 1998). Comparison with protein databases, functional identification, investigation of dose-response relationships and of time dependency of

each protein adduct formation could aid in further elucidation of the toxicity mechanism of PAR as well as of many other chemicals.

i. Intermezzo

As mentioned, cysteine is not the only amino acid that becomes arylated after NADPH-dependent microsomal oxidation of PAR. In mouse liver microsomal incubations, PAR-based radiolabel binding was found in ϵ -amino lysine groups in the intra-luminal endoplasmatic reticulum proteins calreticulin and the Q2 and Q5 isoforms of the microsomal thiol:protein oxidoreductase (TPDO). No adducts of cysteine residues were found (Holtzman, 1995; Zhou *et al.*, 1996). Interestingly, a 'selenium binding protein' that was isolated from mouse kidneys showed 96.8% homology with an isoenzyme of rat liver microsomal TPDO (Jamba *et al.*, 1996). Furthermore, the selenium is very likely bound by cysteine residues as this rat liver TPDO typically contains two typical bis (cysteinyl) sequence motives Cys-X-X-Cys (Jamba *et al.*, 1996). Other 'selenium binding proteins' that contain one to three of such Cys-X-X-Cys motive(s) are thioredoxin, endoplasmatic reticulum protein (ERp72), formate dehydrogenase, and, very interestingly, the 58-kDa 'acetaminophen-binding protein' (Jamba *et al.*, 1996). Now, if firstly, the TPDO isoforms Q2 and Q5 become covalently modified after incubation with PAR, if secondly, no cysteine residues are present in the tryptic digests containing PAR-dependent radiolabel, if thirdly, these TPDO isoforms do contain Cys-X-X-Cys motives, and fourthly, if TPDO isoforms are 'selenium binding proteins', the most logical explanation is that it is just the presence of these Cys-X-X-Cys motives that prevents the cysteine residues from becoming covalently modified by the elusive NAPQI, just by binding selenium. In the past, covalent binding of PAR to 'selenium binding protein' was thought to occur analogously to cysteine binding (Pumford *et al.*, 1992). Analysis of hepatocyte incubations of PAR and PAR analogues for covalent protein binding by mass spectroscopy in our group, however, did not provide any indication for the formation of adducts to selenium containing amino acids such as selenocysteine (Li *et al.*, 1994a).

Therefore, it is proposed that the cysteine residues in these selenium binding (but probably not selenium containing) proteins, are mainly active in reduction of NAPQI via ipso-attack (see the following Intermezzo in this Section).

b. Microsomal Proteins

Typical with respect to covalent protein modification is the finding that liver microsomal glutathione S-transferase (GST) activity becomes seriously increased after administration of PAR to rats, while GSH content in the liver is markedly decreased (Yonamine *et al.*, 1996). Also, microsomal GSH peroxidase activity becomes significantly enhanced within 3 h. The cytosolic GST activity, however, is decreased. NAPQI is known to become covalently bound (dithiothreitol insensitive) to the microsomal GST after addition to rat liver hepatocytes (Weis *et al.*, 1992b). Although the effect on catalytic activity of cytosolic GST was not determined, it is known that PAR becomes preferably covalently bound to a cytosolic GST and to a lesser extent to microsomal GST after a mouse liver (S9)-homogenate incubation (Wendel and Cikryt, 1981). As indicated in the Intermezzo, other microsomal proteins that were shown to become covalently bound to PAR at lysine moieties (Figure 9), albeit in mouse liver microsomal incubations, are calreticulin and the Q2 and Q5 isoforms of thiol:protein disulfide oxidoreductase (Holtzman, 1995; Zhou *et al.*, 1996). In addition, as found in and noted above for the cytosolic fraction, probably after disruption from the endoplasmatic reticulum, glutamine synthetase subunits were found to be covalently modified by PAR (Bulera *et al.*, 1995).

c. Mitochondrial Proteins

Besides cytosolic and microsomal proteins, mitochondrial proteins also have been reported recently to become arylated after a hepatotoxic dose of PAR to mice. Glutamate dehydrogenase (Halmes *et al.*, 1996), carbamyl phosphate synthetase I (Gupta *et al.*, 1997), and aldehyde dehydrogenase (Landin *et al.*, 1996) were identified as

being adducted after administration of hepatotoxic doses of PAR to mice. Covalent modification of these mitochondrial proteins may result in impaired functioning of these proteins, which could give rise to impaired detoxification (oxidation) of aldehydes to acids, leading to LPO, and to impaired metabolism of ammonia (synthesis of glutamine). This could ultimately lead to mitochondrial damage as observed in hepatocytes as well as *in vivo* in mice as early as 1 h following PAR administration, hours before sincere toxicity emerges (Meyers *et al.*, 1988; Ramsay *et al.*, 1989; Burcham and Harman, 1990; Burcham and Harman, 1991; Donnelly *et al.*, 1994).

d. Concluding Remarks

The finding of mitochondrial protein adducts does not seem to be in line with the fact that the Michael adduct 3-glutathion-S-yl-paracetamol is fairly stable. However, as mentioned briefly above, a less stable *ipso*-adduct might be formed and diffuse to compartments other than the site of formation and subsequently form GSH and NAPQI in a reverse reaction. It thus allows NAPQI to react again with GSH or protein thiols distinct from the endoplasmic reticulum. Protein arylation after administration of PAR has been found in proteins even further away from the site of reactive intermediate formation, that is, outside the hepatocyte, for example, in hemoglobin and blood plasma (Axworthy *et al.*, 1988; Bartolone *et al.*, 1989).

One protein that is returning in many reports that studied protein arylation by PAR, PAR-congeners (e.g., 3'-HAA), and other arylating toxicants (bromobenzene) during the last decade seems to be the cytosolic 58 kDa 'acetaminophen-binding protein' (which is not necessarily the same as the cDNA-deduced 56 AP). Although more extensive arylation of the 58-kDa 'acetaminophen-binding protein' does not seem to be correlated with a decrease in toxicity, it could be a preferential and common target for reactive metabolites and serve as electrophile scavenger, as with low 58-kDa 'acetaminophen-binding protein' there is increased arylation of other proteins (Hoivik *et al.*, 1996b). Moreover, an alternative role for the 58 kDa 'acetaminophen-binding protein' was sug-

gested by indications that administration of PAR results in translocation of the arylated 58-kDa 'acetaminophen-binding protein' from hepatic cytosol into the nucleus (Hong *et al.*, 1994). This would be consistent with a role for this protein as electrophilic sensor such that translocation of arylated 58-kDa 'acetaminophen-binding protein' may be a signal to the nucleus for the presence of an electrophile (Hoivik *et al.*, 1996b). Recently, even a nuclear protein was reported to become arylated after PAR administration. Khairallah and Cohen reported covalent adduct formation of PAR to lamin A, one of the three intermediate filaments that form the nuclear lamina (Khairallah *et al.*, 1995). This phenomenon discussed further in the last paragraph of this section.

In conclusion, convincing evidence exists that protein arylation by PAR/NAPQI is a main trigger for processes that lead to disruption of cell homeostasis in hepatocytes. With about 10 essential proteins in the cytosol and three main cell organelles (endoplasmic reticulum, mitochondria, and nucleus) being struck by covalent protein modification, an additional role for protein thiol oxidation in the disruption of the cell physiology cannot be excluded, however, a prominent role is unlikely. In PAR overdose patients, the relation between plasma ALT and plasma 3-(cystein-S-yl)-paracetamol protein adducts, as found by an immunoassay, is highly suggestive of a dominant mechanistic role of protein arylation (Hinson *et al.*, 1990).

i. Intermezzo

The last proposal could be important when looking at the mechanisms underlying the extensive arylation of the so-called 'selenium binding protein', a 56 to 58 kDa cytosolic protein found in liver and kidneys in several species (Pumford et al., 1992; Lanfear et al., 1993; Jamba et al., 1996). This protein is one of the most prominent proteins being arylated in vivo after a (sub)toxic dose of PAR to mice. It was proposed that this 'selenium binding protein' would be arylated by reaction of NAPQI with the selenium analogue of the cysteinyl thiol group, the selenol or selenide (Pumford et al., 1992). However, selenium containing protein adducts have never been identi-

fied. Furthermore, the administration of sodium selenite to mice, prior to toxic doses of hepatotoxic doses of PAR, reduced the covalent binding of PAR to all cytosolic proteins, including the 56 kDa selenium-binding protein (Hinson *et al.*, 1996). While attempting to detect covalent protein binding of NAPQI in peroxidase systems, in rat liver microsomal and rat hepatocyte incubations with PAR and multiple substituted PAR analogues in our laboratory, selenium containing protein adducts were never found (unpublished results). Intensive investigations into the reactions between PAR, GSH, and ebselen, a potent antiinflammatory selenium-containing drug reported to protect against PAR toxicity in hepatocyte incubations, did neither result in the detection of covalent binding of PAR to selenium containing amino acids (Li *et al.*, 1994a). The most likely explanation was the formation of ebselen selenol by GSH, followed by a fast reduction of NAPQI to PAR by the selenol, faster than the reduction by GSH. As studied by Baldew *et al.*, glutathionylselenol can be formed from selenite (SeO_3^{2-}) by GSH in a concerted reaction with glutathione reductase ([Vermeulen *et al.*, 1993] and references therein). *In vivo*, a protective action of a surplus selenite against PAR-induced toxicity has been shown (Schnell *et al.*, 1988). Analogously, an 'intraprotein cysteinylselenol' (ProtSeH) might be formed from a cysteine in a bis (cysteinyl) sequence motive inside a protein (Figure 10).

Combining these findings and suggestions with the proposed mechanism of protein thiol oxidation (Nelson, 1995), it might be hypothesized that an 'intraprotein cysteinylselenol' (ProtSeH) moiety of the 'selenium binding protein' also forms a Meisenheimer complex with NAPQI, followed by attack of GSH and rearrangement, resulting in a 3'-arylated 'selenium binding protein'. This mechanism is theoretically possible because the 'selenium binding protein', unlike various GSH peroxidases, does not contain a selenocysteine encoded by an internal UGA codon (Lanfear *et al.*, 1993). Selenium is only bound and not incorporated. This hypothesis of ipso-attack awaits further investigation. One way to test this hypothesis might be to treat animals with ^{75}Se -sodium selenite (Jamba *et al.*, 1996) and ^{14}C -PAR after a few days, followed by investigation of individual

amino acids of hepatic cytosolic proteins for ^{75}Se - and ^{14}C -label.

4. Covalent Binding to Lipids

Not regarded as a very important event in the development of toxicity, a fourth event, i.e. covalent binding of NAPQI to lipids *in vitro* as well as *in vivo*, has received some attention. Covalent lipid binding (0.1 nmol/mg phospholipid) was about 3% of the covalent protein binding in incubations of PAR with microsomes from 3-methylcholanthrene-induced mice, which is equivalent to one modified phospholipid molecule out of 10^4 . Covalent lipid binding on horseradish peroxidase/ H_2O_2 incubations in the presence of inactivated mouse liver microsomes amounted up to 10 nmol/mg phospholipid (Wendel and Hallbach, 1986). Although covalent lipid binding is also found after *in vivo* administration, it appears to be due to an electrophilic attack on nucleophilic centers in lipids rather than a radical reaction with electro-neutral alkyl residues of the phospholipids (Smith *et al.*, 1984).

5. Nuclear Effects

The last hypothesis on causative events in PAR-induced hepatotoxicity that will be discussed is that of the nuclear mechanism. Ambiguity still exists as to possible genotoxic effects of PAR. Although genotoxic effects were reported to be found in liver as well as other organs, the indicated mechanisms will probably be similar in liver and extrahepatic organs. Therefore, these effects are discussed in this Section on hepatotoxicity. As reviewed recently, two studies indicate chromosomal damage in lymphocytes at therapeutic intake of PAR by human volunteers, whereas one study is negative (Rannug *et al.*, 1995). High doses of PAR have been reported to induce liver tumours in mice and bladder tumours in rats as has been reviewed in extenso recently (Flaks and Flaks, 1983; Flaks *et al.*, 1985; Rannug *et al.*, 1995). Mostly, however, low but chronic levels of exposure are studied with respect to carcinogenic effects of PAR. For these low levels, carcinogenicity studies were negative, and a

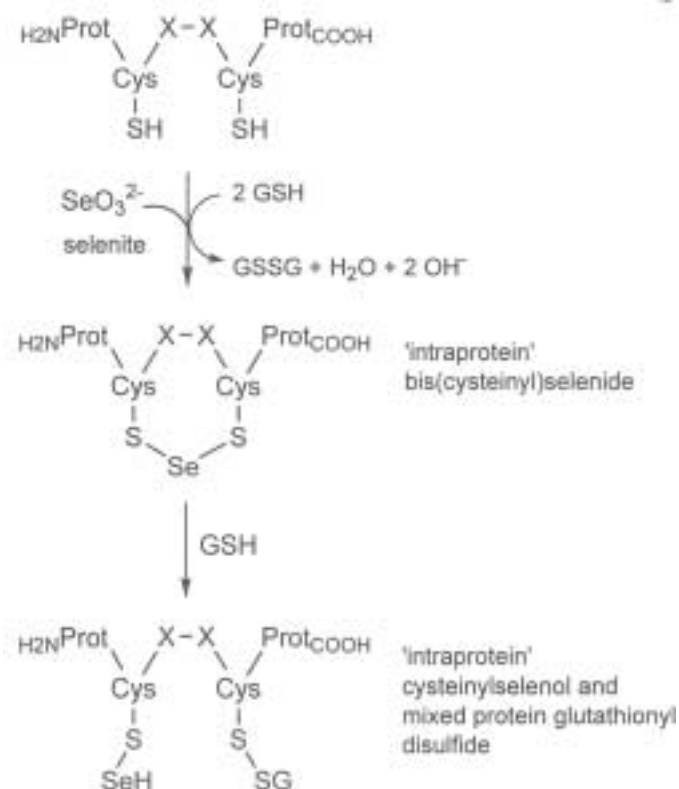


FIGURE 10. Proposed mechanism for protection of protein thiols by selenite. Hypothetical reaction of selenite in the presence of GSH with a protein containing a Cys-X-X-Cys motif (e.g., the 58-kDa 'acetaminophen binding protein'), giving rise to a protein selenol. This reaction is analogous to the reaction of selenite with GSH as proposed by Vermeulen *et al.* (1993). Selenite is suggested to be converted into an 'intraprotein' bis(cysteiny)selenide ($\text{ProtNH}_2\text{-Cys-Se-Cys-ProtCOOH}$), with concomitant consumption of two GSH equivalents. This bis(cysteiny)selenide subsequently reacts with a third GSH equivalent to an 'intraprotein' cysteiny)selenol ($\text{ProtNH}_2\text{-Cys-SeH}$) and three amino acids further to the carboxy terminus a mixed protein glutathionyl disulfide ($X = \text{amino acid}$).

recent review concluded that there was limited evidence for carcinogenicity of PAR in animals and inadequate evidence in humans when exposed to therapeutic levels (Bergman *et al.*, 1996).

In an *in vitro* study, covalent binding of DNA by a PAR metabolite was unequivocally proven after incubations with microsomes from rat hepatic and renal tissues, supported by NADPH or cumene hydroperoxide, and after incubation with HRP and H_2O_2 . The binding levels in the peroxidase system were 200-fold greater than in the microsomal systems. The presence of cysteine or nuclear protein modulated the covalent binding in incubations containing radiolabeled NAPQI. Cysteine exhibited increased binding when present at 0.1 mM and decreased binding when present at 1.0 mM, both compared to incubations without cysteine. By combination of these *in vitro* results with *ex vivo* results, the authors suggested that

DNA binding could occur at therapeutic doses in humans (Rogers *et al.*, 1997). Earlier, covalent binding of PAR to cellular nucleic acids (DNA as well as RNA) as the result of (provoked) respiratory bursts of granulocytes as well as neutrophilic type cells (differentiated from a leukemic cell line) was reported. Although the exact identity of the DNA adduct remained ambiguous, *N*-acetyl-*p*-benzosemiquinone imine (NAPSQI), the one electron oxidation product of PAR was proposed (Corbett *et al.*, 1989; Corbett *et al.*, 1992). The *in vivo* relevance of these findings on direct DNA modification for humans taking PAR at therapeutic doses remains to be established. In addition and as mentioned above, recently it was found that following covalent binding of PAR to the 55- to 58-kDa 'acetaminophen-binding protein', this arylated protein is directed to the nucleus via transnuclear transport (Hong *et al.*, 1994). Al-

though highly speculative, following this translocation, the nucleus might start Stage II of the toxicity process by signaling responses to neighbouring cells for promotion of tissue repair or by signaling macrophages inflicting release of inflammatory mediators (Mehendale *et al.*, 1994b; Hoivik *et al.*, 1996b; Cohen *et al.*, 1997).

In human volunteers, PAR decreased the unscheduled DNA synthesis (UDS) in peripheral lymphocytes and increased the frequency of micronucleated cells in the buccal mucosa. Concomitant intake of ascorbic acid did not decrease the observed genotoxic effects but extended the effects of PAR intake on UDS (Topinka *et al.*, 1989). Metabolic intermediates of high concentrations of phenacetin and PAR were suggested to induce a low frequency of nonneoplastic morphological transformations in a mouse embryo fibroblast cell line (C3H/10T1/2 clone 8) (Patierno *et al.*, 1989). One of these metabolic intermediates of phenacetin as well as of PAR is *p*-benzoquinone (PBQ), a hydrolysis product of and chemically very similar to NAPQI (Miner and Kissinger, 1979; Dahlin *et al.*, 1984; Koymans *et al.*, 1989).

This PBQ was reported recently to interact with microtubule proteins *in vitro*, thereby preventing microtubule formation. *In vivo* this may lead to interference with the formation of a functional spindle apparatus in the mitotic cell, thus leading to abnormal chromosome segregation and aneuploidy induction (Pfeiffer and Metzler, 1996). Aneuploidy is considered a critical event in the multistep process of neoplastic cell transformation (Eastmond, 1993). Moreover, a nuclear protein was reported to become arylated after PAR administration. Khairallah and Cohen reported covalent adduct formation of PAR to lamin A, one of the three intermediate filaments that form the nuclear lamina (Khairallah *et al.*, 1995). This finding is consistent with PAR-induced disruption of the nuclear lamina. Chromatin attachment to the inner nuclear membrane dropped within 2 h after treatment with PAR (Hong *et al.*, 1994). Moreover, unmetabolized PAR was reported to quench the tyrosyl radical in one subunit of the ribonucleotide reductase, thereby slowing the DNA polymerization necessary to fill gaps in DNA strands (Hongslo *et al.*, 1990; Hongslo *et al.*, 1994).

In support of the covalent binding theory, it was reported recently that reactive metabolites of PAR appeared to bind covalently not only to nuclear protein but also to hepatic and renal DNA from mice pretreated with diethylmaleate to deplete GSH and that measurable covalent binding to hepatic DNA was observed up to 1 week after PAR administration (Hongslo *et al.*, 1994). The effects of Ca^{2+} -endonuclease, DNA repair, and inhibitors of GSH depletion on DNA fragmentation and cell death after cytotoxic doses of PAR in cultured mouse hepatocytes were described by Shen *et al.* As accumulation of Ca^{2+} in the nucleus, and fragmentation of DNA *in vitro* and *in vivo* (characteristic of Ca^{2+} -mediated endonuclease activation) are known to unfold well in advance of cytotoxicity and the development of necrosis, the authors suggested that unrepaired damage to DNA contributes to PAR-induced cell death and may play a role in necrosis *in vivo* (Shen *et al.*, 1992).

Recently, data were reported indicating that PAR interferes with nucleotide excision repair in several mammalian cell types, for example, keratinocytes (Skorpen *et al.*, 1998). This constitutes a mechanism by which paracetamol might contribute to genotoxicity in humans (Brunborg *et al.*, 1995). By electron paramagnetic resonance it was demonstrated that PAR added to crude cell extracts of a mammary tumor cell line of mouse destroyed a tyrosyl free radical of the small subunit of ribonucleotide reductase. These results show that PAR reduces DNA synthesis by specific inhibition of ribonucleotide reductase (Hongslo *et al.*, 1990). In a subsequent study, PAR treatment increased single strand breaks in nuclear DNA isolated from the liver but not from the kidneys, 2 h after i.p. injection of PAR at 600 mg/kg in male B6 mice. Only marginal DNA damage was noted at 300 mg/kg. Results also suggested that breaks were induced in DNA from a subpopulation of murine liver cells. Interestingly, the nonhepatotoxic PAR regioisomer (congener), 3'-hydroxyacetanilide (3'-HAA; 600 mg/kg), which also binds covalently to proteins, did not cause DNA single strand breaks. DNA polymerization, necessary to fill the gap in a DNA strand, was concluded to be blocked by reversible inhibition of deoxyribonucleotide (dNTP) synthesis and therefore may also interfere with DNA repair (Hongslo *et al.*, 1994).

At 1 h following an oral dose of 1 g/kg bw to male Wistar rats, DNA synthesis was reported to be inhibited in various organs, that is, spleen, testis, thymus, stomach, small intestine, and bone marrow, but not in liver. This effect was shown to be transient and disappeared within 4 h, except in spleen. This transient inhibition was explained by a reversible inhibition of deoxyribonucleotide reductase (Lister and McLean, 1997). In addition, PAR was reported to inhibit replicative DNA synthesis in V79 Chinese hamster cells, probably by competitive reaction with a tyrosyl radical species involving the transfer of a hydrogen atom at the active site of ribonucleotide reductase (Richard *et al.*, 1991).

As mentioned earlier, mostly low but chronic levels of exposure were studied with respect to potential nuclear effects of PAR. However, DNA effects and an apoptotic-like mechanism in acute PAR toxicity have been receiving attention as well (Ray *et al.*, 1991; Ray *et al.*, 1993; Ray *et al.*, 1996). In 1996, Ray *et al.* established for the first time that in addition to necrosis (toxic cell death), apoptosis (programmed cell death) may be involved in some stages of the highly integrated process of PAR-induced toxicity. PAR-induced cell death was preceded by massive elevation in serum ALT coupled with a rapid loss of genomic DNA (2 to 24 h), fragmentation of DNA (2 to 24 h), apoptotic nuclear condensation (2 to 6 h) followed by massive fragmentation and margination of heterochromatin at later hours (6 to 24 h) and a near total loss of glycogen in pericentral areas (Ray *et al.*, 1996). Although positioned here as a Stage I process, the later steps in apoptosis leading to cell death might as well be positioned in Stage II.

Intermediate between Stage I and Stage II might be the following effects that were found and seem to be independent of metabolic bioactivation. An almost equal antiproliferative effect was observed in a human hepatoma HepG2 subline expressing human CYP2E1 as well as in a comparable subline not expressing CYP2E1 (Dai and Cederbaum, 1995). From this early finding, new investigations emerged. PAR appeared to modulate serum growth factor signal transduction in Hepa 1 to 6 cells, thereby inhibiting cells from completing specific phases of cell division. PAR was found to inhibit *c-myc* expression, activation

of NF- κ B DNA binding and Raf kinase activity via serum growth factor. The serum growth factor appears to play an important role in counteracting the pro-apoptotic effects of transforming growth factor- β (TGF- β). The ability of PAR to inhibit cell division might interfere with organ regeneration and thus exacerbating acute liver damage caused by PAR (Boulares *et al.*, 1999). Another finding that might have direct consequences in the subsequent Stage II is the impairment of expression and secretion of tumor necrosis factor- α (TNF- α) in primary rat liver cell cultures treated with PAR. As TNF- α has both adverse (aggravation of primary damage to hepatocytes) and beneficial effects (stimulation of tissue repair) in toxic liver damage, the consequences of this finding remain to be established (Nastevska *et al.*, 1999).

C. Stage II — Damage in Hepatocytes and Nonparenchymal Cells

As mentioned briefly above, in the development of PAR-induced hepatic injury, several stages have been discerned (Figure 5) (Mourelle *et al.*, 1990; Mehendale, 1991; Mourelle *et al.*, 1991; Mehendale, 1995; Chanda and Mehendale, 1996b). Stage I phenomena could be argued to collectively comprise all of the cellular damaging events but also the cytoprotective mechanisms (thus within the cell), that is, selective protein modification, LPO and so on but also reduction of oxidative metabolites and oxidized protein thiols, superoxide dismutase activity etc. So-called Stage II processes, observed shortly before and during tissue necrosis, discussed here. At Stage II, processes spread from cell to cell in the extracellular matrix embedded in a tissue and from hepatocytes to nonparenchymal cells and even extrahepatic tissues. These processes are triggered by the release of factors by hepatocytes that are chemotactic for Kupffer cells as well as for blood monocytes, that may increase the production of cytokines and of reactive oxygen and nitrogen intermediates and that may enhance fagocytosis. Simultaneously, in Stage II of toxicity, tissue-based protective response mechanisms (tissue repair) are launched (Laskin, 1994; Soni and Mehendale, 1998). These cells can release reactive oxygen species (causing tissue damage and

necrosis) and cytokines. A consequence of the latter might be a possible effect on the blood circulation, which may play an important additional role in the elimination of toxic quantities of PAR (Skoglund *et al.*, 1987; Laskin, 1994). An overview on the role of nonparenchymal cells and inflammatory mediators in hepatotoxicity in general has been published (Laskin, 1994).

As several of the covalently modified proteins are involved in Ca^{2+} sequestration in the endoplasmatic reticulum, impairment of the activities of these proteins might lead to disruption of cellular Ca^{2+} homeostasis and thereby cause cellular injury. In mouse hepatocytes, however, increase in cellular Ca^{2+} was determined to be a secondary event posterior to cytotoxicity and not occurring before 2.5 h after addition of PAR according to Grewal *et al.* (Grewal and Racz, 1993). A second mechanism for cellular injury could be impaired synthesis of plasma membrane proteins. As calreticulin and the Q5 thiol:protein oxidoreductase are involved in posttranslational modification of proteins, any toxin that inactivates these proteins could block the final synthesis of membrane proteins resulting in cytotoxicity (Holtzman, 1995; Zhou *et al.*, 1996).

Many reports described direct effects of NAPQI when added to hepatocytes or isolated mitochondria (Albano *et al.*, 1985; Weis *et al.*, 1992a; Weis *et al.*, 1992b). For example, NAPQI can release Ca^{2+} from isolated mitochondria via pyridine nucleotide hydrolysis. The beneficial use of synthetical NAPQI as a tool in the investigation of subcellular (molecular/biochemical) processes in P450 and PAR-dependent reactions is without doubt, its value in studying the PAR biotransformation-dependent effects in isolated cells or organs remains questionable, however. Due to the reactivity of NAPQI, being a relative strong electrophile as well as an oxidant, the rate and site of formation of NAPQI is of utmost importance (Harman *et al.*, 1991). The incapability of BCNU to increase covalent protein binding in hepatocytes treated with NAPQI (Albano *et al.*, 1985) may be due to saturation of the hepatocyte system with an unrealistically high concentration of NAPQI used (250 μM).

Posttranslational modification of proteins can also be involved in the sequestration of NAPQI (Krishna and Wold, 1993). More likely, however,

is altered protein functioning triggered by modification (arylation, glutathiolation, protein thiol oxidation). One protein, the 55 to 58 kDa 'acetaminophen-binding protein', is sequestered into the nucleus, signalling the presence of a toxic compound (Cohen *et al.*, 1997). Interestingly, but beyond the system of Stage I and Stage II processes, bioactivation-independent effects on mitochondrial functioning in rat liver slices are observed within 30 min after PAR administration, that is, long before cell damage is observed (Nazareth *et al.*, 1991). This can be an effect on mitochondrial DNA processing, a phenomenon similar to the inhibitory effect of PAR on ribonucleotide reductase (see below) where an essential tyrosyl residue was destroyed (Hongslo *et al.*, 1990; Hongslo *et al.*, 1994). One should be careful, however, in interpreting the relevance of these *in vitro* findings for the *in vivo* situation. In a comparative study, *in vivo* mitochondrial effects were found to be dependent on biotransformation whereas *in vitro* mitochondrial effects were not (Meyers *et al.*, 1988). Another, biotransformation independent observation is the complexation of PAR with human insulin (Smith and Ciszak, 1994). Whether this is relevant in an impaired potential of liver regeneration after PAR intoxication remains to be established.

Most research in the 1970s and 1980s focussed on liver parenchymal cells, the hepatocytes. In the last 10 years, however, more attention was paid to nonparenchymal cells such as Kupffer cells, the resident macrophages of the liver, which could be involved in the damage observed after 10 h in liver tissue (Koop *et al.*, 1991; Qu *et al.*, 1992). About 1 to 6 h after PAR ingestion, damage in the hepatic tissue is observed that may be one of the indirect consequences of the early changes in the primary target cells, i.e., the hepatocytes. Solid evidence exists that as a result of signals from damaged hepatocytes, Kupffer cells and the mediators they release contribute to PAR-induced tissue injury (Mehendale *et al.*, 1994a; Laskin *et al.*, 1995). Release of mediators, however, might be also a consequence of early changes in Kupffer cells themselves at high dose administration. This could be due to the CYP2E1-based bioactivation of PAR in the Kupffer cells as these resident macrophages, in contrast to peritoneal macrophages, ex-

hibit CYP2E1 activity (Koop *et al.*, 1991). Although this activity is only about 20% of CYP2E1 activity in hepatocytes (Koop *et al.*, 1991), it is suggested here that CYP2E1-mediated bioactivation in Kupffer cells might contribute significantly to bioactivation of PAR at higher than therapeutic plasma levels. In addition, novel mechanisms in chemically induced hepatotoxicity (downregulation of subunit proteins [connexins], important in gap junction functioning), described by Mehendale *et al.* for CCl₄ (Mehendale *et al.*, 1994a), could be important in the toxicity mechanism of PAR.

Even a role for nitric oxide as a cytotoxic mediator in PAR-induced hepatotoxicity in the rat was demonstrated (Gardner *et al.*, 1998), a finding which was in line with a communication on nitrotyrosine-protein adducts in hepatic centrilobular areas following toxic doses of PAR in mice (Hinson *et al.*, 1998). Besides an increase in serum ALT, these effects were shown to be accompanied by increased serum levels of nitrate plus nitrite (a marker of nitric oxide synthesis). Tyrosine nitration occurs by peroxynitrite, a reactive intermediate formed by an extremely rapid reaction of nitric oxide and superoxide. Peroxynitrite has also hydroxyl radical-like activity. The involvement of Kupffer cells (hepatic macrophages) in Stage II of PAR-toxicity, leading to increased synthesis of nitric oxide and superoxide presumably leading to peroxynitrite nitrotyrosine-protein adducts was hypothesized (Hinson *et al.*, 1998).

However, other phenomena that could be hypothesized to be related to Stage II of PAR-induced liver toxicity, such as adhesion and vascular plugging by neutrophils, were concluded not to be significant determinants of PAR-induced liver swelling and necrosis (Welty *et al.*, 1993). Maybe, for proper understanding of the PAR toxicity mechanism Stage I and Stage II as discerned by Mehendale *et al.* should be further subdivided. For example, a Stage IA could refer to the primary bioactivation process (the phase I biotransformation), whereas a Stage IB could encompass the adduct formation to GSH, proteins and potentially lipids (revolving phase II biotransformation enzymes and chemical reactions with macromolecules) (Mourelle *et al.*, 1990; Mourelle *et al.*, 1991; Mehendale, 1995; Chanda and Mehendale, 1996b).

Recently, the protection against PAR and bromobenzene toxicity in mice by pretreatment (72 h) with a single dose of amphetamine was reported (Salminen *et al.*, 1997). Protection was proposed to be caused by induction of heat shock proteins (hsp) without decreased protein binding. Although not suggested in the original report, this might well be a Stage II phenomenon. Hsp-induction is normally a result of stressors such as severe hyperthermia and chemicals (references in (Salminen *et al.*, 1997)). A comparable protective mechanism might be working as suggested for reported autoprotection when stimulated tissue repair by a nonlethal dose of a toxicant protects against a subsequent, normally lethal dose, of the same chemical (Mehendale *et al.*, 1994b). That is to say that liver cells are prepared for a new noxious stimulus.

D. Summary

Hepatotoxicity of PAR is the result of a cascade of interrelated biochemical events. Each of the eventualities, protein oxidation, covalent modification and inhibition of enzyme activity, lipid peroxidation, transnuclear transport of 'acetaminophen-binding proteins' and inhibition of protein phosphatase activity, although not all simultaneously nor of equal importance, probably have a role in the mechanism causing liver damage after PAR intoxication. A dominant mechanistic role is probably reserved for the formation of 3-(cystein-S-yl)paracetamol protein adducts. These adducts are found in liver fraction with concentration- and time dependency after administration of PAR to mice. Furthermore, the levels of arylated proteins in serum correlate with serum ALT levels. In addition, a threshold level is suggested for adduct formation in the development of toxicity as 3-(cystein-S-yl)paracetamol protein adducts appear in hepatic fractions prior (peak after 2 h) to serum (peak after 6 to 12 h). Moreover, these 3-(cystein-S-yl)paracetamol protein adducts are found in serum of PAR-intoxicated patients and correlate with serum ALT levels.

In the last decade, many protein targets of the reactive metabolite of PAR were identified, some of which as important enzymes. Plasma membrane and mitochondrial fractions appear to con-

tain most covalently modified proteins, whereas a 55- to 58-kDa cytosolic protein appears to be the most intense arylated individual protein. Scavenging of the reactive metabolite by proteins may, in some ways, be much more important and at least more complex than the scavenging by glutathione. The processes in which probably most effort was put into in the nineties, notably adduct formation of the 55- to 58-kDa 'acetaminophen-binding proteins', may not be a molecular toxicological endpoint but a protection mechanism. In addition, a role in signaling of electrophilic damage to the nucleus followed by provocation of tissue damage has been suggested. The mechanistic role of selenium compounds and selenium binding proteins in the protection against PAR hepatotoxicity also needs further investigation.

An emerging number of *in vitro* and *in vivo* results as well as epidemiological reports point to all kinds of nuclear effects. The most prominent findings are impaired DNA repair and even DNA-adduct formation at low target concentrations but also activation of Ca²⁺-endonuclease at high concentrations. In addition to necrosis (toxic cell death), apoptosis (programmed cell death) may also be involved in some stages of the highly integrated process of PAR-induced toxicity. It remains to be established, however, what the relevance as well as the possible consequences of the experimental and epidemiological findings are for PAR-intoxicated patients as well as humans taking PAR at therapeutic levels.

Phenomena that also have retrieved much attention in the last decade and which may be related to the nuclear effects are intra- and inter-cellular signaling. After the cascade of noxious insults in the first hours after administration of a toxic dose of PAR, mainly in the hepatocytes (distinguished as Stage I), another gush of events commences, that is, secretion by hepatocytes and nonparenchymal cells (e.g., Kupffer cells) of signalling factors, inflammatory mediators and reactive oxygen species such as superoxide anion radical, nitric oxide and peroxynitrite (distinguished as Stage II). Consequences of these actualities may be tissue damage but also tissue repair, the balance of which determines recovery or death. These findings could provide important new leads for improvement of clinical treatment of PAR intoxication.

V. MECHANISMS OF RENAL TOXICITY

A. Introduction

Although the hepatotoxicity of PAR is generally accepted to be primed by the formation of NAPQI, a metabolite formed during cytochrome P450 catalysed oxidation of PAR (Albano *et al.*, 1985; Van de Straat *et al.*, 1988b), the cause of renal toxicity is less clear. Prostaglandin endoperoxide synthase (PGES), *N*-deacetylase as well as P450 enzymes are known to be involved in PAR-dependent toxicity as investigated in one or more species. High doses of PAR result in renal cortical necrosis in man and the F344 rat. Cellular injury is primarily confined to the proximal tubule and significant reductions in glomerular filtration rate (Newton *et al.*, 1983; Hu *et al.*, 1993; Blantz, 1996; Trumper *et al.*, 1996). Like their hepatic counterpart, renal microsomes also oxidise PAR to an arylating intermediate via a P450-dependent mechanism. Thus, at least part of the acute PAR-dependent renal damage is probably due to a biochemical mechanism similar to that in liver. In addition, PAR is deacetylated to *p*-aminophenol in rat renal and hepatic cytosol and microsomes (Newton *et al.*, 1983).

In a recent short review article, various aspects related to PAR-dependent renal toxicity have been summed up. Factors that may influence renal toxicity include chronic liver disease, gender and conditions that alter the activity of P450-metabolizing enzyme systems (Blantz, 1996). In contrast to acute renal failure related to high dose intake of PAR, conflicting results have been presented with respect to the question whether chronic intake of PAR by humans contributes to chronic renal disease and analgesic nephropathy. An epidemiological report on this subject concludes that chronic use of PAR is related to chronic renal disease (Sandler *et al.*, 1989), whereas Blantz (Blantz, 1996) refers to another epidemiological investigation that failed to demonstrate a significant incidence of chronic renal disease in healthy individuals who use over-the-counter analgesics such as PAR. Unambiguous conclusions are probably hampered by the absence of a clear mechanism of analgesic nephropathy. Elucidation thereof has been hampered due to the lack of animal models that closely mimic the human disease as

rodents do not appear to be an appropriate model (Schnellmann, 1998).

B. Prostaglandin Synthase and N-Deacetylase

High acute doses of PAR may result in hepatic centrilobular and renal cortical necrosis in man and the F344 rat (Newton *et al.*, 1983). Chronic exposure, however, is correlated much stronger to renal toxicity and probably depends on prostaglandin endoperoxide synthetase (PGES), as illustrated by results obtained in rabbit kidney microsomes. Covalent binding clearly correlated with the zone that microsomes were prepared from (Figure 11) (Mohandas *et al.*, 1981). The very high affinity of PGES for PAR indicates that even at therapeutic doses metabolic activation to nephrotoxic metabolites (presumably NAPQI and/or NAPSQI) may occur (Figure 12) (Larsson *et al.*, 1985).

Human kidney medulla microsomes also catalyzed the PGES-based metabolic activation of PAR at rates similar to those found in rabbit kidneys (Larsson *et al.*, 1985). Similar to protein arylation in liver, covalent binding to an 58-kDa protein preceded nephrotoxicity in mice (Hoivik *et al.*, 1996a). Significant species-, strain-, as well as gender-specific variations in PAR-induced renal toxicity were observed (Newton *et al.*, 1985b; Hu *et al.*, 1993; Hoivik *et al.*, 1995; Mugford and

Tarloff, 1995). An early finding of interest in the pathogenesis of analgesic nephropathy was the reporting of *p*-aminophenol, a well-known nephrotoxic agent, in urine of hamsters that were administered PAR (Gemborys and Mudge, 1981). Oxidation of *p*-aminophenol, possibly via PGES, to the *p*-aminophenoxy free radical may be catalyzed by PGES. Oxidation or disproportionation of this radical will form 1,4-benzoquinoneimine, which can covalently bind to tissue macromolecules (Newton *et al.*, 1983; Fischer *et al.*, 1985c; Fowler *et al.*, 1991). NAPQI was shown to be converted to the *p*-aminophenoxy free radical in a microsomal incubation by combined *N*-deacetylase catalysis and reductive activity of the microsomal mixed-function oxidase system (Fischer *et al.*, 1985c). Subsequent reduction of the *p*-aminophenoxy free radical to *p*-aminophenol seems to be common sense, thus providing a mechanistic explanation for *p*-aminophenol as a urinary metabolite of PAR. Moreover, in F344-rats, deacetylation of PAR to *p*-aminophenol is regarded as a prerequisite to nephrotoxicity (Newton *et al.*, 1985b), although it is suggested here that not PAR but NAPQI is the actual substrate for *N*-deacetylase. In contrast to F344-rats, Sprague-Dawley (SD) rats were susceptible to PAR-mediated renal toxicity, without the involvement of *N*-deacetylase (Figure 12) (Mugford and Tarloff, 1995). Interestingly, in bile of Wistar rats, PAR-GLUC, as well as PAR-SG, were detected after i.p. administration of *p*-aminophenol,

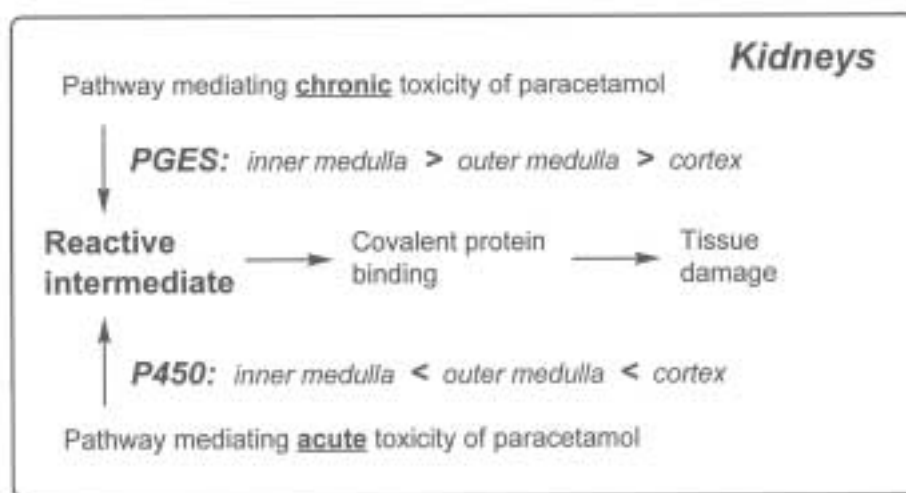


FIGURE 11. Organ distribution of activities in kidneys of rabbit. Distribution in different sections of the kidneys. (Adapted from Mohandas *et al.* [1981].)

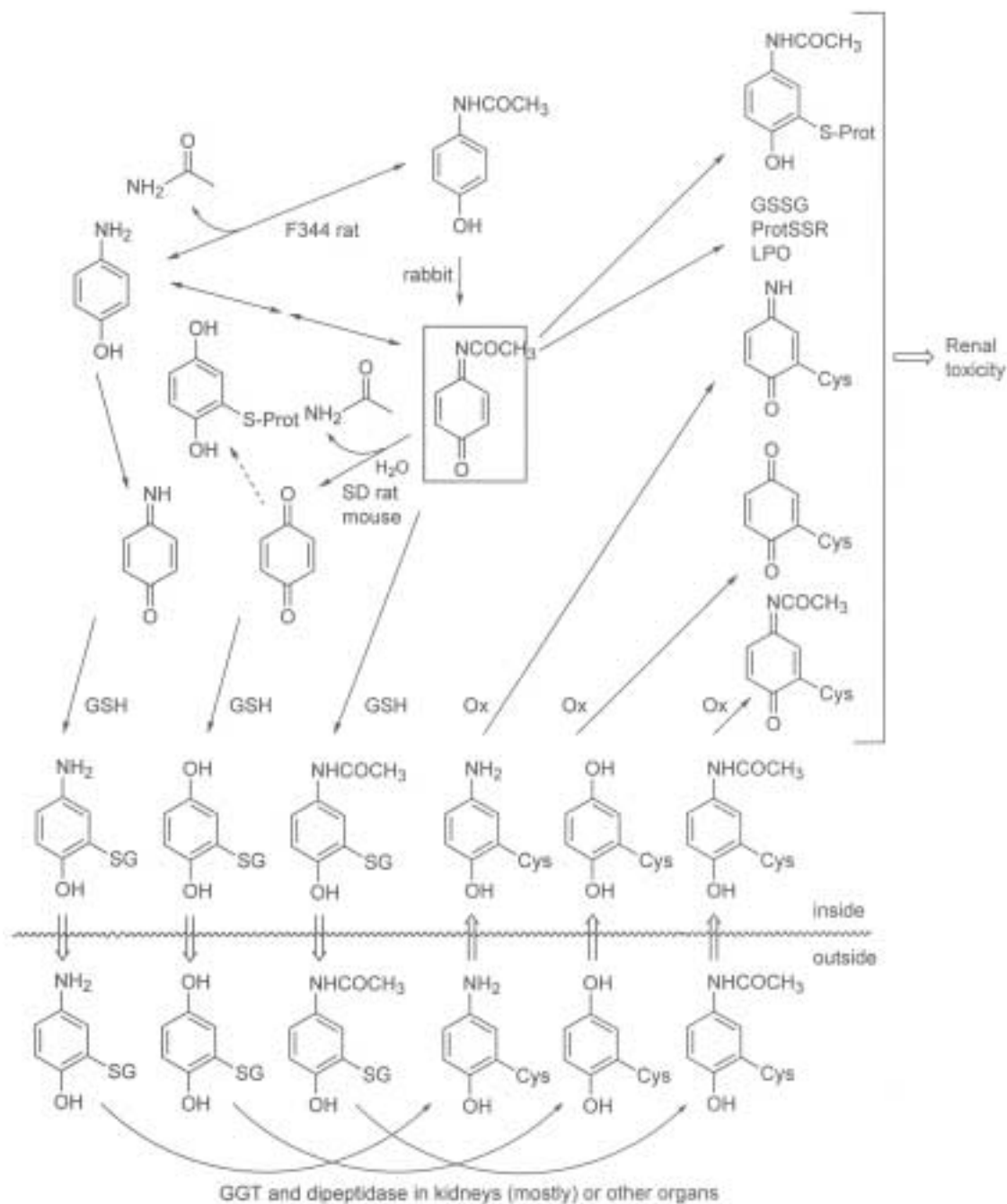


FIGURE 12. Biotransformation of paracetamol in kidneys of various species. Biotransformation of PAR in the renal proximal tubule cell of the rabbit with P450 peroxidase as well as PGES activity and of the Fisher 344 rat with specific *N*-deacetylation favored over CYP2E1 activity. The pathways between NAPQI, quinone imine and *p*-aminophenol may involve free radicals. The route releasing acetamide and leading to pBQ is also important in mice and Sprague Dawley rats. Composed from various references (see text).

supporting the close metabolic relation between PAR and *p*-aminophenol (Klos *et al.*, 1992).

Although based on a limited amount of data, covalent binding of PAR or a metabolite to DNA isolated from the kidneys of mice that were treated

with PAR after fasting and depletion of GSH was also reported (Hongslo *et al.*, 1994). Stronger evidence for DNA binding was obtained recently in *ex vivo* ³²P-postlabeling experiments using renal DNA from PAR-treated mice as well as in *in*

in vitro metabolic activation experiments. Interestingly, in the *in vitro* experiments, horseradish peroxidase (HRP) and H₂O₂ produced binding levels 200-fold greater than microsomes with cumene hydroperoxide or NADPH. These data would further support the hypothesis that acetaminophen metabolites bind covalently to DNA in experimental animals *in vivo* at doses that mimic therapeutic doses in humans (Rogers *et al.*, 1997). These suggestions remain to be established, however. More information with respect to PAR-dependent processes in relation to DNA and the cell nucleus, which may be comparable in liver and kidneys, was presented in Section IV.

C. Cytochrome P450

Although the cytochrome P450 enzymes differ somewhat in liver and kidneys, the consequences of exhaustive cytochrome P450 catalyzed oxidation of PAR to NAPQI are similar in both organs and are discussed in Section IV (protein and nonprotein thiol depletion followed by cellular dysfunction and organ failure). PAR-induced nephrotoxicity in CD-1 mice clearly differs from that described for Fischer rats (F344). The bioactivation mechanism in CD-1 mice is more similar to that in the SD-rat and likely involves renal cytochrome P450-dependent activation and subsequent covalent binding of a reactive metabolite without prior deacetylation (Emeigh Hart *et al.*, 1991a). Proteins of similar size as found in liver (44 and 58 kDa) became arylated in kidneys of mice after administration of PAR. In addition, a 33-kDa protein became arylated. The severity of renal damage and the amount of adducts present could be significantly reduced with the P450 inhibitor piperonyl butoxide (Bartolone *et al.*, 1989). The development of PAR toxicity in mouse kidney slices indicated the existence of an *in situ* activation system (Hoivik *et al.*, 1996a). Moreover, no differences were found in renal damage between bile-canulated and nonbile-canulated rats, indicating the renal toxicity to be independent of hepatic biotransformation (Trumper *et al.*, 1996). Vice versa, hepatic damage was not altered when rats were pretreated with acivicin (AT-125), an inhibitor of γ GT (Trumper *et al.*, 1996). In fact, the renal PAR-based protein arylation and toxic-

ity is caused primarily by renal CYP2E1 catalyzed biotransformation. The increase in PAR-dependent renal toxicity in female CD-1 and C3H/HeJ mice by testosterone pretreatment correlated well to the inducibility of renal CYP2E1 by testosterone (Hu *et al.*, 1993; Hoivik *et al.*, 1995).

The significant sex-related difference observed in the NADPH-dependent bioactivation of PAR in renal microsomes from C3H/HeJ mice further supports the important role of CYP2E1. Moreover, it stresses the importance of gender differences in P450-based bioactivation of PAR (Hu *et al.*, 1993). Summarizing, in CD-1 and C3H/HeJ mice the mechanisms contributing to renal and hepatic toxicity are similar, although independent, and involve P450-dependent bioactivation of PAR. In contrast to bioactivation in liver, important gender differences are observed in renal (CYP2E1-based) bioactivation and toxicity (Hu *et al.*, 1993). See Figure 13 for an overview on interaction of hepatic and renal biotransformation steps via interorgan transport of PAR and metabolites.

In a recent study with renal (as well as hepatic) S9 incubations (cytosolic and microsomal fraction) from male and female SD rats covalent protein binding of [¹⁴C-phenyl]-PAR was reduced more than 85% by omission of NADPH. 1-Aminobenzotriazole (ABT; a suicide inhibitor of P450) reduced covalent binding by only 50% and bis(*p*-nitrophenyl) phosphate (BNPP; a reversible inhibitor of *N*-deacetylase) had no effect. From these data the authors concluded that there must be other NADPH-requiring enzymes, not destroyed by ABT or inhibited by BNPP, that participate in the covalent binding of PAR (Mugford and Tarloff, 1995). It is postulated here, however, that part of the NADPH may be consumed without requiring NADPH-dependent enzymatic activity. PAR might be oxidised by renal peroxidases (such as PGES) to the phenoxy radical NAPSQI, which then can abstract a hydrogen atom from NADPH. The NADP[•] thus formed may react with O₂ to produce the superoxide anion O₂^{•-} (Figures 4 and 7) (Keller and Hinson, 1991). Concomitantly with formation of NADP[•], PAR is formed, being available again for P450-dependent formation of NAPQI, which may result in protein binding. When no NADPH is present at all, peroxidase activity may result in increased

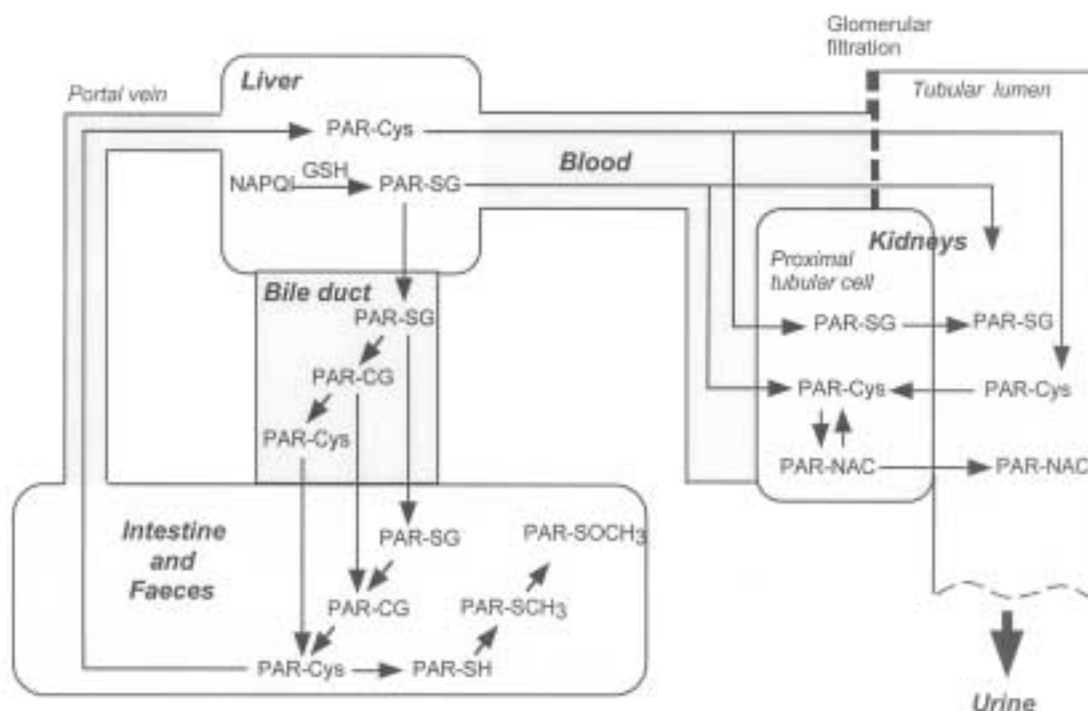


FIGURE 13. Interorgan transport of glutathione-dependent paracetamol conjugates. (Adapted and modified from Commandeur *et al.* [1995].)

formation of NAPSQI, which may either result in dimerisation, trimerisation, etcetera, but also in a radical termination reaction, producing PAR and NAPQI. For substantiation of this hypothesis, detailed investigations studying the concentration dependency of all enzymes and reactants involved are required.

D. Glutathione S-Conjugation

It has been hypothesized recently that a GSH-conjugate of PAR/NAPQI or of a secondary metabolite is involved in renal toxicity of PAR in CD-1 mice (Emeigh Hart *et al.*, 1996). *In vivo* inhibition of γ GT and organic anion transport (by AT-125 and probenecid) significantly decreased renal toxicity after administration of PAR (Figure 13). An extensive review was presented (Commandeur *et al.*, 1995). The observed decrease in PAR nephrotoxicity after inhibition of γ GT (preventing the formation of the PAR-Cys) together with the complete prevention of damage after inhibition of the organic-anion transport with concomitant accumulation of substrates (PAR-Cys and PAR-NAC but not PAR-SG) are indicative for PAR-Cys or PAR-

NAC contributing to the observed renal toxicity in CD-1 mice. As (1) PAR-Cys, and not PAR-NAC, is concentrated in mouse kidneys, (2) PAR-Cys is the predominant urinary metabolite, (3) inhibition of *N*-deacetylation does not alter nephrotoxicity (references in (Emeigh Hart *et al.*, 1996)), and (4) inhibition of β -lyase activity by aminoxyacetic acid did not alter renal toxicity, it is most likely that PAR-Cys but not the mercaptan PAR-SH plays a crucial role in the toxicity.

The PAR-dependent GSH-conjugate referred to above may be the 3-glutathione-S-yl-paracetamol conjugate but also a GSH-conjugate of *p*-aminophenol, being suggested as a secondary metabolite of PAR. In addition, another P450-based PAR-metabolite, *p*-benzoquinone (PBQ), being a hydrolysis metabolite of NAPQI, or its GSH-conjugates could contribute to the renal toxicity (Emeigh Hart *et al.*, 1996). Species differences as reported for PAR-based renal toxicity might be related to differences in GSH conjugation and protein-alkylation. As *p*-benzoquinone is known as a minor metabolite of PAR, mono-, di-, and even tri(glutathione-S-yl)hydroquinone conjugates thereof formed in liver could be transported to the kidneys. For example, after administration of

tri(glutathione-S-yl)hydroquinone, qualitative species differences have been reported for nephrotoxicity, correlating with protein alkylation of a 34 kDa in the S₃ segment of the renal proximal tubule (sensitive male Fisher 344 rats versus resistant BALB/c and B6C3F₁ mice) (Kleiner *et al.*, 1998).

E. Summary

Prostaglandin endoperoxide synthase (PGES), *N*-deacetylase as well as P450s are involved in the renal toxicity after administration or intake of a high dose of PAR. Although important species and gender differences exist, acute renal toxicity may be observed next to hepatotoxicity. In mice and the SD rat, the mechanisms contributing to renal toxicity of PAR involve P450-dependent bioactivation of PAR. In contrast to bioactivation in liver, important gender differences are observed in renal (CYP2E1-based) bioactivation and toxicity. In addition, F344 rats and hamsters exhibit renal deacetylation of PAR to *p*-aminophenol, followed by PGES-dependent formation of the phenoxy radical of *p*-aminophenol. In addition, species differences in the renal disposition of the cysteine- and the *N*-acetylcysteine conjugates may be relevant, although their importance remains to be investigated.

After chronic intake of low doses of PAR, the kidney rather than the liver may be the target organ. Activation by PGES, which exhibits high affinity, may be responsible for chronic renal disease, although epidemiological results are still controversial and rodents do not seem to be appropriate animal models. Rabbits might be better animal models for investigating PAR-dependent chronic renal disease. Recently, findings in mice that suggested covalent binding of PAR to DNA were supported by *in vitro* findings. The clinical relevance of these findings at therapeutic doses remains to be established, however. At the moment, epidemiological findings are ambiguous.

VI. TOOLS TO MODULATE TOXICITY

A. *N*-Acetylcysteine

The most important antidote used in the clinic since long is *N*-acetylcysteine (NAC;

Fluimucil™), the second one probably methionine (Prescott, 1983; Thomas, 1993; Jones, 1998). Both compounds are good examples of mechanism-based chemoprotection as they are GSH precursors and are preferred over GSH as this tripeptide is not absorbed as such by cells but only after breakdown into the corresponding amino acids. However, in human hepatocytes contrasting results were obtained with respect to methionine. As in human hepatocytes several sulfhydryl compounds increase intracellular GSH levels, in the order *N*-acetylcysteine > thioproline > cysteine > 2-oxo-4-thiazolidine carboxylic acid > methionine, only *N*-acetylcysteine, thioproline, and cysteine substantially counteracted PAR-based GSH-depletion (Larrauri *et al.*, 1987). Recently, combined treatment of intoxicated mice with NAC and cimetidine (inhibitor of P450-dependent metabolic oxidation), 2 h after PAR administration, was reported to provide a 100% survival rate and a marked reduction in plasma ALT and AST levels. In comparison, single administration of either NAC or cimetidine caused only a partial improvement of these parameters (Al-Mustafa *et al.*, 1997). These authors referred to a paper that reported the absence of additive effects of NAC and cimetidine in PAR-intoxicated patients. This was suggested (Al-Mustafa *et al.*, 1997) to be due to a different protocol used (initiation of cimetidine treatment 8 h post-PAR overdose) and stresses the importance of the time schedule in these treatments. Furthermore, the combined treatment might be useful as lower plasma levels of NAC could be sufficient, thereby minimizing the risk for potential adverse reactions to NAC such as listed (Thomas, 1993; Al-Mustafa *et al.*, 1997). It should be stressed that NAC appears to be effective only when given within a few hours after PAR poisoning (Thomas, 1993). This period seems to correlate with the Stage I toxicity events as described in Section IV. This appears plausible because NAC is presumed to be mainly active via repletion of GSH and to some extent via repletion of the cofactor for sulfate conjugation but not via intercellular processes that are designated as Stage II hepatotoxicity. An overview of a selection of modulating agents, which are only partially described in the text (see Table 6).

TABLE 6
***In Vitro* and *In Vivo* Modulation of Paracetamol-Induced Renal and Hepatic Effects**

Protective agent	Species/Test system	Mechanism	Effect	Reference
<i>At the level of glucuronidation and sulphation</i>				
oleonic acid	mice, <i>in vivo</i>	incr. glucuronidation	decr. hepatotoxicity	(Liu et al., 1993a; Liu et al., 1993b)
pregnenolone-16 α -carbonitrile	hamster, <i>in vivo</i>	incr. UDPGT / increase UDPGA	decr. hepatotoxicity	(Mathu and Klaassen, 1991)
ranitidine	rat, <i>in vivo</i>	inhibition conjugative route	incr. hepatotoxicity	(Leonard et al., 1985)
ranitidine	rat (Fischer 344), <i>in vivo</i>	inhibition of glucuronidation	incr. hepatotoxicity	(Rogers et al., 1988)
selenite	rat (male SD)	incr. total glucuronidation (maybe preceded by reduction of NADP ⁺ to PAR) ¹⁾	decr. hepatotoxicity	(Schneil et al., 1969)
<i>At the level of deacetylation</i>				
bis(p-nitrophenyl) phosphate (BNPP)	rat (F344): <i>in vivo</i> and <i>in vitro</i>	inhibition PAR deacetylation to PAP	decr. protein arylation; decr. renal toxicity	(Newton et al., 1983; Newton et al., 1983a)
bis(p-nitrophenyl) phosphate (BNPP)	mouse (CD-1)	inhibition deacetylation	no effect	(Eneigh Hart et al., 1991a)
1 α -o-tolyl-phosphate (TOTP)	mouse (CD-1)	inhibition deacetylation	no effect	(Eneigh Hart et al., 1991a)
<i>At the level of PGES bioactivation</i>				
indomethacin	rat, kidney microsomes	inhibition PGES	decr. protein arylation	(Mohandas et al., 1981)
<i>At the level of P450 bioactivation: (unspecified)</i>				
caffeine	rat, <i>in vivo</i>	stimulation P450	incr. hepatotoxicity	(Sato and Izumi, 1989)
carbamazepine	man, <i>in vivo</i>	induction P450	incr. metabolism PAR	(Smith et al., 1986)
chlordecone	mouse, <i>in vivo</i>	induction P450	incr. hepatotoxicity	(Fouss and Hodgson, 1987)
chlordecone	mouse, hepatocytes	induction P450	incr. cytotoxicity	(Fouss and Hodgson, 1987)
cimetidine	man/mouse/rat, <i>in vivo</i>	inhibition P450	decr. hepatotoxicity	(Leonard et al., 1985; Kadri et al., 1988)
cimetidine (+ N-acetylcysteine)	mouse, <i>in vivo</i>	inhibition P450	decr. hepatotoxicity	(Al-Mustafa et al., 1987)
cobaltous chloride	hamster, <i>in vivo</i>	destruction P450	decr. biliary PAR-SG excretion	(Mathu et al., 1989)
cobaltous chloride	mouse, <i>in vivo</i> (not with PAR but with THAA)	suppression P450	decr. covalent binding	(Salminen et al., 1988)
disulfiram	rat, <i>in vivo</i>	inhibition P450	decr. hepatotoxicity	(Jørgensen et al., 1988)
ethanol	mouse, <i>in vivo</i>	induction P450	incr. hepatotoxicity	(Carter, 1987)
ethanol	mouse, <i>in vivo</i>	inhibition P450	decr. hepatotoxicity	(Thummel et al., 1989)
ethanol	man, <i>in vivo</i>	depletion of NADPH	decr. hepatotoxicity	(Thummel et al., 1989)

TABLE 6 (continued)

4-methylpyrazole	rat, in vivo	inhibition P450	decr. hepatotoxicity	(Burk et al., 1990)
methylxanthines	rat (SD), in vivo	inhib/activation P450's	protection/potentiation	(Kalforn et al., 1990)
metynopone	hamster, in vivo	inhibition P450	decr. biliary PAR-SG	(Madhu et al., 1989)
Mirex	mouse, in vivo	induction P450	incr. hepatotoxicity	(Fouse and Hodgson, 1987)
Mirex	mouse, hepatocytes	induction P450	incr. cytotoxicity	(Fouse and Hodgson, 1987)
β -naphthoflavone	rat, hepatocytes	inhibition P450	decr. cytotoxicity	(Kyle et al., 1987)
phenobarbital	rat, hepatocytes	induction P450	incr. cytotoxicity	(Devalia et al., 1992)
piperonyl butoxide	mouse (male CD-1), in vivo	inhibition P450	decr. protein arylation and tissue damage in liver and kidneys	(Bartolone et al., 1989)
piperonyl butoxide	mouse (male CD-1), in vivo	inhibition P450	decr. PAR-SG; decr. protein arylation; decr. hepatotoxicity	(Emsigh Hart et al., 1991a)
piperonyl butoxide	mouse (male CD-1), in vivo	inhibition P450	decr. renal PAR toxicity; however no effect on PAP toxicity	(Emsigh Hart et al., 1991a)
piperonyl butoxide	hamster, in vivo	no effect on hamster P450?	no influence on PAR-SG excretion	(Madhu et al., 1989)
pregnenolone-16 α -carbonitrile	hamster, liver microsomes	modulation P450(s)	decr. NAPOI formed	(Madhu and Klaassen, 1991)
propylene glycol	mouse, in vivo	inhibition P450	decr. hepatotoxicity	(Hughes et al., 1991)
tanidone	rat, in vivo	inhibition P450	decr. hepatotoxicity	(Leonard et al., 1985)
SKF525A	rat liver slices	inhibition P450 only 0-2 h of incubation	decr. cytotoxicity	(Mourille et al., 1990)
<i>At the level of specific CYP bioactivation</i>				
α -naphthoflavone	hamster, in vivo	inhibition CYP1A	decr. biliary PAR-SG	(Madhu et al., 1989)
alpha-fodrin	mouse, in vivo	suppression CYP1A, 2A, 3A	decr. hepatotoxicity	(Liu et al., 1995a)
caffeine	mouse, liver microsomes	stimulation CYP3A family	incr. PAR-SG	(Jaw and Jeffery, 1993)
clotrimazole	mouse, in vivo	inhibition CYP3A	decr. cov. binding and necrosis	(Salminen et al., 1998)
CYP1A2 and CYP2E1 not expressed	CYP1A2 and CYP2E1 double-null mice,	no CYP1A2 and CYP2E1 expressed	decr. hepatotoxicity	(Zaher et al., 1998)
dexamethasone	mouse, in vivo	induction CYP3A	incr. hepatotoxicity	(Madhu et al., 1992)
dialkylsulfide and other org. sulfur compounds	mouse, in vivo	inhibition CYP2E1	decr. cov. binding and hepatic necrosis	(Wang et al., 1996; Salminen et al., 1998)
dialkylsulfide	human cell-line expressing hCYP2E1	inhibition CYP2E1	decr. cytotoxicity	(Dai and Cederbaum, 1995)
DMSO	mouse, in vivo	inhibition CYP2E1	decr. hepatotoxicity	(Jeffery and Haschek, 1988)
DMSO	mouse, in vivo	no CYP2E1 present	no decr. lung toxicity and nasal toxicity	(Jeffery and Haschek, 1988)
ethynylpyrene	rat, hepatocytes	inhibition CYP1A	decr. cytotoxicity	(Bessoms et al., 1997)
flavonoids	rat (SD), liver microsomes	modulation CYP 3A4	PAR-SG up or down	(Li et al., 1994b)
flvoxamine	mouse (male NMR), in vivo	inhibitor CYP1A2	no effect	(Thomson et al., 1995)

TABLE 6 (continued)

isoniazid	rat, <i>in vivo</i>	induction CYP2E1	incr. hepatotoxicity	(Burk <i>et al.</i> , 1990)
3-methylcholanthrene	hamster, <i>in vivo</i>	induction CYP1A	incr. biliary PAR-SG	(Madhu <i>et al.</i> , 1989)
4-methylpyrazole	human cell-line expressing hCYP2E1	inhibition CYP2E1	decr. cytotoxicity	(Dai and Cederbaum, 1995)
methylxanthines (e.g. caffeine)	rat, microsomes and <i>in vivo</i>	modulation NAPQI formation by CYP1A/CYP3A ²⁷	modulation hepatotoxicity	(Lee <i>et al.</i> , 1991a; Lee <i>et al.</i> , 1991b; Lee <i>et al.</i> , 1995a)
oleonic acid	mouse, <i>in vivo</i>	suppression CYP1A, 2A	decr. hepatotoxicity	(Liu <i>et al.</i> , 1992a; Liu <i>et al.</i> , 1995b)
phenethyl isothiocyanate	mouse, <i>in vivo</i>	inhibition CYP1A2 + CYP2E1	decr. hepatotoxicity	(Li <i>et al.</i> , 1997)
phenethyl isothiocyanate	mouse, liver microsomes	inhibition CYP1A2 + CYP2E1	decr. PAR-SG	(Li <i>et al.</i> , 1997)
propylene glycol	mouse (male B6C3F1), hepatic microsomes (light and heavy fractions)	inhibition CYP2E1 but not CYP1A2 (30% activ. retained)	decr. PAR-SG	(Sneider <i>et al.</i> , 1993)
propylene glycol	mouse (male NMRI), <i>in vivo</i>	inhibition CYP2E1	decr. hepatotoxicity	(Thomsen <i>et al.</i> , 1995)
taxol	rat, <i>in vivo</i>	induction CYP3A	incr. hepatotoxicity	(Kostrubsky <i>et al.</i> , 1997a)
TCDD	hamster, <i>in vivo</i>	induction CYP1A	incr. biliary PAR-SG	(Madhu <i>et al.</i> , 1988)
triacetyloleandomycin	rat, <i>in vivo</i>	inhibition CYP3A	decr. hepatotoxicity	(Kostrubsky <i>et al.</i> , 1997b)
watercress	man, <i>in vivo</i>	inhibition CYP2E1 suggested	decr. urinary PAR-Cys and PAR-NAC	(Chen <i>et al.</i> , 1996)
<u>Modulation at the level of reduction of NAPQI</u>				
acetylsalicylic acid	rat, kidney microsomes	inhibition PGES or reduction of NAPQI	decr. protein arylation	(Mohandas <i>et al.</i> , 1991; Ramakrishna Rao <i>et al.</i> , 1990)
ascorbate	mouse, liver microsomes	reduction of NAPQI	decr. protein arylation	(Dahlin <i>et al.</i> , 1984)
ascorbate	hamster, hepatocytes	reduction of NAPQI (or NAPQI as a comproportionation product?)	50% decr. protein arylation ³⁾	(Miller and Jollow, 1984)
ascorbate	hamster, <i>in vivo</i>	reduction of NAPQI	no decr. protein arylation or hepatotoxicity	(Miller and Jollow, 1984)
ascorbate	man, erythrocytes	antioxidant	protection hemoglobin and Na ⁺ /K ⁺ ATP-ase	(Tukel, 1995)
ebosol	rat (Wistar), hepatocytes	reduction of NAPQI to PAR	LDH leakage down; only by simultaneous or pre-treatment	(Li <i>et al.</i> , 1994a)
tocopherol	man, erythrocytes	antioxidant	protection hemoglobin and Na ⁺ /K ⁺ ATP-ase	(Tukel, 1995)
<u>Modulation at the level of GSH-synthesis or GSH-conjugation</u>				
S-adenosylmethionine	man, hepatocytes	elevation of GSH	protection against GSH depletion	(Ponzoa <i>et al.</i> , 1991)
N-acetylcysteine	mouse, <i>in vivo</i>	increased GSH synthesis	prevention hepatic damage	(Corcoran <i>et al.</i> , 1985)
N-acetylcysteine	man, hepatocytes	decr. GSH depletion	decr. cytotoxicity	(Larrouf <i>et al.</i> , 1987)
N-acetylcysteine	mouse, <i>in vivo</i>	increased GSH synthesis	decr. hepatotoxicity	(Corcoran and Wong, 1985)
N-acetylcysteine (+-carnitine)	mice, <i>in vivo</i>	incr. hepatic GSH concentrations	decr. hepatotoxicity	(Al-Mustafa <i>et al.</i> , 1997)
AT-125 (acivicin)	mouse (male CD-1)	inhibition gGT	decr. renal toxicity	(Eneigh Hart <i>et al.</i> , 1996)
1,3-bis(2-chloroethyl)-1-nitrosourea(BCNU)	mouse, hepatocytes	inhibition of GSH peroxidase and GSSG reductase	incr. membrane damage due to incr. ROOH	(Adams and Harman, 1989)

TABLE 6 (continued)

buthionine sulfoximine	mice (male C3H)	inhibition γ -glutamylcysteine synthetase	depletion hepatic and renal GSH	(Drew and Miners, 1984)
buthionine sulfoximine	rat, hepatocytes	inhibition GSH-synthesis	depletion GSH	(Flue et al., 1985)
buthionine sulfoximine	rat, in vivo	inhibition γ -glutamylcysteine synthetase	incr. clearance of PAR and partial clearance of PAR-SLUP	(Gatnisky, 1986)
buthionine sulfoximine	rat, in vivo	incr. UDPGT and GST	incr. plasma concentrations PAR-GLUC	(Manning et al., 1991)
clofibrate	mouse (male CD-1)	incr. hepatic [GSH]	incr. biliary PAR-SG; decr. protein arylation	(Manastou et al., 1994; Manastou et al., 1996)
coenzym A	mouse, in vivo	decr. GSH depletion	decr. mortality	(Berleth et al., 1990)
cysteine	man, hepatocytes	decr. GSH depletion	decr. cytotoxicity	(Larrauri et al., 1987)
cysteine-prodrugs				
2-oxo-4-thiazolidine carboxylate	man, hepatocytes	no decr. GSH depletion!	no decr. cytotoxicity	(Larrauri et al., 1987)
L-2-oxothiazolidine-4-carboxylate	mouse, in vivo	converted to L-cysteine, stimulation of GSH synthesis	decr. toxicity	(Williamson et al., 1982)
thiazolidine-4R-carboxylic acids	mouse, in vivo	stimulation of GSH resynthesis	decr. hepatotoxicity	(Nagasawa et al., 1984)
thiazolidine-saccharides	mouse, in vivo	increase GSH biosynthesis; no GSH depletion	decr. hepatotoxicity	(Roberts et al., 1987b; Roberts et al., 1992; Roberts et al., 1998)
goldthioglucose	mouse, hepatocytes	inhibition of GSH peroxidase and GSSG reductase	incr. membrane damage due to incr. ROOH	(Adams and Harman, 1989)
isopropylester of GSH	mouse, i.p. in vivo	increased GSH levels in various organs	decr. hepatic damage	(Uhlir and Wordal, 1990)
isauconine	rat (SD); hepatocytes	probably GSH depletion	incr. cytotoxicity	(Shrivastava et al., 1994)
malfonine	mouse, in vivo	probable stimulation GSH synthesis	decr. hepatotoxicity	(Neukonen et al., 1986)
phenylpropanolamine	mouse, ICR	depletion of [GSH]; only when 3 hr pre-PAR	incr. hepatotoxicity	(James et al., 1983)
prednisolone	mouse	repletion of GSH \uparrow ; pos. effect on GSH synthesis?	decr. hepatotoxicity	(Speck et al., 1983)
thiopropine	man, hepatocytes	decr. GSH depletion	decr. cytotoxicity	(Larrauri et al., 1987)
Zn ²⁺ (as zinc sulfate)	mouse, ICR	replenishment hepatic GSH	decr. ALT; decr. malondialdehyde	(Woo et al., 1995)
Modulation at the level of oxidative stress				
allopurinol	mouse, in vivo	antioxidant	decr. hepatotoxicity	(Jäschke, 1990)
1,3-bis(2-chloroethyl)-1-nitrosourea(BCNU)	mouse, hepatocytes	inhibition GSSG-reductase and increased sensitivity to oxidative stress	incr. cytotoxicity	(Gerson et al., 1985)
catalase	rat, hepatocytes (BCNU present)	scavenging H ₂ O ₂	decr. cytotoxicity	(Kyle et al., 1987)
curcumin (low concentration)	rat, hepatocytes	antioxidant	decr. cytotoxicity	(Donatus et al., 1990)
desferrioxamine	mouse, hepatocytes	decr. sensitivity to oxidative stress	decr. cytotoxicity	(Gerson et al., 1985)
desferrioxamine	mouse, in vivo	chelation of Fe ²⁺ ions; inhibition LPO	no decr. hepatotoxicity	(Younes et al., 1988)
dichlorophenol inoiphenol (DCPIP)	rat liver slices	antioxidant after 2 h of incubation	decr. cytotoxicity	(Mourelle et al., 1990)
flavones	rat, hepatocytes	no mprotein arylation, antioxidant?	decr. cytotoxicity	(Devalia et al., 1982)

TABLE 6 (continued)

lobenzarit	rat (Wistar), hepatocytes	probably AO effect and/or stimulation GSSG-PED	decr. LDH leakage	(Remirez et al., 1995)
pipridine analogues	rat, hepatocytes	antioxidant; decr LPO	decr. cytotox	(Alexidis et al., 1998)
propylgallate	human cell-line expressing hCYP2E1	no inhibition LPO	no decr. cytotoxicity	(Dai and Cederbaum, 1995)
superoxide dismutase	rat, hepatocytes (BCNU present)	scavenging O ²⁻	decr. cytotoxicity	(Kyle et al., 1987)
zinc	mouse	induction of metallothionein	decr. Fe2 + decr LPO ?	(Chengelis et al., 1988)
<i>Various modulations at stage I of toxicity development</i>				
ascorbate	hepatocytes	decr. protein arylation	no influence in vivo toxicity	(Miller and Jolow, 1984)
acetylasalicylic acid	mouse / rat	unknown post-bioactivation process	decr. hepatotoxicity	(Whitehouse et al., 1978; De Vries et al., 1984)
cytohesamide	rat (SD)	inhibition of protein synthesis (incorporation of acetyl carbon in proteins)	decr. radiolabel in renal protein in [acetyl ¹⁴ C]-PAR dosed rats	(Newton et al., 1985b)
PBN (N-butyl- α -phenylthiourea)	human cell-line expressing hCYP2E1	radical trapping (spin-trapping)	decr. cytotoxicity	(Dai and Cederbaum, 1995)
probenecid	mouse (male CD-1)	inhibition organic-anion transport	decr. renal toxicity	(Erneigh Hart et al., 1996)
<i>Modulation at stage II of toxicity development</i>				
amphetamine	mouse, in vivo	induction heat shock protein in liver	decr. hepatotoxicity	(Salminen et al., 1987)
chlorpromazine	mouse, in vivo	decr. nuclear Ca ²⁺ increase and DNA fragmentation	decr. hepatic apoptosis/necrosis	(Ray et al., 1993)
cholesteryl hemisuccinate	rat, in vivo	decr. apoptosis	decr. hepatic apoptosis/necrosis	(Ray et al., 1996)
dextran sulfate	rat, in vivo	inhibition of macrophages	decr. hepatic necrosis	(Laskin et al., 1995)
fructose	rat, liver slices	increased intracellular ATP in stage II of hepatotoxicity	prevention of damage	(Mourelle et al., 1991; Martin and McLean, 1996)
galatium chloride	rat, in vivo	inhibition of macrophages	decr. hepatic necrosis	(Laskin et al., 1995)
iodoacetate	rat, liver slices	inhibition glycolyse	incr. damage	(Martin and McLean, 1996)
lipopolysaccharide	rat, in vivo	activation of macrophages	incr. hepatic necrosis	(Laskin et al., 1995)
misoprostol	rat, in vivo	decr. microvascular injury	decr. hepatic necrosis	(Lim et al., 1995)
thioacetamide	rat, in vivo	sustained tissue repair	decr. hepatic necrosis	(Chanda et al., 1996; Chanda and Mohandas, 1999a)
venapamil	mouse, in vivo	decr. nuclear Ca ²⁺ increase and DNA fragmentation	decr. hepatic apoptosis/necrosis	(Ray et al., 1993)

- 1) The mechanism depicted between brackets is hypothesized in this review. See Chapter III
- 2) Actual effect (stimulation or inhibition, dependent on previous induction or not and on the sort of induction (CYP1A or CYP3A)
- 3) Remaining 50% inhibitable by cysteine

Explanations:

1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) = a glutathione reductase inhibitor; bis(μ -nitrophenyl) phosphate (BNPP) is an inhibitor of carboxylesterases and thus of *N*-deacetylation; tri-*o*-tolyl-phosphate (TOTP) is an inhibitor of carboxylesterases and thus of *N*-deacetylation; LPO = lipid peroxidation

The question mark '?' is used when no straight explanation of a finding was presented in the reference but deduced in this review.

B. Other GSH Precursors

An interesting idea has been since long to formulate antidotes together with PAR in order to prevent PAR-mediated toxicity (chemoprotection), but at the same time retain analgesic activity. It was found that the addition of methionine to a PAR formulation (PAR/methionine 5/1) did not affect the analgesic potency of PAR in rats. Besides, methionine reduced the acute toxicity (LD_{50}) of paracetamol by 50% in mice (Ponsoda *et al.*, 1991). Moreover, in 10 human volunteers the pharmacokinetics of paracetamol (1500 mg) were not affected by methionine (300 mg) (Ponsoda *et al.*, 1991). The addition of methionine to PAR formulations or even esterifying methionine to PAR, thus delivering methionine simultaneously with potential overdosing, is an option that has been suggested in the past as a simple way to reduce the high mortality after paracetamol overdosage (Neuvonen *et al.*, 1985; Skoglund *et al.*, 1986). Although no information is available regarding current registration, actually in the United Kingdom a PAR preparation containing methionine, named Pameton, has been available (Thomas, 1993).

In addition, it was found that incubation of human hepatocytes with the methionine containing endogenous compound *S*-adenosylmethionine attenuated the GSH depletion of human hepatocytes incubated with toxic concentrations of paracetamol (Ponsoda *et al.*, 1991). Moreover, *in vivo* in mice, *S*-adenosylmethionine significantly decreased the number of PAR-based fatalities when administered 2 to 5 h after PAR (Bray *et al.*, 1992). Thus, *S*-adenosylmethionine might prove to be suitable to improve clinical antidote therapy compared with methionine or *N*-acetylcysteine or to be fitting as antidote in combination with one or both of these.

Cysteine (actually *L*-cysteine), the limiting reagent for GSH biosynthesis, has been investigated as a potential antidote to be formulated together with PAR or to be given as an antidote after intoxication. The administration of cysteine is known to increase GSH levels, but the amino acid can be neurotoxic and even mutagenic at therapeutic doses (references in [Roberts *et al.*, 1998]). Thiazolidine prodrugs of cysteine, constructed from the amino acid and a variety of

alkyl or aryl aldehydes or ketones, such as aldose monosaccharides, have shown protective activity against PAR-dependent hepatotoxicity and function as a slow release of the precursor for the GSH biosynthesis (Nagasawa *et al.*, 1984; Roberts *et al.*, 1987b; Roberts *et al.*, 1992). Recently, even attempts were described to construct prodrugs of cysteine containing disaccharides in order to obtain analogues with a pendant cyclic sugar moiety that may allow selective delivery to carbohydrate receptors, such as the asialoglycoprotein receptor of hepatocytes (Roberts *et al.*, 1998).

C. Selenium Compounds

Another mechanism-based compound that has been investigated more recently for its chemoprotective properties in relation to PAR-mediated hepatotoxicity is ebselen, an antiinflammatory agent (Harman *et al.*, 1992; Li *et al.*, 1994a). Ebselen protected against PAR cytotoxicity when co-incubated in freshly isolated hepatocytes. The protective effect of ebselen was probably not caused by direct reaction with PAR or inhibition of P450 but by reduction of NAPQI by selenol (Li *et al.*, 1994a). It was suggested that the selenol of ebselen, formed after redox reactions with GSH (Haenen *et al.*, 1989; Cotgreave *et al.*, 1992), was much more a reductant than a nucleophile towards NAPQI when compared with GSH as no indication has been found for the formation of a nucleophilic substitution product between NAPQI and ebselen (Figure 14) (Li *et al.*, 1994a). In addition, peroxidase-like activity of ebselen, protection of ebselen as a direct, thiol-independent antioxidant or radical scavenger could add to the protection of ebselen against PAR-induced cytotoxicity (Li *et al.*, 1994a).

It is known that administration of other selenium compounds, such as sodium selenite (Na_2SeO_3), to mice prior to PAR administration, decreases the hepatotoxicity of PAR as well. Concomitantly, inhibition of the PAR-induced hepatic GSH depletion and covalent binding to hepatic protein was observed. It was suggested that sodium selenite protected via enhanced glucuronidation of PAR thereby diverting the amount of PAR converted to NAPQI (Schnell *et al.*, 1988). However, another mechanism, analogous to the mechanism

noted later for ebselen, might also be responsible for the observed protection by sodium selenite against PAR-induced hepatotoxicity in mice. The protective effect of sodium selenite against the renal toxicity of another compound, cisplatin, was suggested to be caused by methylselenol as well as glutathionylselenol, a new metabolite of selenite (Baldew *et al.*, 1992; Vermeulen *et al.*, 1993) (see also Section IV on hepatotoxicity mechanisms). With the coadministration of PAR and ebselen to hepatocytes, glutathionylselenol thus formed might effectively reduce NAPQI, thereby lowering the NAPQI-based covalent modification of sensitive proteins. In the case of administration of sodium selenite to protect against PAR-dependent toxicity (Schnell *et al.*, 1988), selenite is likely converted to glutathionylselenol by a GSH-dependent mechanism while significantly depleting GSH. The fall in the level of GSH could trigger an increase in activities of γ -glutamylcysteine synthetase as actually observed by Schnell *et al.* (Schnell *et al.*, 1988). This might well increase GSH levels again.

This suggestion is substantiated by the recent observation of a decrease in the PAR-based arylation of the 56-kDa selenium binding protein (SP56; see also Section IV on hepatotoxicity) in mice after preadministration of sodium selenite, prior to marginally toxic doses of PAR, in comparison to administration of PAR only. The extent of decrease of binding to this SP56 was, however, not different

from the decrease in covalent binding to other proteins (Hinson *et al.*, 1996). A prior interaction of selenium with the selenium binding protein should have resulted in a selective decrease in the available binding sites of this protein for NAPQI. The absence of such selectivity in the decrease of covalent binding as found by Hinson *et al.* (Hinson *et al.*, 1996) is suggested here to be based on the same mechanism as explained in the paragraphs above for protection of PAR cytotoxicity by ebselen. This is protection by sodium selenite against PAR-induced covalent binding and hepatotoxicity was caused by a decrease in the level of NAPQI and a decrease in the level of general protein modification, due to blocking of cysteine thiols by selenium thus forming thioselenols (R-CH₂-S-SeH). Thioselenols in turn, would reduce NAPQI to PAR. See also Figure 10 in Section IV. Probably many proteins that have free accessible cysteines are chemically prone to attack by selenite and could thus be called selenium binding proteins. Importantly, these are different from selenoproteins that have specifically one or more selenocysteine(s) incorporated in their amino acid backbone.

D. Various Modulating Agents

Treatment of mice for 10 days with the peroxisome proliferating agent clofibrate (500 mg/

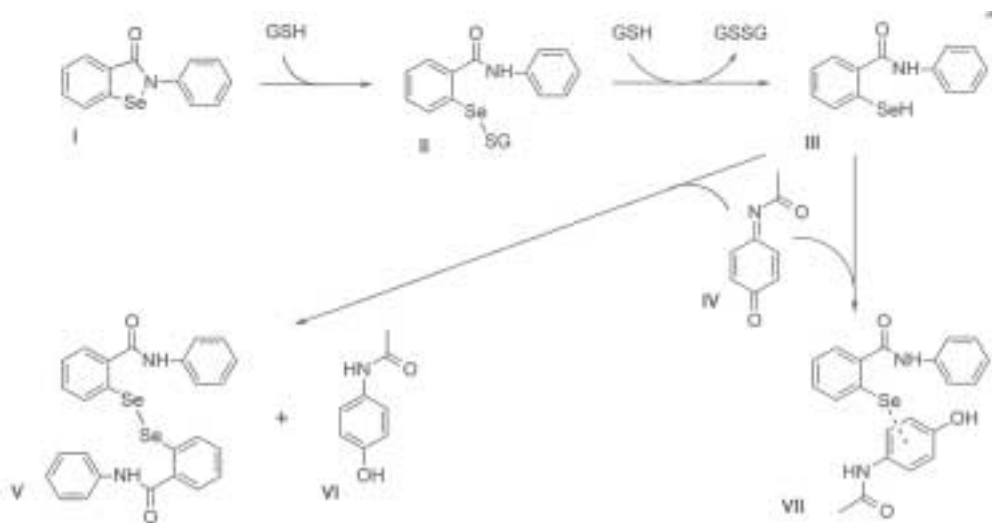


FIGURE 14. Proposed mechanism for protection by ebselen. Proposed mechanism for protection by ebselen againsts PAR-mediated cytotoxicity. (Adapted from Li *et al.* [1994a].)

kg bw/day; i.p.) protected against PAR-induced hepatotoxicity after a PAR challenge after an overnight fast (Nicholls-Grzemeski *et al.*, 1992; Manautou *et al.*, 1994). Although clofibrate pretreatment has no effect on urinary excretion of PAR-GLUC, PAR-SULP, PAR-Cys, or PAR-NAC, the elimination of PAR from plasma and liver and urinary excretion of the unchanged PAR was faster in clofibrate-pretreated mice. This was accompanied by elevated biliary excretion of PAR-SG at 2 h after PAR dosing, and by increased urinary PAR excretion (Manautou *et al.*, 1996). Total covalent binding to hepatic proteins was diminished significantly; however, the level of a 58-kDa PAR binding protein was not diminished by clofibrate pretreatment. Furthermore, no effect of clofibrate pretreatment on microsomal PAR bioactivation to NAPQI was observed. Hepatic nonprotein sulfhydryl levels were increased, leading to the hypothesis of clofibrate protection by increase in hepatic GSH levels (Manautou *et al.*, 1994; Manautou *et al.*, 1996).

Recently, protection against PAR-dependent hepatotoxicity in mice by pretreatment (chemoprevention) with a single dose of amphetamine 72 h before PAR was suggested to be caused by the induction of heat shock proteins (hsp) (Salminen *et al.*, 1997). Although it was suggested in Section IV that hepatocytes might be protected by amphetamine pretreatment by becoming prepared to cope with stressors, future studies to better characterize the relationship between hsp induction and susceptibility to toxicity are warranted.

Many investigations have resulted in protective agents that were effective in *in vitro* systems or in test animals when added before (chemoprevention) or concomitantly with (chemoprotection) addition of or treatment with PAR (like clofibrate or amphetamine), respectively. Therefore, for clinical use, these protective agents are rather useless. However, many of them have been proven to be advantageous as tools in the elucidation of the toxicity mechanism of PAR. For example, ascorbic acid reduced the NAPSQI radical formed after peroxidase-catalyzed oxidation much more efficiently than GSH, thus suggesting that the endogenous ascorbate might be more important in the detoxification of the PAR phenoxy radical than high concentrations of GSH

in tissues with high peroxidase activity (Ramakrishna Rao *et al.*, 1990). Also, ascorbate added to hamster liver microsomal incubations with PAR inhibited covalent binding of PAR. However, administration of ascorbate (that rises hepatic ascorbate levels rapidly in control rats) immediately after PAR, did not decrease covalent binding nor hepatotoxicity in hamsters (Miller and Jollow, 1984).

A completely different mechanism that has not been resolved unambiguously yet is probably responsible for the chemoprotective properties of oleanolic acid, a triterpene, against PAR and a number of other hepatotoxicants. Oleanolic acid, used in China to treat hepatitis, strongly protects against PAR induced hepatotoxicity in mice. The mechanism of this protection against PAR-induced hepatotoxicity, at least in part, appears to be due to the decreased formation of toxic metabolites of PAR by CYP1A and CYP2A, as well as increased detoxication by enhanced glucuronidation of PAR (Liu *et al.*, 1993a; Liu *et al.*, 1995b). In addition, compounds such as phenetyl isothiocyanate (or phenetyl isothiocyanate containing watercress), propylene glycol, taxol and triacetyloleandomycine were found to inhibit or induce CYP1A2, CYP2E1, and/or CYP3A with concomitant decrease or increase of hepatotoxicity (Snawder *et al.*, 1993; Thomsen *et al.*, 1995; Chen *et al.*, 1996; Kostrubsky *et al.*, 1997a; Kostrubsky *et al.*, 1997b; Li *et al.*, 1997). An overview of modulating agents for which sufficient evidence was present to be active via enhancement or inhibition of bioactivating and detoxifying enzymes and that were reviewed earlier (Vermeulen *et al.*, 1992) is presented in Table 6 without further discussion.

E. Modulation of Stage II

Later on in the process of PAR intoxication, Kupffer cells become activated, a process generally associated with release of proinflammatory cytokines with a variety of pathophysiological responses (Blazka *et al.*, 1995). Antibodies against TNF- α and IL-1 α appear to protect against PAR-induced hepatotoxicity in mice significantly. This suggests that TNF- α and IL-1 α are released in response to PAR intoxication and are responsible

for certain pathological manifestations of PAR induced hepatotoxicity (Blazka *et al.*, 1995). For example, the decrease in body temperature after a dose of 500 mg/kg bw PAR was counteracted by the antibodies against TNF- α and IL-1 α .

Recently, even a possible role of the source of cellular energy was hypothesized in the modulation of PAR-based hepatotoxicity. SD rats that were fed a normal rodent chow supplied with 15% glucose in drinking water (as source of energy for the centrilobular hepatocytes) during 7 days suffered from increased lethality to PAR (Chanda and Mehendale, 1996b). Also, a lowered ATP content of cells as found in fructose medium compared to glucose medium, rendered freshly isolated rat hepatocytes less vulnerable to quinone toxicants (Toxopeus *et al.*, 1994). It had been demonstrated before that aerobic oxidation of glucose is decreased in toxic liver diseases although glucose is often used as a ready source of energy for the patients with severe hepatic disorder (references in [Chanda and Mehendale, 1996b]). Thus, as glucose would be an inappropriate substrate to support a rapid and timely cell division and tissue repair and glucose might increase the toxicity of PAR by the inhibition of hepatocellular regeneration and tissue repair (Chanda and Mehendale, 1996b), change of the sources of energy in clinical settings might improve the chances for recovery from a PAR intoxication.

F. Summary

Despite numerous studies that have been performed with the main aim of finding protective agents for PAR-induced hepatotoxicity, to be added before (chemoprevention), concomitantly or following PAR (chemoprotection), very few agents have been used successfully in the clinic. Of the many agents that have been investigated in laboratory animals and were reviewed before, some were highlighted here because of their importance. In addition, most of the recent findings with respect to modulation of PAR-mediated toxicity were discussed. Modulation of PAR-mediated toxicity by structural modification of PAR is discussed in detail in Section VII.

As became clear, adduct formation between NAPQI and numerous cellular proteins is of ut-

most importance in the hepatotoxicity mechanism of PAR. In the first 30 years since PAR became an over-the-counter analgesic, most efforts were put into prevention of primary damage (Stage I). Decreasing the reactive metabolite formation via inhibition of P450 and structural modification of PAR and increasing the efficiency of scavenging of the reactive metabolite via the *N*-acetylcysteine and GSH routes were important goals. A new lead in preventing further primary damage in liver might be protection of susceptible target sites in proteins, maybe via selenium containing compounds. Often, however, most primary damage has taken place already upon admission to an emergency ward. Treatment with the most important mechanism-based antidote since long, *N*-acetylcysteine (a precursor of GSH which itself does not enter the cell) may be only partially effective after the progression of hepatic failure. Therefore, it seems to be clinically very relevant to aim additionally at the Stage II events of intra- and intercellular signaling as to influence the balance of tissue repair and damage in favor of repair.

VII. STRUCTURAL MODIFICATION OF PARACETAMOL

A. Introduction

Next to the many reports describing prevention of liver injury of PAR (1) by decreasing the production of toxic intermediate(s), (2) by increasing the capacity to detoxify the toxic intermediates(s), or (3) by increasing the ability of the tissue to withstand or even repair the molecular damage caused by the toxic species (Corcoran *et al.*, 1985), several reports have been published on approaches (4) to modify PAR-induced organ toxicity by mechanism-based structural modification. Regarding the latter, sometimes, the pharmacological aspects were taken into account. Thus, taking the presumed molecular mechanisms of analgesic activity as well as that of the hepatotoxicity of PAR into consideration, there have been several efforts to improve its analgesic activity while preventing its toxicity by modifying its structure (Figure 15) (Dearden *et al.*, 1980; Harvison *et al.*, 1986b; Skoglund *et al.*,

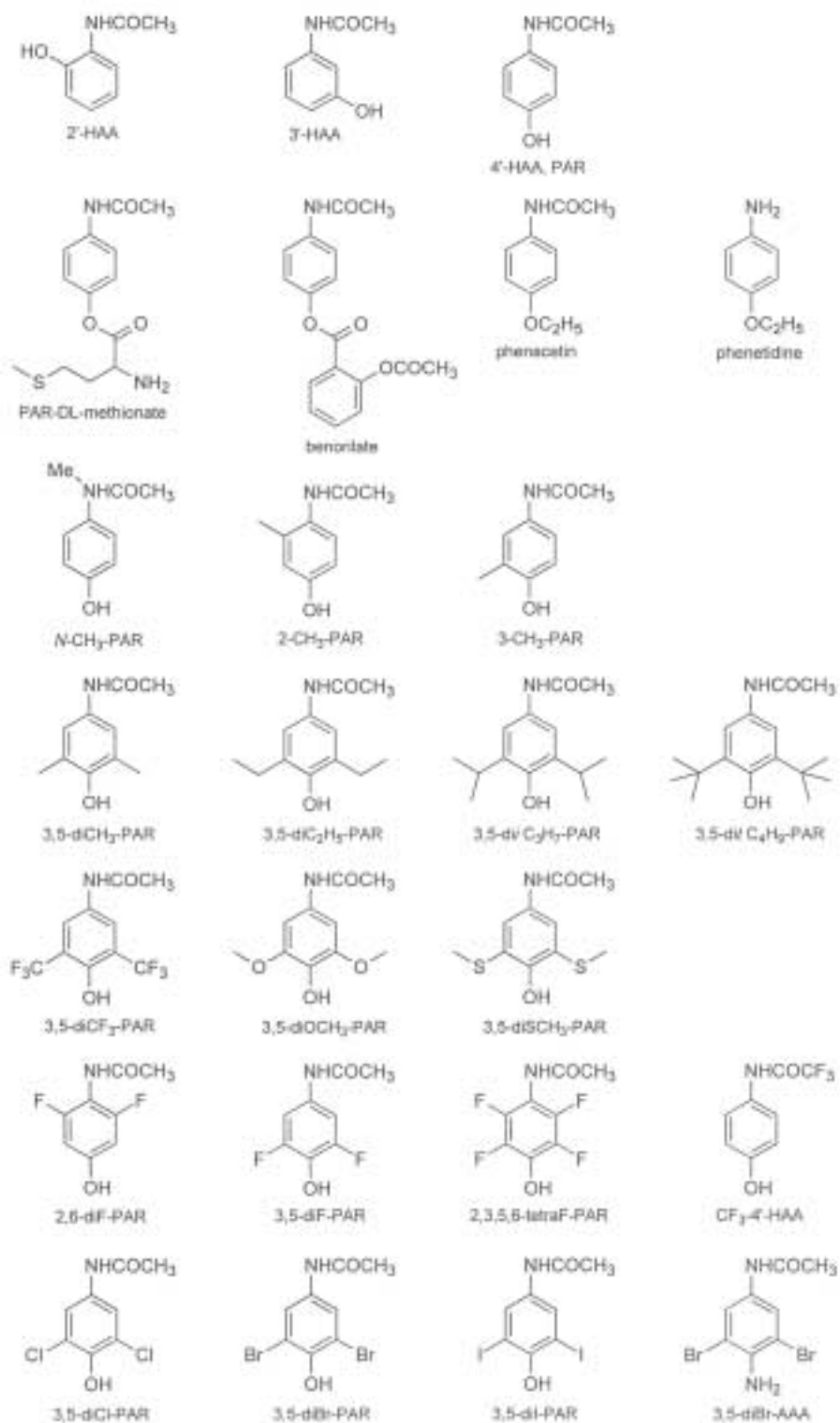


FIGURE 15. Analogues of paracetamol. Regioisomers, *O*-conjugates, an *O*-alkyl derivative, an *O*-alkylated *p*-aminophenol, ring-substituted analogues, *N*-acetyl-substituted analogues, and a ring-substituted *N*-acetyl-*p*-aminoaniline.

1986; Porubek *et al.*, 1987; Van de Straat *et al.*, 1987b; Van de Straat *et al.*, 1987c; Harvison *et al.*, 1988a; Skoglund *et al.*, 1988; Barnard *et al.*, 1993a; Barnard *et al.*, 1993b; Bessems *et al.*, 1995; Bessems *et al.*, 1997). Besides, several patents describe the synthesis of ring-substituted PAR derivatives and *N*-substituted *p*-aminoanilides with halogen substitution(s) *ortho* of the amino group. These patent applications included preliminary results on investigations into supposed analgetic, antipyretic, and/or antiinflammatory properties (Anon., 1966; Pieper *et al.*, 1987; Nickl *et al.*, 1988). However, to our knowledge, thorough descriptions of analgesic and inflammatory properties are rare. Unfortunately, investigations on toxic properties are mostly limited to acute toxicity (LD₅₀).

B. Prodrugs

A special class of PAR derivatives are the so-called prodrugs. A prodrug can be converted in a biological system as to give one or more drugs. Benorilate (Figure 15), an ester of acetylsalicylic acid and PAR, is such a prodrug and might be seen as an early attempt of line 1. By esterifying the hydroxyl group, a delay would be introduced in the speed at which the PAR concentration would build up after an overdose as activity of an esterase would be required. Benorilate, however, is relatively rapidly hydrolysed by esterases to its basic constituents, causing liver GSH depletion and liver necrosis (although somewhat delayed) due to the formation of PAR (De Vries, 1981; De Vries *et al.*, 1981). A second example of a prodrug for which some *in vitro* experiments have been performed with respect to biological system dependent formation of PAR is found in the group of *N*-(substituted 2-hydroxyphenyl and 2-hydroxypropyl)carbamates based on ring-opened derivatives of active benzoxazolones and oxazolidinones. This class of compounds contains compounds such as metaxalone and mephenoxalone (prodrugs of PAR and oxazolidones) and chlorzacetamol (a mutual prodrug of chlorzoxazone (a muscle relaxant) and PAR) (Vigroux *et al.*, 1995).

Several structural changes were directly aimed at prevention of hepatotoxicity by linking PAR

covalently to compounds facilitating GSH resynthesis (line 2). It was shown that esterification of the phenolic group of PAR with *N*-acetyl-DL-methionate (Figure 15) prevented both the hepatotoxicity of PAR in mice (Skoglund *et al.*, 1986; Skoglund *et al.*, 1988). Furthermore, replenishment of GSH-levels occurred significantly faster in the group treated with the PAR-ester compared with the PAR-only dosed group due to the availability of methionine as a precursor of GSH. Pharmacokinetics and pharmacodynamics of this PAR-methionine ester and PAR were similar (Skoglund *et al.*, 1986; Skoglund *et al.*, 1988). Interestingly, in the same experiments, simultaneously administered *N*-acetyl-cysteine was more effective in the prevention of GSH depletion 1 h after dosing than PAR-methionine or simultaneous dosing of PAR and free *N*-acetyl-DL-methionate. However, it was less effective in promoting *de novo* GSH synthesis toward 16 h. Lastly, there was no statistically significant difference between PAR-methionine ester and free *N*-acetyl-DL-methionate with respect to effects on GSH depletion or hepatic cell integrity (Skoglund *et al.*, 1986).

C. Phenacetin

Before PAR was discovered, analgesic and antipyretic activity was found to be present in phenacetin, a PAR analogue in which the phenolic hydroxyl group of PAR was masked by an ethyl substituent. Phenacetin was used for many years as an analgesic and antipyretic drug until renal damage became epidemiologically associated with long-term therapeutic treatment (Sandler *et al.*, 1989). In contrast to what was found later for PAR, hepatotoxicity was no problem with phenacetin, which is further substantiating the pro-toxicant properties of the phenolic hydroxyl group. Actually, PAR was suggested to be responsible for the therapeutic effects of phenacetin and in such low concentrations was considered to be nontoxic for the kidneys. However, as phenacetin is almost completely metabolised in the liver while the toxic effects are expressed elsewhere, further activation of phenacetin metabolites, such as *p*-phenetidine (*N*-deacetylated phenacetin), PAR (*O*-dealkylated phenacetin), and *p*-aminophenol (*N*-deacetylated and *O*-dealkylated

phenacetin) proximate to the site of toxicity is eligible. For example, protein binding was observed after activation of *p*-phenetidine by human kidney medulla microsomes (Larsson *et al.*, 1985).

D. Regioisomers

Two regioisomers (congeners, positional isomers) of PAR (4'-hydroxyacetanilide; 4'-HAA) studied are 2'-hydroxyacetanilide (2'-HAA) and 3'-hydroxyacetanilide (3'-HAA). The latter has been used frequently to study the role of covalent binding in cytotoxicity as 3'-HAA is nonhepatotoxic, nevertheless giving rise to reactive metabolites that arylate hepatic proteins in murine hepatocytes as well as *in vivo* in mice and hamsters (Roberts and Jollow, 1978; Rashed and Nelson, 1989a; Tirmenstein and Nelson, 1989; Rashed *et al.*, 1990; Roberts *et al.*, 1990; Holme *et al.*, 1991). The metabolism of 3'-HAA by rat liver P450 leads to the formations of two main products of aromatic hydroxylation that subsequently can be further oxidized to their respective *ortho*- and *para*-benzoquinone derivatives and form conjugates with GSH as illustrated in Figure 16 (Streeter *et al.*, 1984a; Rashed and Nelson, 1989b; Rashed and Nelson, 1989a). Similarly 2'-HAA that is metabolized to 2',5'-dihydroxyacetanilide was found not to be hepatotoxic in mice (Roberts and Jollow, 1979; Roberts and Jollow, 1980; Hamilton and Kissinger, 1986). A review on the P450 catalyzed oxidation of a variety of PAR analogues has been published by Koymans *et al.* (Koymans *et al.*, 1993).

An apparently important phenomenon in the toxicity of PAR and PAR analogues is the dual capacity of the once-formed oxidized reactive metabolites to both oxidize and covalently bind protein and nonprotein thiols. This is illustrated, for example, by the findings regarding the formation of specific glutathione conjugates and the differences in protein binding and hepatotoxicity observed between hepatotoxic PAR (4'-HAA) and nonhepatotoxic 3'-hydroxyacetanilide (3'-HAA). As illustrated in Figure 16 and Table 7, after microsomal oxidation of 3'-HAA via 2',5'-HAA-, 3',4'-HAA, and 2',3'-HAA, at least two acetamido-benzoquinones are likely formed, that is, 2-acetamido-*p*-benzoquinone (2-APBQ), and

4-acetamido-*o*-benzoquinone (4-AOBQ). These benzoquinones are *very* soft electrophiles, compared to NAPQI being a soft electrophile, which might give rise to less critical types of covalent binding when compared with NAPQI (Streeter *et al.*, 1984a; Rashed and Nelson, 1989a; Rashed and Nelson, 1989b). Once formed they react readily with soft nucleophiles like GSH (Rashed and Nelson, 1989a; Rashed and Nelson, 1989b) or protein thiols close to their site of formation. The latter was proposed by Nelson *et al.* (Nelson *et al.*, 1990) and substantiated by earlier findings. In mouse liver microsomal incubations, NADPH-dependent covalent binding of radioactivity from [¹⁴C]-3'-HAA compared with [¹⁴C]-PAR to microsomal protein was almost four times as rapid (Streeter *et al.*, 1984a). This may prevent extensive cytosolic GSH depletion as observed in hamsters (Roberts *et al.*, 1990) and protect some critical target proteins in cytosol or mitochondria from damage (Rashed *et al.*, 1990). In contrast, NAPQI, which is little a less soft electrophile, may react less readily with GSH. Also, compared with the benzoquinones, it may react more readily with non-cystein thiols, such as from histidine and lysine (see Figure 9). An overview of the biotransformation of the isomeric analogues of PAR was presented (Rashed *et al.*, 1990). In mice, although 3'-HAA treatment produces only slightly smaller total levels of covalent binding to liver cytosolic and mitochondrial proteins than PAR, no inhibition of plasma membrane Ca²⁺-ATPase in liver, less extensive mitochondrial GSH depletion, and lesser decrease of cytosolic glyceraldehyde-3-phosphate dehydrogenase activity was observed (Tirmenstein and Nelson, 1989; Myers *et al.*, 1995; Dietze *et al.*, 1997).

A possible explanation for this disparity in protein binding and toxicity between PAR (4'-HAA) and 3'-HAA might be the remarkable difference between their oxidative metabolites, being a quinone imine (NAPQI) and benzoquinones (4-AOBQ and 2-APBQ), respectively. Although NAPQI as well as 4-AOBQ and 2-APBQ are reactive soft electrophiles, only NAPQI is capable of forming the special benzoquinone imine *ipso*-adduct (as depicted in Table 7) with soluble and nonsoluble thiols. In case of soluble thiols (GSH), formation of this *ipso*-adduct, could facilitate transport of this latent form

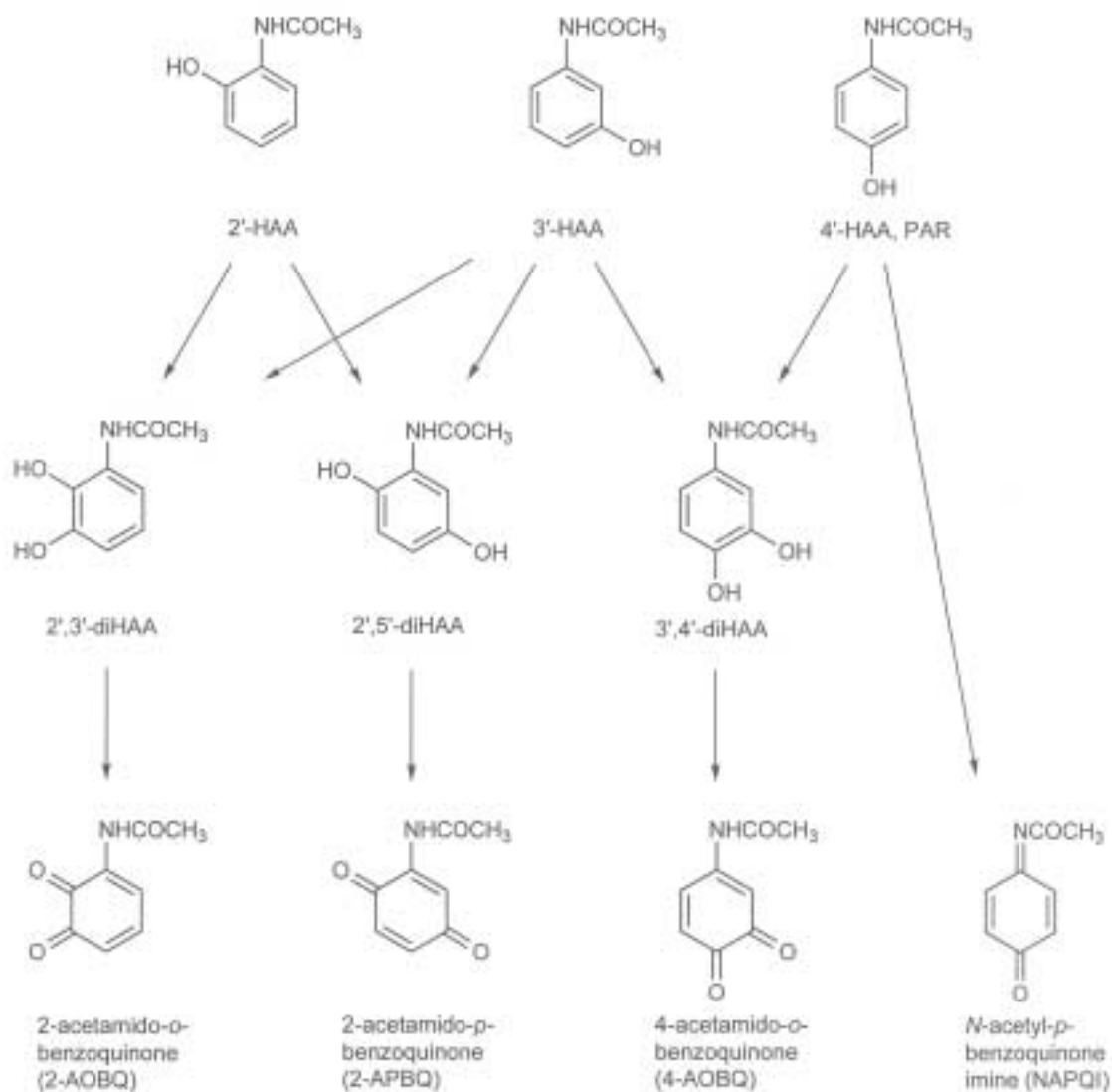


FIGURE 16. Oxidative biotransformation of paracetamol and its regioisomers — A comparison. (See text for references.)

of PAR away from the site of formation (microsomal environment). In this quasistable form, NAPQI might even escape from hepatocytes, where most of the oxidative biotransformation activity is located, and participate in the arylation of protein thiols in erythrocytes, which has been observed in mice that were administered PAR (Axworthy *et al.*, 1988).

Furthermore, glutathiolated hydroquinone structures and covalently bound hydroquinones may be formed that are labile to further (auto)oxidation processes causing formation of di-glutathiolated quinones, analogously to (halogenated) hydroquinones and *tert*-butylhydroquinone (Lau *et al.*, 1996; Peters *et al.*, 1996). The finding of a di-GSH conjugate upon

chemical reaction of the 3'-HAA metabolite 2-APBQ (Figure 16 and Table 7) with GSH (Rashed and Nelson, 1989a) is only conceivable when one of the mono-GSH conjugates is autooxidized before a second GSH molecule is trapped. The differences between PAR and 3'-HAA in oxidative metabolite formation, GSH depletion, and protein arylation are excellently reviewed (Rashed *et al.*, 1990). Recently, some more investigations were undertaken to compare especially which proteins were covalently adducted/inhibited or not on administration of PAR or 3'-HAA to mice (Tirmenstein and Nelson, 1991; Myers *et al.*, 1995; Dietze *et al.*, 1997; Matthews *et al.*, 1997; Salminen *et al.*, 1998). Treatment of mice with a large dose of radiolabeled 3'-HAA

was shown to result in selective arylation of proteins in cytosol and microsomes but not in mitochondria. A major 3'-HAA protein adduct was observed in microsomes at 50 kDa with peak levels appearing at 1 h. Minor adducts were observed at 47 kDa in microsomes and 56-kDa in cytosol (Matthews *et al.*, 1997). Further *in vivo* and *in vitro* studies revealed the 50-kDa microsomal protein to be CYP2E1, based on comigration, immunoblotting, loss of catalytical CYP2E1 activity and inhibition of the 50-kDa binding by the CYP2E1 inhibitor diallylsulfide, leading to the postulation that 3'-HAA is a suicide inactivator of CYP2E1 (Matthews *et al.*, 1997; Halmes *et al.*, 1998; Salminen *et al.*, 1998). Furthermore, the finding that some of the covalent protein binding of 3'-HAA to CYP2E1 is less stable (partial loss by ultrafiltration and electrophoresis) emerged the hypothesis that the heme as well as the apoprotein might be adducted (Myers *et al.*, 1995; Salminen *et al.*, 1998). It should be noted that the panlobular binding and some of the centrilobular binding (not and partially decreased by treatment of mice with diallylsulfide) in mice that were administered 3'-HAA could be due to bioactivation by non-CYP2E1 P450 enzymes, as there are, for example, CYP1A2, CYP2A, CYP2B, and CYP2C. These P450s have been shown, at least in humans, to be expressed uniformly throughout the liver acinus (reference in Salminen *et al.*, 1998). Other papers on 3'-HAA are referred to elsewhere in this review.

E. N-Methylparacetamol and 2,6-Dimethylparacetamol

N-Methylparacetamol (*N*-methyl-PAR), synthesized on the basis of the expectation that *N*-oxidation would be hindered by *N*-methylation (Nelson *et al.*, 1978), indeed appeared to be not hepatotoxic. The prevention of toxicity was presumably due to the fact that *N*-methyl-PAR is a poor substrate for the hepatic P450 (Harvison *et al.*, 1986b). The same mechanism probably applies to another methylated analogue, viz. 2,6-dimethyl-PAR (Fernando *et al.*, 1980; Porubek *et al.*, 1987; Birge *et al.*, 1988). Torsion of the *N*-acetyl group out of plane is suggested to prevent either the positioning in the P450 active site

or the second hydrogen abstraction from the nitrogen. This suggestion is supported by the finding in cultures of mouse hepatocytes, where 2,6-diCH₃-PAR, in contrast to PAR and 3,5-diCH₃-PAR, did not impel changes in synthesis of two specific proteins (Bruno *et al.*, 1992). A similar mechanistic reason was suggested (next to the oxidation potential) for 2,6-diF-PAR, which will be discussed further on in this Section (Barnard *et al.*, 1993a; Barnard *et al.*, 1993b). These findings were substantiated when chemically synthesized 2,6-diCH₃-NAPQI appeared to be an efficient inhibitor of calmodulin-activated Ca²⁺-pump ATPase activity, basal (calmodulin-independent) Ca²⁺-pump ATPase activity, as well as Na⁺,K⁺-pump ATPase activity (Nicotera *et al.*, 1990) whereas 3,5-diCH₃-NAPQI, which primarily oxidizes protein thiols, caused selective inhibition of only the calmodulin-activated Ca²⁺ pump ATPase activity. The hypothetical formation of 2,6-diCH₃-NAPQI *in situ* in hepatocytes or *in vivo* would certainly exhibit more dramatic toxicity effects than observed. Both oxidation and arylation of protein thiols can alter the functional properties of important proteins. Of the two reactions, arylation (NAPQI and 2,6-diCH₃-NAPQI) appeared to be the less specific and more damaging event (Nicotera *et al.*, 1990). Another analogue of PAR, methylated *ortho* toward the *N*-acetyl group is 2-CH₃-PAR. It was calculated that *ortho*-methylation will cause some torsion of the *N*-acetyl group, although not enough to prevent P450 binding that much as was observed with 2,6-diCH₃-PAR (unpublished observations). In line with at least some P450 catalyzed oxidation of 2-CH₃-PAR, some hepatotoxicity was observed with 2-CH₃-PAR, although significantly less compared with PAR (Harvison *et al.*, 1986b).

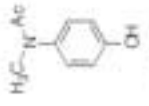
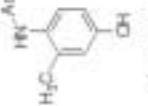
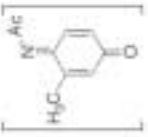
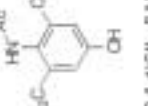
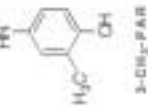
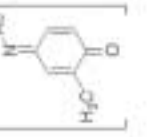
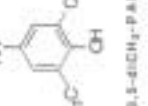
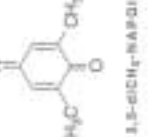
F. 3,5-Dialkylated Paracetamol Analogues

As another possibility for the prevention of PAR-induced hepatotoxicity, notably monosubstitution at the 3-position or disubstitution at the 3- and 5-position of the aromatic nucleus of PAR by alkyl groups, was evaluated. In view of the essential role of irreversible GSH depletion and the covalent binding to critical protein nucleo-

TABLE 7
Main *In Vitro* and *In Vivo* Biotransformation Products of Paracetamol and Analogues

PAR Analogues	Phase I Metabolites	Phase II Conjugates	Effect	References

TABLE 7 (continued)

 <chem>CC(=O)Nc1ccc(Cl)c(Cl)c1</chem> N-CH ₂ -PAR	<p>No PARQ substrate due to out of phase location of the acetyl moiety</p>	(Harrison et al., 1982a)
 <chem>CC(=O)Nc1ccc(Cl)c(Cl)c1C(=O)N</chem> 2-CH ₂ -PAR		(Harrison et al., 1982b)
 <chem>CC(=O)Nc1ccc(Cl)c(Cl)c1C</chem> 2,4-dichloro-PAR	<p>Less favorable PARQ oxidation probably due to out of phase location of the acetyl moiety</p>	(Fernando et al., 1980)
 <chem>CC(=O)Nc1ccc(Cl)c(Cl)c1C(=O)N</chem> 2-CH ₂ -PAR		(Alonsson et al., 1983b; Van de Graaf et al., 1980)
 <chem>CC(=O)Nc1ccc(Cl)c(Cl)c1C</chem> 2,4-dichloro-PAR		(Fernando et al., 1980; Van de Graaf et al., 1983; Nussens et al., 1979)

philic groups such as thiols in the hepatotoxicity of PAR, it has been proposed that protecting of the electrophilic 3- and 5-positions in NAPQI would prevent PAR toxicity (Van de Straat *et al.*, 1986). As expected, 3,5-dialkyl substitution, in contrast to 3-monoalkyl substitution, efficiently prevented GSH depletion, lipid peroxidation and the toxicity of PAR, measured as LDH leakage in rat hepatocytes. Hepatotoxicity was also absent upon administration of 3,5-dialkylated PAR analogues to mice, in contrast to PAR and 3-monoalkylated PAR (Van de Straat *et al.*, 1987c). The P450-catalyzed oxidation of the mono- and dialkylated PAR-analogues to their corresponding NAPQI-analogues did not appear to be significantly influenced by this mono- or dialkyl substitution, but only 3,5-dialkylation was found to prevent the conjugation of the respective quinone imines with GSH. Oxidation of GSH to GSSG, was still found to occur in microsomal incubations with the 3,5-dialkyl derivatives (Van de Straat *et al.*, 1986). This reaction is apparently less critical to the hepatocyte, because GSSG is normally reduced back again to GSH rapidly by GSSG reductase.

The toxic potential of PAR was not completely eliminated by 3,5-dialkylation, however, as synthetic 3,5-diCH₃-NAPQI appeared to inhibit calmodulin-activated Ca²⁺-pump ATPase activity in red blood cells, probably due to its oxidant properties (Nicotera *et al.*, 1990). Furthermore, 3,5-diCH₃-NAPQI was found to be deacetylated to a dimethylated benzoquinone imine, thereby reestablishing conjugative properties (Rossi *et al.*, 1988). Moreover, 2,6-diCH₃-PAR as well as 3,5-diCH₃-PAR exhibited cytotoxic properties concomitantly with covalent protein binding in hepatocytes (Porubek *et al.*, 1987). The cytotoxic properties of 2,6-diCH₃-PAR are likely due to electrophilic as well as oxidant properties of a NAPQI-like metabolite, whereas those of 3,5-diCH₃-PAR should be likely attributed to the oxidant properties of a NAPQI analogue. The 3,5-diCH₃-PAR associated covalent protein binding could be due to hydrolytic removal of the complete *N*-acetyl group, as well as deacetylation of the reactive metabolite 3,5-diCH₃-NAPQI, resulting in a dimethylated quinone or a dimethylated quinone imine, respectively. Both compounds possess covalent binding properties toward thiol groups. The increased selective pro-

tein arylation to a cytosolic 58-kDa protein by 2,6-diCH₃-PAR in cultured hepatocytes from phenobarbital induced compared with uninduced mice (Birge *et al.*, 1989) is in line with the fact that the active site of phenobarbital inducible P450 is wider than that of β NF-inducible P450 (Lewis *et al.*, 1987). Because in 2,6-diCH₃-PAR the *N*-acetyl group is not coplanar (unpublished data from our group), it is not expected to fit into the active site of CYP1A1.

Differences were observed with respect to the time course of protein adduct formation, the susceptibilities of the modification of cysteine residues by 3,5-diCH₃-NAPQI or NAPQI after incubation in freshly isolated hepatocytes (Weis *et al.*, 1992b). Comparable results were obtained after *in situ* formation of these quinone imines by co-incubation of hepatocytes with 3,5-diCH₃-PAR or PAR with a peroxidase model system (Weis *et al.*, 1996).

As a variation on substitution of the positions *ortho* to the phenolic hydroxyl group, disubstitution with the electron donating methylether- and methylthioether-substituents R = -OCH₃ and R = -SCH₃ (Figure 15) resulted in better cyclooxygenase inhibiting properties (an *in vitro* test for analgesic activity) than PAR, which was most likely due to their lower oxidation potentials than PAR (Bessems *et al.*, 1995). Analogous to 3,5-diCH₃-PAR, these compounds displayed lower cytotoxicity than PAR, which is probably due to their blockade of the aromatic positions, normally prone to *S*-glutathiolation. Interestingly, structural modulation by various alkyl-, methylether- and methylthioether substituents (R = -CH₃, -C₂H₅, -*t*C₄H₉, -OCH₃, -SCH₃) did not block the phase I bioactivation in an *in vitro* peroxidase system as revealed by ESR detection of their respective phenoxy free radicals (Bessems *et al.*, 1998). This substantiates the general assumption that cyclooxygenase inhibition is dependent on one-electron oxidation of phenolic compounds. The formation of the respective phenoxy free radicals was in line with *ab initio* calculations that showed that hydrogen abstraction from the phenolic hydroxyl group of the PAR analogues used in the calculations (R = -CH₃ and R = -OCH₃) is energetically more favorable than hydrogen abstraction from the acetylamino nitrogen (Bessems *et al.*, 1998).

G. 3,5-Dihalogenated and Other Dihalogenated Paracetamol Analogues

Another group of PAR analogues is formed by halogenated compounds. For example, 4-hydroxytrifluoroacetanilide (Figure 15) was analgesically inactive, probably because of the relative instability due to rapid hydrolysis to *p*-aminophenol (Aboul-Enein *et al.*, 1982). However trifluoroacetanilide and 4-ethoxytrifluoroacetanilide (the trifluoro analogue of phenacetin) both exhibited analgesic activity. Trifluoroacetanilide might be analgesically active due to metabolic hydroxylation to 4-hydroxytrifluoroacetanilide that acts on the site of action, thus bypassing its hydrolysis to *p*-aminophenol (Aboul-Enein *et al.*, 1982). Unfortunately, no information was presented at that moment on the toxicity of these trifluorinated analogues.

More information regarding the analgesic activity and toxicity of trifluorinated and fluorinated analogues of PAR is retrieved from more recent literature. 2,6-Difluorination of PAR prevented PAR-induced hepatotoxicity when administered to mice. Furthermore, no thioether metabolites were found in the urine (Barnard *et al.*, 1993a; Barnard *et al.*, 1993b). The reduced hepatotoxicity is probably due to impaired oxidation by P450, due to noncoplanarity of the *N*-acetyl group in 2,6-diF-PAR, together with an increased oxidation potential of two electron withdrawing fluorine atoms at *meta* positions of the phenolic hydroxyl group (Barnard *et al.*, 1993a). Trifluor-methylation of the *N*-acetyl group of PAR caused such an increase in lipophilicity of PAR that a shift in toxicity from the liver to the central nervous system was observed combined with markedly different routes of metabolism, for example, involving extensive *N*-de-tri-fluoroacetylation to *p*-aminophenol (Barnard *et al.*, 1993a). A small disparity is observed between the results with 3,5-difluorinated PAR as presented by Bessems *et al.* and those presented by Barnard *et al.* In rat hepatocyte incubations, 3,5-diF-PAR appeared to be approximately as toxic as PAR (Bessems *et al.*, 1997), whereas *in vivo* in mice, 3,5-diF-PAR was less toxic than PAR (Barnard *et al.*, 1993a). However, the *in vivo* differences in LD₅₀, serum ALT, and hepatic GSH between PAR and 3,5-diF-

PAR are not more than twofold (Barnard *et al.*, 1993a), which is relatively small for *in vivo* differences.

Interestingly, 3,5-dihalogenation (R = -F, -Cl, -Br and -I; Figure 15) resulted in worse *in vitro* cyclooxygenase inhibition as well as *in vivo* analgesic activity than PAR, which was most likely due to their higher oxidation potentials than PAR (Bessems *et al.*, 1995). For the dihalogenated compounds that were studied as such (R = -F, -Cl, and -Br), cytotoxicity was comparable to that of PAR. Moreover, structural modification by these halogens (R = -F, -Cl, and -Br) did not block the phase I bioactivation in an *in vitro* peroxidase system as revealed by ESR detection of the respective phenoxy free radicals (Bessems *et al.*, 1998). As for the series of 3,5-disubstituted analogues with electron-donating substituents mentioned before, this substantiates the general assumption that cyclooxygenase inhibition is dependent on one-electron oxidation of phenolic compounds. Again, the formation of the respective phenoxy free radicals was in line with *ab initio* calculations that showed that hydrogen abstraction from the phenolic hydroxyl group of the PAR-analogues used in the calculations (R = -F and R = -Cl) is energetically more favorable than hydrogen abstraction from the acetylamino nitro- gen (Bessems *et al.*, 1998).

Moreover, in hepatocytes from β NF-induced rats, the toxicity of a whole series of 3,5-dihalogenated PAR analogues (3,5-diF-, 3,5-diCl-, and 3,5-diBr-PAR; Figure 15) was in the same order of magnitude as the cytotoxicity of PAR. It should be noted, however, that an increase in the size of the substituent seemed to be related to a decrease in cytotoxicity (Bessems *et al.*, 1997). This trend might be caused by structure-related detoxification by glucuronidation of the 3,5-dihalogenated PAR-analogues. Glucuronidation increases with the size of the substituents adjacent to the phenolic hydroxyl group, which seems feasible as the reactivity toward the cofactor uridine 5'-diphosphoglucuronic acid (UDPGA) is known to be dependent on the nucleophilicity of the structure, both due to electronic and steric factors, but also on lipid solubility (Mulder *et al.*, 1990). As no redox-reaction is involved (glucuronidation is an SN₂ reaction), the ease of the *O*-glucuronidation is not directly related to the oxidation potential.

The latter varies only between 0.032 and 0.034 Volt for 3,5-diF-PAR, 3,5-diCl-PAR, and 3,5-diBr-PAR (Bessems *et al.*, 1995).

Analogous to PAR, the observed cytotoxicity was due to bioactivation of substantial amounts of the 3,5-dihalogenated PAR analogues, predominantly by CYP1A, as observed also in microsomal incubations. As expected to be formed via a nucleophilic (SN₂) addition-elimination mechanism, in microsomal incubations GSH-conjugates and in hepatocyte incubations covalent protein were observed for most halogenated analogues (Bessems *et al.*, 1996; Bessems *et al.*, 1997). For 3,5-diCl-PAR and 3,5-diBr-PAR but not 3,5-diF-PAR, protein adducts at regio-specific aromatic positions were formed (Bessems *et al.*, 1997). As far as GSH adduct formation of 3,5-diBr-PAR is concerned, these *in vitro* results are supported by a study in which analogous S-conjugate formation was studied in male rats with the analgesic 'amino analogue' of 3,5-diBr-PAR, 4-amino-3,5-dibromoacetanilide. The mercapturic acid degradation product of 3-bromo-4-amino-5-glutathionyl-acetanilide was found as the main urinary metabolite (Prox *et al.*, 1987).

H. Nuclear Effects

A specific activity of PAR that was described is inhibition of replicative DNA synthesis. As this might eventually cause apoptosis or necrosis it is regarded highly significant. A structure activity study of PAR analogues on this inhibitory activity as measured in V79 Chinese hamster cells revealed that PAR, just like *p*-cresol, *m*-amino-phenol, and *p*-hydroxyphenol, has moderate replicative DNA synthesis inhibiting potencies, whereas 2,4-diaminophenol, *o*-aminophenol, *p*-aminophenol and *p*-methylaminophenol, exhibited high-inhibiting potencies and a variety of other analogues exhibited low to no inhibiting potencies. Based on these results it was hypothesized that the observed inhibitory activity variation of the PAR analogues was based on the relative abilities of these compounds to undergo hydrogen atom loss at the phenolic oxygen and on the relative stabilities of the resulting free-radical species (Richard *et al.*, 1991).

I. Summary

A large number of reports describe investigations into structural modification of PAR in order to improve its analgesic and safety properties. Valuable knowledge on the analgesic and toxic activities of PAR and their underlying mechanism was obtained. Analgesic activity appears to be confined to acetanilides with an –OH or an –NH₂-group at *para*-position and the acetanilide moiety 'in plane'. The phenolic structure could also be created by *in situ* oxidation of an acetanilide although absence of the –OH would change lipophilicity and thus disposition significantly. Substitution *ortho* to the –OH groups with electron-donating substituents could improve the analgesic properties as the cyclooxygenase inhibition probably involves oxidation of this –OH group.

No radicals were detected in rat liver cytochrome P450-containing microsomal or reconstituted systems in which rat liver CYP1A1 or CYP2E1 (for which PAR is known to be a substrate) were present. The failure to experimentally detect phenoxy radicals in cytochrome P450-catalyzed oxidation of any of the eight 3,5-disubstituted PAR analogues, even of those analogues that provide very stable phenoxy radicals, indicates that the quantity of these radicals remains below the detection level of the ESR-analysis. This could indicate that phenoxy radicals do not leave the active site of the P450 involved at all or in very low quantities. In addition, the reducing effects that agents like NADPH and protein thiol groups have on phenoxy radicals rather than the physical instability of the respective radicals might prevent detection by ESR. Thus, the findings substantiate the fact that formation of PAR-dependent free phenoxy radicals is relevant in tissues rich in peroxidase activity such as the kidneys, whereas it is probably irrelevant in P450-dependent hepatotoxicity.

Hepatotoxic activity appears to be confined to compounds that are capable of forming quinoid structures, which are susceptible to both irreversible and, importantly, reversible attack by soluble and nonsoluble thiols. If only irreversibly bound S-conjugates are formed, such as with the metabolites of 3'-HAA (a regioisomer of PAR), this covalent binding seems to be confined mainly to

microsomal proteins in hepatocytes. If also reversible adduct formation is possible, such as with NAPQI (a reactive PAR metabolite), the number of proteins and the possible sites where adduct formation becomes feasible is importantly increased. The evidence is even growing that by GSH conjugation or protein-SH adduct formation, the reactive metabolite is not unerringly detoxified. Maybe these *S*-conjugates and -adducts exist in two forms, as a stable Michael adduct and as an *ipso*-conjugate, with the latter being prone to release of NAPQI distinct from the endoplasmatic reticulum or even at extracellular molecular targets.

It should be mentioned that although hepatotoxic activities can be diminished by blocking the proximate electrophilic sites *ortho* to the -OH group of PAR (or -NH₂ group of an amino analogue) by, for example, alkyl substituents, toxicity to other organs may become manifest (e.g., renal toxicity). This may be due to renal *N*-deacetylase activity, giving rise to toxic aminophenols. The *N*-acetyl group could be modified as to prevent *N*-deacetylation. However, variations might influence the lipophilicity of the parent compound which could direct it to other compartments of the body. Also, substitution *ortho* to the -OH (or -NH₂) by substituents that can be removed too easily by nucleophilic thiols in an addition-elimination reaction, after the formation of corresponding NAPQI analogues, is unwarranted. In addition, the substituents themselves should not be inert to biotransformation because this could direct biotransformation to *N*-deacetylation of the parent compound to nephrotoxic aminophenols. Examples of these compounds might be PAR derivatives that are disubstituted *ortho* to the -OH group with -OCH₃, -SCH₃, -COOH or -OC₂H₅ groups, leaving some possibilities of phase I biotransformation at this position (e.g., *O*- and *S*-dealkylation). Interestingly, *in vitro* experiments already provided strong indications for analgesic activity as well as decreased hepatotoxicity for 3,5-diOCH₃-PAR and 3,5-diSCH₃-PAR (Bessemers *et al.*, 1995). The *in vivo* relevance of these findings as well as the potential toxicity for other organs remains to be established, however.

A new lead for structural modification of PAR in order to obtain a safer analgesic substance

should combine the two traces, one that is aimed at retainment of improvement of analgesic properties, the other one that is aimed at decrease of the hazardous properties.

VIII. GENERAL CONCLUSIONS

As established in numerous investigations, the acute and fulminant liver toxicity due to a large dose of paracetamol (PAR) is mainly dependent on P450-catalyzed oxidative biotransformation to *N*-acetyl-*p*-benzoquinone imine (NAPQI). The reactive NAPQI subsequently reacts with soluble (glutathione; GSH) and nonsoluble (protein) thiols. The analgesic and antipyretic properties are due to the anticyclooxygenase properties of PAR. The inhibition of the cyclooxygenase activity of prostaglandin endoperoxide synthase (PGES) results in decreased formation of prostaglandins.

Significant species differences exist with respect to susceptibility to PAR-dependent hepatic and renal toxicity, the latter possibly occurring together with acute liver failure. Mice and hamsters are relatively sensitive, whereas rat, rabbit, and guinea pig are rather resistant. The sensitivity positively and negatively correlates with urinary excretion of toxication pathway-related metabolites, that is, breakdown products of the glutathione conjugate of PAR (PAR-SG) and secondary metabolites thereof, and inactivation pathway-related metabolites such as the sulfate and the glucuronide conjugate of PAR and 3-hydroxy-paracetamol (3-OH-PAR) or secondary metabolites thereof, respectively. Except for differences in the glutathione-conjugation pathway, species differences in *N*-deacetylase activity may also render species vulnerable to renal toxicity of PAR, as illustrated by the renal *N*-deacetylation of *p*-aminophenol, a well-known nephrotoxicant, in rabbits.

In combination with species differences in phase II biotransformation enzymes (expression level as well as localization), diversity in hepatic phase I oxidative enzymes is importantly correlated with sensitivity of PAR toxicity. In mice and hamsters, phase I biotransformation seems to be mostly limited to the P450 enzymes CYP2E1 and CYP1A2, both activating P450s. In contrast, rats

seem to exhibit a variety of P450s that are active in oxidation of PAR, including P450s, such as CYP1A1 and CYP2C11 that catalyze the oxidative activation as well as the inactivation of PAR to NAPQI and 3-OH-PAR, respectively. In addition, some rat P450s such as CYP2B1 exhibit significant regioselective catalytic oxidation in favour of 3-OH-PAR. Moreover, it should be noted that acute renal toxicity is largely dependent on species and even gender specific activation of PAR by renal CYP2E1 with male mice being sensitive in contrast to female mice. Although the sensitivity of the human species has not been compared in detail with that in other species, in man, probably CYP3A4 is mainly involved in oxidative biotransformation of PAR at therapeutic intake whereas CYP2E1 and CYP1A2 become increasingly involved at high intake levels. A combination of species differences in phase II biotransformation with activity of the second phase I biotransformation group of enzymes, that is, the peroxidases (such as PGES), may cause species selective formation of radical metabolites from PAR via *p*-aminophenol. With respect to the mechanism of oxidation of PAR to NAPQI by P450s, a direct two-electron oxidation mechanism is most likely. A single hydrogen abstraction mechanism, resulting in a phenoxy radical intermediate (NAPSQI), and followed by a second hydrogen abstraction or hydroxyl radical recombination, resulting in NAPQI and 3-OH-PAR, respectively, has been suggested but lacks as yet experimental proof.

Uncertainty still exists as to the mechanism of targetting of NAPQI to sites distinct from the site of formation, presumably and largely the liver, although some formation may occur, in, for example, the kidneys. Possibly two structures exist for covalent binding of NAPQI to sulfhydryl groups, that is, the relatively inert one formed after 1,4-Michael addition and a relatively unstable *ipso*-adduct at the C1-carbon (to which the *N*-acetyl group is linked). NAPQI could be released from the *ipso*-adduct, either with GSH or protein, at sites distinct from the endoplasmic reticulum or even at extracellular molecular targets. Thus, cytoplasmatic, mitochondrial, plasma membrane as well as nuclear proteins could be covalently modified as such in the early hours after the administration of a toxic dose of PAR, a

stage that has been designated as Stage I of PAR-toxicity.

The formation 3-(cystein-*S*-yl)paracetamol protein adducts, as even found in plasma of PAR-intoxicated patients, probably exhibits a dominant mechanistic role in acute hepatic necrosis. These PAR-arylated proteins appear time and dose dependently in liver fractions mice after the administration of PAR. In addition, the adduct levels found in serum correlates with serum ALT levels and peak several hours later than the adduct levels in liver fractions, suggesting a threshold level of adduct formation in liver for the development of hepatotoxicity. Plasma membrane and mitochondrial fractions appear to contain most covalently modified proteins, whereas a 55- to 58-kDa cytosolic protein appears to be the most intense arylated individual protein. Many microsomal, mitochondrial, cytosolic, and even nuclear proteins that have been shown the past decade to become arylated, catalyze important biochemical events and may exhibit cellular signaling functions. The major alkylated proteins, of which the actual functions remain to be elucidated, have been studied thoroughly in the 1990s and designated as the 55- to 58-kDa 'acetaminophen-binding proteins'. One of these proteins appeared in several tissues such as liver and kidneys in mice after addition of a toxic dose of PAR. These proteins may act as scavengers of reactive metabolites of xenobiotics with oxidant properties. Moreover, it has been suggested that the 58-kDa 'acetaminophen-binding protein' may have a nuclear signaling function.

An emerging number of *in vitro* results as well as epidemiological reports point to all kinds of nuclear effects. The most prominent findings are impaired DNA repair and even DNA adduct formation at low target concentrations. In addition to necrosis, also apoptosis may be involved in some stages of the highly integrated process of PAR-induced toxicity. It remains to be established, however, what the relevance as well as the possible consequences of the *in vitro* findings as well epidemiological findings are for PAR-intoxicated patients as well as humans taking PAR at therapeutic levels.

It was envisaged in the last decade also that many extracellular events emerged after the administration of a toxic dose of PAR to mice after

the first hours and spread in liver tissue. These events, designated as Stage II phenomena, include the excretion of growth factors, inflammatory mediators, and reactive oxygen species by hepatocytes as well as nonparenchymal cells such as the Kupffer cells. Some of these mediators stimulate tissue repair, whereas others provoke tissue damage. Up to now it is not clear whether a relation exists between the nuclear effects and these Stage II phenomena. However, these findings may provide interesting leads for clinical treatment of acute hepatic failure.

Many agents that have been investigated for modulation of liver toxicity of PAR are of little value in the clinic as they are to be administered before (chemoprevention) or concomitantly with PAR (chemoprotection). They are aimed at the inhibition of oxidative bioactivation of PAR of increased phase II detoxification. However, most primary damage (Stage I) often has taken place before admittance to a hospital. Therefore, a new guide might be to protect susceptible molecular targets by stimulation or improvement of the functioning of the 'acetaminophen-binding proteins', for example, by selenium-containing compounds.

Lastly, structural modification was a lead for numerous investigations into improvement of analgesic and safety properties of PAR. Valuable knowledge on the analgesic and toxic activities of PAR and their underlying mechanisms was obtained. For example, 3,5-disubstitution of PAR with electron-donating substituents facilitates one-electron oxidation, leading to phenoxy free radicals in phase I biotransformation (including peroxidase reactions), which has implications for analgesic activity as well as the hazardous properties compared with PAR. The opposite holds for electron-withdrawing substituents. Phenoxy free radical formation is relevant in the toxicity mechanism in tissues rich in peroxidase, such as the kidneys, whereas it is probably irrelevant in the P450-dependent hepatotoxicity. A new lead for structural modification of PAR in order to obtain a safer analgesic substance should combine two traces. The analgesic trace prescribes an acetanilide with an -OH (or eventually an -NH₂) at *para*-position and the *N*-acetyl group 'coplanar'. The *N*-acetyl group could be modified as to prevent *N*-deacetylation. However, variations might influence the lipophilicity of the parent

compound that could direct it to other compartments of the body with potential toxicity. The 'toxicologically safe' trace prescribes substituents *ortho* to the -OH (or maybe -NH₂) group that are not liable to addition-elimination reactions by sulfhydryls after formation of a corresponding NAPQI analogue. In addition, the substituents themselves should not be inert to biotransformation as this could direct biotransformation to *N*-deacetylation of the parent compound to aminophenols. Substitution by alkyl groups via an ether or thioether bond appear to fulfil both analgesic and safety requirements. *In vitro* experiments already provided strong indications for some of these compounds exhibiting analgesic properties combined with decreased hepatotoxicity although the *in vivo* relevance of these findings as well as the potential toxicity for other organs remains to be established.

In general, it is concluded that notwithstanding the huge amount of investigations on chemoprevention, on chemoprotection and on various analogues of PAR, which were all aimed at modulation mainly of PAR-dependent hepatotoxicity, very few clinically useful results have been obtained. However, it is also accomplished that perception of the molecular mechanisms of the PAR-dependent toxicity, mainly in liver, but also in other organs, is extremely valuable. Mechanism-based development of chemoprotective agents and progress in the development of structural analogues with an improved therapeutic index may be expected. In addition, this understanding of the molecular toxicological aspects of the model-toxicant PAR, with dose- and time-dependent covalent modification of critical and non-critical proteins are important in the comprehension of toxicity mechanisms of many clinically relevant and clinically nonrelevant chemical substances. The perception of these molecular and biochemical mechanisms may help in the development of improved methods for early treatment of intoxications as well as refined methods in toxicological risk assessment of chemicals (e.g., the delineation of safe levels of covalent modification of specific proteins). Lastly, insight into the physiological processes that follow primary damage close to the site of formation of reactive metabolites is valuable for the elaboration of chemoprotective agents that can be used

clinically in situations when fulminant liver failure has progressed significantly.

Note: When this manuscript had reached its final stage, a paper appeared in which for the first time, evidence was presented for the formation of a labile *ipso* adduct between NAPQI and both protein and nonprotein cysteinyl thiols (Chen W., Shockcor J.P., Tonge R., Hunter A., Gartner C. and Nelson S.D. (1999) Protein and nonprotein cysteinyl thiol modification by *N*-acetyl-*p*-benzoquinone imine via a novel *ipso* adduct, *Biochemistry* **38**:8159–8166). Another important paper was published at the same time and confirming earlier communications on the formation of nitrotyrosine-protein adducts in centrilobular cells of the liver after administration of PAR, as mentioned in the manuscript. In addition, it was reported that macrophage inactivators/depletors gadolinium chloride or dextran sulfate diminished hepatotoxicity as caused by paracetamol (Michael S.L., Pumford N.R., Mayeux P.R., Niesman M.R. and Hinson J.A. [1999] Pretreatment of mice with macrophage inactivators decreases acetaminophen hepatotoxicity and the formation of reactive oxygen and nitrogen species, *Hepatology* **30**:186–195).

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