IDENTIFICATION OF HUMAN HEPATIC CYTOCHROME P450 SOURCES OF N-ALKYLPROTOPORPHYRIN IX AFTER INTERACTION WITH PORPHYRINOGENIC XENOBIOTICS, IMPLICATIONS FOR DETECTION OF XENOBIOTIC-INDUCED PORPHYRIA IN HUMANS

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ABSTRACT:

Porphyrinogenicity of certain xenobiotics depends upon mechanism-based inactivation of specific cytochrome P450 (P450) enzymes, followed by formation of N-alkylprotoporphyrin IX (N-alkyl-IPP). Examination of the porphyrinogenicity of xenobiotics in animals and extrapolation of the results to humans is associated with ambiguity due, in part, to differences between P450 enzymes. The goal of this study was to develop an in vitro test for the detection of N-alkylPeps, produced in human liver after administration of xenobiotics found to be porphyrinogenic in animals. This goal was achieved using fluorometry to detect N-alkylIPP formation following mechanism-based inactivation by porphyrinogenenic xenobiotics of single cDNA-expressed human P450 enzymes in microsomes prepared from baculovirus-infected insect cells (Supersomes) and in human liver microsomes. The following combinations of P450 enzymes were major sources of N-alkylPPs in Supersomes: CYP3A4 [3-[(arylthio)-ethyl]sydnone (TTMS)]; CYP1A2 and 2C9 [3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-ethylpyridine (4-ethyl DDC)]; and CYP2C9, 2D6, and 3A4 [allylisopropylacetamide (AIA)]. Whereas similarities were found between results with human enzymes in Supersomes and their rat orthologs in rat liver microsomes, some differences were found. The results with TTMS and AIA, but not with 4-ethyl DDC, were the same in individual human enzymes expressed in Supersomes and human liver microsomes. We conclude that some differences exist between human liver P450 enzymes and their rat orthologs in liver microsomes. It would therefore be prudent when dealing with xenobiotics in which porphyrinogenicity depends upon N-alkylIPP formation to supplement animal data with studies using human P450 enzymes.

Xenobiotics which interfere with control of heme biosynthesis and allow porphyrins to accumulate are referred to as porphyrinogenic. The porphyrinogenic effects of several xenobiotics (e.g., TTMS, 4-ethyl DDC, and AIA) depend on their ability to cause mechanism-based inactivation of P450 enzymes (Ortíz de Montellano et al., 1981a; Ortíz de Montellano and Grab, 1986; Ortíz de Montellano and Mico, 1981c). These xenobiotics, upon selective P450 enzyme mediated biotransformation, form reactive intermediates at the P450 active site. The reactive intermediates interact with the heme moiety resulting in N-alkylation of a pyrrole ring (Marks et al., 1988; Ortíz de Montellano and Correia, 1995). P450 inactivation and dissociation of N-alkylheme is followed by loss of iron, yielding N-alkylprotoporphyrin IX (N-alkylPP), some of which are potent inhibitors of ferrochelatase (EC 4.99.1.1) (De Matteis et al., 1980; Cole et al., 1981). Ferrochelatase inhibition results in decreased heme production and less control over the rate-limiting enzyme aminolevulinic acid synthase (EC 2.3.1.37; aminolevulinic acid synthase). Increased aminolevulinic acid synthase activity results in porphyrin accumulation and porphyria (De Matteis and Marks, 1996). The N-alkylPP formed after mechanism-based inactivation of P450 by AIA, viz. N-AIAPP, does not inhibit ferrochelatase. The porphyrinogenic effect of AIA is explained as follows: since P450 can be reconstituted after undergoing mechanism-based inactivation by AIA as a result of the exchange of the N-alkylated heme moiety (N-AIAPP) for a fresh heme molecule, AIA functions as a heme-destructive catalyst that depletes regulatory free heme (Ortíz de Montellano et al., 1985).

The porphyrinogenicity of TTMS, 4-ethyl DDC, and AIA, has been shown in animals to be dependent on mechanism-based inactivation of specific P450 enzymes (Marks et al., 1988). Our previous in vivo studies have elucidated the important rat liver P450 enzymes responsible for N-alkylPP formation. CYP3A2 is the major source of N-vinylprotoporphyrin IX (N-vinylPP) after the administration of TTMS to rats whereas CYP2C11 is the major source of N-ethylprotoporphyrin IX (N-ethylPP) and N-AIApproporphyrin IX (N-AIAPP) after the

1 Abbreviations used are: TTMS, 3-[(arylthio)-ethyl]sydnone; 4-ethyl DDC, 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-ethylpyridine; AIA, allylisopropylacetamide; P450, cytochrome P450; N-alkylPP, N-alkylprotoporphyrin; N-AIAPP, N-AIA protoporphyrin; N-vinylPP, N-vinylprotoporphyrin; N-ethylPP, N-ethylprotoporphyrin; TLC, thin layer chromatography; LLD, lower limit of detection.

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2 The N-alkyl moiety is derived from AIA by addition of a hydroxyl group to the internal carbon of the alky group and a porphyrin nitrogen to the terminal carbon of the alky group. The N-alkyl moiety is converted in a secondary reaction into a lactone by reaction of the hydroxyl with an amide group (Ortíz de Montellano et al., 1984).
administered 4-ethyl DDC and AIA, respectively (Wong et al., 1998, 1999a).

Currently during toxicity testing, the porphyrinogenicity of xenobiotics is tested in animals, and it is sometimes a problem to relate these results to humans. In the case of xenobiotics, which owe their porphyrinogenicity to mechanism-based inactivation of selective P450 enzymes with formation of N-alkylPPs, the problem stems, in part, to differences in the P450 enzymes between animals and humans. This is a particular problem when a xenobiotic is found to be porphyrinogenic in one species but not in others (Frater et al., 1993). Therefore, an understanding of the human P450 enzymes targeted by these xenobiotics is required to establish whether porphyria elicited in animals is likely to occur in humans. Using microsomes prepared from human lymphoblastoid cell lines containing single cDNA-expressed human P450 enzymes, McNamie et al. (1997) used selective enzyme activities to determine which human P450 enzymes are susceptible to mechanism-based inactivation after administration of TTMS, 4-ethyl DDC, and AIA. TTMS and 4-ethyl DDC caused mechanism-based inactivation of human P450 enzymes 1A2 and 3A4, whereas only 4-ethyl DDC caused mechanism-based inactivation of CYP2C9; neither xenobiotic caused mechanism-based inactivation of CYP2D6. AIA did not inactivate any of the human P450 enzymes. However, there are several different ways in which xenobiotics can cause mechanism-based inactivation, and hepatic porphyrin accumulation will only occur if N-alkylPP formation accompanies mechanism-based inactivation (Marks et al., 1988). Therefore, it is important to determine which human P450 enzymes targeted for mechanism-based inactivation also elicit N-alkylPP formation.

In previous studies, we determined that interaction of porphyrinogenic xenobiotics with in vitro preparations containing hepatic P450 enzymes in quantities similar to those in commercially available microsomal products from human lymphoblastoid cells (0.5–1.0 nmol total P450) did not yield N-alkylPPs in sufficient quantity to be detected by a combination of thin-layer chromatography and UV-visible spectrophotometry (Wong and Marks, 1999b). Our primary goal is the development of an in vitro test system for the isolation and detection of N-alkylPPs, which may be produced in human liver after administration of xenobiotics, previously found to be porphyrinogenic in animals. We therefore explored a variety of methods to determine which specific P450 enzymes were responsible for N-alkylPP formation from in vitro preparations containing human hepatic P450 enzymes. Our first objective was to use the most sensitive method to determine which specific P450 enzymes were responsible for N-alkylPP formation using single cDNA-expressed human P450 enzymes in microsomes prepared from baculovirus-infected insect cells (Supersomes). The second objective was to use the most sensitive method to determine whether N-alkylPP formation could be detected in human liver microsomes after interaction with xenobiotics in vitro.

Materials and Methods

Source of Chemicals. TTMS and 4-ethyl DDC were obtained from Color Your Enzyme (Bath, Ontario, Canada). AIA was obtained as a gift from F. Hoffman-LaRoche (Vaudreuil, Quebec, Canada). Solvents (acetone, dichloromethane, and methanol) were purchased from VWR Canada (Mississauga, Ontario, Canada). Inducers (β-naphthoflavone, dexamethasone, and phenobarbital sodium) and NADPH were purchased from Sigma-Aldrich (St. Louis, MO). Human liver microsomes and Supersomes were purchased from Gentest Corp. (Woburn, MA).

Treatment of Animals. Male Sprague-Dawley rats (250 to 300 g) were obtained from Charles River Canada, Inc. (St. Constant, Quebec, Canada). All rats received Purina Laboratory Chow (Purina, St. Louis, MO) and water ad libitum and were housed under controlled conditions (22°C, 14:10 h light/dark cycle) prior to treatment.

Treatment of Male Rats with P450 Inducers. Rats received either dexamethasone (100 mg/kg, dissolved in 0.5 ml dimethyl sulfoxide) or phenobarbital sodium (80 mg/kg, dissolved in 0.5 ml deionized water) administered i.p. once daily for 4 days. β-Naphthoflavone (40 mg/kg, dissolved in 0.5 ml dimethyl sulfoxide) was administered i.p. once daily for 3 days (McNamee et al., 1997).

Preparation of Rat Hepatic Microsomes. Twenty-four hours after final treatment with specific P450 enzyme inducer, rats were sacrificed by decapitation, and their livers were perfused in situ with 150 ml of ice-cold 1.15% (w/v) KCl to remove the blood. Livers were excised, then minced and homogenized in 3 volumes of ice-cold phosphate-buffered KC1 [1.15% (w/v) KC1, 10 mM K2HPO4, pH 7.4] using a Potter-Elvehjem apparatus. The homogenate was centrifuged at 9000g for 20 min. The supernatant was centrifuged at 106,000g for 60 min at 4°C. The resulting eight microsomal pellets were resuspended in ice-cold 0.1 M K2HPO4 buffer (pH 7.4) containing 1.5 mM EDTA and re centrifuged at 106,000g for 60 min at 4°C. Each microsomal pellet was resuspended in 4.0 ml of 0.1 M K2HPO4 buffer (pH 7.4) containing 1.5 mM EDTA to yield a protein concentration of 4 to 6 mg/ml (Wong and Marks, 1999b). Microsomal P450 content was determined on 1 ml of microsomal suspension prior to mechanism-based inactivation by the difference spectrophotometric method of Omura and Sato (1964).

Mechanism-Based Inactivation of Rat Hepatic Microsomal P450 with Porphyrinogenic Xenobiotics. For studies with TTMS, 4-ethyl DDC, and AIA, liver microsomal suspensions from dexamethasone-, β-naphthoflavone-, or phenobarbital-pretreated rats were used, respectively. Microsomal suspensions were incubated with 2.0 mM NADPH and TTMS (0.5 mM), 4-ethyl DDC (1.0 mM), or AIA (10 mM) for 30 min at 37°C in a shaking water bath. When one microsomal pellet was used, the final volume was 4 ml. With consecutive reductions in amount of microsomes used, the final volume was reduced in a corresponding manner.

Isolation of N-alkylPP from Rat Hepatic Microsomes. Following incubation, the reaction mixture (4 ml) was mixed with 5 volumes of ice-cold 5% H2SO4/methanol (v/v) (20 ml) and shaken in the dark at 4°C for 18 h. The mixture containing N-alkylPP dimethyl esters was filtered, diluted with an equal volume of deionized water (24 ml), and then extracted twice with dichloromethane (2 × 12 ml) in a separatory funnel. The dichloromethane extract was washed with 5% sodium bicarbonate (24 ml). Zinc acetate (12 μmol) in 1.0 ml methanol was added to the dichloromethane solution to form the Zn N-alkylPP dimethyl ester, and the solution was evaporated to dryness. The N-alkylPP dimethyl ester forms complexes with a variety of metals leading to the formation of a mixture of metallo-complexes, particularly during TLC. Preparation of the zinc complex circumvents this problem. The residue was dissolved in dichloromethane (3 ml), and an aliquot (1 ml) was taken for UV-visible spectrophotometry, and an aliquot (2 ml) was taken for fluorometry (see below). The dichloromethane aliquots were combined and applied to an Analtec silica gel G TLC plate (2000 μm; Analtech, Newark, DE) and developed in dichloromethane/methanol (195:30) for 60 min. A single green band (Rf 0.68–0.74) that fluoresced red under long wavelength ultraviolet light was eluted from the plate with aceton and evaporated to dryness under nitrogen, as previously described by Wong and Marks (1999b). The residue was dissolved in dichloromethane (1.0 ml) for both UV-visible spectrophotometry and fluorometry.

Quantitation of Zn N-alkylPP Dimethyl Ester by UV-Visible Spectrophotometry and Fluorometry prior to and following TLC. The electronic absorption spectrum of the Zn N-alkylPP dimethyl ester in dichloromethane (1.0 ml) was determined, and the Zn N-alkylPP dimethyl ester concentration was calculated using the molar extinction coefficient of 128,000 m-1 cm-1 at 432 nm (Ortiz de Montellano et al., 1981b). The dichloromethane solution (1.0 ml) was diluted with 1.0 ml of dichloromethane, and the fluorescence spectrum of the Zn N-alkylPP dimethyl ester in dichloromethane was determined. Using an excitation wavelength of 432 nm (Soret band), the emission spectrum of the sample was scanned from 600 to 800 nm. The emission spectrum characteristics of samples containing Zn N-alkylPP dimethyl esters included peaks at 660 and 720 nm for N-vinylPP, 635 and 715 nm for N-ethylPP, and 650 and 712 nm for N-AIAPP. The Zn N-alkylPP dimethyl ester concentration was determined from standard curves obtained by using known concentrations determined by UV-visible spectrophotometry.
Mechanism-Based Inactivation of Human P450 Enzymes in Super-

omes with Porphyrinogenic Xenobiotics. Microsomes prepared from bac-

lovirus-infected insect cells (Supersomes) possessing single cDNA-expressed hu-

man P450 enzymes (1A2, catalogue no. P203; 2C9, catalogue no. P258; 2D6, 
catalogue no. P217; or 3A4, catalogue no. P202) were obtained from Gentest Corp.

All Supersomes were prepared from cell lines coexpressing P450 and oxidoreduc-
tase cDNA. Following previously described methods (McNamee et al., 1997), 
when required, Supersomes were rapidly thawed in a 37°C shaking water bath. 
The Supersomes suspension (1.0 ml for CYP1A2, 2C9, and 2D6 and 0.5 ml for 
CYP3A4) was added to 5-ml Erlenmeyer flasks, containing 2.0 mM NADPH and 
TTMS (0.5 mM), 4-ethyl DDC (1.0 mM), or AIA (10 mM). Samples were 
incubated for 30 min in a 37°C shaking water bath.

Isolation of N-alkylIPPs from Supersomes Containing CYP1A2, 2C9, 
2D6, and 3A4. Following incubation, the reaction mixture was mixed with 5 

volumes of ice-cold 5% H2SO4:methanol (v/v) (5 ml) and shaken in the dark 
at 4°C for 18 h. The mixture containing N-alkylIPP dimethyl esters was filtered, 
diluted with an equal volume of deionized water (6 ml), and then extracted 
twice with dichloromethane (2 × 3 ml) in a 60-ml separatory funnel. The 
dichloromethane extract was washed with 5% sodium bicarbonate (6 ml). Zinc 
acetate (12 μmol) in 1.0 ml of methanol was added to the dichloromethane 
solution to form the Zn-N-alkylIPP dimethyl ester. The volume of solution was 
reduced to 2 ml by a stream of nitrogen prior to fluorometric quantitation. 
Since the Supersome preparation of CYP3A4 was contained in a volume of 0.5 
ml, the above procedure was carried out by reducing volumes of reagents by 
50%. Wherever the presence of an N-alkylIPP was detected, the experiment was 
repeated with a second Supersome preparation.

Mechanism-Based Inactivation of Human P450 Enzymes in Human 
Liver Microsomes and Isolation of N-alkylIPPs. The procedure described 
above for Supersomes was used for human liver microsomes, which were also 
obtained from Gentest Corp. Human liver microsomes possessing elevated 
CYP3A4 (catalogue no. HG112) contained 6.28 nmol of total P450 in 0.5 ml, 
of which 53% was CYP3A4, 14% CYP2C9, 7.5% CYP2B6, 11.5% CYP2C19, 1.2% 
CYP2D6, and 11.2% CYP2E1. The human liver microsomes were 
obtained from a two-year-old white female who had died of head trauma. 
During hospitalization, she had received mannitol, phenytoin, ibuprofen, naf-
cillin, phenobarbital, ranitidine, dobutamine, dopamine, and lorazepam. Human 
liver microsomes possessing elevated CYP1A2 and CYP2C9 (catalogue no. 
HG56) contained 4.98 nmol of total P450 of which 25% was CYP1A2, 29% 
CYP2C9, 12.5% CYP3A4, 11.6% CYP2A6, 5% CYP2C19, 2.2% CYP2D6, 
and 13.2% CYP2E1. The human liver microsomes were obtained from a 
57-year-old white female who had died of an aneurism. Her medical history 
included hypertension and hepatitis A. Medication given during hospitalization 
was not available. Whenever an N-alkylIPP was detected, the experiment was 
repeated with a second batch of human liver microsomes with the same 
catalogue number.

Results and Discussion

To achieve our primary goal, namely to develop an in vitro test 
system for the isolation and detection of N-alkylIPPs, which may be 
produced in the human liver after administration of xenobiotics, our 
first studies were directed to establishing a sensitive technique for 
detecting N-alkylIPP formation.

Isolation and Detection of N-alkylIPPs Formed in Rat Liver 
Microsomes. The lower limit of detection (LLD) of N-alkylIPPs by 
UV-visible spectrophotometry and fluorometry was determined, using 
known amounts of N-alkylIPP in dichloromethane. The LLD was 
determined by setting the criterion that the peak heights were at least 
1.5 times greater than that of the noise level. The fluorometer cuvettes 
required 2 ml of solution as compared with the spectrophotometer cuvettes, 
which required 1 ml. The LLD for UV-visible spectrophotometry 
was found to be 0.16 nmol/ml of N-alkylIPP, whereas flu-

orometry detected as little as 0.04 nmol/2 ml of N-alkylIPP. Therefore, 
it was concluded that fluorometry has greater sensitivity than UV-
visible spectrophotometry for detection of N-alkylIPP.

To determine which of four methods was the most sensitive, rat 
liver microsomal suspensions were incubated with NADPH and 
TTMS for 30 min at 37°C, and N-alkylIPP formation was quantified 
by each of the four methods. The first method explored (UV-visible 
spectrophotometry without N-alkylIPP isolation by TLC) could not 
be relied upon for N-alkylIPP quantitation because the mixture, prior to 
TLC, still contained impurities with absorbencies that masked the 
characteristic spectrum of N-alkylIPP. On the other hand, fluorometry 
without TLC could be relied upon to quantitate N-alkylIPPs in this 
mixture, prior to TLC. Using the Soret peak of 432 nm as the 
excitation wavelength, the N-alkylIPP emission spectrum displayed 
two peaks at approximately 660 and 720 nm, which were not subject 
to interference by other components in the mixture. While TLC could 
separate N-alkylIPPs from interfering components in the mixture, the 
procedure results in a 60 to 70% loss of N-alkylIPP, and we concluded 
that since fluorometry could be used without losses incurred by TLC, 
fluorometry alone was the most sensitive method for the detection of 
N-alkylIPPs formed in vitro.

To determine whether the isolation and detection of N-alkylIPPs 
from in vitro preparations containing human hepatic P450 enzymes 
was feasible for future studies, we determined the minimum amount 
of P450 required for the detection of N-alkylIPP using fluorometry 
without TLC. We determined the lowest amount of P450 required to 
produce a detectable amount of N-alkylIPP by using the LLD for 
fluorometry (0.04 nmol/2 ml) and interpolating the value from the 
graph. TTMS was able to elicit detectable N-alkylIPP formation from 
rat hepatic liver microsomes prepared from dexamethasone-treated 
liver (0.01 g), containing 0.2 nmol of total P450 (Fig. 1). For N-
ethylIPP and N-AIAPP detection, after the administration of 4-ethyl 
DDC and AIA, respectively, we required rat liver microsomes con-
taining approximately 0.5 and 1.0 nmol of total P450. Since, 
commercially available microsomal products (Gentest Supersomes) con-
tain 0.5 to 1.0 nmol human P450 enzyme, we concluded that these in 
vitro preparations contained sufficient P450 for use in detecting 
N-alkylIPP formation following mechanism-based inactivation by por-
phyrinogenic xenobiotics.

Isolation and Detection of N-alkylIPP from Microsomes Pre-
pared from Baculovirus-Infected Insect Cells Possessing Single 
cDNA-Expressed P450 Enzymes. The most abundantly expressed 
P450 enzymes of the human liver are 1A2, 2C9, 2D6, and 3A4, and 
these enzymes are responsible for the biotransformation of most drugs 
that undergo P450-mediated metabolism in the human liver.
Results and Discussion

Mechanism-based inactivation of P450 can involve a number of different pathways. However, with respect to hepatic porphyria, only the inactivation of those P450 enzymes which undergo N-alkylation are relevant. The formation of N-vinylPP, N-ethylPP, and N-AIAPP all lead to porphyrin accumulation. Therefore, our first objective was to identify the specific human P450 enzymes responsible for N-alkylPP formation after interaction with TTMS, 4-ethyl DDC, and AIA.

In our earlier studies, we used microsomes prepared from human lymphoblastoid cell lines containing single cDNA-expressed human P450 enzymes to determine which P450 enzymes are susceptible to mechanism-based inactivation by porphyrinogenic xenobiotics (Guengerich, 1995). Mechanism-based inactivation of P450 can involve a number of different pathways. However, with respect to hepatic porphyria, only the inactivation of those P450 enzymes which undergo N-alkylation are relevant. The formation of N-vinylPP, N-ethylPP, and N-AIAPP all lead to porphyrin accumulation. Therefore, our first objective was to identify the specific human P450 enzymes responsible for N-alkylPP formation after interaction with TTMS, 4-ethyl DDC, and AIA.

In our earlier studies, we used microsomes prepared from human lymphoblastoid cell lines containing single cDNA-expressed human P450 enzymes to determine which P450 enzymes are susceptible to mechanism-based inactivation by porphyrinogenic xenobiotics (McNamee et al., 1997). Since that time, microsomes from baculovirus-infected insect cells possessing single cDNA-expressed P450 enzymes (Supersomes) became available. We elected to use the microsomes from the baculovirus-infected insect cells because commercial preparations contained considerably higher amounts of P450 than the microsomes from the lymphoblastoid cells, thus enhancing the potential for formation of N-alkylPP from the heme moiety of P450. We believed that the change from a lymphoblastoid cell preparation to a baculovirus-infected insect cell preparation was justifiable since our major goal was to detect N-alkylPP formation in an in vitro system. We recognize that differences would be anticipated between these two systems for a variety of reasons (e.g., a difference in the P450 to P450 reductase ratio). Thus, microsomes prepared from baculovirus-infected insect cells (Supersomes) possessing single cDNA-expressed human P450 enzymes (1A2, 2C9, 2D6, or 3A4) were incubated with TTMS, 4-ethyl DDC, and AIA, plus NADPH, and N-alkylPP formation was quantitated using fluorometry. Controls included both the omission of NADPH or porphyrinogenic xenobiotic from each of the four different human P450s.

When TTMS (0.5 mM), 4-ethyl DDC (1.0 mM), or AIA (1.0 or 10 mM) were incubated in vitro with Supersomes possessing cDNA-expressed human CYP1A2, only 4-ethyl DDC elicited N-alkylPP formation in an NADPH-dependent manner (Figs. 2 and 3). The average ratio of N-ethylPP formation to the amount of CYP1A2 present in Supersomes (nmol/nmol) was found to be 0.60 (ratio of first trial = 0.94 and ratio of second trial = 0.25). The above results indicate that N-ethylPP formation occurred concurrently with mechanism-based inactivation of CYP1A2 in the microsomes from baculovirus-infected insect cells. The fact that neither TTMS nor AIA elicited N-alkylPP formation indicates that neither TTMS nor AIA caused mechanism-based inactivation of CYP1A2, or if they do, then mechanism-based inactivation takes place by a pathway other than N-alkylation of one of the pyrrole rings. These results correspond with results obtained by Wong et al. (1998, 1999a) using rat liver microsomes from β-naphthoflavone-pretreated rats in which 4-ethyl DDC did not elicit N-alkylPP formation when incubated with 4-ethyl DDC (1.0 mM) and AIA (10 mM), respectively.

These results indicate that N-ethylPP and N-AIAPP formation occurred concurrently with mechanism-based inactivation of CYP2C9 in the microsomes from baculovirus-infected insect cells. The fact that TTMS did not elicit N-vinylPP formation indicates that TTMS either did not cause mechanism-based inactivation of CYP2C9, or if it did, then mechanism-based inactivation takes place by a pathway other than N-alkylation of one of the pyrrole rings. It is of interest to compare these results with results previously obtained with CYP2C enzymes in rats in which both rat CYP2C11 and CYP2C6 have been shown to be enzymes responsible for N-ethylPP and N-AIAPP formation, whereas CYP2C11 but not CYP2C6 was responsible for N-vinylPP formation (Table 1). Thus, the interaction between TTMS and human CYP2C9 resembles that between TTMS and rat CYP2C6 but not rat CYP2C11.

When TTMS (0.5 mM), 4-ethyl DDC (1.0 mM), or AIA (10 mM) were incubated in vitro with Supersomes possessing cDNA-expressed human CYP2D6, N-alkylPP formation was clearly observed with AIA (Figs. 2 and 3), and the ratio of N-AIAPP formation to the amount of CYP2D6 present in Supersomes (nmol/nmol) was found to be 0.23 (ratio of first trial = 0.24 and ratio of second trial = 0.22). On the other hand, N-alkylPP formation after TTMS and 4-ethyl DDC was at the lowest limit of detection of 0.04 nmol, and the ratio of both N-vinylPP and N-ethylPP to the amount of CYP2D6 was found to be 0.08. We are unable to compare these results with P450s in rat liver since no CYP2D6 orthologs are present in rat liver.

When 0.5 mM TTMS, 1.0 mM 4-ethyl DDC, or AIA (10 mM) were incubated, in the presence of NADPH, with Supersomes containing cDNA-expressed human CYP3A4, TTMS and AIA elicited N-vinylPP and N-AIAPP formation, respectively, upon interaction with Supersomes containing cDNA-expressed human CYP2C9 in an NADPH-dependent manner; on the other hand, TTMS did not elicit N-vinylPP formation (Fig. 2). The average ratio of N-alkylPP formation to CYP2C9 present in Supersomes (nmol/nmol) was found to be 0.29 (ratio of first trial = 0.39 and ratio of second trial = 0.16) and 0.21 (ratio of first trial = 0.26 and ratio of second trial = 0.16) when incubated with 4-ethyl DDC (1.0 mM) and AIA (10 mM), respectively.

These results indicate that N-ethylPP and N-AIAPP formation occurred concurrently with mechanism-based inactivation of CYP2C9 in the microsomes from baculovirus-infected insect cells. The fact that TTMS did not elicit N-vinylPP formation indicates that TTMS either did not cause mechanism-based inactivation of CYP2C9, or if it did, then mechanism-based inactivation takes place by a pathway other than N-alkylation of one of the pyrrole rings. It is of interest to compare these results with results previously obtained with CYP2C enzymes in rats in which both rat CYP2C11 and CYP2C6 have been shown to be enzymes responsible for N-ethylPP and N-AIAPP formation, whereas CYP2C11 but not CYP2C6 was responsible for N-vinylPP formation (Table 1). Thus, the interaction between TTMS and human CYP2C9 resembles that between TTMS and rat CYP2C6 but not rat CYP2C11.
1987), that mechanism-based inactivation of rat CYP3A2 by 4-ethyl DDC was found to involve fragmentation of the heme moiety to reactive metabolites that irreversibly bind to the P450 apoprotein. It is of interest to compare the above results with those obtained by Wong et al. (1998, 1999a) using rat liver microsomes. Thus, TTMS but not 4-ethyl DDC yielded N-alkylPP formation from rat CYP3A2. On the other hand, results obtained for AIA with CYP3A4 did not correspond with those of Wong et al. (1998, 1999a), who did not detect N-alkylPP formation with rat CYP3A2 in liver microsomes (Table 1). This finding suggests that there are sufficient differences between the active sites of CYP3A4 and 3A2 to result in different pathways for biotransformation of AIA. An alternative explanation is the difference in the P450 to P450 reductase ratio between the two P450 enzymes, one of which is present in Supersomes and the other in rat microsomes.

A comparison of N-alkylPP formation between Supersomes containing single cDNA-expressed human P450 enzymes (1A2, 2C9, or 3A4) and rat liver (Wong et al., 1998; Wong and Marks 1999a) after treatment with TTMS, 4-ethyl DDC and AIA can be found in Table 1. The results obtained with human CYP1A2 correspond with that of rat CYP1A2. The results obtained with TTMS and 4-ethyl DDC with human CYP3A4 correspond with those obtained with rat CYP3A2, whereas the results with AIA do not correspond. The results obtained with TTMS, AIA, and 4-ethyl DDC with human 2C9 correspond with those observed with rat CYP2C6 for all three xenobiotics.

FIG. 3. Fluorescence spectra resulting from the incubation of 4-ethyl DDC and NADPH with Supersomes containing CYP1A2 (A); control, NADPH omitted (B); and control, 4-ethyl DDC omitted (C); fluorescence spectra resulting from the incubation of AIA and NADPH with Supersomes containing CYP2D6 (D); control, NADPH omitted (E); control, AIA omitted (F); fluorescence spectra resulting from the incubation of TTMS and NADPH with Supersomes containing CYP3A4 (G); control, NADPH omitted (H); control, TTMS omitted (I).

<table>
<thead>
<tr>
<th>P450 Enzyme</th>
<th>TTMS</th>
<th>4-Ethyl DDC</th>
<th>AIA</th>
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<tbody>
<tr>
<td>Rat 1A2</td>
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</tr>
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<tr>
<td>Rat 2C6</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Human 2C9</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Rat 2C11</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Human 2C9</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

TABLE 1

A comparison of N-alkylPP formation between Supersomes containing single cDNA-expressed human P450 enzymes (1A2, 2C9, or 3A4) and rat liver (Wong et al., 1998; Wong and Marks 1999a) after treatment with TTMS, 4-ethyl DDC and AIA.
other hand, the results obtained with 4-ethyl DDC and AIA with human CYP2C9 correspond with those obtained with rat CYP2C11, but this is not the case with TTMS. The differences observed between rat and human P450 enzyme orthologs indicate that it would be useful when dealing with xenobiotics in which porphyrinogenicity depends upon interaction with P450 enzymes accompanied by N-alkylPP formation to supplement animal data with studies using cDNA-expressed single P450 enzyme systems (Rodrigues, 1999). Therefore, our second objective was to determine whether N-alkylPP formation could be detected in human liver microsomes after interaction with xenobiotics in vitro.

When TTMS (0.5 mM) or AIA (10 mM) were incubated with human liver microsomes possessing elevated CYP3A4 (HG112), both TTMS and AIA elicited N-alkylPP formation in an NADPH-dependent manner (Figs. 4 and 5). The amount of N-vinylPP formation from TTMS and N-AIAPP formation from AIA was found to be 0.83 nmol (trial one = 0.91 nmol, trial two = 0.84 nmol, and trial three = 0.73 nmol) and 0.38 nmol (trial one = 0.48 nmol and trial two = 0.28 nmol), respectively. In view of the fact that we obtained both N-vinylPP and N-AIAPP formation from Supersomes possessing only CYP3A4, these results were anticipated. When 4-ethyl DDC or AIA was incubated with human liver microsomes possessing elevated CYP1A2 and 2C9 (HG56), only AIA elicited N-alkylPP formation in an NADPH-dependent manner (Figs. 4 and 5). The amount of N-AIAPP formation was found to be 0.37 nmol (trial one = 0.42 nmol and trial two = 0.32 nmol). Given the fact that AIA elicited N-alkylPP formation from Supersomes possessing CYP2C9, these results were anticipated. However, the absence of N-ethylPP formation after the interaction of 4-ethyl DDC with human liver microsomes (HG56) was not anticipated, since N-ethylPP formation was observed with Supersomes containing either CYP1A2 or 2C9. A possible explanation for this result is the following: although these human liver microsomes (HG56) contain elevated CYP1A2 and 2C9, they also contain 0.62

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**Fig. 4.** N-Alkyl formation after the incubation of TTMS and AIA with human liver microsomes (HG112) and 4-ethyl DDC and AIA with human liver microsomes (HG56).

Data are expressed in units as indicated and each bar represents the mean of two or three experiments. Individual values are given under Results and Discussion.

**Fig. 5.** Fluorescence spectra resulting from the incubation of TTMS and NADPH with human liver microsomes (HG112) (A); control, NADPH omitted (B); control, TTMS omitted (C); fluorescence spectra resulting from the incubation of AIA and NADPH with human liver microsomes (HG56) (D); control, NADPH omitted (E); control, AIA omitted (F).
nmol or 12.5% CYP3A4. Moreover, 4-ethyl DDC caused mechanism-based inactivation of rat CYP3A2, which is not accompanied by N-alkylPP formation (Correia et al., 1987). Thus the major portion of 4-ethyl DDC may undergo biotransformation by CYP3A4 diverting the xenobiotic from CYP1A2 and CYP2C9.

In summary, we have achieved our primary goal and have developed an in vitro test system for the isolation and detection of N-alkylPPs, which are produced in the human liver after the administration of xenobiotics, previously found to be porphyrinogenic in one or several animal species. Fluorometry was found to be a sensitive method to 1) determine which specific P450 enzymes in Supersomes were the source of N-alkylPP formation and to 2) detect N-alkylPP formation in human liver microsomes after interaction with xenobiotics in vitro. We have shown that some differences exist between human liver P450 enzymes and their rat orthologs with respect to N-alkylPP formation following mechanism-based inactivation by porphyrinogenic xenobiotics. It would therefore be prudent when dealing with xenobiotics in which porphyrinogenicity depends upon interaction with P450 enzymes and N-alkylPP formation to supplement animal data with studies in human liver microsomes and cDNA-expressed individual P450 enzyme preparations. This would be particularly important where xenobiotics are shown to be porphyrinogenic due to the production of N-alkylPPs in the livers of some animal species but not others (Frater et al., 1993).

References