XENOBIOTICA , 1997, VOL. 27, NO. 7, 681–692

Irgasan® DP 300 (5-chloro-2-(2,4-dichlorophenoxy)-phenol) induces cytochrome P450s and inhibits haem biosynthesis in rat hepatocytes cultured on Matrigel

H. JINNO†*, N. HANIOKA†, S. ONODERA‡, T. NISHIMURA† and M. ANDO†

† Division of Environmental Chemistry, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158, Japan
‡ Faculty of Pharmaceutical Sciences, Science University of Tokyo, 1–3 Kagurazaka, Shinjyuku-ku, Tokyo, 163, Japan

Received 31 January 1997

1. The effect of Irgasan DP 300 (5-chloro-2-(2,4-dichlorophenoxy)phenol) on cytochrome P450 (P450) induction and haem biosynthesis was studied in rat hepatocytes cultured on Matrigel.

2. Irgasan DP 300 significantly induced 7-benzyloxyresorufin *O*-debenzylase activity, followed by 7-pentoxyresorufin *O*-dependentylase and 7-ethoxyresorufin *O*-deethylase activities. 4-Nitrophenol hydroxylase, testosterone 6β-hydroxylase and methoxyresorufin *O*-demethylase activities were also slightly increased. The maximum induction of these enzyme activities was obtained at the same concentration of 125 μM in the culture medium.

3. Immunochemical blots using anti-rat cytochrome P450 antibodies revealed that Irgasan DP 300 preferably induced CYP2B1/2 along with a slight increase in 3A. These results indicate that Irgasan DP 300 is a phenobarbital-type inducer.

4. In the absence of exogenous 5-aminolevulinic acid (ALA), slight increases in protoporphyrin IX (2·6-fold) and coproporphyrin III (1·3-fold) were observed in the Irgasan DP 300-treated cultures. In contrast, when 75 μM ALA was present, Irgasan DP 300 (250 μM) caused an extensive accumulation of uroporphyrin I (13-fold).

5. Irgasan DP 300 inhibited rat hepatic uroporphyrinogen III synthase *in vitro*.

6. These results indicate that Irgasan DP 300 produced accumulation of hydroxymethylbilane in rat hepatocytes by inhibiting uroporphyrinogen III synthase, and consequently an accumulation of uroporphyrin I.

Introduction

Irgasan DP 300 (5-chloro-2-(2,4-dichlorophenoxy)phenol) (figure 1) is widely used as a bacteriostatic ingredient in household products, such as soap and toothpaste. In addition to Irgasan DP 300 being an environmental pollutant (Levin and Nilsson 1977, Miyazaki *et al*. 1984), it has been of interest because of the chemical structure 'predioxin', or polychloro-2-phenoxyphenol, which is readily cyclized to polychlorodibenzo-*p*-dioxins by pyrolysis and photolysis (Rappe and Nilsson 1972, Langer *et al*. 1973, Nilsson *et al*. 1974, Kanetoshi *et al*. 1988).

The toxic effect of Irgasan DP 300 is rather low, with an acute oral LD_{50} of approximately 4000 mg/kg in rat and mouse (Lyman and Furia 1969) and an acute i.p. LD_{50} = 1090 mg/kg in mouse (Kanetoshi *et al*. 1992). Another biological effect of concern is the induction of cytochrome P450-dependent monooxygenase

* Author for correspondence.

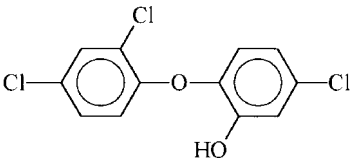

Figure 1. Chemical structure of Irgasan DP 300.

activities, such as aminopyrine *N*-demethylase activity in mouse (Miller *et al*. 1981), and 7-ethoxycoumarin *O*-deethylase and *p*-nitrophenetole *O*-deethylase activities in rat (Kanetoshi *et al*. 1992). To date, however, no information has been available on the P450 isozymes induced by Irgasan DP 300.

The present study examined the induction of P450 by Irgasan DP 300 by assessing both enzyme activity and the apoprotein, using rat hepatocytes cultured on Matrigel, a laminin-rich reconstituted basement membrane. This extracellular matrix was adopted on the basis of the maintenance and induction of P450 during primary culture (Schuetz *et al*. 1988, Kocarek *et al*. 1993). We also demonstrate the impact of Irgasan DP 300 on hepatocellular haem biosynthesis, which has been demonstrated to be disturbed by a variety of chemicals such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxins (TCDD) (Poland and Glover 1973, Goldstein *et al*. 1973), polychlorinated biphenyl (PCB) congeners (Vos and Koeman 1970, Goldstein *et al*. 1974) and diphenyl ether herbicides (Matringe *et al*. 1989).

Materials and methods

Materials

Irgasan DP 300 (99·5%) was obtained from Ciba-Geigy (Basle, Switzerland). Matrigel was purchased from Nippon Becton Dickinson (Tokyo, Japan). Resorufin, 7-ethoxyresorufin, 7-pentoxyresorufin and 6β-hydroxytestosterone were purchased from Sigma (St Louis, MO, USA). 7-Methoxyresorufin and 7-benzyloxyresorufin were from Molecular Probes (Eugene, OR, USA). Testosterone, 4-nitrophenol and 4-nitrocatechol were from Wako Pure Chemicals (Osaka, Japan). Goat anti-rat CYP1A1/2, 2B1/2, 2E1 and 3A2/1 antibodies were purchased from Daiichi Pure Chemicals (Tokyo, Japan). Porphobilinogen (PBG), uroporphyrin I (URO I), uroporphyrin III (URO III) and coproporphyrin III (COPRO III) were purchased from Porphyrin Products (Logan, UT, USA), protoporphyrin IX (PROTO IX) from Sigma, and 5-aminolevulinic acid (ALA) from Wako. All other chemicals were of the highest purity commercially available.

Isolation and culture of rat hepatocytes

Culture dishes (60 mm diameter; Iwaki Glass, Chiba, Japan) were coated with 100 μl undiluted Matrigel as described by Li *et al*. (1991). Hepatocytes were prepared from male Wistar rats (Charles River Japan, Kanagawa, Japan), weighing 180–210 g, by the two-step collagenase perfusion method (Seglen 1976). The cells were plated at a density of 2×10^6 cell/dish in 4 ml Williams' E medium supplemented with 10% newborn calf serum, 10 nM insulin, 10 nM dexamethsone and 30 mg/l kanamycin. Hepatocytes were incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. After 4 h in culture, media were renewed with Williams' E medium with the same supplement. After a total preincubation period of 24 h, media were refreshed and the chemicals dissolved in dimethyl sulphoxide (DMSO) were added to the cultures (final DMSO concentration 0·5% v/v). Thereafter, media and the chemicals were refreshed every 24 h.

Enzyme assays

After the exposure to chemicals for 48 h, hepatocyte cultures were washed twice with ice-cold PBS. The cells and Matrigel were then scraped with a rubber policeman in 4 ml 0·2 M sucrose–0·05 M Tris HCl, pH 7·4 (buffered sucrose) and placed on ice. After the cells were freed from Matrigel by repeated

pipetting, the cells were precipitated by centrifugation, resuspended in 1 ml buffered sucrose, and lysed by sonication using a Shimadzu USP-300 ultrasonic processor (Kyoto, Japan). Enzyme activities in the cell lysate were assayed, except for testosterone 6β-hydroxylase (TS6βH) activity, which was assayed in intact hepatocytes.

7-Methoxyresorufin *O*-demethylase (MROD), 7-ethoxyresorufin *O*-deethylase (EROD), 7-pentoxyresorufin *O*-depentylase (PROD) and 7-benzylozyresorufin *O*-debenzylase (BROD) activities were determined fluorometrically from the amount of resorufin produced (Hanioka *et al*. 1995). The substrate concentrations employed were 2 μM for MROD, 4 μM for EROD and PROD, and 1 μM for BROD.

4-Nitrophenol hydroxylase (PNPH) activity was assayed in 0·6 ml incubation medium consisting of phosphate buffer (0·1 M, pH 6·8), ascorbic acid (1 mM), NADPH-generating system (1 mM $NADP^+$, 10 mM glucose 6-phosphate, 2 U glucose 6-phosphate dehydrogenase and 5 mM $MgCl_2$), cell lysate (0·1 ml) and 4-nitrophenol (100 μM). The reaction was initiated by the addition of a NADPH-generating system and carried out at 37 °C for 30 min. The resulting 4-nitrocatechol was determined by hplc according to Tassaneeyakul *et al*. (1993).

TS6βH activity was assayed in intact hepatocytes (Wortelboer *et al*. 1990). The cells were washed twice with prewarmed Hanks' balanced salt solution (HBSS) and incubated in 4 ml HBSS containing 250 μM testosterone at 37 °C. After 15 min, the reaction was stopped by cooling the medium on ice. An aliquot (1 ml) of the medium was extracted with 6 ml dichloromethane, and the solvent was evaporated to dryness under a stream of N_2. The residue was dissolved in 50% (v/v) methanol/water and analysed by LC Module 1 (Waters, Tokyo). The samples were injected into a 150×4·6 mm i.d. Inertsil ODS-80A (5 μm) column (GL-Science, Tokyo). The metabolites were eluted with aqueous 25% methanol for 10 min, followed by a 25-min linear gradient to 40% methanol and 3·5% acetonitrile at a flow rate of 1·0 ml/min. 6β-Testosterone was detected by the absorbance at 254 nm. The peak areas were calculated with a Shimadzu CR-4A Chromatopac.

Immunoblot analysis of P450s

Hepatocytes microsomes were prepared by centrifugation of the cell lysates for 30 min at 105000 *g* (Schuetz *et al*. 1988). Separation of the microsomal proteins was carried out with the SDS-polyacrylamide gel electrophoresis (Laemmli 1970), using a 4% stacking gel and a 7·5% separating gel. The resolved proteins were electrophoretically transferred to nitrocellulose membranes according to the method of Towbin *et al*. (1979) and immunochemically stained using goat anti-rat P450 antibodies. The nitrocellulose membranes were blocked overnight at 4 °C in Tris-buffered saline containing 0·5% Tween 20 and 3% bovine serum albumin. Thereafter the membranes were incubated with a primary antibody for 1 h at room temperature, followed by 1-h incubation with anti-goat IgG conjugated with horseradish peroxidase. Colour was developed with 4-chloro-1-naphthol and hydrogen peroxide.

Hplc analysis of porphyrins in hepatocyte cultures

After the exposure to chemicals for 24 h, hepatocytes were scraped off the dishes in the culture medium and briefly sonicated. To a portion (1 ml) of the cell lysate was added an equal volume of freshly prepared acetone/concentrated HCl (97·5/2·5, v/v). After centrifugation, the resulting supernatant was assayed for porphyrins by the hplc method of Bonkovsky *et al*. (1986). The hplc consisted of two Shimadzu LC-10AD pumps, a SCL-10A system controller, a SIL-10A autoinjector and a RF-550 fluorescence monitor. The samples were diluted twice with 56% 0·1 M ammonium dihydrogenphosphate-44% methanol, pH 3·4 (solvent A), and an aliquot (200 μl) was injected into an Inertsil ODS-80A column. A 15-min linear gradient from 70% solvent A–30% methanol to 100% methanol was employed at a flow rate of 1·0 ml/min. Porphyrins were detected by fluorescence with excitation at 398 nm and emission at 620 nm.

URO I and URO III were co-eluted under these hplc conditions. For the separation of the isomers, 12% acetonitrile in 1 M ammonium acetate, pH 5·16 was employed as the mobile phase (Tsai *et al*. 1987).

Assay of uroporphyrinogen III synthase (UROgen III-S) activity in vitro

The inhibition of UROgen III-S by Irgasan DP 300 was demonstrated *in vitro* according to the methods of Tsai *et al*. (1987) and Clement *et al*. (1982). The cytosolic fraction was prepared from the male Wistar rat by differential centrifugation (20 min, 9000 *g*; 60 min, 105000 *g*) as described previously (Hanioka *et al*. 1995). The intact and the heated (60 °C, 1 h) cytosolic fractions were applied as enzyme sources of UROgen III-S and hydroxymethylbilane (HMB) synthase respectively. UROgen III-S activity was measured in 0·6 ml incubation medium consisting of Tris-HCl buffer (0·05 M, pH 7·8), KCl (0·06 M), the intact cytosol (5 μl), the heated cytosol (115 μl) and PBG (2·5–12·5 μM). Irgasan DP 300 was dissolved in methanol and added to the incubation medium (final methanol concentration 1·7%). The reaction was initiated by the addition of PBG and carried out at 37 °C for 15 min. The reaction was terminated by adding 0·4 ml 10% (w/v) tricloroacetic acid–0·5% (w/v) iodine. After centrifugation, URO I and URO III in the supernatant were analysed by hplc as described above.

Table 1. Cytochrome P450-dependent monooxygenase activities of rat hepatocytes treated with Irgasan DP 300.

Treatment	Concentration (μM)	Enzyme activity (pmol/min/mg protein)					
		MROD	EROD	PROD	BROD	PNPH	TS6βH
Irgasan DP 300	15·6	3·4 ± 0·4	16·7 ± 1·1†	1·5 ± 0·1	5·9 ± 0·8	29·8 ± 2·0	86·1 ± 2·2
	31·3	3·5 ± 0·3	18·1 ± 0·8†	5·5 ± 0·5	13·7 ± 5·4	36·8 ± 5·6	95·4 ± 3·9
	62·5	4·2 ± 0·6	25·0 ± 1·1‡	11·3 ± 1·2†	44·6 ± 8·3†	44·0 ± 6·4†	105·4 ± 3·9
	125	7·7 ± 0·6‡	49·4 ± 4·1‡	43·5 ± 12·9†	126·6 ± 28·4†	58·2 ± 7·4†	117·9 ± 9·2†
Methylcholanthrene	5·0	72·3 ± 9·3‡	662·5 ± 81·0‡	2·9 ± 0·6†	37·6 ± 6·9†	29·7 ± 1·6	153·1 ± 9·0‡
Phenobarbital	750	10·6 ± 2·2†	70·3 ± 15·1†	103·0 ± 19·6†	296·2 ± 55·2†	147·1 ± 45·2†	294·3 ± 10·2‡
Untreated		3·4 ± 0·3	13·2 ± 0·8	0·2 ± 0·1	3·5 ± 0·3	24·8 ± 3·5	88·7 ± 11·3

Results are mean ± SD from three independent cultures.
† $p < 0·05$, ‡ $p < 0·01$, significantly different from corresponding controls.
See text for the definition of enzyme abbreviations.

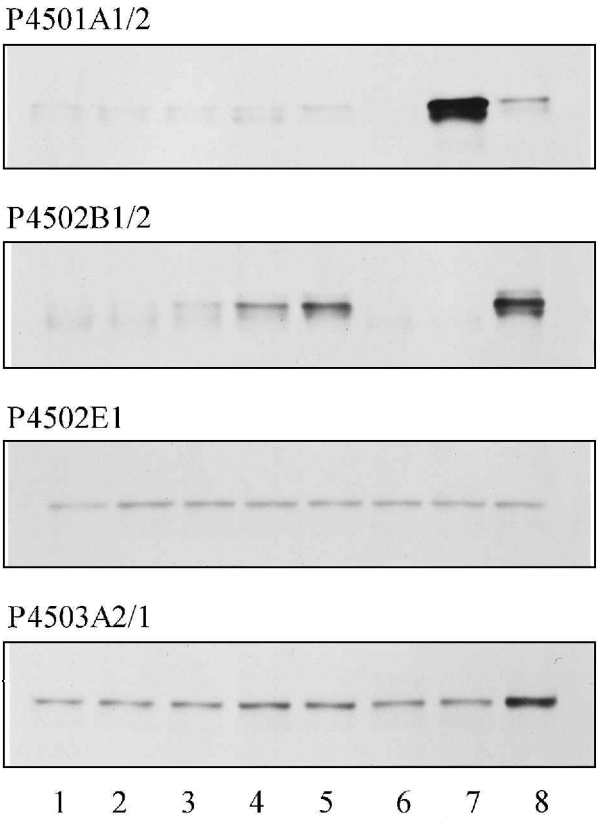

Figure 2. Immunoblot analysis of P450 induction in rat hepatocytes. Proteins from pooled microsomes were subjected to electrophoresis, electrophoretically transferred to nitrocellulose membranes and immunochemically detected with anti-rat P450 antibodies. Each lane contained 5 μg (1A1/2 and 2B1/2) or 10 μg (2E1 and 3A2/1) of microsomal protein. Lanes 1–5 contained microsomal protein from Irgasan DP 300-treated hepatocytes: 1, 7·8 μM; 2, 15·6 μM; 3, 31·3 μM; 4, 62·5 μM; 5, 125 μM. Lanes 6–8 contained proteins from control, MC (5 μM)- and PB (750 μM)-treated hepatocytes respectively.

Other methods

Protein concentrations were measured by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

Results

Induction of P450s in Irgasan DP 300-treated hepatocyte cultures

As summarized in table 1, Irgasan DP 300 induced P450-dependent monooxygenase activities in rat hepatocytes. Of the enzyme activities assayed, the BROD activity was the greatest, followed by PROD and EROD activities. The PNPH, TS6βH and MROD activities were also slightly increased (< 2-fold) in the Irgasan DP 300-treated hepatocytes. The maximum induction of these enzyme activities was obtained at the same concentration of 125 μM. At 250 μM, all of the activities were reduced to the almost undetectable level, reflecting the cytotoxity of Irgasan DP 300 (LC_{50} = 150 μM; data not shown).

The induction of P450 was further demonstrated at the apoprotein level by immunoblot analysis using anti-rat P450 antibodies (figure 2). In accordance with

Table 2. Porphyrins accumulated in rat hepatocyte cultures treated with Irgasan DP 300.

Treatment	Concentration (μM)	Porphyrins (pmol/dish)					
		−ALA			+ALA (75 μM)		
		URO	COPRO III	PROTO IX	URO	COPRO III	PROTO IX
Irgasan DP 300	15·6	nd	104±24	32±10	122±11	938±68	1412±49
	31·3	nd	90±7	33±6	131±14	879±40	1324±52
	62·5	nd	90±15	42±9†	187±70	965±66	1475±48
	125	nd	111±5†	68±14‡	246±27‡	1037±104	1388±67
	250	nd	2±4‡	4±6‡	1614±127‡	92±31‡	743±67‡
Untreated		nd	84±15	26±4	120±20	986±23	1376±26

Results are mean±SD from three independent cultures.
† $p < 0{\cdot}05$; ‡ $p < 0{\cdot}01$, significantly different from corresponding controls. nd, Not detected.
See text for the definition of porphyrin abbreviations.

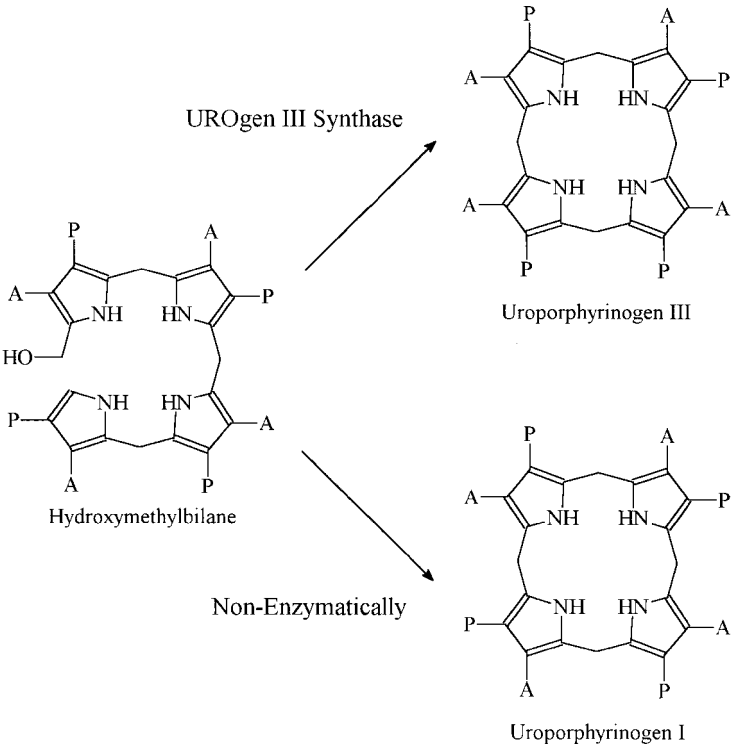

Figure 3. Formation of UROgen I and UROgen III from hydroxymethylbilane.

the increase in BROD activity, Irgasan DP 300 preferably induced CYP2B1/2 along with a slight increase in 3A in rat hepatocytes. In contrast, 2E1 was not affected by Irgasan DP 300, despite the induction of PNPH activity. Of interest was that 1A2 was detected at low levels in the Irgasan DP 300-treated hepatocytes. For this P450 isozyme, the maximum induction was observed at 62·5 μM.

The results of both the enzyme assay and the immunoblot analysis clearly indicate that Irgasan DP 300 was similar to phenobarbital (PB) as an inducer.

Inhibition of hepatocellular haem biosynthesis by Irgasan DP 300

Porphyrins accumulated in the hepatocyte culture were assayed after 24-h exposure to Irgasan DP 300. In the absence of exogenous ALA, slight increases of PROTO IX (2·6-fold) and COPRO III (1·3-fold) were observed in the hepatocyte cultures treated with Irgasan DP 300 at 125 μM (table 2). In contrast, when 75 μM ALA was present, Irgasan DP 300 caused an extensive accumulation of URO (13-fold) at the concentration of 250 μM. As shown in figure 3, two isomers of URO, URO I and URO III, could be formed in the haem biosynthetic pathway. However, URO I and URO III not separated from each other because both of the isomers co-eluted under the hplc conditions used for the porphyrin profile analysis (ammonium phosphate–methanol as mobile phase). Complete separation of the isomers was achieved using ammonium acetate–acetonitrile as the mobile phase and URO I was the isomer which accumulated in hepatocyte cultures treated with Irgasan DP 300

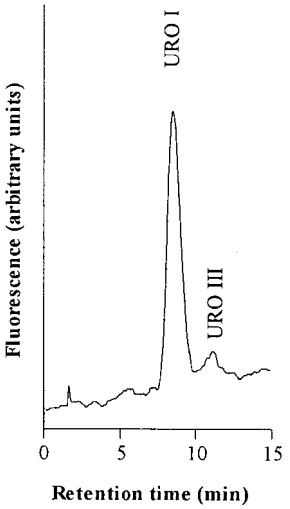

Figure 4. Hplc chromatogram of URO in hepatocyte cultures treated with Irgasan DP 300 (250 μM) and ALA (75 μM). URO I and URO III were separated using 12% acetonitrile in 1 M ammonium acetate, pH 5·16 as the mobile phase.

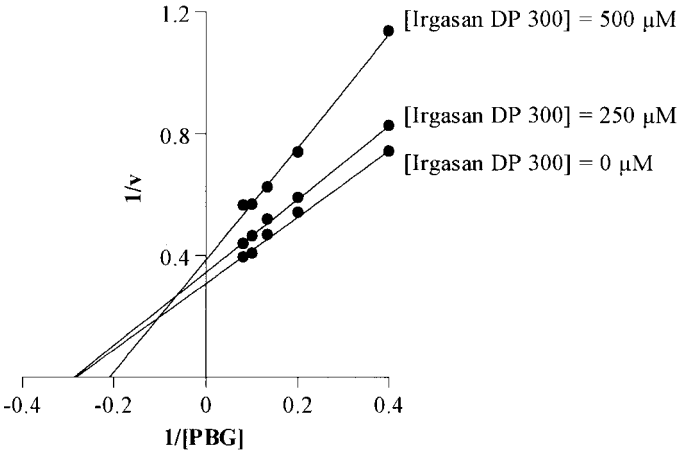

Figure 5. Lineweaver–Burk plot for the UROgen III-S activity in the presence of Irgasan DP 300. Each point represents the mean ± SD from three determinations.

in the presence of exogenous ALA (figure 4). These results suggested that Irgasan DP 300 possibly caused the accumulation of HMB as a result of UROgen III-S inhibition.

In vitro *inhibition of UROgen III-S activity by Irgasan DP 300*

To test the possible inhibition of UROgen III-S activity by Irgasan DP 300, a coupled enzyme assay (Tsai *et al.* 1987) was carried out using cytosolic fractions prepared from rat liver. In this assay system, PBG (substrate) was first converted to HMB by HMB synthase in the heat-treated cytosolic fraction, then cyclized to UROgen III by UROgen III-S in the intact cytosolic fraction. Figure 5 shows a Lineweaver–Burk plot for the UROgen III-S activity observed in the presence of

Irgasan DP 300. This plot demonstrated that Irgasan DP 300 directly inhibited UROgen III-S in a non-competitive or a mixed (competitive/non-competitive) manner.

Discussion

Both the induction of P450 and the inhibition of haem biosynthesis have been demonstrated in experimental animals intoxicated with polychlorinated aromatic hydrocarbons. For example, TCDD and certain members of the PCB family specifically induce CYP1A1/2 in the liver (Goldstein and Linko 1984, Dragnev *et al*. 1995), mediated by the aryl hydrocarbon (*Ah*) receptor (reviewed by Landers and Bunce 1991), and additionally cause hepatic URO III accumulation (Poland and Glover 1973, Goldstein *et al*. 1973, 1974, Vos and Koeman 1970). However the mechanisms involved in the inhibition of UROgen III decarboxylase have not been fully elucidated (Cantoni *et al*. 1984). Diphenyl ether herbicides, another class of polychlorinated aromatic hydrocarbons, have been shown to inhibit proto-porphyrinogen IX oxidase, resulting in the accumulation of PROTO IX (Matringe *et al*. 1989). Some of the diphenyl ethers have also been demonstrated to induce hepatic P450s (Jacobs *et al*. 1992, Krijt *et al*. 1993). In view of the structural similarity to Irgasan DP 300, we studied the effect of Irgasan DP 300, a typical 2-phoenoxyphenol, on hepatic P450 levels and haem biosynthesis.

In previous *in vivo* studies, Irgasan DP 300 has been reported to increase aminopyrine *N*-demethylase activity in mouse (Miller *et al*. 1981), suggesting that Irgasan DP 300 is a PB-type inducer. Kanetoshi *et al*. (1992) reported that Irgasan DP 300 significantly induced *p*-nitrophenetole *O*-deethylase (2·3-fold), biphenyl 2-hydroxylase (2·3-fold), 7-ethoxycoumarin *O*-deethylase (2·0-fold), *p*-nitroanisole *O*-demethylase (1·7-fold) and biphenyl 4-hydroxylase (1·3-fold) activities in rat. From these latter results, however, it is impossible to identify the P450 isozymes induced by Irgasan DP 300. In the present study, the induction of P450 was first demonstrated by enzyme assays of MROD, EROD, PROD, BROD, PNPH and TS6βH activities, which have been established as sensitive and highly selective markers for the induction of P450 isozymes. Yang *et al*. (1988) reported that purified rat CYP1A1 and 1A2 preferentially metabolize ethoxyresorufin and methoxy-resorufin respectively. Dealkylation of pentoxyresorufin and benzyloxyresorufin has been shown to be catalysed by purified rat 2B1 (Wolf *et al*. 1986, Yang *et al*. 1988). The predominant contribution of 2E1 to 4-nitophenol hydroxylation in the rat is generally accepted (Dicker *et al*. 1990). Rat 3A has been purified and shown to metabolize testosterone to the 6β-hydroxy derivative (Halvorson *et al*. 1990). Of these enzyme activities, BROD activity was the most increased in the Irgasan DP 300-treated hepatocytes (table 1), indicating the possible induction of 2B1/2. The results obtained from our immunochemical study were mainly consistent with those from enzyme assays. Irgasan DP 300 preferably induced 2B1/2 along with a slight increase in 3A (figure 2), in accordance with the increase in BROD activity. Thus, Irgasan DP 300 was identified as a PB-type inducer in rat hepatocytes. 2E1 was not affected by Irgasan DP 300, despite the induction of PNPH activity. P450 isozymes other than 2E1, such as 2B1/2 and 3A, might catalyse 4-nitrophenol hydroxylation to a minor extent, and contribute to this discrepancy between PNPH activity and 2E1 apoprotein.

Exogenous ALA substantially altered the impact of Irgasan DP 300 on hepatocellular haem biosynthesis. In the absence of exogenous ALA, Irgasan DP

300 slightly increased PROTO IX and COPRO III in the hepatocyte cultures. When ALA (75 μM) was added alone, the hepatocytes accumulated mostly PROTO IX and COPRO III, in accordance with previous results in mouse (Sinclare *et al.* 1990), rat and chick embryo hepatocytes (De Matteis *et al.* 1988). In the present study, Irgasan DP 300 (250 μM), together with exogenous ALA, induced an extensive accumulation of URO at the expense of COPRO III and PROTO IX. The URO isomer accumulated in the culture was URO I. In the haem biosynthetic pathway, HMB is cyclized to UROgen III by UROgen III synthase, associated with the intermolecular rearrangement of the D-pyrrole group (Battersby *et al.* 1982, Hart and Battersby 1985). Another isomer UROgen I, a reduced form of URO I, is generated from HMB by spontaneous cyclization, or non-enzymatic reaction (Battersby *et al.* 1982). Therefore, the accumulation of URO I we observed suggests that Irgasan DP 300 may interfere with the UROgen III synthesis from HMB by UROgen III synthase. As a test of this hypothesis, a coupled-enzyme assay of UROgen III synthase activity was carried out *in vitro*. Although the mode of inhibition was not fully elucidated, it was demonstrated that Irgasan DP 300 directly inhibited UROgen III synthase. Thus, we showed that Irgasan DP 300 inhibited hepatocellular UROgen III synthase, resulting in the accumulation of URO I. The accumulation of URO I has also been reported in the rare human disease of congenital erythropoietic porphyria (CEP) which is characterized by the deficient activity of UROgen III synthase (Deybach *et al.* 1981). The clinical syndrome of CEP is dominated by extreme photosensitivity and haemolytic anemia. Irgasan DP 300 may induce such manifestations *in vivo*.

Acknowledgement

This study was supported by grants from the Environment Agency, Japan.

References

Battersby, A. R., Fookes, C. J. R., Gustafson-Potter, K. E., McDonald, E. and Matcham, G. W. J., 1982, Biosynthesis of porphyrins and related macrocycles. Part 18. Proof by spectroscopy and synthesis that unrearranged hydroxymethylbilane is the product from deaminase and the substrate for cosynthetase in the biosynthesis of uroporphyrinogen-III. *Journal of Chemical Society Perkin Transaction I*, 2427–2444.

Bonkovsky, H. L., Wood, S. G., Howell, S. K., Sinclair, P. R., Lincoln, B., Healey, J. F. and Sinclair, J. F., 1986, High-performance liquid chromatographic separation and quantitation of tetrapyrroles from biological materials. *Analytical Biochemistry*, **155**, 56–64.

Cantoni, L., dal Fiume, D., Rizzardini, M. and Ruggieri, R., 1984, *In vitro* inhibitory effect on porphyrinogen carboxylyase of liver extracts from TCDD treated mice. *Toxicology Letters*, **20**, 211–217.

Clement, R. P., Kohashi, M. and Piper, W. N., 1982, Rat hepatic uroporphyrinogen III cosynthase: purification, properties, and inhibition by metal ions. *Archives of Biochemistry and Biophysics*, **214**, 657–667.

De Matteis, F., Harvey, C., Reed, C. and Hempenius, R., 1988, Increased oxidation of uroporphyrinogen by an inducible liver microsomal system. Possible relevance to drug-induced uroporphyria. *Biochemical Journal*, **250**, 161–169.

Deybach, J. C., De Verneuil, H., Phung, N., Nordmann, Y., Puissant, A. and Boffety, B., 1981, Congenital erythropoietic porphyria (Gunther's disease): enzymatic studies on two cases of late onset. *Journal of Laboratory and Clinical Medicine*, **97**, 551–558.

Dicker, E., McHugh, T. and Cederbaum, A. I., 1990, Increased oxidation of *p*-nitrophenol and aniline by intact hepatocytes isolated from pyrazole-treated rats. *Biochimca et Biophysica Acta*, **1035**, 249–256.

DRAGNEV, K. H., NIMS, R. W., FOX, S. D., LINDAHL, R. and LUBET, R. A., 1995, Relative potencies of induction of hepatic drug-metabolizing enzyme genes by individual PCB congeners. *Toxicology and Applied Pharmacology*, **132**, 334–342.

GOLDSTEIN, J. A., HICKMAN, P. and JUE, D. L., 1974, Experimental hepatic porphyria induced by polychlorinated biphenyls. *Toxicology and Applied Pharmacology*, **27**, 437–448.

GOLDSTEIN, J. A., HICKMAN, P. and VOS, J. G., 1973, Hepatic porphyria induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in the mouse. *Research Communication of Chemical and Pathological Pharmacology*, **6**, 919–928.

GOLDSTEIN, J. A. and LINKO, P., 1984, Differential induction of two 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-inducible forms of cytochrome P-450 in extrahepatic versus hepatic tissues. *Molecular Pharmacology*, **25**, 185–191.

HALVORSON, M., GREENWAY, D., EBERHART, D., FITZGERALD, K. and PARKINSON, A., 1990, Reconstitution of testosterone oxidation by purified rat cytochrome P450p (IIIA1). *Archives of Biochemistry and Biophysics*, **277**, 166–180.

HANIOKA, N., JINNO, H., TAKAHASHI, A., NAKANO, K., YODA, R., NISHIMURA, T. and ANDO, M., 1995, Interaction of tetrachloroethylene with rat hepatic microsomal P450-dependent monooxygenases. *Xenobiotica*, **25**, 151–165.

HART, G. J. and BATTERSBY, A. R., 1985, Purification and properties of uroporphyrinogen III synthase (co-synthetase) from *Euglena gracilis*. *Biochemical Journal*, **232**, 151–160.

JACOBS, J. M., SINCLAIR, P. R., GORMAN, N., JACOBS, N. J., SINCLAIR, J. F., BEMENT, W. J. and WALTON, H., 1992, Effects of diphenyl ether herbicides on porphyrin accumulation by cultured hepatocytes. *Journal of Biochemical Toxicology*, **7**, 87–95.

KANETOSHI, A., KATSURA, E., OGAWA, H., OHYAMA, T., KANESHIMA, H. and MIURA, T., 1992, Acute toxicity, percutaneous absorption and effects on hepatic mixed function oxidase activities of 2,4,4′-trichloro-2′-hydroxydiphenyl ether (Irgasan DP300) and its chlorinated derivatives. *Archives of Environmental Contamination and Toxicology*, **23**, 91–98.

KANETOSHI, A., OGAWA, H., KATSURA, E., KANESHIMA, H. and MIURA, T., 1988, Formation of polychlorinated dibenzo-*p*-dioxins upon combustion of commercial textile products containing 2,4,4′-trichloro-2′-hydroxydiphenyl ether (Irgasan® DP300). *Journal of Chromatography*, **442**, 289–299.

KOCAREK, T. A., SCHUETZ, E. G. and GUZELIAN, P. S., 1993, Expression of multiple forms of cytochrome P450 mRNAs in primary cultures of rat hepatocytes maintained on matrigel. *Molecular Pharmacology*, **43**, 328–334.

KRIJT, J., VAN HOLSTEIJN, I., HASSING, I., VOKURKA, M. and BLAAUBOER, B. J., 1993, Effect of diphenyl ether herbicides and oxadiazon on porphyrin biosynthesis in mouse liver, rat primary hepatocyte culture and HepG2 cells. *Archives of Toxicology*, **67**, 255–261.

LAEMMLI, U. K., 1970, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.

LANDERS, J. P. and Bunce, N. J., 1991, The Ah receptor and the mechanism of dioxin toxicity. *Biochemical Journal*, **276**, 273–287.

LANGER, H. G., BRADY, T. P. and BRIGGS, P. R., 1973, Formation of dibenzodioxins and other condensation products from chlorinated phenols and derivatives. *Environmental Health Perspectives*, **5**, 3–7.

LEVIN, J. O. and NILSSON, C. A., 1977, Chromatographic determination of polychlorinated phenols, phenoxyphenols, dibenzofurans and dibenzodioxins in wood-dust from worker environments. *Chemosphere*, **6**, 443–448.

LI, D., SCHUETZ, E. T. and GUZELIAN, P. S., 1991, Hepatocyte culture in study of P450 regulation. *Methods in Enzymology*, **206**, 335–344.

LOWRY, O. H., ROSENBROUGH, N. J., FARR, A. L. and RANDALL, R. J., 1951, Protein measurement with Folin phenol reagent. *Journal of Biological Chemistry*, **193**, 265–275.

LYMAN, F. L. and FURIA, T., 1969, Toxicology of 2,4,4′-trichloro-2′-hydroxydiphenyl ether. *Industrial Medicine and Surgery*, **38**, 64–71.

MATRINGE, M., CAMADRO, J. M., LABBE, P. and SCALLA, R., 1989, Protoporphyrinogen oxidase as a molecular target for diphenyl ether herbicides. *Biochemical Journal*, **260**, 231–235.

MILLER, T. L., LORUSSO, D. J. and DEINZER, M. L., 1981, Induction of enzymes of the hepatic mixed-function oxidase system by hydroxychlorodiphenyl ether contaminants of technical PCP. *Federation Proceedings*, **40**, 630.

MIYAZAKI, T., YAMAGISHI, T. and MATSUMOTO, M., 1984, Residues of 4-chloro-1-(2,4-dichlorophenoxy)-2-methoxybenzene (triclosan methyl) in aquatic biota. *Bulletin of Environmental Contamination and Toxicology*, **32**, 227–232.

NILSSON, C. A., ANDERSON, K., RAPPE, C. and WESTERMARK, S. O., 1974, Chromatographic evidence for the formation of chloro-dioxins from chloro-2-phenoxyphenols. *Journal of Chromatography*, **96**, 137–147.

POLAND, A. and GLOVER, E., 1973, 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin: a potent inducer of δ-aminolevulinic acid synthetase. *Science*, **179**, 476–477.

RAPPE, C. and NILSSON, C. A., 1972, An artifact in the gas chromatographic determination of impurities in pentachlorophenol. *Journal of Chromatography*, **67**, 247–253.

SCHUETZ, E. G., LI, D., OMIECINSKI, C. J., MULLER-EBERHARD, U., KLEINMAN, H. K., ELSWICK, B. and GUZELIAN, P. S., 1988, Regulation of gene expression in adult rat hepatocytes cultured on a basement membrane matrix. *Journal of Cellular Physiology*, **134**, 309–323.

SEGLEN, P. O., 1976, Preparation of isolated rat liver cells. *Methods in Cell Biology*, **13**, 29–83.

SINCLAIR, P. R., BEMENT, W. J., LAMBRECHT, R. W., GORMAN, N. and SINCLAIR, J. F., 1990, Chlorinated biphenyls induce cytochrome P450IA2 and uroporphyrin accumulation in cultures of mouse hepatocytes. *Archives of Biochemistry and Biophysics*, **281**, 225–232.

TASSANEEYAKUL, W., VERONESE, M. E., BIRKETT, D. J. and MINERS, J. O., 1993, High-performance liquid chromatographic assay for 4-nitrophenol hydroxylation, a putative cytochrome P-4502E1 activity, in human liver microsomes. *Journal of Chromatography*, **616**, 73–78.

TOWBIN, H., STAEHELIN, T. and GORDON, J., 1979, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceeding of the National Academy of Sciences, USA*, **76**, 4350–4354.

TSAI, S., BISHOP, D. F. and DESNICK, R. J., 1987, Coupled-enzyme and direct assays for uroporphyrinogen III synthase activity in human erythrocytes and cultured lymphoblasts. *Analytical Biochemistry*, **166**, 120–133.

VOS, J. G. and KOEMAN, J. H., 1970, Comparative toxicologic study with polychlorinated biphenyls in chicken with special reference to porphyria, edema formation, liver necrosis, and tissue residues. *Toxicology and Applied Pharmacology*, **17**, 656–668.

WOLF, C. R., SEILMAN, S., OESCH, F., MAYER, R. T. and BURKE, M. D., 1986, Multiple forms of cytochrome P-450 related to forms induced marginally by phenobarbital. Differences in structure and in the metabolism of alkoxyresorufins. *Biochemical Journal*, **15**, 27–33.

WORTELBOER, H. M., DE KRUIF, C. A., VAN IERSEL, A. J., FALKE, H. E., NOORDHOEK, J. and BLAAUBOER, B. J., 1990, The isozyme pattern of cytochrome P450 in rat hepatocytes in primary culture, comparing different enzyme activities in microsomal incubations and intact monolayers. *Biochemical Pharmacology*, **40**, 2525–2534.

YANG, H. Y, NAMKUNG, M. J. and JUCHAU, M. R., 1988, Cytochrome P-450-dependent biotransformation of a series of phenoxazone ethers in the rat conceptus during early organogenesis: evidence for multiple P-450 isoenzymes. *Molecular Pharmacology*, **34**, 67–73.