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USE OF 7-ALKOXYPHENOXAZONES, 7-ALKOXYCOUMARINS AND 7-ALKOXYQUINOLINES AS FLUORESCENT SUBSTRATES FOR RAINBOW TROUT HEPATIC MICROSOMES AFTER TREATMENT WITH VARIOUS INDUCERS*

MARY L. HAASCH,†‡ WENDY K. GRAF,† ELLEN M. QUARDOKUS,† RICHARD T. MAYER§ and JOHN J. LECH†

†Medical College of Wisconsin, Pharmacology and Toxicology Department, Milwaukee, WI 53226; and NIEHS Marine and Freshwater Biomedical Research CORE Center, University of Wisconsin-Milwaukee Great Lakes Research Facility, Milwaukee, WI 53204; and §U.S. Department of Agriculture, Agricultural Research Service, U.S. Horticultural Research Laboratory, Orlando, FL 32803, U.S.A.

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Abstract—Various fluorescent substrates have been used as specific indicators of induction or activity of different cytochrome P450 isozymes in both fish and mammalian species. In an attempt to identify additional definitive fluorescent substrates for use in fish, we examined a series of 7-alkoxyphenoxazones, 7-alkoxycoumarins and 7-alkoxyquinolines as substrates in O-dealkylation assays with hepatic microsomes from rainbow trout (*Oncorhynchus mykiss*). Microsomes were prepared after 48 hr of treatment with β-naphthoflavone (β-NF), pregnenolone-16α-carbonitrile (PCN), phenobarbital (PB), isosafrole (ISF), or dexamethasone (DEX). Total P450 spectra were obtained, and spectral binding studies were performed. Microsomal O-dealkylation rates were greater after ISF treatment than after β-NF treatment for 7-methoxy-, 7-ethoxy-, 7-propoxy- and 7-benzyloxyphenoxazones but not for 7-butoxyphenoxazone. DEX treatment resulted in a significant elevation of pentoxyphenoxazone metabolism (about a 144-fold increase) compared with microsomes induced by β-NF (11-fold) and ISF (37-fold). The rates of dealkylation of the alkoxyphenoxazones by ISF-treated microsomes occurred in the following order: methoxy > ethoxy > propoxy > benzxyloxy > butoxy > pentoxy. When β-NF-treated microsomes were used, the 7-alkoxyphenoxazones were metabolized as follows: methoxy > ethoxy > propoxy > butoxy > benzyloxy ≈ pentoxy, while the order of metabolism of the 7-alkoxycoumarins was: ethoxy ⩾ butoxy > propoxy ≈ methoxy > benzyloxy > pentoxy. None of the other treatments significantly increased the rate of metabolism of any of the alkoxycoumarins. Treatment with β-NF did not significantly elevate the rate of metabolism of any of the alkoxyquinolines. DEX treatment produced significant elevations in the rate of metabolism of benzyloxy-, ethoxy-, and butoxy- ≈ pentoxy- ≈ propoxyquinoline, in that order. ISF treatment significantly elevated the rate of metabolism of benzyloxy-, methoxy- and butoxyquinoline, in that order. These results suggest that some of these new fluorescent substrates can be used to characterize induction of rainbow trout hepatic microsomal monooxygenase activity by ISF and DEX, in addition to the commonly used ethoxyphenoxazone and ethoxycoumarin for the characterization of induction by β-NF or other 3-methylcholanthrene-type P450 inducers. Distinction between ISF-type and β-NF-type inducers in rainbow trout hepatic microsomes may best be made using 7-methoxycoumarin as a substrate. Distinction between ISF-type and DEX-type inducers and between β-NF-type and DEX-type inducers may best be made using 7-methoxyphenoxazone as a substrate. With β-NF induction 7-methoxycoumarin, with ISF induction 7-methoxyphenoxazone, and with DEX induction 7-ethoxyquinoline were metabolized to the greatest extent compared with controls and all other substrates tested.

Key words: cytochrome P450; fluorescent substrates; trout; microsomes; induction; hepatic

Multiple isozymes of cytochrome P450s exist in animals, and these isozymes function in both activation and inactivation of xenobiotics as well as endogenous substrates [1]. Rainbow trout liver has been shown to contain at least five different isozymes [2, 3]. The aromatic hydrocarbon-inducible form, P450 LM4b [4] (or CYP1A1), has been sequenced and appears to be orthologous to both rat CYP1A1 and CYP1A2, but has greater similarity to CYP1A1 [5]. Another trout isozyme structurally related to the rat PB‖-inducible form CYP2B1, based on

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‡ Corresponding author: Mary L. Haasch, Ph.D., Medical College of Wisconsin, Department of Pharmacology and Toxicology, 8701 Watertown Plank Road, Milwaukee, WI 53226. Tel. (414) 257-8635; FAX (414) 778-4289.

‖ Abbreviations: β-NF, β-naphthoflavone; cDNA, complementary DNA; DEX, dexamethasone; DMFA, dimethylformamide; ISF, isosafrole; 3-MC, 3-methylcholanthrene; MO, monooxygenase; PB, phenobarbital; PCN, pregnenolone-16α-carbonitrile and PCB, polychlorinated biphenyl.

immunological cross-reactivity [3] and cDNA sequence [6], has been described. Rainbow trout genomic DNA has been shown to contain sequences that hybridize to rat CYP2B1 cDNA, lending support to the suggestion that trout have an isozyme related to the CYP2 family of isozymes [7]. One of the constitutive trout cytochrome P450 isozymes, designated LMC5, can catalyze the 6β-hydroxylation of steroid hormones, such as progesterone and testosterone [8]. LMC5 may be structurally related to the human CYP3A4 [8]. LM2 [2], another constitutive trout cytochrome P450 isozyme that was first isolated from β-NF-fed trout, has high activity in aflatoxin B_1 biotransformation and in the metabolism of lauric acid, an endogenous substrate [9, 10].

In fish, the multiple forms of cytochrome P450 isozymes each have slightly different, but usually overlapping substrate specificities [11]. Several isozymes can also be induced by the same agent [12]. In addition to the various immunological and molecular techniques for detecting and characterizing cytochrome P450 isozymes, detection of induction of the catalytic activity of specific isozymes has depended on the availability of practical and sensitive assays. These assays usually rely on metabolism of a substrate to a fluorescent product that can be measured spectrofluorimetrically. Unfortunately, in many cases the substrates used do not adequately discern differences between induced or between constitutive isozymes [13]. In addition, it has been shown in rats that the induction of alkoxyphenoxazone metabolism may be a valuable diagnostic indicator of the presence of a variety of pollutants, including polycyclic aromatic hydrocarbons, organochlorine pesticides, polyhalogenated biphenyls and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [14]. This approach has been applied to fish hepatic cytochrome P450 induction as a biomarker of environmental chemical contamination [15–17], but unfortunately very little work has been done in characterizing fluorescent substrate use in fish species.

In mammals such as the rat and mouse, increased metabolism by the 3-MC-inducible cytochrome P450 isozyme (e.g. CYP1A1) can be detected by the "model" specific substrate, 7-ethoxyphenoxazone (7-ethoxyresorufin) [18]. Induction by PB-type inducers has been shown to be specific for the O-dealkylation of 7-pentoxyphenoxazone [19, 20]. Hepatic MO enzymes in fish are quite similar to those in mammals [10, 21] but appear to respond differently to some inducers [7, 10, 21–23]. Examination of fish cytochrome P450 induction by phenobarbitone (5 days, 80 mg/L) or Aroclor 1254 (5 days, 100 mg/kg, i.p.) with 7-pentoxy- and 7-benzyloxyphenoxazone has shown that these substrates are not specific for PB-type induction in either trout or catfish, since a decrease from control rates of metabolism was shown with these substrates [23, 24]. However, in the above study, use of Aroclor 1254, which contains both coplanar (3-MC-type) and noncoplanar (PB-type) PCB congeners, may have caused a reduction of the LM2 isozyme, resulting from a concomitant 2-fold induction of the 1A1 isozyme as evidenced by 7-ethoxyphenoxazone metabolism [24]. Reduction of one P450 isozyme after induction of another has been seen previously in β-NF-treated trout [10]. Another study has shown that 7-pentoxyphenoxazone is not specific for PB-type induction in rainbow trout, since treatment with β-NF (96 hr, 100 mg/kg, i.p.) resulted in an approximately 5-fold increase in the rate of metabolism of 7-pentoxyphenoxazone by rainbow trout hepatic microsomes [25]. Treatment of rainbow trout with β-NF or other 3-MC-type inducers results in the induction of both ethoxyresorufin and ethoxycoumarin-*O*-deethylase activities in conjunction with increases in CYP1A1 apoprotein and mRNA levels [26]. These results demonstrate a need for more specific fluorescent substrates for the investigation of hepatic microsomal MO activities after treatment of rainbow trout with various types of cytochrome P450 inducers. In this regard, a series of 7-alkoxyphenoxazones, 7-alkoxycoumarins and 7-alkoxyquinolines have been synthesized [13, 19, 27, 28] and can now be used to determine their metabolic specificity as substrates for trout hepatic microsomal metabolism.

The objective of the present studies was to investigate rainbow trout hepatic microsomal rates of metabolism using the new fluorescent substrates—the alkoxyphenoxazones, the alkoxycoumarins, and the alkoxyquinolines—after treatment with various classes of hepatic MO inducers. As a supplement to the immunological and molecular studies that have been done in rainbow trout, these studies should expand our knowledge of the qualitative and quantitative differences between mammalian and teleostean hepatic MO induction responses and substrate specificities, and provide information on hepatic P450 gene expression and isozyme content of rainbow trout.

MATERIALS AND METHODS

Chemicals

Phenobarbital, NADPH, dexamethasone and β-naphthoflavone were purchased from the Sigma Chemical Co. (St. Louis, MO). Isosafrole was purchased from the Aldrich Chemical Co., Inc. (Milwaukee, WI) and pregnenolone-16α-carbonitrile from the Upjohn Co. (Kalamazoo, MI). Alkoxyphenoxazones, alkoxycoumarins, and alkoxyquinolines were synthesized as previously described [13, 19, 27, 28]. It should be noted that the hexoxy-derivative was not available for these studies. All other chemicals were of the highest quality commercially available.

Animals and treatments

Rainbow trout (*Oncorhynchus mykiss*) were purchased from the Evergreen Trout Farm (Pound, WI) and were maintained on a 12 hr light/12 hr dark photoperiod at 10–15°, under flow-through conditions, in dechlorinated Milwaukee city water for at least 2 weeks prior to the initiation of the experiments. Trout were fed daily at a rate approximately equal to 3% of their body weight with Silvercup® trout pellets (Murray Elevators, Murray, UT) until the day prior to the start of the experiments. The trout used in these experiments

were maintained under conditions in compliance with the IACUC of the Medical College of Wisconsin Animal Resource Center, which is AAALAC accredited, and also with the approval of the IACUC of the University of Wisconsin-Milwaukee, Milwaukee, WI.

Rainbow trout were treated, by i.p. injection, either as sham-injected controls or as DMFA controls (1 mL/kg). DMFA was also used as the carrier for the various inducers including: β-NF (10 mg/kg), PCN (25 mg/kg), PB (40 mg/kg), ISF (175 mg/kg), or DEX (2 mg/kg). The trout were killed by a sharp blow to the head after 48 hr and livers were excised and rinsed in 1.15% KCl. Microsomes were prepared by differential ultracentrifugation as previously described [29] and stored in cryotubes at −80° until the enzyme assays could be performed.

O-Dealkylation fluorescence assays

Fluorescence of the products 7-hydroxycoumarin, 7-hydroxyphenoxazone (resorufin) and 7-quinolinol was measured using a Perkin–Elmer (Norwalk, CT) model LS 50 spectrofluorimeter. (The metabolism of the benzyloxy-derivatives is not technically an O-dealkylation, but it has been labeled as such throughout the manuscript for convenience.) The buffer for all reactions was 66 mM Tris–HCl (pH 7.4), and all of the reactions were carried out at room temperature. Substrates were prepared in DMSO as 20 mM stocks and added to the reaction mixtures to give final concentrations of 1 μM with phenoxazones, 50 μM with coumarins, and 100 μM with quinolines. The 7-ethoxy form of each type of substrate was tested for linearity with protein content, optimal pH and temperature, using sham-injected control trout microsomes. The microsomal protein content was determined using the Bio-Rad Protein Reagent [30] with bovine serum albumin as the standard. Microsomal protein content of all reactions ranged from 73.4 to 299.8 μg per reaction, which was within the linear range for the reactions. NADPH was added to the reactions to initiate metabolism of the substrates. All fluorescence assays were performed on at least three and up to seven separate pools of two microsomal preparations from individual animals.

7-Alkoxyphenoxazones were measured using a 1 mM final NADPH concentration, an excitation wavelength of 510 nm, an emission wavelength of 586 nm, and a slit width of 10 nm for both excitation and emission. Data were collected at 1.0-sec intervals over 80 sec with a response time of 2.0 sec. The internal standard consisted of 10 pmol of resorufin. The wavelengths and final NADPH concentrations used were based on previous ethoxyphenoxazone assays performed with rainbow trout microsomes [29] and were different from those used previously with rat microsomes [28].

7-Alkoxycoumarins were measured using a 0.2 mM final NADPH concentration, an excitation wavelength of 380 nm, an emission wavelength of 460 nm, and a slit width of 5 nm for both excitation and emission. Data were collected at 1.0-sec intervals over 120 sec with a response time of 2.0 sec. The internal standard consisted of 400 pmol of umbelliferone. The wavelengths and final NADPH concentrations used were based on previous ethoxycoumarin assays done with rainbow trout microsomes [29] and were different from those used previously with rat microsomes [28].

7-Alkoxyquinolines were measured using a 0.5 nM final NADPH concentration, an excitation wavelength of 510 nm, an emission wavelength of 410 nm as described previously [28], and a slit width of 10 nm for both excitation and emission. Data were collected at 1.0-sec intervals over 120 sec with a response time of 2.0 sec. The internal standard consisted of 25 pmol of 7-quinolinol.

Total cytochrome P450 and P450-substrate binding studies

Total cytochrome P450 content [31] was estimated as previously described [26]. The binding of the fluorescent substrates to microsomal cytochrome P450 was determined as previously described [28] except that a 66 mM Tris–HCl (pH 7.4) buffer was used. The final concentrations of the substrate were 8 and 80 μM, and the microsomal protein concentration was 2 mg/mL. Substrate-binding studies and total cytochrome P450 measurements were performed using an SLM-Aminco (Urbana, IL) DW2000 spectrophotometer.

RESULTS

Cytochrome P450 levels and substrate binding spectra

Cytochrome P450 concentrations are shown in Table 1. Only the β-NF treatment produced a significant increase in P450 concentration. While treatment with ISF appeared to decrease cytochrome P450 levels (about 50% of the sham-injected control value), these values were not significantly different from control values due to individual variability.

Cytochrome P450 binding spectra were obtained with control microsomes and various 7-substituted substrates. Aniline and dexamethasone were included to provide examples of known Type II and Modified Type II binding spectra, respectively. The majority of the 7-substituted substrates bound to control microsomes in a manner similar to Modified Type II binding (Table 2).

Structure–activity relationships and induction of hepatic microsomal MO activity

Figures 1–3 portray the structure–activity relationships for the series of 7-alkoxyphenoxazones, 7-alkoxycoumarins and 7-alkoxyguinolines, respectively, for sham-injected controls, DMFA controls, or after induction with β-NF, PCN, PB, ISF, or DEX for 48 hr. Each graph represents the relationship of metabolism of rainbow trout hepatic microsomes to the length of the ether side-chain for each type of substrate. The type of inducing agent and the substrate used result in different patterns of metabolic activity. It is evident that not all members of any homologous series are metabolized at the same rate by trout hepatic microsomal preparations. The cytochrome P450 inducers PCN and PB did not produce any significant increases in the metabolism of any of the fluorescent substrates examined. Table 3 presents information regarding the ability of the different substrates to discriminate between ISF, β-

Table 1. Cytochrome P450 concentrations in hepatic rainbow trout microsomes after treatment with various inducers

Treatment	Cytochrome P450 (nmol P450/mg microsomal protein)
Sham-injected controls	0.354 ± 0.035 (N = 6)
DMFA vehicle controls	0.380 ± 0.048 (N = 7)
β-NF	1.253 ± 0.108* (N = 4)
PCN	0.362 ± 0.068 (N = 5)
PB	0.323 ± 0.076 (N = 5)
ISF	0.171 ± 0.041 (N = 5)
DEX	0.377 ± 0.045 (N = 3)

Values (means ± SEM) are from the indicated number of pools of two individual liver microsomal preparations.

* Significantly different from control values by one-way ANOVA and one-tailed Dunnett's *t*-test $P < 0.05$.

Table 2. Cytochrome P450 binding spectra of non-treated trout hepatic microsomes

Substrate	Binding spectra: Trough minimum (nm)	Binding spectra: Peak maximum (nm)	Type
Aniline	394	432	II*
Dexamethasone	398	420	Mod. II†
7-Ethoxyphenoxazone	410	475	–‡
7 Methoxycoumarin	389	417	Mod. II
7-Ethoxycoumarin	480	380	–
7-Propoxycoumarin	410	440	Mod. II
7-Benzyloxycoumarin	370	470	–
7-Methoxyquinoline	370	470	–
7-Ethoxyquinoline	390	490	–
7-Propoxyquinoline	424	403	–
7-Butoxyquinoline	400	460	–
7-Pentoxyquinoline	420	460	–
7-Benzyloxyquinoline	350	421	–

* Type II: trough, 390 nm; peak 430 nm.

† Modified Type II: trough, 365–410 nm; peak, 409–445 nm.

‡ Resembles Modified Type II: includes larger trough and peak range.

NF and DEX as inducers. It is apparent that certain derivatives of the different substrate classes can act as discriminators between different types of inducers (see below). Because the activity of control (sham-injected control and DMFA vehicle control) microsomal preparations was generally quite low, the O-dealkylation rates of the controls were placed in a separate table (Table 4) for easy comparison.

Phenoxazones. The rates of phonoxazone metabolism were greater after 48-hr ISF-treatment than after β-NF-treatment for all phenoxazone homologues except 7-butoxyphenoxazone (Fig. 1, Table 3). DEX treatment resulted in a significant elevation (about 144-fold) in the metabolism of only 7-pentoxyphenoxazone (Fig. 1, Table 3). 7-Benzyloxyphenoxazone had the highest discrimination factor between ISF and β-NF induction (Table 3), while 7-methoxyphenoxazone showed the highest discrimination between ISF and DEX or β-NF and DEX (Table 3). The alkoxyphenoxazones were metabolized by ISF-treated microsomes in the following order: methoxy > ethoxy > propoxy > benzyloxy > butoxy > pentoxy (Fig. 1). When β-NF-treated microsomes were used, the 7-alkoxy-

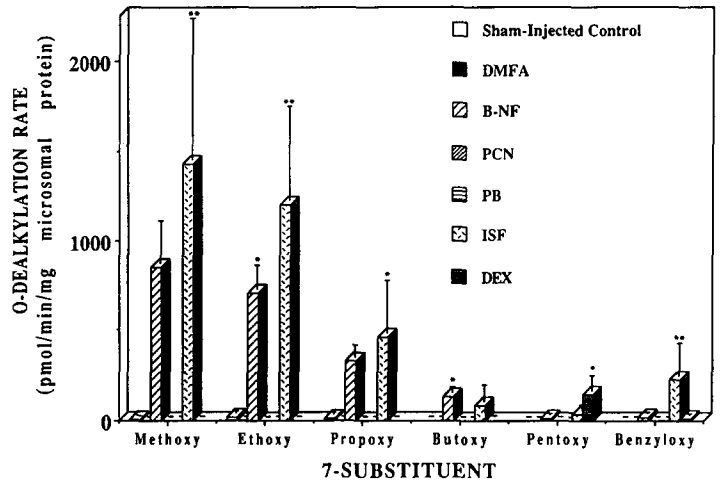

Fig. 1. O-Dealkylation of 7-alkoxyphenoxazones (7-alkoxyresorufins) by rainbow trout hepatic microsomes after treatment with various inducers. Columns and error bars represent the mean ± SEM for the indicated number of pools of two individual microsomal preparations (N = 5). Asterisks indicate values significantly different from both sham-injected control and DMFA vehicle control values by one-way ANOVA and one-tailed Dunnett's *t*-test: (*) $P \leq 0.05$, and (**) $P \leq 0.01$.

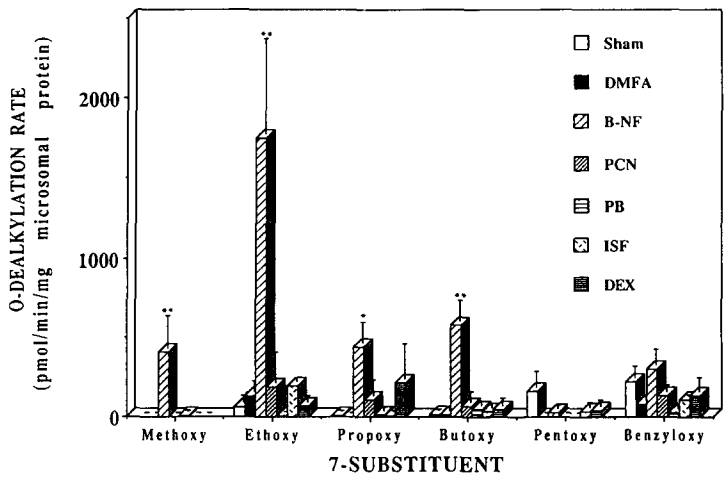

Fig. 2. O-Dealkylation of 7-alkoxycoumarins by rainbow trout hepatic microsomes after treatment with various inducers. Columns and error bars represent the mean ± SEM for the indicated number of pools of two individual microsomal preparations (N = 5). Asterisks indicate values significantly different from both sham-injected control and DMFA vehicle control values by one-way ANOVA and one-tailed Dunnett's *t*-test: (*) $P \leq 0.05$, and (**) $P \leq 0.01$.

phenoxazones were metabolized in the following order: methoxy > ethoxy > propoxy > butoxy > benzyloxy ≈ pentoxy. Other inducers did not produce significant differences on microsomal metabolic activity toward any of the phenoxazone homologues.

Microsomal O-dealkylation rates of the phenoxazones by control microsomes (sham-injected and DMFA-treated) were very low for all of the homologues (Fig. 1 and Table 4). Activity was highest for the ethoxy- (18.2 pmol/min/mg microsomal protein) and propoxy- (14.1 pmol/min/mg microsomal protein) derivatives and lowest for the pentoxy (no activity detected) derivative for the sham-injected controls (Table 4). While there appeared to be a trend for decreased enzyme activity towards the phenoxazones in the DMFA-treated microsomes compared with the sham-injected control microsomes, the differences were not significant, and, in addition, there was no discernible trend with either the coumarins or the quinolines. These results taken together suggest that DMFA treatment did not adversely affect enzyme activity with any of the substrate classes. While these control rates of

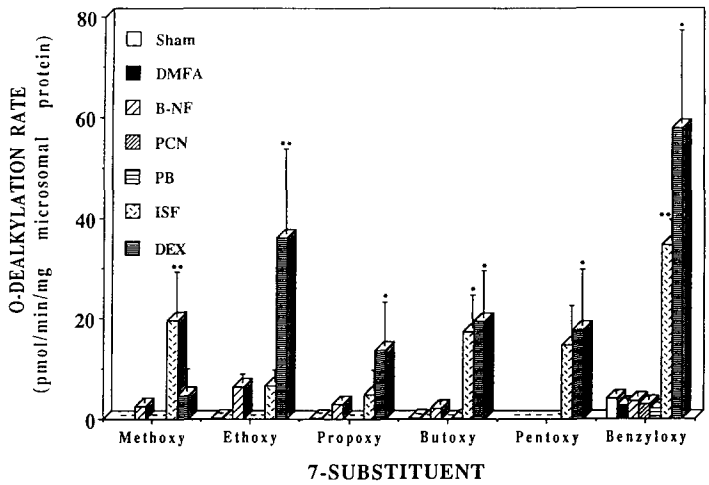

Fig. 3. O-Dealkylation of 7-alkoxyquinolines by rainbow trout hepatic microsomes after treatment with various inducers. Columns and error bars represent the mean ± SEM for the indicated number of pools of two individual microsomal preparations (N = 5). Asterisks indicate values significantly different from both sham-injected control and DMFA vehicle control values by one-way ANOVA and one-tailed Dunnett's *t*-test: (*) $P \leq 0.05$, and (**) $P \leq 0.01$.

Table 3. Induction of microsomal O-dealkylation of 7-alkoxyphenoxazones, 7-alkoxycoumarins and 7-alkoxyquinolines by isosafrole (ISF), β-naphthoflavone (β-NF) or dexamethasone (DEX) and discrimination between inducers

Substrate	Fold induction*			ISF vs β-NF discrimination factor†	ISF vs DEX discrimination factor	βNF vs DEX discrimination factor
	ISF	β-NF	DEX			
7-Methoxyphenoxazone	289.5	172.7	0.2	1.7	1447.5	863.5
7-Ethoxyphenoxazone	66.3	38.8	0.1	1.7	663.0	388.0
7-Propoxyphenoxazone	32.7	23.2	0.1	1.4	327.0	232.0
7-Butoxyphenoxazone	91.1	140.4	1.6	1.5	56.9	87.8
7-Pentoxyphenoxazone	36.8	11.2	143.7	3.3	4.0	12.8
7-Benzyloxyphenoxazone	194.8	17.4	10.8	11.2	18.0	1.6
7-Methoxycoumarin	1.0	403.0	1.0	403.0	1.0	403.0
7-Ethoxycoumarin	3.4	30.7	1.1	9.0	3.1	27.9
7-Propoxycoumarin	0.9	52.9	26.0	58.8	28.9	2.0
7-Butoxycoumarin	2.9	49.1	4.1	16.9	1.4	12.0
7-Pentoxycoumarin	0.2	0.1	0.3	2.0	1.3	3.0
7-Benzyloxycoumarin	0.5	1.3	0.6	2.7	1.2	2.2
7-Methoxyquinoline	19.6	2.6	4.7	7.5	4.2	1.8
7-Ethoxyquinoline	28.2	27.6	154.9	1.0	5.5	5.6
7-Propoxyquinoline	20.3	12.6	56.6	1.6	2.8	4.5
7-Butoxyquinoline	105.6	13.1	118.3	8.1	1.1	9.0
7-Pentoxyquinoline	14.6	1.0	17.7	14.6	1.2	17.7
7-Benzyloxyquinoline	8.8	0.9	14.6	9.8	1.7	16.2

* Values were calculated from data represented in Figs. 1–3.

† The discrimination factor compares induction for each substrate as the greater fold-induction of one inducer divided by the lesser fold-induction of the other inducer. A discrimination factor of 1 indicates that there is no difference between treatment groups in the rate of metabolism for a particular substrate.

metabolism are quite low, it is possible that the enzyme reponsible for the constitutive metabolism of these derivatives is inducible by either β-NF or ISF. This hypothesis is supported by immunodetection data, which indicated that CYP1A1 isozyme was induced about 5-fold by β-NF treatment (48 hr, i.p., data not shown).

Coumarins. The metabolism rates of the coumarins were greatest after β-NF treatment compared with those of the other inducers (Fig. 2). Using microsomes from β-NF-treated trout, the extent of metabolism of the 7-alkoxycoumarins was in the following order: ethoxy ≫ butoxy > propoxy ≈ methoxy > benzyloxy > pentoxy. While DEX

Table 4. Control (sham-injected or DMFA-treated) O-dealkylation rates of rainbow trout hepatic microsomes representing potential constitutive metabolism

	O-Dealkylation rate (pmol/min/mg microsomal protein)					
	Phenoxazones		Coumarins		Quinolines	
No. of carbons	Sham (N = 3)	DMFA (N = 4)	Sham (N = 5)	DMFA (N = 7)	Sham (N = 3)	DMFA (N = 4)
1	4.936	**1.017**	0.000	0.000	0.000	0.000
	(2.703)	**(0.788)**	(0.000)	(0.000)	(0.000)	(0.000)
2	**18.152**	0.007	**56.982**	**133.281**	0.233	0.000
	(9.942)	(0.005)	**(48.989)**	**(66.627)**	(0.127)	(0.000)
3	**14.133**	0.000	8.292	0.000	0.242	0.000
	(7.741)	(0.000)	(8.292)	(0.000)	(0.133)	(0.000)
4	0.944	**1.017**	11.815	15.445	0.163	0.000
	(0.517)	**(0.788)**	(8.189)	(15.445)	(0.045)	(0.000)
5	0.000	0.000	**161.149***	0.000	0.000	0.000
	(0.000)	(0.000)	**(101.149)**	(0.000)	(0.000)	(0.000)
7	1.176	0.107	**220.081**	**81.774**	**3.957**	**2.867**
	(0.644)	(0.082)	**(72.447)**	**(21.960)**	**(0.711)**	**(0.239)**

Values are given as means (± SEM), and the highest O-dealkylation rates for each treatment group of each substrate series are in bold face type. Values for the number of samples (e.g. N = 3) represent the number of pools of two microsomal preparations from individual animals, which were used to determine the experimental values.

* Significant difference between sham-injected control values and corresponding DMFA-treated control values by one-way ANOVA and one-tailed Dunnett's *t*-test, $P \leq 0.05$.

treatment resulted in an apparent 26-fold increase in the metabolism of propoxycoumarin compared with that of the sham-injected controls, statistically this increase was not significant (Fig. 2). The rates of metabolism of pentoxycoumarin were lower in all inducer-treated microsomes than in the controls.

Discrimination factors were determined for comparison between different inducer treatment groups. A discrimination factor is defined as the greater fold-induction of one inducer divided by the lesser fold-induction of the other inducer. A discrimination factor of 1 indicates that there is no difference between inducers with regard to the metabolism rate for a particular substrate. The highest discrimination factor (403; Table 3) between β-NF and ISF and between β-NF and DEX occurred with methoxycoumarin for which only β-NF produced an appreciable increase in metabolism. For discrimination between ISF and DEX induction, the substrate propoxycoumarin produced the highest discrimination factor (28.9; Table 3). These numbers are based on low, 0.2- to 3.4-fold increases for ISF and 0.3- to 26-fold increases for DEX, while β-NF induction resulted in increases of 0.1- to 403-fold. These results suggest that distinction between ISF-type and β-NF-type inducers in rainbow trout hepatic microsomal preparations may best be made using 7-methoxycoumarin (Table 3).

Sham-injected control metabolic activity against pentoxycoumarin was relatively high (Table 4), possibly indicating constitutive metabolism that was not induced by any of the classes of inducers used in this experiment.

Quinolines. As a group, the alkoxyquinolines, except for methoxyquinoline, were the best substrates for detection of induction by DEX (Fig. 3). Significant increases in O-dealkylation rates due to DEX induction occurred for all of the quinoline derivatives; the order of metabolism was benzyloxy- > ethoxy- > butoxy- ≈ pentoxy- ≈ propoxyquinoline. Induction by ISF produced significant increases in the metabolism of methoxy-, butoxy- and benzyloxyquinoline relative to control metabolism. The greatest induction due to DEX occurred for ethoxy- (155-fold) and butoxyquinoline (118-fold; Table 3); the latter also showed the greatest increase (105-fold) in metabolism after ISF induction.

The best discrimination between β-NF and DEX induction occurred with either pentoxy- or benzyloxyquinoline, while none of the quinolines markedly discriminated between ISF and DEX (Table 3). The highest discrimination factor between ISF and β-NF (14.6) occurred with pentoxyquinoline (Table 3). None of these discrimination factors were high enough to instill confidence in the ability to distinguish between the various inducers.

The only quinoline that was metabolized by control microsomes was benzyloxyquinoline. Metabolic rates were 4.0 and 2.9 pmol/min/mg microsomal protein for sham-injected and DMFA vehicle controls, respectively (Fig. 3, Table 4). While metabolic activity toward the other quinolines was nearly non-existent in control microsomes, results with benzyloxyquinoline suggest the presence of a basal NADPH-dependent metabolism that was inducible by either ISF or DEX (Fig. 3).

DISCUSSION

Until recently, 3-MC-type inducers were the only inducers of rainbow trout hepatic microsomal MO activity that could be assessed by fluorescent

substrate assays. There exist other types of inducers of Phase I metabolism, which have been characterized, historically, by the induction of a different spectrum of increased metabolic reactions for each class of inducers. This spectrum of activities is due to the participation of different cytochrome P450 isozymes. Generally, each cytochrome P450 isozyme exhibits broad and overlapping substrate specificity, but the spectrum of metabolic activities that may be induced can be distinctive. These potentially distinctive patterns of metabolism can be used to accurately identify and quantitate isozymes. The identification of these distinctive patterns of metabolism can be important for our understanding of the mechanisms of induction, as well as the categorization of novel inducers.

For many years it has been known that the extent or mode of substrate binding to cytochrome P450 could be correlated with kinetic data for substrate metabolism [32]. Various types of difference spectra have been characterized, including a Type I spectral change with a peak at about 385 nm and a trough at about 420 nm; a reverse Type I that is the mirror image of the Type I; and a Type II spectral change with a broad trough between 390 and 410 nm and a peak varying between 425 and 435 nm [32]. These spectra have additionally been described as Type II with a trough at 390 nm and a peak at 430 nm and Modified Type II with a trough between 365 and 410 nm and a peak between 409 and 445 nm [33]. These spectral categories correspond to the types of ligand interaction with the cytochrome P450 isozyme. Type I compounds are almost invariably good substrates, and Type II compounds are usually poor substrates that can inhibit the activity of Type I substrates [32]. Because of the potential contribution of multiple forms of cytochrome P450 in control microsomes, due to the presence of constitutive isozymes, it is possible that the addition of a compound may produce essentially no spectral response (due to a mixture of different spectral changes of different isozymes to the same compound) [32]. This type of mixed activity may account for the majority of substrates eliciting a similar spectra to a Modified Type II response with trout hepatic microsomes.

The results of the structure–activity studies indicate that at least some members among the phenoxazones, coumarins or quinolines are metabolized to a certain extent by control microsomes, and that this metabolism in some cases can apparently be induced by β-NF, ISF or DEX. After β-NF induction, the coumarins as a group were better substrates than either the phenoxazones or the quinolines. This was largely due to the metabolism of methoxycoumarin. However, the phenoxazones were also metabolized quite readily. As a group, the phenoxazones were metabolized more readily after ISF induction and the quinolines were metabolized more readily after DEX induction. Within groups, after β-NF induction, methoxy- and butoxyphenoxazone produced the largest relative increases in metabolism. These differences in reactivity among the different substrates may provide insight into the active sites of the cytochrome P450 isozymes involved in their metabolism.

While significant increases in the metabolism of the alkoxyquinolines as a group occur after DEX induction, no single alkoxyquinoline produces high discrimination of DEX induction versus induction by β-NF or ISF. Therefore, no single alkoxyquinoline will be a substrate of choice for discriminating between different inducers in rainbow trout. Alternatively, the combined use of the alkoxyquinoline fluorescent substrates, such as the use of ethoxyquinoline, which indicates a stronger DEX induction compared with β-NF or ISF, and either butoxy- or pentoxyquinoline, which indicates a similar DEX, β-NF and ISF induction, may be able to provide an indication of the type of inducer present in a situation where induction is occurring but the class of inducer is unknown.

The utility of some of these new fluorescent substrates is demonstrated by the high degree of induction of microsomal metabolism that occurred after only 48 hr of treatment. The largest fold-induction (403-fold) after β-NF treatment occurred in the metabolism of 7-methoxycoumarin. This is in contrast to the rat in which the largest 48-hr induction (70-fold) occurred in the metabolism of propoxyphenoxazone followed by ethoxy- (58-fold) and butoxyphenoxazone (41-fold) [28]. After ISF treatment of trout, a 290-fold induction occurred in the metabolism of 7-methoxyphenoxazone, while after DEX treatment the largest induction of metabolism occurred for 7-ethoxyquinoline (155-fold) and 7-pentoxyphenoxazone (144-fold). Because 7-pentoxyphenoxazone is currently commercially available (Pierce: Rockford, IL; Sigma: St. Lous, MO; and Molecular Probes: Eugene, OR), it may become the substrate of choice for monitoring DEX-type induction of rainbow trout hepatic microsomal preparations, but because the discrimination factors are low, 7-pentoxyphenoxazone will not be useful for distinction between inducers.

The induction of hepatic cytochrome P450-mediated alkoxyphenoxazone metabolism by prototype inducers is highly species dependent [34, 35]. For example, the metabolism of methoxy-, ethoxy- and propoxyphenoxazone was increased in the rat, mouse, golden Syrian hamster, rabbit, Mongolian gerbil and Japanese quail after 3-MC induction [34]. In contrast, the metabolism by PB-induced microsomes was highest in the rat, mouse and rabbit, intermediate in the gerbil, and low in the hamster and quail [34]; it was found not to be inducible in the cotton rat [35]. Rainbow trout, like the hamster and quail, have been found previously to be refractive to PB-type induction ([7], reviewed in Ref. 22), and the results of this study support those findings.

It has been shown in rats that β-NF treatment results in the induction of both P450 1A1 and 1A2 and that ISF treatment selectively induces 1A2 [36]. It has also been shown that after β-NF treatment of rats, liver microsomes have a markedly increased rate of metabolism of 7-methoxy- and 7-ethoxycoumarin, whereas ISF treatment results in increased metabolism of 7-ethoxycoumarin only, suggesting that the activity against 7-methoxycoumarin is mediated primarily by P450 1A1 [37]. The results of the present investigation, in which the metabolism of 7-methoxy- and 7-ethoxycoumarin was increased only

with β-NF induction and not with ISF induction, suggest that either trout do not have an isozyme that is orthologous to rat P450 1A2 or that the isozyme is not inducible in trout. Conversely, it has been shown that in rat extrahepatic tissues, such as the intestine but not the kidney or lung, the metabolism of 7-methoxycoumarin is a sensitive and specific probe for induction of P450 2B1 activity and that the metabolism of 7-methoxycoumarin is mediated by constitutive cytochrome P450 forms of the liver, lung, small intestine and kidney, in that order [38]. Our results in trout, in which hepatic 7-methoxycoumarin metabolism was very low in control microsomes but was markedly induced by β-NF, suggest that the isozyme(s) responsible for 7-methoxycoumarin metabolism in trout is either not orthologous to the rat isozymes or is regulated differently.

In human placenta, the metabolism of all of the 7-alkoxyquinoline derivatives is induced by maternal cigarette smoking [39]. The authors suggested that the metabolism of the 7-alkoxyquinolines was mediated by CYP1A1 but concluded that none of the 7-alkoxyquinoline derivatives were P450 isozyme specific in human tissues. In addition, 3-MC-induced rat microsomes have been shown to metabolize 7-ethoxy- and 7-propoxyquinoline, suggesting a role for CYP1A1 in alkoxyquinoline metabolism [28]. Our evidence with rainbow trout microsomes indicates that the alkoxyquinolines are metabolized by an isozyme that is induced by DEX and ISF, but not by β-NF (CYP1A1 inducer in trout). Taken together, these results may indicate that trout have a DEX- and ISF-inducible isozyme that is uniquely regulated compared with mammalian cytochrome P450 isozymes. Definitive identification of this isozyme will require DNA sequence information [6].

In trout, relatively high rates of constitutive metabolism were observed for ethoxy- and propoxyphenoxazone, for ethoxy-, pentoxy- and benzyloxycoumarin, and for benzyloxyquinoline. Since the constitutive metabolism of ethoxy- and propoxyphenoxazone was induced similarly by both β-NF and ISF, it is possible that these two substrates are metabolized by the same isozyme. In contrast, using the same reasoning, it is possible that ISF induced a unique P450 isozyme that is not induced by β-NF and that is capable of metabolizing benzyloxyphenoxazone. Similarly, it is possible that DEX induced a unique isozyme that is not induced by either ISF or β-NF.

In regard to the alkoxycoumarins, it appears that the isozyme induced by β-NF is not the same as the isozyme responsible for the constitutive metabolic activity towards pentoxy- and benzyloxycoumarin. Similarly, the metabolism of the alkoxyquinolines also provides some interesting clues as to the constitutive and inducible cytochrome P450 isozymes present in rainbow trout. The isozyme responsible for the constitutive metabolism of benzyloxyquinoline may be the same as the DEX- and ISF-inducible isozyme (and to a minor extent β-NF-inducible) since the metabolism of benzyloxy-, pentoxy-, butoxy-, propoxy- and perhaps ethoxyquinoline was induced similarly by both inducers for each of these substrates. In contrast, since the metabolism of methoxyquinoline after ISF induction was much greater than the metabolism after DEX induction, it is possible that these inducers enhance the expression of two different isozymes. It is also possible that the presence of one or more inducible isozymes in the microsomal membrane may affect the metabolic activity of other isozymes (either constitutive or induced) in the vicinity by selective modulation [40]. Monitoring the various metabolic activities after concurrent induction with one or more types of inducers may provide insight into this possible selective modulation. This type of information about the induction of possible unique cytochrome P450 isozymes by DEX and ISF could provide the basis for the purification, identification, and characterization of new P450 families in rainbow trout. Coordinately, the monitoring of cytochrome P450 isozyme purification with these new fluorescent substrates may provide insight into the lability of different isozymes and the content of different isozymes during each step of the purification process.

The goal of the present investigation was to identify new fluorescent substrates that can be used to assess cytochrome P450 induction in rainbow trout hepatic microsomes. In this regard, the results of the structure–activity relationships and the derived discrimination factors reveal that distinction between ISF- and β-NF-type inducers can be made using 7-methoxycoumarin, while distinction between ISF- and DEX-type inducers can be made using 7-methoxy-, 7-ethoxy- and 7-propoxyphenoxazone (in that order). Similarly, distinction between β-NF- and DEX-type inducers can be made using 7-methoxyphenoxazone, 7-methoxycoumarin and 7-ethoxy or 7-propoxyphenoxazone (in that order). These results indicate that the class of an unknown inducer may be assessed by using a battery of fluorescent substrates. The accuracy of the resulting classification will be dependent on the number of substrates used and will be enhanced by the advent of additional substrates. This latter factor may be particularly important for PB- or PCN-type inducers, since induction by these classes of compounds was not detected in rainbow trout microsomes by any of the phenoxazones, coumarins or quinolines tested.

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