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ACCELERATED COMMUNICATION

Clotrimazole Induction of Cytochrome P-450: Dose-Differentiated Isozyme Induction

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SUMMARY

Treatment of male rats for 3 days with the *N*-substituted imidazole, clotrimazole, produced up to a 4-fold induction of hepatic microsomal cytochrome P-450. The monooxygenase activities induced varied with the dose administered. At low doses (<25 mg/kg), *p*-nitroanisole demethylase and aniline hydroxylase activities were induced. Only at higher doses were other monooxygenase activities (erythromycin and ethylmorphine demethylases and cytochrome P-450 metabolic-intermediate complex formation from troleandomycin) induced. Microsomal UDP-glucuronosyltransferase activity toward morphine was induced at low doses in a manner similar to that of *p*-nitroanisole demethylase. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of microsomes indicated that low doses of clotrimazole caused the intensification of a 48,000 molecular weight protein band, whereas at high doses, there was a marked intensification of an additional 50,500 molecular weight protein, the same molecular weight band as was intensified in phenobarbital- and dexamethasone-induced microsomes. The observations suggest a phenomenon of "dose-differentiated" isozyme induction for cytochrome P-450.

For convenience, inducers of hepatic drug-metabolizing enzymes were often classed into one of two groups, phenobarbital- or polycyclic hydrocarbon-like. Some agents, such as polyhalogenated biphenyls, appear to have the inducing properties of both groups (1–3). The "mixed" induction was originally thought to be due to different biphenyl isomers each with either phenobarbital- or polycyclic hydrocarbon-inducing properties (4, 5). However, subsequent investigations showed that a single isomer can have both properties (6, 7). Other inducers do not readily fit either classification and other classes of agents (e.g., certain steroids such as dexamethasone and PCN) have since been identified (8). In addition, isosafrole (9), clofibrate (10), and isoniazid (11) among others are able to selectively induce forms of cytochrome P-450 that are not induced, or are only induced in minor amounts, by phenobarbital- or polycyclic hydrocarbon-like agents.

The majority of induction investigations are studied with doses and regimens that maximize induction. Induction so investigated shows bidirectional degeneracy; many isozymes are induced by a single agent and a single isozyme may be induced by many inducers (8, 12). Where many isozymes are induced by a single agent, a facet that appears to have been overlooked is that several different induction mechanisms may exist and that different concentrations of inducer may be required to elicit them.

This paper provides evidence of dose-differentiated isozyme induction; low doses of a single agent (clotrimazole) induce one set of enzyme activities, higher doses induce an additional set. With the *N*-substituted imidazole given at low doses (<25 mg/kg), an isozyme or isozymes efficient at *p*-nitroanisole demethylation and aniline hydroxylation is (are) induced. At higher doses, an isozyme or isozymes capable of erythromycin and ethylmorphine demethylation and metabolic-intermediate complex formation from troleandomycin is (are) induced.

Materials and Methods

Clotrimazole and the biochemicals were obtained from Sigma Chemical Co. (the melting point of the clotrimazole was checked at 142–144°). Erythromycin was a gift from Abbott Laboratories and troleandomycin from Pfizer Inc.

Following treatment with various doses of clotrimazole in 30% polyethylene glycol intragastrically for 3 days, adult male Sprague-Dawley rats were killed by decapitation 48 hr after the last dose, and liver microsomal fractions were prepared using established conditions (13). Protein concentrations were determined by the method of Lowry *et al.* (14).

The microsomal cytochrome P-450 concentration, metyrapone binding, and extent of metabolic-intermediate complex formation from troleandomycin *in vitro* were quantitated using extinction coefficients of 91 mM^{-1} cm^{-1} (15), 68.5 mM^{-1} cm^{-1} (16), and 64 mM^{-1} cm^{-1} (17), respectively. The maximum extent of metabolic-intermediate complex formation was determined by dual wavelength spectroscopy (456 versus 490 nm) in intermittently oxygenated NADPH-supplemented micro-

ABBREVIATIONS: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PCN, pregnenolone 16α-carbonitrile.

somal incubations containing 0.133 mM troleandomycin. Ethylmorphine and erythromycin demethylations were determined from the formaldehyde produced (18) in incubations where the substrate concentrations were 8 mM and 1 mM, respectively. *p*-Nitroanisole demethylase was determined from the *p*-nitrophenol produced (19). Aniline hydroxylase activity was determined using a modified method of Mieyal and Blumer (20) and the rate of color development was enhanced by oxygen gassing of the alkaline phenol solution. Microsomal UDP-glucuronosyltransferase activities were determined using reverse phase high performance liquid chromatography to determine the glucuronides formed (21). PAGE in the presence of 0.1% SDS was performed using conditions previously described (22).

Results

Clotrimazole administered at doses up to 90 mg/kg for 3 days induced cytochrome P-450, but the induction was disproportionate to the dose (Fig. 1). With doses of 75 or 90 mg/kg, the cytochrome P-450 concentration was 4 times that of untreated animals. Metyrapone binding to ferrous cytochrome P-450 increased with the increasing cytochrome concentration, showing that the induced form(s) bind(s) metyrapone. In addition, at the highest level of induction almost all (95%) of the cytochrome bound metyrapone, whereas in untreated animals, the extent of binding was 25%. Thus, the forms present in the uninduced animal that do not bind metyrapone (0.7 nmol/mg) are gradually lost as the induction progresses, since only 0.2 nmol/mg is unable to bind metyrapone at the highest induction state.

With only small increases in cytochrome P-450 at lower doses (<25 mg/kg), there was a large increase in *p*-nitroanisole demethylase activity (Fig. 2). There was little or no additional *p*-nitroanisole demethylase activity seen with high doses over that seen at low doses, indicating that the majority of the cytochrome P-450 present after high doses was not efficient at catalyzing this reaction. Aniline hydroxylase activity was also not induced to any greater extent by high doses over that seen at low doses. For this reaction, the activity decreased toward that found in untreated animals as the dose was increased from 25 to 90 mg/kg.

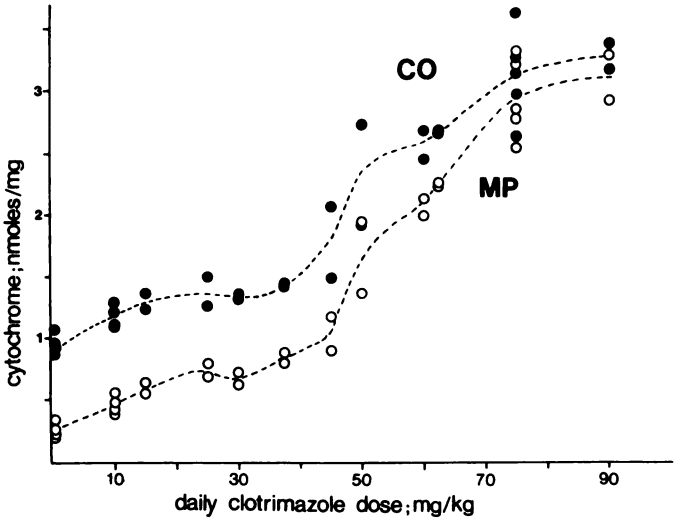

Fig. 1. The effect of clotrimazole dose on hepatic cytochrome P-450 concentration and metyrapone binding. Cytochrome P-450 (●) and metyrapone binding (○) concentrations were determined after CO gassing and metyrapone (100 μM) addition to dithionite-reduced microsomal fractions. Spectral complexes were determined by difference spectroscopy. Each point represents a microsomal sample from a single animal.

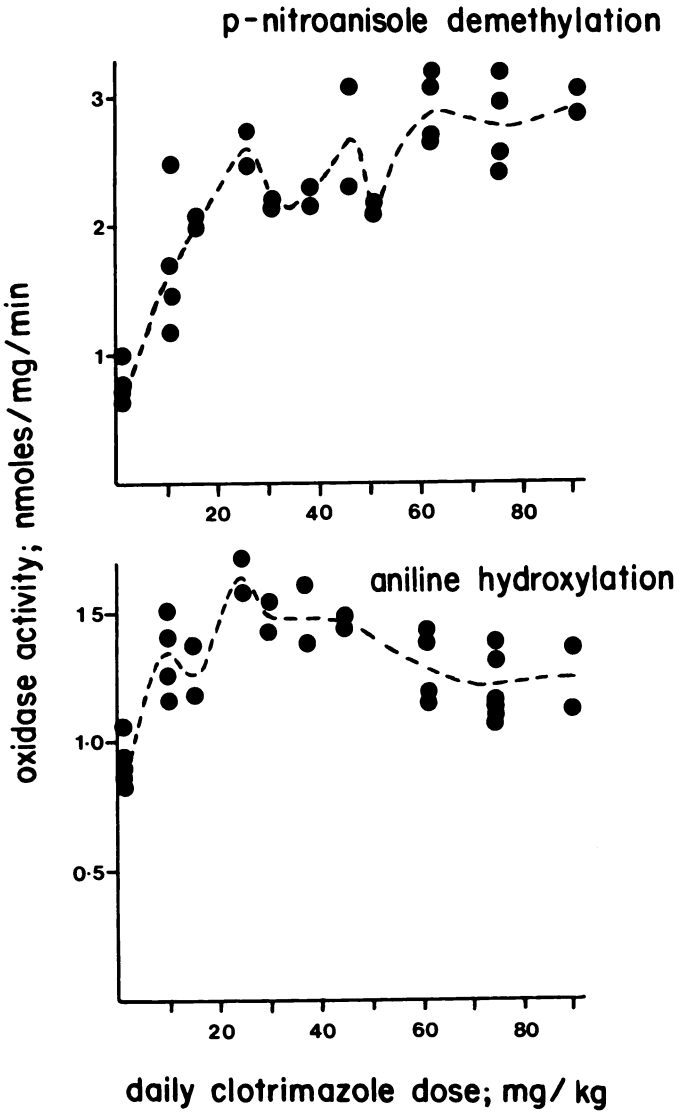

Fig. 2. The effect of clotrimazole dose on hepatic microsomal *p*-nitroanisole and aniline oxidations. *p*-Nitroanisole demethylation (*top*) and aniline hydroxylation (*bottom*) rates were determined in microsomes from rats treated for 3 days with various doses of clotrimazole. Each point represents a microsomal sample from a single animal.

Three monooxygenase activities paralleled the total (carbon monoxide detectable) cytochrome P-450 changes (Fig. 3), erythromycin and ethylmorphine demethylases, and cytochrome P-450-metabolic intermediate complex formation from troleandomycin. All three showed very little increase with clotrimazole doses of up to 25–35 mg/kg, but slight increases in dose above this produced dramatic enhancement of these reactions.

In addition to cytochrome P-450, microsomal UDP-glucuronosyltransferase enzymes are known to be readily induced by a variety of drugs and xenobiotics (23, 24). Clotrimazole treatment induced the transferase activity toward morphine but not toward 1-naphthol (Fig. 4). These reactions are catalysed by different isozymes. The induction of the morphine conjugation rate was large at low doses, much like *p*-nitroanisole demethylase and aniline hydroxylase activities. At higher doses (>50 mg/kg), there was a decline in the extent of induction, reminiscent of the changes seen with aniline hydroxylase activity.

SDS-PAGE of hepatic microsomes from animals treated with

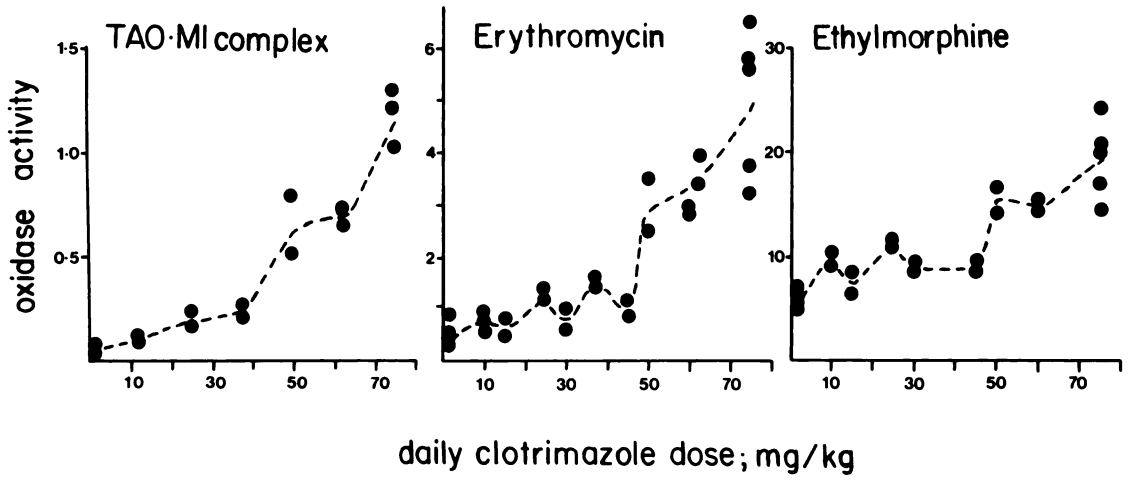

Fig. 3. The effect of clotrimazole dose on hepatic microsomal ethylmorphine, erythromycin, and troleandomycin metabolism. Ethylmorphine demethylation (*right*) erythromycin demethylation (*center*), and maximum extent of metabolic intermediate complex formation from troleandomycin (*TAO·MI complex, left*) were determined as described in Materials and Methods. Each point represents a microsomal sample from a single animal.

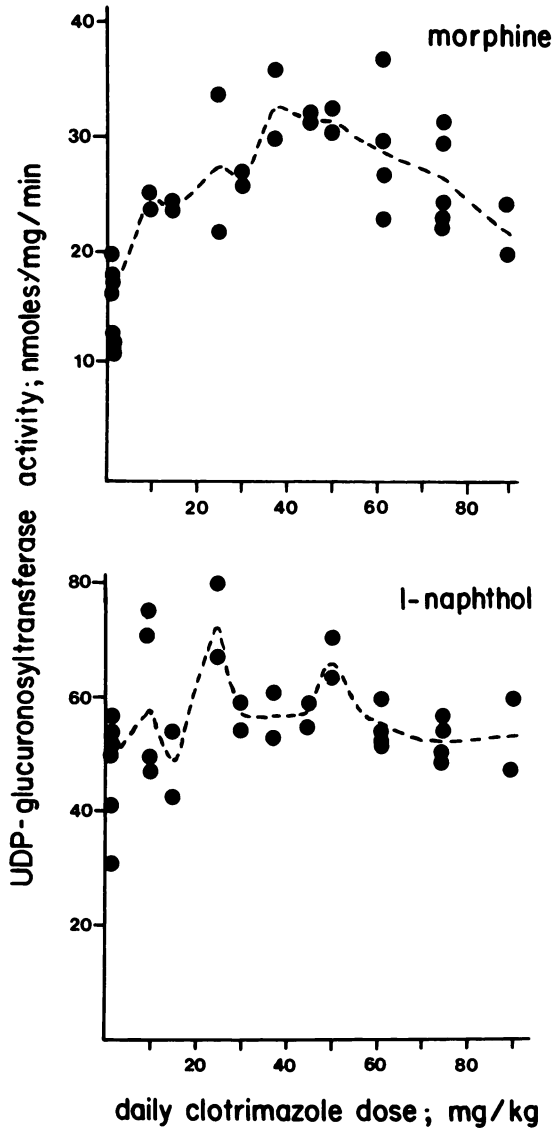

Fig. 4. The effect of clotrimazole dose on hepatic microsomal UDP-glucuronosyltransferase activity. UDP-glucuronosyltransferase activity toward morphine (*top*) and 1-naphthol (*bottom*) was performed as described in Materials and Methods. Each point represents a microsomal sample from a single animal.

high and low doses of clotrimazole are shown in Fig. 5. The microsomes from the rats treated with the low dose showed an intensification of a protein band with a molecular weight of 48,000 compared to microsomes from untreated rats. Microsomes from the animals treated with a high dose also showed this band but, in addition, showed a major intensification in the 50,500 molecular weight region. Microsomes from animals treated with dexamethasone and phenobarbital were also co-chromatographed for comparative purposes and showed induction of the higher molecular weight protein band, with dexamethasone showing more intensification than phenobarbital. The high molecular weight protein band present after high doses of clotrimazole approximates the high density band present after dexamethasone treatment. The lower molecular weight protein band was not intensified by phenobarbital or dexamethasone.

Discussion

Induction of hepatic enzymes by polycyclic hydrocarbons is believed to be mediated by a cytosolic receptor that translocates to the nucleus. For phenobarbital and other inducers, there is no convincing evidence for a similar high affinity receptor that could support such a mechanism, and several concepts, often involving endogenous inducer or regulator molecules for which steroids or steroid metabolites are prime candidates, have been suggested. Treatment of animals with the inducers, isosafrole, SKF 525-A, or troleandomycin also results in cytochrome P-450 inhibition by heme sequestration as a metabolic-intermediate complex, which could result in the accumulation of an endogenous inducer normally removed by oxidative metabolism. However, dexamethasone and PCN induce the same isozyme as troleandomycin but without forming a metabolic-intermediate complex, indicating that complex formation is not an essential component. It may be that any sustained inhibition can cause induction. Agents that inhibit cytochrome P-450 by destruction, such as allylisopropylacetamide, can also act as inducing agents (25). *N*-Substituted imidazoles are among the most potent cytochrome P-450 inhibitors known (26, 27) and a few reports of their inducing properties, generally reported as phenobarbital-like, have emerged (28–30). Induction of morphine but not naphthol UDP-glucuronosyltransferase activity in the present study would also support this classification.

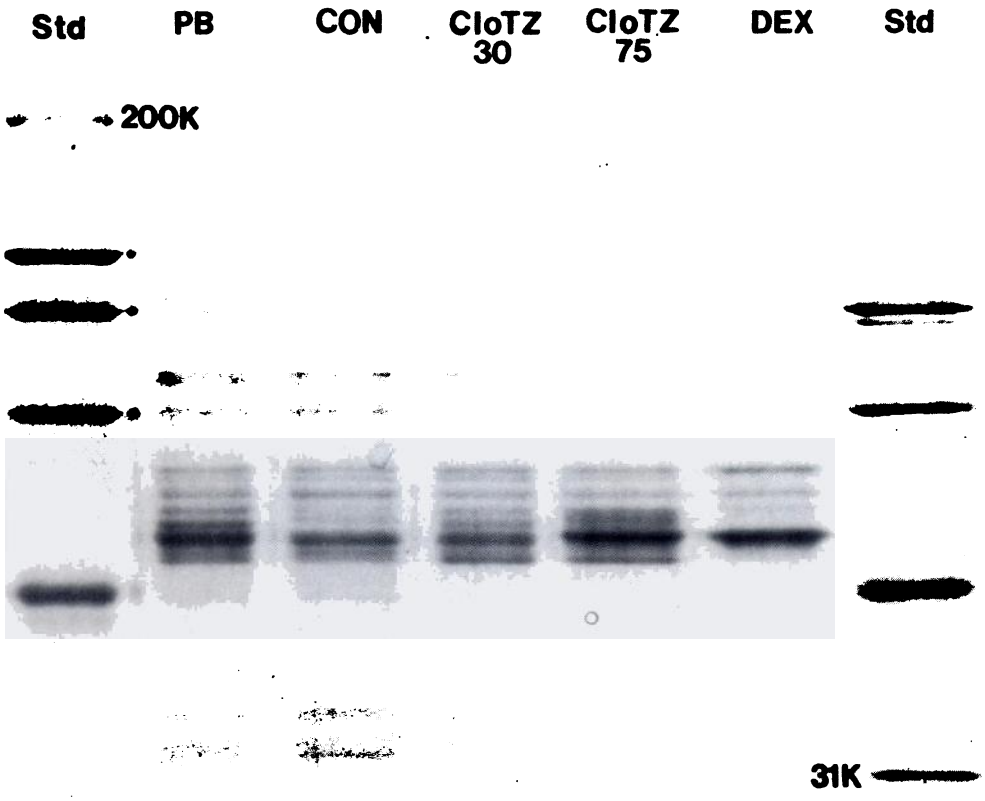

Fig. 5. SDS-PAGE of hepatic microsomes from rats treated with inducing agents. Microsomes from untreated (*CON*) rats and rats treated with phenobarbital (*PB*) (80 mg/kg, intraperitoneally for 3 days), dexamethasone (*DEX*) (100 mg/kg in Tween 80 intraperitoneally for 3 days), or clotrimazole (*CloTZ*) at doses of 30 or 75 mg/kg for 3 days were electrophoresed (25 μg of microsomal protein/track) together with protein standards (*Std*) of 45,000, 66,200, 116,300, and 200,000 MW (*left*) and 31,000, 45,000, 66,200, and 92,500 MW (*right*).

Rarely does induction of cytochrome P-450 result in induction of only one isozyme, although one may predominate. If more than one isozyme is induced, the possibility exists that more than one mechanism may be operating and there is no *a priori* reason why the different mechanisms that might be responsible for the induction of each isozyme should operate at, or be triggered by, the same inducer concentration. Dose-differentiated responses are not unknown in pharmacology. The results presented above demonstrate that different effects are observed at low and high doses of clotrimazole. Whether these are both direct effects or mediated through physiological changes caused by the drug is unknown. The cytochrome P-450-dependent enzyme activities induced at high doses of clotrimazole are similar to those seen with troleandomycin and the synthetic steroids, dexamethasone and PCN. Since neither clotrimazole nor troleandomycin has a steroid structure, they may be exerting their inductive effect via a common physiological change. The changes in UDP-glucuronosyltransferase activities seen with clotrimazole (increase in morphine but not naphthol conjugation rates) are similar to those seen with PCN (31) and troleandomycin (21), suggesting again that induction may be dependent on a common physiological effect. It should be noted, however, that the dose at which the UDP-glucuronosyltransferase is maximally induced (40–50 mg/kg) is lower than that at which the troleandomycin-like cytochrome P-450-dependent activities are maximally induced, suggesting that the induction of the two microsomal enzymes is independent of one another. The similarity in the extent of induction of morphine conjugation, by clotrimazole doses of 30 and 75 mg/kg, eliminates changes in this enzyme from being responsible for the selective intensification of the higher molecular weight protein band at high clotrimazole doses. This is further supported by the difference in molecular weight of the band that is intensified and the reported subunit molecular weight (56,000) of this enzyme (32).

The enzyme activities selectively induced at high clotrimazole doses coupled with intensification of a similar molecular weight protein band in the SDS-polyacrylamide gel strongly suggest that this is the same isozyme that is induced by PCN, dexamethasone, and troleandomycin. In a recent study (33), induction of this isozyme by phenobarbital and phenobarbital-like inducers was found to involve both decreased degradation and increased synthesis. Whether both of these mechanisms also contribute to high dose clotrimazole induction of this isozyme remains for elucidation. In addition, either or both mechanisms could also be responsible for the low dose response.

The enzyme activities maximally induced by low doses of clotrimazole, *p*-nitroanisole demethylation, and aniline hydroxylation are known to be catalyzed by many cytochrome P-450 isozymes (12, 34, 35). High turnover rates for aniline hydroxylation have been associated with a 51,000 molecular weight isozyme induced by isoniazid and ethanol, isozyme "j" (36). However, induction of this isozyme by ethanol is also associated with a high oxidase activity toward dimethylnitrosamine (37) and clotrimazole induction shows no increase in the microsomal dimethylnitrosamine demethylation rate (unpublished results). Thus, although it is helpful to interpret activity changes in the light of known isozyme specificities, it is possible that clotrimazole is also inducing an isozyme or isozymes that is (are) currently unknown or has (have) yet to be characterized.

Overall, the findings for differential induction of enzyme activities by different doses of a single agent, clotrimazole,

serve as a caution to the whole field of induction of drug-metabolizing enzymes. Induction studies are often undertaken at the highest tolerated doses. It may be that the use of several doses may unravel some of the complexity of induction, particularly with respect to the apparent lack of selectivity (degeneracy) of isozyme induction (i.e., more than one isozyme induced) that is seen with most non-polycyclic hydrocarbon-inducing agents.

References

1. Alvares, A. P., D. R. Bickers, and A. Kappas. Polychlorinated biphenyls: a new type of inducer of cytochrome P-448 in the liver. *Proc. Natl. Acad. Sci. USA* **70**:1321–1325 (1973).
2. Dent, J. G., K. J. Netter, and J. E. Gibson. Effects of chronic administration of polybrominated biphenyls on parameters associated with hepatic drug metabolism. *Res. Commun. Chem. Pathol. Pharmacol.* **13**:75–82 (1976).
3. Ryan, D. E., P. E. Thomas, and W. Levin. Purified liver microsomal cytochrome P-450 from rats with the polychlorinated biphenyl mixture Aroclor 1254. *Mol. Pharmacol.* **13**:521–532 (1977).
4. Poland, A., and E. Glover. Chlorinated biphenyl induction of aryl hydrocarbon hydroxylase activity: a study of the structure-activity relationship. *Mol. Pharmacol.* **13**:924–938 (1977).
5. Goldstein, J. A., P. Hickman, H. Bergman, J. D. McKinney, and M. P. Walker. Separation of pure polychlorinated biphenyl isomers into two types of inducers on the basis of induction of cytochrome P-450 or P-448. *Chem. Biol. Interact.* **17**:69–87 (1977).
6. Parkinson, A., S. H. Safe, L. W. Robertson, P. E. Thomas, D. E. Ryan, L. M. Reik, and W. Levin. Immunochemical quantitation of cytochrome P-450 isozymes and epoxide hydrolase in liver microsomes from polychlorinated or polybrominated biphenyl-treated rats. A study of structure-activity relationships. *J. Biol. Chem.* **258**:5967–5976 (1983).
7. Dannan, G.A., F. P. Guengerich, L. S. Kaminsky, and S. D. Aust. Regulation of cytochrome P-450. Immunochemical quantitation of eight isozymes in liver microsomes of rats treated with polybrominated biphenyl congeners. *J. Biol. Chem.* **258**:1282–1288 (1982).
8. Wrighton, S. A., P. Maurel, E. G. Schuetz, P. B. Watkins, B. Young, and P. S. Guzelian. Identification of the cytochrome P-450 induced by macrolide antibiotics in rat liver as the glucocorticoid responsive cytochrome P-450. *Biochemistry* **24**:2171–2178 (1985).
9. Dickins, M., J. W. Bridges, C. R. Elcombe, and K. J. Netter. A novel haemoprotein induced by isosafrole pretreatment in the rat. *Biochem. Biophys. Res. Commun.* **80**:89–96 (1978).
10. Tamburini, P. P., H. A. Masson, S. K. Bains, R. J. Makowski, B. Morris, and G. G. Gibson. Multiple forms of hepatic cytochrome P-450. Purification, characterization and comparison of a novel clofibrate-induced isozyme with other major forms of cytochrome P-450. *Eur. J. Biochem.* **139**:235–246 (1984).
11. Ryan, D. E., L. Ramanathan, S. Iida, P. E. Thomas, M. Haniu, J. E. Shively, C. S. Lieber, and W. Levin. Characterization of a major form of rat hepatic microsomal cytochrome P-450 induced by isoniazid. *J. Biol. Chem.* **260**:6385–6393 (1985).
12. Guengerich, F. P., G. A. Dannan, S. T. Wright, M. V. Martin, and L. S. Kaminsky. Purification and characterization of liver microsomal cytochromes P-450: electrophoretic, spectral, catalytic, and immunochemical properties and inducibility of eight isozymes isolated from rats treated with phenobarbital or β-naphthoflavone. *Biochemistry* **21**:6019–6030 (1982).
13. Franklin, M. R., and R. W. Estabrook. On the inhibitory action of mersalyl on microsomal drug oxidation: a rigid organization of the electron transport chain. *Arch. Biochem. Biophys.* **143**:318–329 (1971).
14. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
15. Omura, T., and R. Sato. The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J. Biol. Chem.* **239**:2370–2378 (1964).
16. Liu, Z., and M. R. Franklin. Cytochrome P-450 ligands: metyrapone revisited. *Arch. Biochem. Biophys.* **241**:397–402 (1985).
17. Pershing, L. K., and M. R. Franklin. Cytochrome P-450 metabolic-intermediate complex formation and induction by macrolide antibiotics: a new class of agents. *Xenobiotica* **12**:687–699 (1982).
18. Nash, T. The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. *Biochem. J.* **55**:416–421 (1953).
19. Netter, K. J., and O. Seidel. An adaptively stimulated *O*-demethylating system in rat liver microsomes and its kinetic properties. *J. Pharmacol. Exp. Ther.* **146**:61–65 (1964).
20. Mieyal, J. J., and J. L. Blumer. Acceleration of the autooxidation of human oxyhemoglobin by aniline and its relation to hemoglobin catalyzed aniline hydroxylation. *J. Biol. Chem.* **251**:3442–3446 (1976).
21. Liu, Z., and M. R. Franklin. Separation of four glucuronides in a single sample by HPLC and its use in the determination of UDP-glucuronosyltransferase activity towards four aglycones. *Anal. Biochem.* **142**:340–346 (1984).
22. Bornheim, L. M., and M. R. Franklin. Metabolic-intermediate complex formation reveals major changes in rat hepatic cytochrome P-450 subpopulations in addition to those forms previously purified after phenobarbital, β-naphthoflavone, and isosafrole induction. *Mol. Pharmacol.* **21**:527–532 (1981).
23. Lilienblum, W., A. K. Walli, and K. W. Bock. Differential induction of rat liver microsomal UDP-glucuronosyltransferase activities by various inducing agents. *Biochem. Pharmacol.* **31**:907–913 (1982).
24. Watkins, J. B., Z. Gregus, T. N. Thompson, and C. D. Klaassen. Induction studies on the functional heterogeneity of rat liver UDP-glucuronosyltransferase. *Toxicol. Appl. Pharmacol.* **64**:439–446 (1982).
25. Deloria, L., J. Low, and G. J. Mannering. Loss and recovery of hepatic monooxygenase activities after the administration of allylisopropylacetamide (AIA) to mice, in *Microsomes, Drug Oxidations, and Chemical Carcinogenesis* (M. Coon, A. Conney, R. Estabrook, H. Gelboin, J. Gillette, and P. O'Brien, eds.), Vol. II. Academic Press, New York, 861–864 (1980).
26. Wilkinson, C. F., K. Hetnarski, and L. J. Hicks. Substituted imidazoles as inhibitors of microsomal oxidation and insecticide synergists. *Pest. Biochem. Physiol.* **4**:299–312 (1973).
27. Leibman, K. C., and E. Ortiz. New potent modifiers of liver microsomal drug metabolism: 1-arylimidazoles. *Drug Metab. Dispos.* **1**:775–779 (1973).
28. Kahl, R., D. E. Friederici, G. F. Kahl, W. Ritter, and R. Krebs. Clotrimazole as an inhibitor of benzo[*a*]pyrene metabolite-DNA adduct formation *in vitro* and of microsomal mono-oxygenase activity. *Drug Metab. Dispos.* **8**:191–196 (1979).
29. Murray, M., C. F. Wilkinson, and K. Hetnarski. Cytochrome P-450 and monooxygenase activity in hepatic microsomes from *N*-phenylimidazole-treated rats. *Toxicol. Lett.* **25**:191–198 (1985).
30. Lavrijsen, K., J. Van Houdt, D. Thijs, W. Meuldermans, and J. Heykants. Induction potential of antifungals containing an imidazole or triazole moiety. *Biochem. Pharmacol.* **35**:1867–1878 (1986).
31. Watkins, J. B., and C. D. Klaassen. Induction of UDP-glucuronosyltransferase activities in Gunn, heterozygous, and Wistar rat livers by pregnenolone-16α-carbonitrile. *Drug Metab. Dispos.* **10**:590–594 (1982).
32. Bock, K. W., D. Josting, W. Lilienblum, and H. Pfeil. Purification of rat microsomal UDP-glucuronosyltransferase. Separation of two enzyme forms inducible by 3-methylcholanthrene or phenobarbital. *Eur. J. Biochem.* **98**:19–26 (1979).
33. Schuetz, E. G., S. A. Wrighton, S. H. Safe, and P. S. Guzelian. Regulation of cytochrome P-450p by phenobarbital and phenobarbital-like inducers in adult rat hepatocytes in primary monolayer culture and *in vivo*. *Biochemistry* **25**:1124–1133 (1986).
34. Ryan, D. E., S. Iida, A. W. Wood, P. E. Thomas, C. S. Lieber, and W. Levin. Characterization of three highly purified cytochromes P-450 from hepatic microsomes of adult male rats. *J. Biol. Chem.* **259**:1239–1250 (1983).
35. Ryan, D. E., P. E. Thomas, D. Korzeniowski, and W. Levin. Separation and characterization of highly purified forms of liver microsomal cytochrome P-450 from rats treated with polychlorinated biphenyls, phenobarbital, and 3-methylcholanthrene. *J. Biol. Chem.* **254**:1365–1374 (1979).
36. Ryan, D. E., D. R. Koop, P. E. Thomas, M. J. Coon, and W. Levin. Evidence that isoniazid and ethanol induce the same microsomal cytochrome P-450 in rat liver, an isozyme homologous to rabbit liver cytochrome P-450 isozyme 3a. *Arch. Biochem. Biophys.* **246**: 633–644 (1986).
37. Tu, Y. Y., and C. S. Yang. Demethylation and denitrosation of nitrosamines by cytochrome P-450 isozymes. *Arch. Biochem. Biophys.* **242**:32–40 (1985).

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