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PRELIMINARY COMMUNICATIONS

STRUCTURE-ACTIVITY STUDY ON INDUCTION OF HEPATIC DRUG METABOLIZING ENZYMES BY AZO COMPOUNDS

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Enzymes engaging in hepatic drug metabolism are of prime importance in this modern era of chemical saturation in our living sphere. Numbers of compounds have been known to induce hepatic drug metabolizing enzymes including cytochromes P-450 and P-448 (1,2). The inducers of cytochrome P-448 have been paid much attention to, because P-448 has been shown to engage in the metabolic activation of carcinogenic polycyclic aromatic hydrocarbons (3). Ichikawa and Yamano in 1967 found that sudan III (1-phenylazophenylazo-2-naphthol) induces cytochrome P-450 and microsomal FeX in rabbits (4). In those days, presence of multiple species of P-450 type cytochromes have not been known. By reevaluating the effect of sudan III in rats, we found that sudan III induces cytochrome P-448 and UDP-glucuronyltransferase activity in liver microsomes. The compounds which induce cytochrome P-448 can be classified into two general groups: One group consists of polycyclic aromatic hydrocarbons which have planer structure with more than two six-membered aromatic rings, i.e. larger than naphthalene, such as 3-methylcholanthrene (3-MC) and benzo(a)pyrene (BP). Structurally similar β-naphthoflavone can be classified into this group. The other includes polyhalogenated aromatics such as 2,3,7,8-tetrachlorodibenzo-p-dioxine and polychlorinated biphenyl. Sudan III is unique in this respect. It does not possess halogens and it only posesses naphthol moiety, the two ring structure. Therefore, it is of interest to study structure-activity relationship of various azo compounds and their ability to induce hepatic drug metabolism. In spite of the fact that many azo dyes are used as food colorings and the colorings of cosmetics, little,if any, information is available in the literature on the effect of the azo dyes on liver enzymes. In this study, we investigated the effect of azo dyes on the contents of cytochrome P-450 or P-448 as they represent the enzymes of phase I drug metabolism, and on the activity of UDP-glucuronyltransferase, an enzyme of phase II drug metabolism. We chose some 19 relatively simple azo compounds to study their inductive effect on these enzymes. Only lipophilic azo dyes with 1-phenylazo-2-naphthol moiety induced P-448 and UDP-glucuronyltransferase activity. It is proposed that these azo compounds form an intramolecular hydrogen bonding (5), providing the third six-membered ring, and this enables these dyes to induce P-448 and UDP-glucuronyltransferase activity. The inductive potency of these dyes equals or exceeds that of 3-MC.

MATERIALS AND METHODS

Materials: UDP-glucuronic acid ammonium salt and Lubrol WX were purchased from Sigma. 3-Methylcholanthrene, from Eastman Kodak and sodiumphenobarbital, from Fujinaga Seiyaku Co. (Japan). 19 azo compounds used are listed as follows. Among them, I,II,VI,X,XI,XIV,XV,XVI,XVII,XVIII and XIX were commercially available. They were recrystalized from acetone and benzene or

alcohol for lipophilic dyes and from H_2O for soluble ones. Azo compounds III, IV and V were generous gifts from Dr. Yukio Mori of Gifu College of Pharmacy, Gifu, Japan. Others were synthesized in this laboratory by coupling diazotized toluidines and naphthols.

Azo compounds used in this experiment:

I. Azobenzene
II. p-Aminoazobenzene
III. 3,3'-dihydroxymethylazobenzene
IV. 3'-methyl- 4 -dimethylaminoazobenzene
V. 3'-hydroxymethyl- 4 -dimethylaminoazobenzene
VI. Sudan I
VII. 1-o-toluneazo-2-naphthol
VIII. 1-m-toluneazo-2-naphthol
IX. 1-p-toluneazo-2-naphthol
X. Sudan II
XI. Sudan III
XII. 1-p-toluneazo-4-naphthol
XIII. 1-m-toluneazo-4-naphthol
XIV. Oil Yellow OB
XV. Methyl Orange
XVI. Ponseau R
XVII. Amaranth
XVIII. Ponseau SX
XIX. Trypan Blue

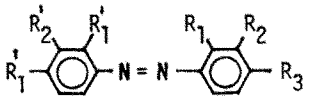
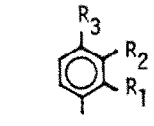
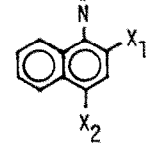
Treatment of Animals: Groups of rats were injected (i.p.) with azo dyes (40 mg/kg/day) and 3-methylcholanthrene (3-MC) (40 mg/kg/day) dissolved in corn oil, and phenobarbital (80 mg/kg/day) dissolved in physiological saline solution for 4 days. The animals were killed on the 5th day and microsomes were prepared and cytochrome P-450 contents were determined according to the method of Omura and Sato (6). Microsomal protein concentrations were determined by the method of Lowry (7).

UDP-glucuronyltransferase assay: p-Nitrophenol was used as the substrate. Activity was determined by continuously recording the decrease in optical absorption of free p-nitrophenol at 400 nm, using Cary 219 spectrophotometer. The assay mixture contained, in final concentrations, 0.3-0.5 mg microsomal protein, 0.02 % Lubrol WX, 40 mM $MgCl_2$, 0.25 mM p-nitrophenol and 1.5 mM UDP-glucuronic acid (UDPGA) in 0.1 M tris-HCl buffer, pH 7.56. The reaction was started by the addition of UDPGA.

RESULTS AND DISCUSSION

Table 1. indicates the effect of various azo compounds on rat hepatic microsomal cytochrome P-450 levels and on UDP-glucuronyltransferase (UDPGT) activity with p-nitrophenol as the aglycon. Effects of phenobarbital (PB) and 3-methylcholanthrene are also indicated for comparison. It was found that those lipophilic azo dyes which posess 2-naphthol or 2-aminonaphthalene moiety, such as sudan I, II, III, 1-o-toluneazo-2-naphthol, 1-m-toluneazo-2-naphthol, 1-p-toluneazo-2-naphthol and oil yellow OB (VI, X, XI, VII, VIII, IX and XIV respectively) are potent inducers of cytochrome P-448 as well as UDPGT activity. They induced cytochrome P-448 levels 2-2.7 fold and UDPGT activity, 4-7 fold of control levels. Therefore, most of them are equal or more potent inducer of these enzymes as compared to 3-MC. If the OH of the naphthol moiety shifts from 2-position to 4-position, the azo compounds loose their ability to induce these enzymes: 1-p-toluneazo-4-naphthol (XII) and 1-m-toluneazo-2-naphthol (XIII), isomers of 1-p-toluneazo-2-naphthol and 1-m-toluneazo-2-naphthol respectively, do not induce cytochrome P-448 nor UDPGT activity. Therefore, the position of OH on naphthalene moiety seems to be very important in the induction of these enzymes by azo compounds. Soluble azo compounds, such as amaranth, ponseau R and ponseau SX do not induce P-448 nor UDPGT activity, although they have 2-naphthol or 1-naphthol moieties in their structures. Lipophilicity seems to be one

Table 1. Effect of various azo compounds on hepatic microsomal cytochrome P-450 and UDP-glucuronyltransferase in rats.

INDUCERS								Cyt. P-450 λ max	Cyt. P-450 % levels (±10%)	UDPGT % activity (±15%)
Physiological Saline								450	100	100
Corn Oil								450	100	100
Phenobarbital								450	217*	237*
3-Methylcholanthrene								448	198*	390*
Lipophilic Azo Compounds										
No.	Basic Structure		Substituting Groups							
	(A)		R_1	R_2	R_3	R_1'	R_2'	R_3'		
I			H	H	H	H	H	H	450	114	154
II			H	H	NH_2	H	H	H	450	111	134
III			H	CH_2OH	H	H	CH_2OH	H	450	95	99
IV			H	H	$N(CH_3)_2$	H	CH_3	H	450	97	137
V			H	H	$N(CH_3)_2$	H	CH_2OH	H	450	84	108
	(B)		R_1		R_2		R_3				
VI			H		H		H		448	242*	580*
VII			CH_3		H		H		448	203*	709*
VIII		X_1=OH	H		CH_3		H		448	266*	679*
IX		X_2= H	H		H		CH_3		448	188*	715*
X			CH_3		H		CH_3		448	221*	392*
XI			H		H		-N=N- 		448	171*	457*
XII		X_1 = H	H		H		CH_3		450	102	83
XIII		X_2=OH	H		CH_3		H		450	107	131
XIV		X_1=NH_2 X_2= H	CH_3		H		H		448	221*	392*
Hydrophilic Azo Compounds											
XV	Methyl Orange								450	82	113
XVI	Ponseau R								450	95	118
XVII	Amaranth								450	83	142
XVIII	Ponseau SX								450	100	143
XIX	Trypan Blue								450	73	102
2-Naphthol									450	117	110

Control levels of cytochrome P-450 for microsomes from physiological saline- and corn oil-treated rats were 0.65 ± 0.05 and 0.64 ± 0.07 nmol/mg microsomal protein respectively. Those for UDP-glucuronyltransferase activities were, 18.6 ± 2.4 and 20.1 ± 3.1 nmol/mg/min.

of the requirments. 2-Naphthol alone does not induce these enzymes, indicating that azo bond is needed for the induction. Among lipophilic azo compounds, those which do not contain naphtalene moiety, i.e. the ones with azobenzene moiety as the basic structure (Group (A) of table 1) do not induce these enzymes, indicating that lipophilicity is not enough for the induction. Apparently, naphthalene moiety in addition to azo linkage is required. Taken altogether, the smallest effective unit for the induction seems to be 1-azo-2-naphthol. The OH on the 2-naphthol moiety can be replaced by NH_2.

With the structures differing from carcinogenic polycyclic aromatic hydrocarbons like 3-MC or polyhalogenated aromatics like 2,3,7,8-tetrachlorodibenzo-p-dioxine, these azo compounds may form third group of inducers of P-448 and UDPGT activity. However, it has been known to organic chemists that 1-arylazo-2-naphthol exists as a hydrazone tautomer, i.e. phenolic OH and and azo nitrogen form hydrogen bond (5), resulting in a new six-membered ring adjacent to naphthalene (Fig. 1). Although the third ring thus formed is heterocyclic,the shape of the overall planer structure with three six-membered rings resembles the bay region structure of polycyclic carcinogens. We tentatively call this structure, "azo-bay region" structure. Even if 1-arylazo-2-naphthol formes metal complex, the azo-bay region structure would not be altered (8). The bay region structure has been shown to be important in chemical carcinogenesis. We thought that this structure may also play an important role in induction of P-448 and UDPGT activity. Needless to say that those chemical carcinogens possessing bay region are good inducers of cytochrome P-448 and UDPGT activity (1, 2). However, what is required for the induction may simply be a planer structure consisting of three six-membered rings, and may not necessarily have to be the bay region structure. In any case, for these azo compounds to possess inductive ability, the formation of the third ring seems to be absolute requirement, and this results in the formation of "azo-bay region". If this is true, this can explain why OH at the 2-position is so important. If OH was at other position, the azo compounds are not able to form bay region structure and therefore unable to induce these enzymes like in the case of XII and XIII. Whether the "bay region theory" for carcinogenesis can be applicable to "azo-bay region" awaites further investigations. It is of interest to note that the induction of P-448 coincides with that of UDPGT activity. The possible link between genes for these enzymes is suggested. Lastly, since many of azo compounds which were found here to have profound effects on liver drug metabolism are currently in use as colorings of cosmetics such as lipsticks, and they are likely to be ingested, reevaluation of these dyes regarding the safety of use may be necessary.

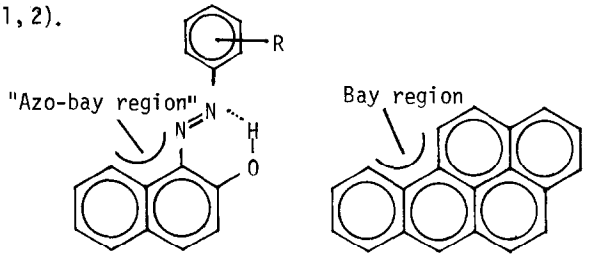


Fig. 1. Bay region structure of 1-arylazo-2-naphthol and benzo(a)pyrene.

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