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INFLUENCE OF 2-(α-HYDROTETRAFLUROETHYL)BENZIMIDAZOLE ON THE ACTIVITY OF MOUSE LIVER MICROSOMAL MONOOXYGENASES

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UDC 615.31:547.785.5].015.44:612.351.11

The system of liver microsomal mixed-function oxygenases participates in the metabolism of fat-soluble xenobiotics, which is accompanied by their conversion to more polar compounds, capable of being excreted from the organisms through the kidneys or through the bile [1]. The central oxygenase in the system of microsomal enzymes is cytochrome P-450 [5]. In recent years the existence of several isoforms of the cytochromes, differing in certain physicochemical properties and induced by different chemical compounds, has been detected. The cytochrome induced by 3-methylcholanthrene (I) forms a complex with carbon monoxide, with an absorption maximum at 448 nm. This cytochrome differs substantially in its affinity for substrates and enzymatic activity from the cytochrome P-450 induced by phenobarbital (II) [2]. It has been established that the aminopyrine demethylase activity is associated with cytochrome P-450, while the benzpyrene monooxygenase activity is associated with cytochrome P-448; it is precisely the latter isoform that participates in the metabolic activation of procarcinogenic polycyclic aromatic hydrocarbons and their conversion to proximal carcinogens [5]. Various inhibitors and inducers of microsomal monooxygenases are of great value in elucidating the role of isoforms of cytochromes P-450 in the processes of carcinogenesis and mutagenesis; they can be used as molecular probes for determining the relationship of various forms of cytochrome P-450 with different enzymatic activity.

Together with effective inducers of monooxygenase, inhibitors of these enzymes are also known. They include imidazoles and their derivatives [14], benzimidazoles [10, 12], and certain other compounds, which suppress the activity of the microsomal monooxygenase in various species of animals.

The purpose of our work was to study the influence of 2-(α-hydrotetrafluoroethyl)benzimidazole (III) on microsomal enzymes in experiments *in vivo* and *in vitro*, as a model of compounds distinguished by increased π-acceptor properties and high hydrophobicity with a small molecular volume [6].

EXPERIMENTAL CHEMICAL SECTION

2-(α-Hydrotetrafluoroethyl)benzimidazole (III) was produced by a modified method of [11], by the reaction of ortho-phenylenediamine with hexafluoropropene in anhydrous DMFA. The reaction was conducted in a closed volume in the presence of excess hexafluoropropene and 10 moles % KF, producing III; quantitative yield, mp 184°C.

NMR spectra (CH_3CN), δ, ppm ^{19}F — 0.3 m (3F, CF_3), 120.74 m (1F, CHF); ^1H — 7.7 s (1H, $H_{(5)}$), 7.6 s (1H, $H_{(6)}$), 7.31 m (3H, $H_{(7)}$), $H_{(4)}$, NH), 6.35 and 6.13 two quadruples (1H, CHF). IR spectrum, ν, cm^{-1}: 3200-2600 (NH), 1630 (C=N). UV spectrum, λ, nm (D): 275 (1.472), 252 (1.419).

EXPERIMENTAL BIOLOGICAL SECTION

In the work we used male C57BL/6 mice weighing 18-20 g. For the induction of microsomal enzymes, the animals were injected intraperitoneally with I (Fluka, Federal Republic of

Branch of the Scientific Research Institute for Biological Testing of Chemical Substances, A. N. Nesmeyanov Institute of Heteroorganic Compounds, Academy of Sciences of the USSR, Moscow. Translated from Khimiko-farmatsevticheskii Zhurnal, Vol. 21, No. 3, pp. 265-269, March, 1987. Original article submitted January 23, 1986.

 0091-150X/87/2103-0156$12.50

Germany) in olive oil in a dose of 50 mg/kg for two days. The animals of the control group were injected with the same amount of oil. The microsomal fraction was isolated by differential centrifugation. For this purpose a liver homogenate was centrifuged at 10,000g for 20 min on a K-24 centrifuge. The precipitate was discarded and the supernatant centrifuged at 105,000g for 1 h on a Beckman L8-55 centrifuge; the precipitate was suspended in the isolation medium and repeatedly centrifuged at 105,000g.

The activity of aminopyrene demethylase (APDM) was determined according to the method of [8] in our modification, based on measurement of the formaldehyde formed as a result of the reaction. Samples containing the microsomal fraction (2 mg protein per 2 ml), NADPH-generating system (Na glucose-6-phosphate 8 mM fron Reanal, Hungary) and its dehydrogenase (0.5 unit/ml from Boehringer, Federal Republic of Germany), as well as 0.4 mM NADP from Reanal (Hungary), 4 mM aminopyrine in phosphate buffer pH 7.4, and 4 mM $MgCl_2$, were incubated for 10 min at 37°C. The reaction was stopped by adding trichloroacetic acid to a final concentration of 5%, and after precipitation of the protein by centrifuging at 3000g, the amount of formaldehyde was determined in the supernatants using Nash's reagent [13]. Samples containg aliquots of the centrifugate and Nash's reagent were incubated for 20 min at 58°C in an ultrathermostat, and after this the extinction was measured at 412 nm on a Shimadzu UV-240 spectrophotometer (Japan). The amount of the formaldehyde remaining was calculated according to a calibration curve, constructed with known amounts of it.

The activity of benzpyrene monooxygenase (BPMO) was determined according to a modified method of [7], based on measurement of the amount of 3-hydroxybenzpyrene (IV) — a reaction product possessing fluorescent properties, which is formed after incubation of microsomes with benzpyrene (V). The microsomal fraction (50-100 µg per ml) was incubated with 80 µM V from Fluka (Federal Republic of Germany) and 0.4 mM NADPH from Reanal in 0.05 M Tris-HCl buffer solution, pH 7.4, containing 3 mM $MgCl_2$, for 1-5 min at 37°C. The reaction was stopped by adding a one tenth volume of a mixture containing 10% Triton X-100, 1 N NaOH, and 1% EDTA. The reaction product IV was determined fluorometrically at λ_{excit} 465 nm, λ_{fl} 522 nm, on a Shimadzu RF-510 spectrofluorometer. The amount of the reaction product formed was found graphically according to a calibration curve constructed with known concentrations of IV, added to samples identical with the incubation sample.

Experiments *in vivo* were conducted on noninbred male mice weighing 18-24 g. The mice of the control group were injected intraperitoneally with a solution of hexenal (VI) in a dose of 70 mg/kg once, while the experimental animals were injected once intraperitoneally with III in doses of 25, 50, and 100 mg/kg. The mice were injected intraperitoneally with VI in a dose of 70 mg/kg 5 min after III. The influence of III on the duration of the soporific action of VI was estimated according to the time of stay of the animals in a lateral position. The action of each dose of the preparations was investigated on no less than six animals. Statistical treatment of the data was performed using the Student tables [3].

RESULTS AND DISCUSSION

The results of a study of the influence of III on the monooxygenase activity of C57BL/6 mouse liver microsomes are cited in Table 1, from which it is evident that the preparations studied produced a concentration-dependent inhibition of the basal activity of APDM and BPMO. In a concentration of $5 \cdot 10^{-4}$ M the activity of APDM was approximately 50% inhibited, whereas the basal activity of BPMO was decreased by approximately 50% at a 10^{-3} M concentration of the preparation. It is of theoretical importance that the compound III affected not only the basal activity of BPMO but also the activity of BPMO after induction with I; moreover, the inhibiting action against a background of the inducer was somewhat more pronounced in comparison with the inhibition of the basal activity of BPMO.

In the following series of experiments we studied the effects of III on the cytochrome P-450 system in experiments *in vivo* after a single intraperitoneal injection of the preparation. The results of these experiments are presented in Table 2. As it follows from Table 2, the preparation in a dose of 25 mg/kg increased the time of hexenal sleep by a factor of approximately two, in a dose of 50 mg/kg by a factor of three, and in a dose of 100 mg/kg by a factor of four. The increase in the sleeping time is evidently due to binding of the preparation to cytochrome P-450; moreover, with increasing dose of the preparation, its inhibiting action increases . These results are similar to the data of studies of the influence of one of the strongest inhibitors of cytochrome P-450, which suppresses xenobiotic metab-

TABLE 1. Influence of Compound III on the Monooxygenase Activity of Mouse Liver Microsomes

Concentration of preparation, M	Activity, %		
	APDM*	BPMO** (basal)	BPMO*** (induced)
$1 \cdot 10^{-5}$	98,7	95,5	—
$1 \cdot 10^{-4}$	91,8	83,3	102
$5 \cdot 10^{-4}$	48,8	65,2	45,3
$1 \cdot 10^{-3}$	36,3	48,3	33,8
$2 \cdot 10^{-3}$	25,1	24,6	14

*Basal activity 7 nmoles/(min•mg).
**Basal activity 0.7 nmole/(min•mg).
*** Activity induced by I 4.4 nmoles/(min•mg).

TABLE 2. Influence of Compound III on the Duration of the Soporific Activity of IV

Series	Group of animals	Number of mice	Dose of III, mg/kg	Time of stay of mice in a lateral position, min (VI, 70 mg/kg)	Number of mice put to sleep
I (single injection)	Experimental	6	25	78,8±3,4*	...
		7	50	108,2±10,0**	...
		7	100	144,3±17,5**	...
	Control	6	—	35,1±10,3	...
II (three injections)	Experimental	10	50	19,5	2
		10	100	20,2±3,1	5
		10	200	18,4±1,4	7
	Control	10	—	39,4±2,8	10

*P < 0 02.
**P < 0.01.

olism both *in vitro* and *in vivo* — 2'-diethylaminoethyl ester of 2,2-diphenylvaleric acid (VII): rats that were given VII in a dose of 15 mg/kg and then barbiturates slept approximately three times as long as the control animals [9].

Let us note that the action of inhibitors of microsomal enzymes *in vivo* is complex, and in addition to the direct influence on cytochrome P-450, it may be associated with a change in the permeability of the cell membranes, a decrease in the volume of blood passing through the liver, and a disruption of the absorption of substances in the gastrointestinal tract [2].

A characteristic feature of the biological effects of many inhibitors of xenobiotic metabolism is the biphasic nature of their action on the biotransformation of drugs [4]. After a single administration, like VII, they suppress the activity of the microsomal monoxygenases and increase the time of action of pharmacological preparations. In the case of repeated administration, the same substances have an inducing effect on the cytochrome P-450 system, increasing the rate of metabolism and shortening the time of their action. Thus, daily administration of VII over a period of three days led to an increase in the ethylmorphine-N-demethylase activity, an increase in the content of cytochrome P-450, and a shortening of the time of soporific action of barbiturates [9]. Therefore, in a number of experiments we studied the influence of three injections of III in doses of 50, 100, and 200 mg/kg on hexenal sleep. From Table 2 it is evident that administration of preparation VI led to a decrease in the sleeping time; moreover, at a dose of 50 mg/kg, only 2 out of 10 mice went to sleep, and at a dose of 100 mg/kg 5 animals out of 10, and at a dose of 200 mg/kg 7 mice out of 10. These data suggest that in the case of three administrations of the preparation

there is an induction of cytochrome P-450, resulting in an increase in the rate of metabolism of VI and a shortening of the sleeping time, or the mice do not go to sleep at all.

The mechanism of the inhibiting action of preparation III may be associated with the formation of intermediate complexes with various forms of cytochrome P-450. Considering the pronounced hydrophobic properties of the preparation, we can suggest that it is capable of changing the properties of the membranes of the endoplasmic reticulum and thereby influencing the interaction of the enzymes included in the multicomponent monooxygenase system.

It was shown in [12] that the hydrophobic nature of the substituent at position 2 in the series of homologs of 2-n-alkylbenzimidazoles plays a leading role in the inhibitory action of these compounds on the benzpyrene monooxygenase activity in liver microsomes of rats treated with II. The authors believe that the inhibitory effect of such compounds is evidently associated with their ability to pass through the hydrophobic barrier and/or interact with hydrophobic binding sites in the cytochrome P-448 molecule.

An analogous relationship between the inhibitory activity and the octanol/water distribution coefficient was described earlier for alkyl-benzimidazoles, which inhibit the APDM activity in microsomes induced by II [10].

We should note, however, that despite the ability of 2-p-alkylbenzimidazoles to inhibit the benzpyrene monooxygenase activity in microsomes obtained from the livers of rats treated with II, these compounds possess weak activity or were entirely ineffective with respect to BPMO in microsomes isolated from rats that were given I. Evidently such a difference is due to the different nature of the cytochromes in these two microsomes preparations: in one case it is cytochrome P-450, and in the other P-448.

On the basis of these results the authors of [12] hypothesized that the sensitive catalytic site of cytochrome P-448 in microsomes induced by I differs structurally from the corresponding portion of cytochrome P-450 in microsomes induced by II.

The data obtained in our work show that the fluoro-derivative of benzimidazole (III) inhibits not only the basal activity of APDM and the cytochrome P-448-dependent activity of BPMO but also the BPMO activity after induction of cytochrome P-448 by I. In this case the BPMO activity in the microsomes of mice induced by I was six times as high in our experiments in comparison with the basal activity of BPMO. These data are evidence of the promise of the search for preparations that modify the activity of microsomal monooxygenases among fluoro-derivatives of benzimidazole.

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