Biochemical Pharmacology, Vol. 27, pp. 167–171. Pergamon Press, 1978. Printed in Great Britain.

MORPHOLOGICAL AND ENZYMATIC ALTERATIONS IN THE RAT LIVER CAUSED BY ADMINISTRATION OF A HYPOCHOLESTEROLEMIC AGENT AT-308 AND ITS RELATED COMPOUNDS

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(*Received* 9 *November* 1976; *accepted* 27 *April* 1977)

Abstract—The administration of a hypocholesterolemic agent, 3-[4-(1-ethoxycarbonyl-1-methylethoxy)-phenyl]-5-(3-pyridyl)-1,2,4-oxadiazole (AT-308), to rats induced hepatomegaly accompanying a significant proliferation of microbodies. AT-308 and some related compounds were given to rats to investigate the relationship between chemical structures of the test compounds and their several biological effects including hypocholesterolemic effect, proliferation of hepatic microbodies, induction of liver catalase activity and drug-metabolizing enzyme activity as manifested by pentobarbital sleeping time. The results obtained are as follows. First the administration of AT-308 or clofibrate to rats fed a basal or high cholesterol diet produced a significant increase in the number of hepatic microbodies, especially of anucleoid type. The administration of 3-[4-(2-diethyl-aminoethoxy)phenyl]-5-(3-pyridyl)-1,2,4-oxadiazole (AT-293) induced a slight increase in the number of hepatic microbodies, and 3,5-di-(3-pyridyl)-1,2,4-oxadiazole (AT-232) did not produce such an increase at all. Second, the liver catalase activity increased in the AT-308- and clofibrate-treated groups, but the increase of the latter was less prominent than that of the former. Third, the administration of AT-308 or clofibrate, but not of AT-293 or AT-232, shortened the duration of pentobarbital anesthesia. These results suggest that the proliferation of hepatic microbodies with AT-308 administration may be ascribable to a clofibrate-like side chain in the molecule of AT-308 and not to its 1,2,4-oxadiazole moiety.

Clofibrate, a widely used hypocholesterolemic agent, was reported to be of relatively low toxicity. Hepatomegaly was the most remarkable manifestation in rats treated with it [1, 2] and there was a marked proliferation in the number of hepatic microbodies (peroxisomes) together with an increase of protein content in the liver [3, 4]. Although the precise physiological significance of hepatic microbodies and of oxidative enzymes in them is not fully elucidated, there is a suggestion that the proliferation of hepatic microbodies induced by some hypocholesterolemic agents has a close relation to their hypocholesterolemic activity [4–6]. The administration of a hypocholesterolemic agent, 3-[4-(1-ethoxycarbonyl-1-methylethoxy)-phenyl]-5-(3-pyridyl)-1,2,4-oxadiazole (AT-308) or a related compound produced an enlargement of the liver like that of clofibrate [7].

The present study was undertaken to investigate the relationship between chemical structures of the test compounds and their several biological effects including hypocholesterolemic effect, proliferation of hepatic microbodies, induction of liver catalase activity and drug-metabolizing enzyme activity as manifested by pentobarbital sleeping time.

MATERIALS AND METHODS

Materials. The derivatives of 1,2,4-oxadiazole (AT-308), 3-[4-(2-diethylaminoethoxy)phenyl]-5-(3-pyridyl)-1,2,4-oxadiazole (AT-293) and 3,5-di-(3-pyridyl)-1,2,4-oxadiazole (AT-232) were synthesized by Dr. S. Yurugi of the Medicinal Research Laboratories of our company. Clofibrate was supplied by Shizuoka Caffeine Kogyosho Co. The structures of the test compounds are shown in Fig. 1.

Animals and treatment. Male Sprague Dawley (JCL:SD, CLEA Japan Inc.) rats, from 5 to 7 weeks in age, were used. Rats were maintained on a powdered basal diet (CE-2, CLEA Japan Inc.) before experiments. The rats received the powdered basal diet or a high cholesterol diet containing the test compounds for 7 days. A high cholesterol diet consists of 1% cholesterol, 0.2% sodium cholate and 5% olive oil in the powdered basal diet. After rats were fed the experimental diets for 7 days, pentobarbital (Nembutal, Dainippon Pharmaceutical Co.) was injected intravenously at a dose level of 40 mg/kg to the rats, and the duration of sleep was determined by judging a loss of righting reflex. Rats were killed by decapitation and the liver was immediately excised and weighed.

Determination of plasma cholesterol. Plasma cholesterol was determined according to the method of Abell *et al.* [8].

Assay of liver catalase and urate oxidase activities. Prior to measurement of enzyme activity, the homogenate was treated with deoxycholate and then diluted with water. The activities of catalase (EC 1.11.1.6) and urate oxidase (EC 1.7.3.3) were determined spectrophotometrically as described by Ganshow and Schimke [9] and London and Hudson [10] respectively. The calculation of enzyme activities was made according to the method of Krishnakantha and Kurup [11]. Total protein was measured with Folin's reagent according to the method of Lowry *et al.* [12].

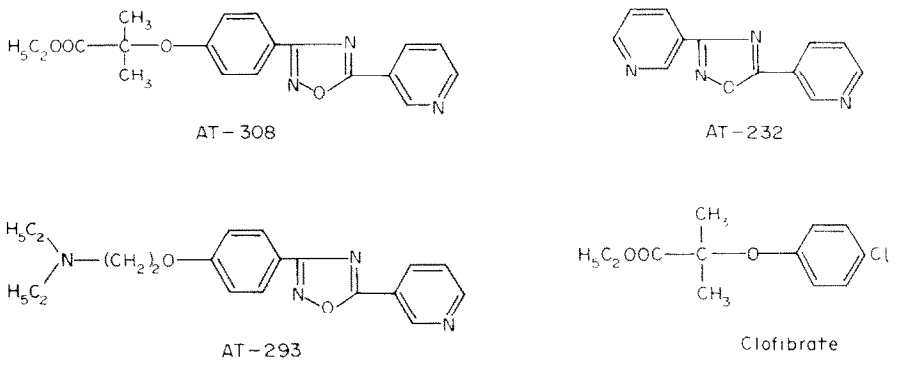

Fig. 1. Structural formulas of 3-[4-(1-ethoxycarbonyl-1-methylethoxy)phenyl]-5-(3-pyridyl)-1,2,4-oxadiazole (AT-308), 3-[4-(2-diethylaminoethoxy)-phenyl]-5-(3-pyridyl)-1,2,4-oxadiazole (AT-293), 3,5-di(3-pyridyl)-1,2,4-oxadiazole (AT-232) and ethyl α-*p*-chlorophenoxyisobutyrate (clofibrate).

Morphologic procedures. After removal from the lobus sinister medialis of the liver, three thin slices were cut and diced into the usual tissue cubes of about 1 mm diameter. These were fixed in 2% osmium tetroxide (0.05 M phosphate buffer, pH 7.4) for 2 hr and then washed in the same buffer for 24 hr. The tissue was dehydrated in a graded series of ethanol and embedded in Epon 812. The blocks were sectioned on an LKB 4800 Ultrotome 1 with a glass knife. After staining with uranyl acetate [13] and lead citrate [14], the sections were examined with a JEM 100B (JEOL Ltd.) electron microscope at 80 kV. Morphometric analyses were conducted on four sections of three randomly chosen tissue blocks from each animal. Three photographs were consistently taken in the hepatocyte central zone of the hepatic lobule that was preselected. The initial plate magnification was 4,802× and final print magnification was 10,564×. The microscope was calibrated by photographing a carbon grating replica with 28,500 lines in. prior to each photographic session throughout the study. Magnification was found to vary by less than 5 per cent.

Statistical method. Student's *t*-test was used for statistical analysis.

RESULTS

Liver microbodies. The administration of AT-308 or clofibrate induced a significant proliferation of microbodies in the rat liver (Table 1). A representative photograph of hepatocytes treated with AT-308 is shown in Fig. 2. With regard to microbody profile, the number of microbodies, especially of anucleoid type, increased and the size of the microbodies was enlarged (Table 1). As the proliferation of microbodies was observed in both normocholesterolemic and hypercholesterolemic rats treated with AT-308 or clofibrate, experiments were performed thereafter only in normocholesterolemic rats fed the basal diet. There was little increase of microbodies in rats treated with AT-293 and a decrease in the AT-232-treated group.

Liver catalase and urate oxidase activities and pentobarbital sleeping time. Liver catalase activity of rats treated with AT-308 increased to approximately the same level as with clofibrate, whereas liver urate oxidase activity did not change or slightly decreased (Table 2). The increased activity of liver catalase of rats treated with AT-308 was associated with a decrease in pentobarbital sleeping time, whereas liver catalase activity and pentobarbital sleeping time remained unchanged in AT-293- or AT-232-treated rats (Fig. 3).

DISCUSSION

Hepatomegaly was the most remarkable manifestation in rats treated with clofibrate. The administration of a hypocholesterolemic agent AT-308 or its related compounds (AT-293 and AT-232) produced a similar enlargement of the liver [7]. The etiology of the hepatomegaly induced by these agents is not precisely understood at present. A possible relationship between hepatic microbodies and cholesterol metabolism was suggested by some reports [4, 5, 15–19], but another investigator showed that the proliferation of hepatic microbodies induced by clofibrate was independent of its hypocholesterolemic effect [20]. All of the agents used in our experiments had hypocholesterolemic activity. The administration of AT-308, clofibrate, AT-293 and AT-232 caused a decrease of plasma cholesterol in hypercholesterolemic rats, as reported in the previous paper [7]. In the case of normocholesterolemic rats, AT-308, clofibrate and AT-293 depressed the plasma cholesterol level (see Table 1). The administration of AT-308 produced, as did clofibrate, proliferation of hepatic microbodies together with an increase of liver catalase activity, whereas little or no increase of hepatic microbodies and liver catalase activity was found in rats treated with AT-293 or AT-232. Therefore, it is presumed that all of the hypocholesterolemic agents do not necessarily produce proliferation of hepatic microbodies. However, we do not presume that the proliferation of hepatic microbodies induced by AT-308 or clofibrate is independent of their hypocholesterolemic effects.

There are two types of microbodies, nucleoid and anucleoid, in the liver. It has been shown that liver urate oxidase is almost exclusively localized in the core of the microbodies and catalase is in the matrix [21–24]. The administration of AT-308 produced mainly an increase in the number of anucleoid microbodies and caused an increase of liver catalase,

Table 1. Effect of AT-308 or related compounds on liver microbodies of rats fed basal or high cholesterol diet for 7 days

Group	Dose (per cent in diet)	Diet cholesterol	Plasma cholesterol (mg/100 ml)	No. of hepatocytes examined	No. of microbodies (/100 μ^2)			Diameter of microbodies (nm)
					Nucleoid type	Anucleoid type	Total	
Control			70 ± 4.9* (6)	30 (2)†	3.8 ± 0.2	4.3 ± 0.2	8.1 ± 0.3	488 ± 19
AT-308	0.05		62 ± 4.4 (6)	30 (2)	5.4 ± 0.3‡	9.8 ± 0.6‡	15.2 ± 0.8‡	616 ± 21‡
	0.25		55 ± 2.8§ (6)	30 (2)	6.8 ± 0.3‡	20.6 ± 0.9‡	27.8 ± 1.0‡	767 ± 20‡
Clofibrate	0.25		48 ± 2.8¦ (6)	30 (2)	5.8 ± 0.3‡	22.4 ± 1.0‡	28.3 ± 1.1‡	763 ± 23‡
AT-293	0.10		49 ± 3.3‖ (5)	38 (2)	2.9 ± 0.3¦	6.4 ± 0.3‡	9.3 ± 0.4§	
AT-232	0.25		93 ± 4.8¦ (7)	28 (2)	1.8 ± 0.2‡	3.1 ± 0.3‖	4.9 ± 0.4‡	
Control		+•	379 ± 25 (7)	9 (2)	3.0 ± 0.4	4.0 ± 0.4	7.0 ± 0.7	
AT-308	0.10	+	236 ± 20‡ (7)	13 (2)	2.7 ± 0.4	10.3 ± 0.7‡	13.0 ± 0.8‡	
Clofibrate	0.10	+	265 ± 25‖ (7)	7 (2)	1.6 ± 0.2§	15.4 ± 2.3‡	17.0 ± 2.4‡	

* Mean ± standard error.
† Number in parentheses in no. of rats examined.
‡ Statistically significant at P = 0.001 against the respective control group.
§ Statistically significant at P = 0.05 against the respective control group.
¦ Statistically significant at P = 0.01 against the respective control group.
• A high cholesterol diet containing 1% cholesterol, 0.2% sodium cholate and 5% olive oil in the CE-2 diet.

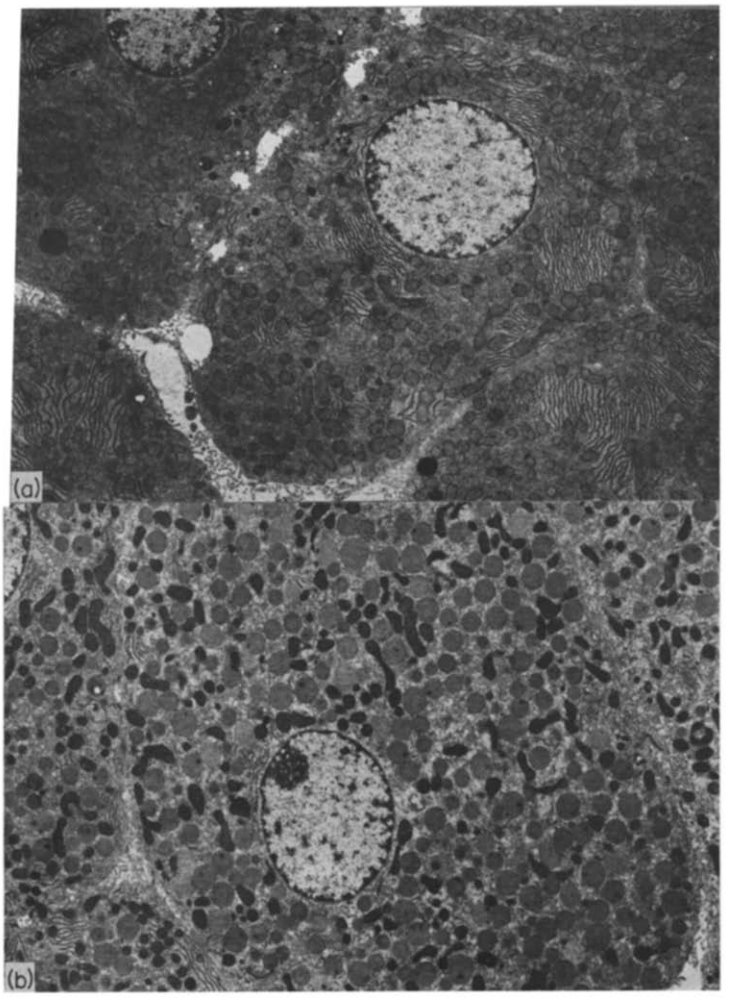

Fig. 2. Representative photograph of hepatocyte by electron microscopy. (a) Control; (b) AT-308 was given to the rat at a dietary concentration of 0.25% for 7 days. The number of microbodies, especially the anucleoid type, increased and the size of the microbodies was enlarged. Final print magnifications were both 4800×.

not of urate oxidase activity, similar to clofibrate [25]. The increase in liver catalase activity did not correlate well in degree with the increase in the number of hepatic microbodies. Such as inconsistency was also noticed in rats treated with methyl clofenapate, clofibrate or nafenopin and was ascribed to the low concentration of active catalase in newly formed microbodies [26].

Although microbodies are well recognized as specialized organells rich in H_2O_2-producing and -consuming enzymes, their biological significance is not precisely understood, and there is some argument

Table 2. Effect of AT-308 or clofibrate on liver catalase and urate oxidase activities*

Group	Dose (per cent in diet)	Catalase activity (m-moles/min/mg protein)	Urate oxidase activity (nmoles/min/mg protein)
Control		0.438 ± 0.017	1.75 ± 0.09
AT-308	0.01	0.490 ± 0.000†	1.55 ± 0.09
	0.05	0.563 ± 0.022‡	1.53 ± 0.14
	0.25	0.585 ± 0.022‡	1.30 ± 0.05†
Clofibrate	0.25	0.605 ± 0.022‡	1.29 ± 0.08†

* Rats were given the compounds for 7 days. Each group consisted of four rats. Catalase activity was assayed in media containing 20 mM H_2O_2 and 50 mM phosphate buffer, pH 7.0. Urate oxidase activity was assayed in media containing 0.357 mM uric acid, 0.65 mM lithium carbonate and 0.1 M borate buffer, pH 9.2.

† Statistically significant at $P = 0.05$ against the respective control group.

‡ Statistically significant at $P = 0.01$ against the respective control group.

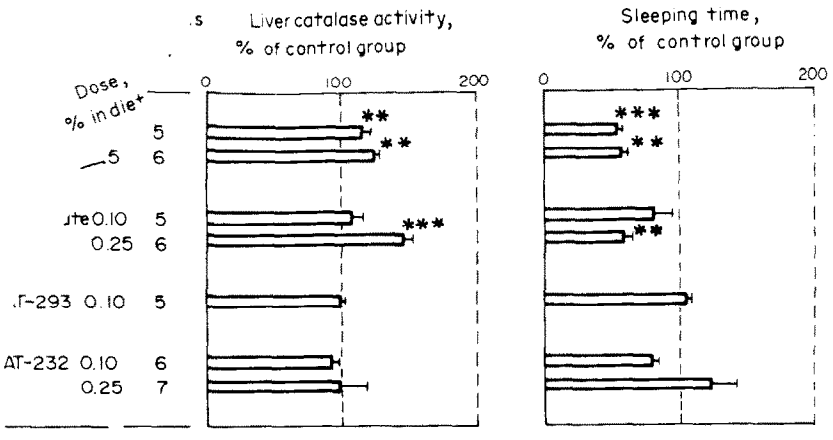

Fig. 3. Effect of AT-308 and its related compounds on liver catalase and pentobarbital sleeping time. After rats were fed the experimental diets for 7 days, pentobarbital was injected intravenously at a dose level of 40 mg/kg to the rats, and the duration of sleep was determined by judging the loss of the righting reflex. Then, the rats were killed by decapitation and the liver was immediately excised for liver catalase assay. Values were statistically significant at P = 0.05(*), 0.01(**) and 0.001(***) against the respective control groups.

as to whether or not the proliferation of hepatic microbodies is a sign of toxicity in animals [27, 28]. Another effect of clofibrate on liver is the induction of microsomal drug-metabolizing enzymes [29]. Treatment with AT-308 was found to reduce pentobarbital sleeping time significantly, as did clofibrate, whereas the pentobarbital sleeping time remained unchanged in rats treated with AT-293 or AT-232. Therefore, it is presumed that all of the hypocholesterolemic agents do not necessarily induce microsomal drug-metabolizing enzymes.

It seems likely that the proliferation of hepatic microbodies in rats treated with AT-308 is ascribable to the phenoxyisobutyrate moiety common to the molecule of both AT-308 and clofibrate, and not to its 1,2,4-oxadizole moiety. It seems pertinent to note here that the treatment with AT-308 inhibited hepatic cholesterogenesis at the stage of transformation of acetate into mevalonate, similar to clofibrate (data are not cited), and that AT-293 blocked the enzymatic reduction of desmosterol to cholesterol [7]; AT-232 failed to depress cholesterogenesis at that stage (data are not cited). Therefore, if there is a correlation between some hypocholesterolemic agents and their proliferative effect on hepatic microbodies, one of the possible causes lies in the inhibition of cholesterogenesis at the stage from acetate to mevalonate. In any case, further studies would be desirable to clarify these relationships.

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