Inhibitory Effect of 2-Diethylaminoethyl 2,2-Diphenylvalerate Hydrochloride (SKF 525-A) on Aromatic Hydroxylation and Phenolic Glucuronidation

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SUMMARY

Kinetics of the inhibition of two microsomal enzymes of guinea pig liver by SKF 525-A was studied; namely, aryl 4-hydroxylase toward aniline and UDP-glucuronyl transferase toward phenolic glucuronidation of *o*-aminophenol. In *vitro* conversion from aniline to *p*-aminophenol by aryl 4-hydroxylase was noncompetitively inhibited with K_i of 1.7×10^{-4} M, while apparent K_m of the reaction was 1.8×10^{-3} M. Excretion of phenol or *p*-aminophenol in the urine of rats and guinea pigs treated with benzene or aniline, respectively, was markedly decreased by the simultaneous injection of SKF 525-A, indicating the inhibitor was effective *in vivo* as well as *in vitro*. Formation of *o*-aminophenyl glucuronide by UDP-glucuronyl transferase *in vitro* had apparent K_m of 7.1×10^{-5} M, and was inhibited by SKF 525-A also in a noncompetitive manner with K_i of 3.6×10^{-4} M. The *in vivo* effect of the inhibitor on phenolic glucuronidation was demonstrated with rats and guinea pigs.

INTRODUCTION

Since the discovery of 2-diethylaminoethyl 2,2-diphenylvalerate hydrochloride (SKF 525-A), a number of studies have been carried out on the action of the compound upon the metabolism of various drugs [see Gillette (1) for a review]. Yet, the mechanism of the action still remains puzzling (2).

Recently Anders and Mannering (3) reported that *N*-demethylase activity of rat liver microsomes on ethylmorphine hydrochloride was inhibited by SKF 525-A in a competitive manner, suggesting that SKF 525-A combines with the active site of *N*-demethylase as an alternative substrate. According to Leibman (4), conversion from trichloroethylene to chloral hydrate by liver microsomes was noncompetitively inhibited by SKF 525-A.

In this paper, we are reporting the facts that both aryl 4-hydroxylase (EC 1.14.1.1) and UDP-glucuronyl transferase (EC 2.4.1.17) in the microsomal fraction (5, 6) of guinea pig liver are noncompetitively inhibited by SKF 525-A. Experiments with rats and guinea pigs confirmed that *in vivo* formation of phenol, *p*-aminophenol, and *o*-aminophenyl glucuronide from benzene, aniline, and *o*-aminophenol, respectively, was also suppressed by SKF 525-A.

MATERIALS AND METHODS

Animals. Male Hartley guinea pigs (about 200 g) and female Wistar rats (about 50 g) were maintained on commercial laboratory chow and water *ad libitum*. Vegetable leaves were given to guinea pigs to prevent vitamin C deficiency.

Tissue preparations. Guinea pig livers were homogenized with 5 volumes of 0.25 M sucrose in glass homogenizers of Potter-Elvehjem type. The homogenate was spun at 10,000 *g* for 15 min and the supernatant fraction was used for enzyme assays. All procedures were carried out in the cold (0–4°).

Enzyme assays. Activity of aryl 4-hy-

droxylase toward aniline was measured as previously described (7) taking advantage of endogenous glucose-6-phosphate dehydrogenase (EC 1.1.1.49) in the soluble fraction. The assay mixture containing 100 μmoles of Tris-acetate buffer (pH 8.0), 8 μmoles of nicotinamide, 2.5 μmoles of $MgCl_2$, 0.12 μmole of NADP, 7.5 μmoles of glucose 6-phosphate, 10,000 *g* supernatant fraction equivalent to 33.3 mg of wet guinea pig liver, and various amounts of aniline and SKF 525-A in a total volume of 1 ml was incubated for 30 min at 37°. UDP-glucuronyl transferase activity was assayed by a modification of the method of Dutton (8). The assay mixture containing 16.6 μmoles of Tris-acetate buffer (pH 7.5), 0.38 μmole of ascorbate, 5 μmoles of $MgCl_2$, 1 μmole of UDPGA, 10,000 *g* supernatant fraction equivalent to 16.6 mg of wet guinea pig liver, and various amounts of *o*-aminophenol and SKF 525-A in a total volume of 0.5 ml was incubated for 30 min at 37°. Determination of *o*-aminophenol formed was conducted after the method of Levvy and Storey (9). The added amount of UDPGA was proved to be enough to saturate the reaction.

Urinalysis. Urine samples were collected as previously described (7). At the end of each time period for urine collection, animals were forced to urinate by gentle squeezing.

Creatinine was determined after Folin (10). Determination of phenol (7) and *p*-aminophenol (11) after hydrolysis of urine (7) was conducted as described. Measurement of *o*-aminophenyl glucuronide was based on the method of Levvy and Storey (9) and conducted as follows: 1 ml of diluted urine was mixed with 1 ml of 1 M trichloroacetic acid-NaH_2PO_4 buffer (pH 2.1). Then, 0.5 ml each of 0.05% sodium nitrite, 0.5% ammonium sulfamate, and 0.1% naphthylethylenediamine solutions were added at 10-minute intervals after thorough mixing. The mixture was incubated for 2 hours at 37° and the resulting color was measured at 555 mμ. In the usual assay, urine was diluted so that creatinine concentration was less than 4 μg/ml. Under this condition, normal components of urine did not disturb *o*-aminophenyl glucuronide determination.

Chemicals. SKF 525-A was a gift from Smith, Kline and French Laboratories, Philadelphia, Pennsylvania. UDPGA was purchased from Sigma Chemical Co., St. Louis, Mo. *o*-Aminophenyl glucuronide for the standard was prepared after Williams (12).

RESULTS

Kinetics of in Vitro Inhibition

SKF 525-A inhibits both aryl 4-hydroxylase and UDP-glucuronyl transferase activities in a noncompetitive manner. Typical cases are shown in Figs. 1 and 2. The apparent K_m of aryl 4-hydroxylase was $1.8 \pm 0.4 \times 10^{-3}$ M (mean of 5 experiments ± SD), which is somewhat higher than K_m values for drug hydroxylation reported elsewhere (13–15). The K_i for SKF 525-A was graphically determined (16) to be $1.7 \pm 0.4 \times 10^{-4}$ M. In the case of UDP-glucuronyl transferase, the apparent K_m was $7.1 \pm 0.9 \times 10^{-5}$ M, and the K_i for SKF 525-A was $3.6 \pm 0.7 \times 10^{-4}$ M.

In Vivo Suppression of Hydroxylation and Glucuronidation

It has been previously observed in our laboratory that rats have shown less variable urinary flow rates than guinea pigs as measured by the amounts of creatinine in urine. Coefficient of variation of creatinine was 18.4% in the urine collected from a group of 7 rats for a 2-hr period (12 determinations). It was 27.7% in the urine collected from a group of 5 guinea pigs for the equivalent time period (9 determinations). Therefore, it was considered wise to use rats as well as guinea pigs for the time course study on urinary excretion of metabolites.

Table 1 shows that SKF 525-A can suppress the aromatic hydroxylation and phenolic glucuronidation *in vivo* as well as *in vitro*. When 50 mg/kg of SKF 525-A was injected intraperitoneally in rats together with aniline administration, the amount of excreted *p*-aminophenol during the first 2 hr was about 50% of the con-

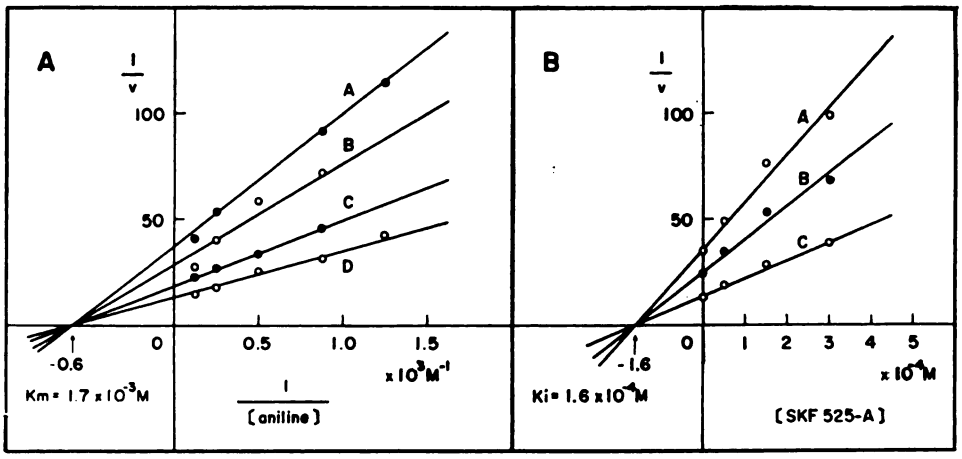

FIG. 1. *Kinetics of aryl 4-hydroxylase*

Incubation was as described under Materials and Methods. (A) Double reciprocal plot of aniline concentration versus rate of hydroxylation with varying concentrations of SKF 525-A. Concentrations of SKF 525-A were as follows: *A*, 3.0×10^{-4} M; *B*, 1.5×10^{-4} M; *C*, 0.5×10^{-4} M; *D*, zero.

(B) Plot of SKF 525-A concentration versus reciprocal of rate of hydroxylation with varying concentrations of aniline. Concentrations of aniline were as follows: *A*, 1×10^{-3} M; *B*, 2×10^{-3} M; *C*, the extrapolated theoretical line for an infinite concentration of aniline.

trol. Ten hours after aniline administration, excretion of *p*-aminophenol in urine was almost negligible in both SKF 525-A and control groups. Duration of suppression by SKF 525-A was rather short compared with the observation of Cook *et al.* (17), Rogers *et al.* (18), and Rogers and Fouts (19); thus overall suppression in 10 hr was

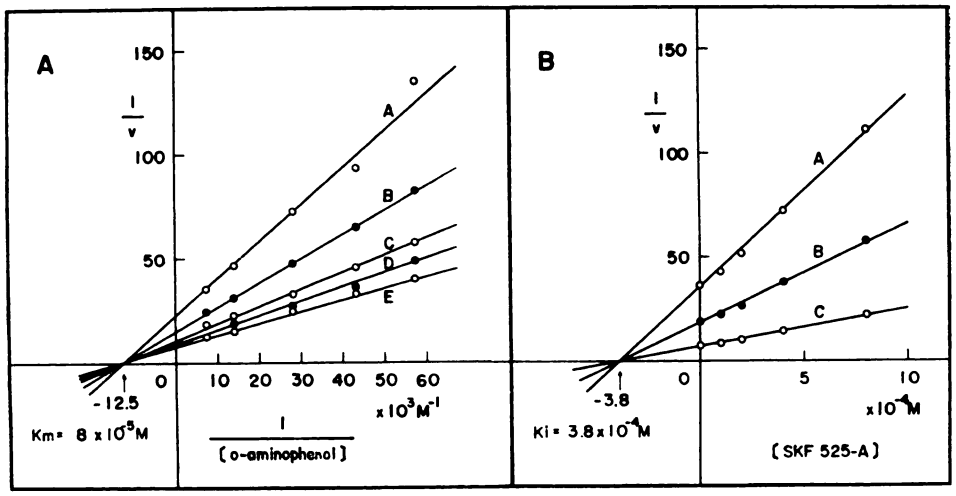

FIG. 2. *Kinetics of UDP-glucuronyl transferase*

Incubation was as described under Materials and Methods. (A) Double reciprocal plot of *o*-aminophenol concentration versus rate of glucuronidation with varying concentrations of SKF 525-A. Concentrations of SKF 525-A were as follows: *A*, 8×10^{-4} M; *B*, 4×10^{-4} M; *C*, 2×10^{-4} M; *D*, 1×10^{-4} M; *E*, zero.

(B) Plot of SKF 525-A concentration versus reciprocal of rate of glucuronidation with varying concentrations of *o*-aminophenol. Concentrations of *o*-aminophenol were as follows: *A*, 2×10^{-5} M; *B*, 5×10^{-5} M; *C*, the extrapolated theoretical line for an infinite concentration of *o*-aminophenol.

TABLE 1

Inhibitory effect of SKF 525-A on in vivo hydroxylation and glucuronidation

Female Wistar rats (about 50 g, 6 animals per group) or male Hartley guinea pigs (about 200 g, 5 animals per group) were intraperitoneally injected with 2 ml/kg of 0.85% saline (control group) or 2 ml/kg of 25 mg/ml SKF 525-A solution in 0.85% saline (SKF 525-A dose, 50 mg/kg-"SKF" group). Immediately thereafter, 51 mg/kg of aniline (diluted 40 times with corn oil), 141 mg/kg of benzene (diluted 4 times with corn oil) or 100 mg/kg of *o*-aminophenol (100 mg was suspended in 2 ml of corn oil) was intraperitoneally injected. Amounts of metabolites excreted in urine during the time periods were determined as described under Materials and Methods and expressed as milligrams per kilogram of body weight of animals. Numbers in the table are means together with standard deviations. Difference between control and "SKF" values are analyzed by *t* test.

Substrate	Metabolite determined	Species	Group	Expt. No.	Metabolite (mg/kg) excreted during		
					First 2 hr	Next 8 hr	Total 10 hr
Aniline	*p*-Aminophenol	Rat	Control	3	8.0 ± 1.6	11.7 ± 1.6	19.7 ± 0.9
			SKF 525-A	3	4.2 ± 1.3	13.0 ± 1.7	17.2 ± 0.9
					$P < 0.05$	$P > 0.1$	$0.025 < P < 0.05$
		Guinea pig	Control	4	3.1 ± 0.9[a]	1.5 ± 0.8[b]	4.5 ± 1.6
			SKF 525-A	4	1.8 ± 0.5[a]	1.7 ± 0.7[b]	3.6 ± 1.1
					$P < 0.05$	$P > 0.1$	$P > 0.1$
Benzene	Phenol	Rat	Control	3	3.9 ± 0.6	13.7 ± 1.3	17.6 ± 0.6
			SKF 525-A	3	1.9 ± 0.1	11.6 ± 1.1	13.5 ± 1.2
					$P < 0.01$	$0.05 < P < 0.1$	$P < 0.01$
o-Aminophenol	*o*-Aminophenyl glucuronide	Rat	Control	4	28.1 ± 6.7[c]	6.5 ± 1.7[d]	34.6 ± 7.2[e]
			SKF 525-A	4	18.6 ± 2.3[c]	6.4 ± 1.4[d]	25.0 ± 3.6[e]
					$P < 0.05$	$P > 0.1$	$P < 0.05$
		Guinea pig	Control	5	40.3 ± 10.2	28.3 ± 8.0	68.6 ± 14.1
			SKF 525-A	5	17.3 ± 14.4	54.0 ± 9.7	71.4 ± 10.3
					$P < 0.025$	$P < 0.01$	$P > 0.1$

[a] First 4 hr.
[b] Next 6 hr.
[c] First hour.
[d] Next 4 hr.
[e] Total 5 hr.

slight. According to Parke and Williams, the ability of rats to convert aniline to *o*-aminophenol is as low as one-sixth of that to form *p*-aminophenol (20). A similar observation was made when benzene, a probable alternative substrate of the hydroxylation reaction, was given to rats, and excretion of phenol in urine followed. Conversion from *o*-aminophenol to *o*-aminophenyl glucuronide in rats proceeded much faster. More than 70% of glucuronide observed was excreted within 1 hr after the administration, and after 5 hours, only a negligible amount was present in urine. With the simultaneous injection of SKF 525-A, excretion of glucuronide in 1 hr after the administration was as low as 66% of the control.

Results obtained from guinea pigs were essentially the same as those obtained from rats. However, guinea pigs excreted much less *p*-aminophenol then rats. *o*-Aminophenyl glucuronide excretion in guinea pigs was slower than in rats, but the quantity excreted was greater.

DISCUSSION

Although *N*-demethylase activity was competitively inhibited by SKF 525-A (3), the present report describes noncompetitive inhibition by SKF 525-A on aryl 4-hydroxylase. Bearing in mind that the hydroxymethyl compound is assumed to be an intermediate of *N*-demethylation (21), the data seem to indicate that aromatic hydroxylation and *N*-demethylation (or possibly *N*-dealkylation) are catalyzed by different enzymes. Mitoma *et al.* (5) reported only 20% inhibition of acetanilide hydroxylation in the presence of 1×10^{-3} M SKF 525-A, and Fouts and Brodie (22) needed 2.5 to 3×10^{-3} M SKF 525-A to obtain 50% inhibition, forming a sharp contrast to our result with aniline hydroxylation in which the K_i is as low as 1.7×10^{-4} M. In agreement with this low K_i value, *in vivo* aromatic hydroxylation was suppressed by the administration of 50 mg/kg of SKF 525-A to rats and guinea pigs. Rogers and Fouts (19) detected 44 μg of SKF 525-A per gram of rat liver 10 min after intraperitoneal injection of 80 mg/kg of the compound, and 28 μg/g with the dose of 40 mg/kg. Kakemi and his associates (personal communication) injected intraperitoneally 80 mg/kg of SKF 525-A to rats and found that SKF 525-A concentration in the liver was 68 μg/g 1 hr after the injection and that the concentration decreased very rapidly as a function of time. The theoretical initial concentration in the liver was about 100 μg/g [calculated from the results of Kakemi *et al.*]. Thus, SKF 525-A concentration in the liver within 2 hr after intraperitoneal injection of 50 mg/kg of the compound may roughly be estimated to be in the range of 10–65 μg/g or 0.3 to 1.7×10^{-4} M.

The present report on the inhibitory effect of SKF 525-A on *o*-aminophenol glucuronidation extends observations by Cooper *et al.* (23) reporting inhibition on morphine glucuronidation, although substrate specificity of liver UDP-glucuronyl transferase is not as yet thoroughly elucidated (24).

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