IN VITRO METABOLISM OF ACNU, 3-[(4-AMINO-2-METHYL-5-PYRIMIDINYL)METHYL]-1-(2-CHLOROETHYL)-1-NITROSOUREA HYDROCHLORIDE, A WATER-SOLUBLE ANTITUMOR NITROSOUREA

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In vitro decomposition of ACNU, 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-1-(2-chloroethyl)-1-nitroso-urea hydrochloride, in various conditions was studied with the use of the ^{14}C -labeled compound.

Metabolite A, 3,4-dihydro-7-methylpyrimido[4,5-*d*]pyrimidin-2(1*H*)-one (an intramolecular cyclized product), was formed spontaneously in the phosphate buffer (pH 7.4) with simultaneous liberation of the alkylating moiety.

With rat liver enzyme preparations, formation of three metabolites was observed. Those were metabolite B, 1-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-3-(2-chloroethyl)urea (a denitrosated product), metabolite C, 1-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-5-hydroxy-3-nitroso-2-imidazolidinone (a product *via* oxidative dechlorination), and metabolite D, 1-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-5-hydroxy-2-imidazolidinone (a denitrosated product of metabolite C).

Formation of metabolite B was catalyzed with both cytosolic and microsomal enzymes, not inhibited with SKF-525A, and partly dependent on nicotinamide adenine dinucleotide phosphate (NADPH). These results suggest that at least two enzymatic steps would be involved in the formation of this product.

Metabolites C and D were produced by the microsomal preparation, being dependent on O_2 and NADPH, inhibited by CO and SKF-525A, and enhanced by phenobarbital pretreatment. When metabolite C was incubated with cytosolic and microsomal preparations, more efficient formation of metabolite D with the former than the latter was observed. From these results, it was assumed that oxidative dechlorination of ACNU to metabolite C would be catalyzed with the microsomal mixed function oxidase, and metabolite D would be produced *via* denitrosation process of metabolite C.

Keywords—3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-1-(2-chloroethyl)-1-nitroso-urea hydrochloride (ACNU); antitumor nitroso-urea; *in vitro* degradation; rat liver homogenate; oxidative dechlorination; denitrosation

INTRODUCTION

In a previous work, the occurrence of the four major metabolites of ACNU, 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-1-(2-chloroethyl)-1-nitroso-urea hydrochloride, a potent antitumor nitroso-urea, in urine of rats and mice^{1,2)} was reported. Those were M-A, 3,4-dihydro-7-methyl-pyrimido[4,5-*d*]pyrimidin-2(1*H*)-one (a

cyclized pyrimidopyrimidine), M-B, 1-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-3-(2-chloroethyl)urea (a denitrosated derivative), M-C, 1-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-5-hydroxy-3-nitroso-2-imidazolidinone (a nitroso-imidazolidinone), and M-D, 1-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-5-hydroxy-2-imidazolidinone (an imidazolidinone).

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Since some of these metabolites are structurally unique comparing to those of other nitrosourea agents, such as 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU), Me-CCNU, and 1,3-bis(2-chloroethyl)-nitrosourea (BCNU), it will be worthy to investigate the mechanism of formation in the animal body.

The present paper concerns about *in vitro* formation of metabolites and their metabolic pathways.

MATERIALS AND METHODS

1. Materials — 2-Chloroethyl-2-¹⁴C-(2.81 mCi/mmol) and aminomethylpyrimidinyl-methyl-¹⁴C-(5.25 mCi/mmol)-ACNUs, and aminomethylpyrimidinyl-methyl-¹⁴C-M-B (2.78 mCi/mmol) were used. M-C-¹⁴C was prepared with the nitrosation of ¹⁴C-M-D isolated from the rat urine after administration of pyrimidine-¹⁴C-ACNU. For nitrosation, sodium nitroxide was added to the M-D fraction, and pH was adjusted to 9 to 10 with sodium carbonate. Thus formed M-C was purified with thin layer chromatography. Various authentic compounds for identification of metabolites were synthesized by Dr. H. Nakao of Chemical Research Laboratories of this Company.

SKF-525A was kindly supplied by Smith, Klein and French Company. Other chemicals used in this study were purchased from various commercial sources.

2. Animals — Male Donryu rats weighing about 250 g were used. Phenobarbital and methylcholanthrene treatments were carried out with the following conditions *i.e.*, 70 mg/kg/d for 5 d for the former and 20 mg/kg/d for 2 d for the latter.

3. Preparation of Enzyme Sources — Rats were killed by decapitation and the liver was removed. The homogenate was prepared with Potter-Elvehjem homogenizer equipped with a Teflon pestle in the solution composed of 10 mM phosphate buffer, pH 7.4 in 1.15% KCl. Centrifugation was carried out with a refrigerated centrifuge for 20 min at $9000 \times g$. The resulting supernatant was further centrifuged for 60 min at

$105000 \times g$ with an ultracentrifuge. The pellet was resuspended in the buffer and the suspension was used as the microsomal fraction.

4. Incubation Procedure — Incubation mixture contained 0.05 M phosphate buffer (pH 7.4), 1.0 μ mol of nicotinamide adenine dinucleotide phosphate (NADP), 25 μ mol of glucose-6-phosphate, 1.2 units of glucose-6-P dehydrogenase, 1.8 μ mol of the substrate, and 50 μ l of an appropriate enzyme preparation in 100 μ l. Incubations were carried out at 37 °C for 1 h, and stopped with the addition of 9 volumes of ice-cooled ethanol. The mixture was centrifuged and a part of the concentrated supernatant was spotted on thin-layer chromatography (TLC) for determining quantities of products. Two-dimensional TLC was carried out with Kiesel gel plate (Art. 5715, Merck), and chloroform-methanol (3:1) and *n*-butanol-methanol-water (9:1:1) were used as the first and the second solvent, respectively. The radioactive spots were scraped and the materials were extracted with 90% methanol for determination of the radioactivity by a liquid scintillation counting.

5. Substrate Difference Spectra — Substrate difference spectra were determined using a Shimadzu MPS-5000 spectrophotometer by the method of Schenkman *et al.*³⁾

RESULTS

1. Decomposition of ACNU in the Aqueous Solution

Pyrimidine-¹⁴C-ACNU in 50 mM phosphate buffer (pH 7.4) at 37 °C was decomposed rapidly with first order kinetics at a half-life ($t_{1/2}$) of 24 min. M-A was formed almost quantitatively with concomitant formation of a small amount of M-B (Fig. 1). The rate of the decomposition was affected by pH (Fig. 2) and temperature (Fig. 3).

These data suggest that ACNU decompose at physiological pH and temperature to form M-A and to liberate the alkylating moiety of ACNU.⁴⁾

2. Enzymatic Decomposition of ACNU

ACNU was transformed enzymatically to produce various kinds of metabolites, *i.e.*, M-B, -C and -D, in the presence of the NADPH-generating system (Table I). Additionally, it was observed

that ACNU showed type-I difference spectrum with rat liver microsomes (Fig. 4) with binding constant (K_s) of 3×10^{-5} M (Fig. 5). These results indicate that, at least partly, cytochrome P-450 linked mixed function oxidase system could participate in the transformation processes of ACNU. As shown in Table I, formation of M-C and -D were dependent on NADPH, and inhibi-

FIG. 1. Time Course of the Conversion of Pyrimidine- ^{14}C -ACNU to Metabolites in the Phosphate Buffer (pH 7.3, 50 mM at 37 °C)

FIG. 2. Effect of pH on Decomposition of ACNU in Aqueous Solution

ACNU was incubated in phosphate buffer (pH 7.4, 50 mM) at 37 °C for 20 min.

ted with SKF-525A and carbon monoxide. These formations were potentiated by the phenobarbital (PB)-pretreatment and suppressed by the 3-methylcholanthrene (3-MC)-treatment. Under anaerobic conditions, M-C and -D formations were greatly suppressed. These properties imply that the microsomal mixed function oxidase was responsible for the conversion of ACNU to M-C and -D. The M-C and -D were formed with microsomal preparations, but not with cytosolic fractions (Table II).

Rate of formation of M-B was accelerated with the addition of NADPH-generating system and inhibited partly with carbon monoxide, and also potentiated by pretreatment with inducers. However, the inhibition by SKF-525A and O_2 -requirement was not observed. M-B formation was found in both microsomal and cytosolic preparations (Table II), whereas the reaction with the latter was not affected with carbon monoxide, and SKF-525A. It was suggested, therefore, that at least two systems will participate in the conversion of ACNU to M-B.

It was found that the rate of formation of M-C and -D in microsomes was very low in comparison with their formation with $9000 \times g$ -supernatant (Table II). Addition of the cytosol

FIG. 3. Effect of Temperature on Decomposition of ACNU in Aqueous Solution

ACNU was incubated in phosphate buffer (pH 7.4, 50 mM) at various temperature.

TABLE I. *Effect of Various Factors on the Conversion of ACNU to M-B, -C and -D in the Rat Liver 9000 × g-Supernatant*

Conditions	Conversion of ACNU		
	M-B	to M-C	M-D
		(nmol/g-liver	30 min)
Complete	398 (100%)	116 (100%)	140 (100%)
N ₂ -saturated	430 (108%)	16 (11%)	21 (15%)
CO-saturated	276 (69%)	57 (49%)	78 (55%)
NADPH omitted	270 (68%)	9 (8%)	6 (4%)
SKF-525A (1 mM)	435 (109%)	17 (15%)	9 (6%)
PB treated	570 (143%)	170 (147%)	312 (223%)
3-MC treated	574 (144%)	79 (68%)	69 (49%)

Incubations were carried out at 37 °C for 30 min and products were assayed with TLC as described in Materials and Methods. CO was bubbled for 1 min before starting the reaction. The values represent means of three incubations.

FIG. 4. *Substrate Difference Spectrum of ACNU to the Rat Liver Microsomes*
Substrate concentration of 1.8 mM was used.

resulted in the enhancement of the microsomal activity, but the enhancement was not observed with heat-denatured cytosol.

Ethylenediaminetetraacetic acid (EDTA, 1 mM), which will inhibit microsomal lipid peroxidation, enhanced the M-C and -D formation with microsomes, but the enhancement was not observed in 9000 × g-supernatant (Table III). These results showed that a part of the effect of the cytosol on the microsomal reactions could be replaced by the addition of EDTA.

¹⁴C-M-C was converted to M-D with various fractions of rat liver, whereas ¹⁴C-M-B was not converted anymore (Table IV). In a preliminary

FIG. 5. *K_s of ACNU for Rat Liver Microsomes*

experiment, it was revealed that *i.v.* injected ¹⁴C-M-B to rats did not receive further metabolism *in vivo*.

DISCUSSION

In the present study, decomposition of ACNU *in vitro* was investigated in aqueous solutions and rat liver enzymes. According to the results, the metabolic pathway of ACNU was proposed as shown in Fig. 6.

Under the physiological conditions, ACNU molecule (Fig. 1). On the fates of the alkylating moiety of various nitrosoureas, it has been reported that chloroethylnitrosourea derivatives decomposed spontaneously to form M-A which lacks the chloroethyl moiety of ACNU in the

TABLE II. Conversion of Ethylene- ^{14}C -ACNU to M-B, -C and -D in the Rat Hepatic Cell Fractions

Fractions	Conversion of ACNU		
	M-B	to M-C	M-D
		(nmol/g-liver	30 min)
9000 $\times$ g-supernatant	247	194	180
Microsomes	402	49.6	8.8
Cytosol	260	n.d. ^{a)}	n.d. ^{a)}
Microsomes + cytosol	254	160	139

Incubations were carried out with methods described in legend of Table I. a) n.d. means not detected.

TABLE III. Effect of EDTA on Conversion of ACNU to M-C and -D in the Rat Hepatic Cell Fractions

Conditions	Conversion of ACNU	
	M-C	M-D
	(nmol/g-liver	30 min)
(Without EDTA)		
9000 $\times$ g-supernatant	164	160
Microsomes	62	6
(With 1 mM EDTA)		
9000 $\times$ g-supernatant	160	157
Microsomes ^{a)}	113	96

Incubations were carried out with methods described in legend of Table I. a) Microsomes were prepared in the buffer containing 1 mM EDTA.

TABLE IV. Conversion of ^{14}C -M-B and -C to M-D in the Rat Hepatic Cell Fractions

Fractions	Formation of M-D	
	M-B	M-C
	(nmol/g-liver	30 min)
Heat-denature 9000 $\times$ g-supernatant	n.d. ^{a)}	80
9000 $\times$ g-supernatant	n.d.	2037
Microsomes	— ^{b)}	392
Cytosol	—	1940

Incubations were carried out with methods described in legend of Table I. a) n.d. means not detected. b) — means not determined.

decompose to produce an isocyanate, and chloroethyldiazohydroxide derived from the alkylating moiety, of which the latter disposed immediately to chloroethyl cation.⁵⁻⁷⁾ Nakamura *et al.*⁴⁾ reported that ACNU generated 2-chloroethanol and acetaldehyde in the aqueous solution, and Asami *et al.*⁸⁾ showed that these compounds were formed *via* chloroethyl cation. These processes will be the most important in correlation with manifestation of antitumor activity of nitrosoureas. On the remaining moieties, it has been also pronounced that the isocyanate derivatives show carbamoylation to biological macromolecules.^{9,10)} In the case of ACNU, however, M-A, which does not show any effects in biological systems, was formed almost quantitatively from the parent compound. These results led us to consider that, after hydrolyzing ACNU in the aqueous systems, an isocyanate intermediate, once formed, will cyclize

intramolecularly to form inactive M-A. In this regard, the process of M-A formation seems important relative to the occurrence of the pharmacological action of ACNU.

It was shown that ACNU was metabolized with microsomes to produce M-C and -D, and M-D was derived from M-C, not from M-B, indicating that M-C should be formed from ACNU by an oxidative process and convert, thereafter, to M-D by a denitrosation with hepatic mixed function oxidases. However, velocity of these reactions is relatively slow with the microsomal preparation comparing with that with the $9000 \times g$ -supernatant, and required enhancement of the cytosolic fraction for full activity. It has been demonstrated that various microsomal drug metabolizing activities were stimulated by the addition of cytosolic fractions.^{11,12)} Wills,¹³⁾ and Kamataki and Kitagawa¹⁴⁾ reported that induction of lipid peroxidation in microsomes lead to

FIG. 6. *Metabolic Pathway of ACNU*

decrease the various microsomal activities. Kamataki *et al.*¹⁵⁾ and Talcott *et al.*¹⁶⁾ described that heat-labile inhibitor of lipid peroxidation exists in hepatic soluble fraction and proposed that a cytosolic inhibitor would be the cause of the stimulation of the microsomal drug metabolizing activities by the cytosol. In the present study, M-C formation in microsomes was enhanced with the addition of EDTA (1 mM), and the effect could not be observed in 9000 × *g*-supernatant. These results suggest that the lipid peroxidation might be a cause of the lower conversion rate of ACNU to M-C and -D in microsomes.

For conversion of ACNU to M-C, it seemed to require at least two steps, *i.e.* oxidation and dechlorination. To ascertain these possibilities, proposed intermediates described below, were synthesized, and incubated with 9000 × *g*-supernatant as a preliminary observation. It was shown that these compounds were not

metabolized with this enzyme fraction. Additionally, glutathione, which has been known as an activating agent for a dechlorination reaction,^{17,18)} did not potentiate M-C formation with 9000 × *g*-supernatant. From these preliminary results, it seems likely that both oxidation and dechlorination reactions might be catalyzed by unseparable single enzymatic reaction just like that reported on 1,1,2-trichloropropane,¹⁹⁾ chloroform^{20,21)} and

halothane.²²⁾ It was shown that M-B was formed with rather complicated processes than that of other metabolites as shown in Tables I and III. Hill *et al.*²³⁾ also reported a similar phenomenon on BCNU in microsomal denitrosation and described that the system catalyzing the reaction apparently has properties different from those of cytochrome P-450-linked activities.

In consequence, it was demonstrated that ACNU was spontaneously decomposed to form M-A in aqueous solution, and that the chloroethyl moiety of ACNU was modified by the hepatic microsomal mixed function oxidase before the occurrence of the hydrolytic degradation of ACNU. This report firstly revealed that chloroethyl moiety of the antitumor nitrosourea compound becomes the substrate of the hepatic mixed function oxidase and the compound can undergo dechlorination enzymatically.

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