Studies on the Metabolic Fate of NK-104, a New Inhibitor of HMG-CoA Reductase (5): In Vitro Metabolism and Plasma Protein Binding in Animals and Human

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Summary: NK-104 is a new and very potent competitive inhibitor of HMG-CoA reductase. NK-104 bound to plasma protein of mouse, rat, rabbit, dog and monkey, at a binding ratio of more than 96%. NK-104 also highly bound to human serum albumin and α_1 -acid glycoprotein. However, no interaction in protein binding was found between NK-104 and selected highly protein binding drugs at their therapeutic concentrations in human plasma.

The *in vitro* metabolism of ^{14}C -NK-104 was investigated using the liver microsomes of rat, rabbit, guinea pig, dog, monkey and human. The radioactivity was mostly due to unchanged NK-104 except for monkey. A relatively large amount of M-13 (8-hydroxy NK-104) was observed in monkey, but not in other animal species. The kinetic study of NK-104 metabolism suggested that M-13 was formed with relatively low intrinsic clearance. Based on three different *in vitro* approaches, namely 1) chemical inhibition, 2) immunoinhibition and 3) metabolism by recombinant human P450, it is concluded that CYP2C9 and CYP2C8 are the enzymes responsible for the metabolism of NK-104. In addition, no inhibitory effect on CYP mediated 4-hydroxylation of tolbutamide (CYP2C9) in the presence of NK-104 was detected.

In conclusion, NK-104 was highly bound to plasma protein whereas no mutual interaction in protein binding was found between NK-104 and various commonly used drugs. Furthermore, NK-104 was scarcely metabolized in the liver microsomes, and no drug/drug interaction between NK-104 and tolbutamide was noted in *in vitro* metabolism.

Key words: NK-104, HMG-CoA reductase inhibitor, Protein binding, Microsome, S-9, Cytochrome P-450, CYP2C9, Drug/drug interaction

Introduction

NK-104 is a highly potent inhibitor of HMG-CoA reductase, the rate limiting enzyme in cholesterol biosynthesis.¹⁾ This agent is a promising therapeutic drug for hyperlipidemia in humans. As previously reported,^{2,3)} this compound cause a significant reduction in serum total cholesterol, LDL cholesterol and triglyceride levels in animals and humans. We recently demonstrated that ^{14}C -NK-104 following oral administration to rats was selectively distributed as unchanged form to the liver, a target organ of this drug.⁴⁾ Moreover, the pharmacokinetics of NK-104 after oral administration in humans include good absorption, a relatively long half-life and possibly high bioavailability.⁵⁾ The favorable metabolic and pharmacokinetic characteristics of this drug support efficacy in clinical studies.³⁾ However, no studies have been published regarding the plasma protein binding and *in vitro* metabolism of NK-104.

In the present study, we have examined the characteristics of the binding of NK-104 to plasma proteins of hu-

man and animals. The *in vitro* metabolism of NK-104 was investigated using liver microsomes of human and several animals. Evidence is presented that a single metabolic pathway of NK-104 to M-13 (8-hydroxy NK-104) is catalyzed by the human cytochrome P-450 family of enzymes.

Recently, to obviate the drug/drug interaction, considerable attention has been paid to the plasma protein binding and *in vitro* metabolism. In view of the widespread clinical use of NK-104 and the importance of drug/drug interaction, we set out to clarify the mutual displacement from the plasma protein site of NK-104 and various common drugs *in vitro*. Thus the mutual displacement of protein binding and the inhibitory effect on cytochrome P-450 enzymes between NK-104 and other drugs were also investigated using several markers and a chemical inhibitor. In addition, we examined the potential inhibitory effects of NK-104 using known P-450 functional markers of CYP2C9.

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Materials and Methods

1. Chemicals and Reagents

NK-104 (monocalcium bis [(3R,5S,6E)-7-[2-cyclopropyl-4-(4-fluorophenyl)-3-quinolyl] 3,5-dihydroxy-6-heptenoate]) and its metabolites (NK-104 lactone, M-3, M-8 and M-13) which were described in our previous report⁶⁾ were synthesized by Nissan Chemical Industries, Ltd.. The presumed metabolic pathways of NK-104 are shown in **Fig. 1**. Commercially available HSA (human serum albumin; Fraction V, Sigma) and α_1 -AGP (α_1 -acid glycoprotein; Fraction VI, Sigma) were used. For plasma protein interaction studies the following agents were used: warfarin, diazepam, digitoxin, phenylbutazone, phenytoin, furosemide, ibuprofen, nitrendipine and glibenclamide, as competitive drugs of protein binding. All were purchased commercially. Nitrendipine was synthesized in our laboratory. Sul-faphenazole, ketoconazole and 4-hydroxytolbutamide as the chemical inhibitors or model substrate markers of P-450 isoform were purchased from Ultrafine Chemicals (Manchester, UK). SKF-525A hydrochloride was purchased from Research Biochemicals Incorporated (Natick, USA). Polyclonal antiserum for human P-450

NADPH reductase, human CYP2C and CYP1A1/1A2, and monoclonal antiserum for human CYP2D6 and CYP3A4 were purchased from GENTEST Co. Ltd. (Woburn, USA). All other chemicals and solvents were purchased commercially and were of HPLC grade or extra pure grade.

2. Radioisotope Labeled Chemicals

[Fluorobenzene- U - ^{14}C] NK-104 (Code CFQ 8596) was synthesized by Amersham Co., Ltd. (Little Chalfort, UK). The specific radioactivity of labeled compound was 2.2 MBq/mg, and the radiochemical purity was more than 98% during the experimental period. ^{14}C -Labeled warfarin (Amersham, 2.07 GBq/m mol), ^{14}C -diazepam (Amersham, 2.04 GBq/m mol), tritium labeled digitoxin (NEN, 677.1 GBq/m mol), 3H -propranolol (NEN, 777 GBq/m mol), 3H -nitrendipine (NEN, 2904.5 GBq/m mol), 3H -glibenclamide (NEN, 1850 GBq/m mol), and [Ring- U - ^{14}C] tolbutamide (Amersham, 2.22 GBq/m mol) were purchased commercially. The radiochemical purity of each product was more than 97%.

3. Microsomes and Human cDNA-expressed Cytochrome P-450s

Pooled liver microsomes (Lot HHM-0249, 0256,

Fig. 1 Postulated metabolic pathway of NK-104

0250, 0219) of male and female human and pooled liver S-9 (Lot HHS-0249) were purchased from KAC Co. Ltd. (Kyoto, Japan). The liver microsomes of male and female rats, male beagle dogs, male rabbits, male guinea pigs and male cynomolgus monkeys were purchased from Charles River Japan Inc. (Yokohama, Japan). Recombinant microsomes derived from human AHH-1 cells expressing human P-450 (Control, CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9-*arg*, 2C9-*cys*, 2C19, 2D6-*val*, 2D6-*met*, 2E1, 3A4 and 4A11) were purchased from GENTEST Co. Ltd.. The NADPH generating system (β -NADP, Glucose-6-phosphate and Glucose-6-phosphate dehydrogenase) was obtained commercially.

4. Protein Binding Studies

1) *In Vitro*

Wistar strain male rats, JW strain male rabbits, HRA-male beagle dogs (Japan Laboratory Animal Inc. Tokyo, Japan), C.D strain male mouse (Charles River Japan Inc.) and male cynomolgus monkeys (Philippine born, Japan SLC. Inc. Shizuoka, Japan) were used. The plasma samples were obtained from animals ($n=4$), which had fasted overnight. Mouse plasma was withdrawn from fifteen mice, pooled, and divided into three groups. The human plasma was obtained from healthy volunteers ($n=4$). NK-104 was mixed with plasma to give a final concentration of 0.1, 0.3 and 1.0 $\mu\text{g/ml}$. The binding to plasma protein was measured in the equilibrium dialysis system (Takara seisakusyo) with Spectra/Por2 membrane (Spectrum) having a molecule cutoff of 12000-14000. The membrane was pretreated by soaking in deionized water and used. In each dialysis cell, fresh plasma was dialyzed against isotonic phosphate buffer, pH 7.4 at 37°C. HSA and α_1 -AGP were also used to prepare each protein solution in 0.05 M phosphate buffer (pH 7.4). The binding assay of the isolated human plasma protein was carried out as described above.

2) *Ex Vivo*

Wistar strain male rats were used and five animals were assigned to each test group. The dosing solution at a dose of 3 mg/kg was administered orally by gavage. The rats were anesthetized by ether inhalation and sacrificed by exsanguination from the abdominal vein at 0.5, 1, 2 and 4 hr after administration, respectively. For the assessment of the *ex vivo* protein binding, the plasma binding assay was carried out as for the *in vitro* studies.

3) *Displacement of Protein Binding*

The interaction between NK-104 and nine competitive protein binding drugs in human plasma was studied. The human plasma samples were obtained from healthy volunteers for each drug study ($n=4$).

For examining the influence of competitive drugs on the protein binding of NK-104, NK-104 was mixed with plasma to give a final concentration of 0.3 $\mu\text{g/ml}$. Concurrently, competitive drugs at therapeutic plasma concentrations were also mixed as follows: warfarin 3 and 15 $\mu\text{g/ml}$, diazepam 15 and 75 $\mu\text{g/ml}$, digitoxin 0.1 and

0.5 $\mu\text{g/ml}$, phenylbutazone 100 and 500 $\mu\text{g/ml}$, phenytoin 20 and 100 $\mu\text{g/ml}$, furosemide 0.5 and 2.5 $\mu\text{g/ml}$, ibuprofen 50 and 250 $\mu\text{g/ml}$, and nitrendipine 0.1 and 0.5 $\mu\text{g/ml}$, glibenclamide 100 and 500 $\mu\text{g/ml}$.

In the study of the influence of NK-104 on competitive drugs, NK-104 was mixed with plasma at a final concentration of 0.3 and 1.0 $\mu\text{g/ml}$. Concurrently, competitive drugs were also mixed as follows: ^{14}C -warfarin 3 $\mu\text{g/ml}$, ^{14}C -diazepam 15 $\mu\text{g/ml}$, ^3H -digitoxin 0.1 $\mu\text{g/ml}$, ^3H -propranolol 1.0 $\mu\text{g/ml}$, ^3H -nitrendipine 0.1 $\mu\text{g/ml}$ and ^3H -glibenclamide 100 $\mu\text{g/ml}$. The binding assay of plasma protein was carried out with the measurement of radioactivity.

5. *In Vitro* Metabolism of NK-104

1) *Metabolism of NK-104 by Liver Microsomes and Hepatic S-9*

Using pooled liver microsomes of rat, dog, rabbit, guinea pig, monkey and human, the *in vitro* metabolism of ^{14}C -NK-104 was investigated at the concentration of 2.5 μM . The incubation mixture was prepared as follows: ^{14}C -NK-104, 0.1M sodium phosphate buffer solution (pH 7.4) and NADPH generating system were added to a tube and pre-incubated at 37°C for 5 min. Then ice-cooled liver microsomes were added individually to the incubation mixture at a final concentration of 1 mg protein/ml to initiate the metabolic reaction. After incubation at 37°C for 2 h, the reaction was quenched by adding the methanol solution. The reaction-terminated samples were centrifuged at 10,000 rpm for 3 min and the supernatant was analyzed by HPLC-RLG (radioluminography) as previously reported.⁴⁾

The *in vitro* metabolism study using human hepatic S-9 was carried out in the same manner as the liver microsome studies.

2) *Kinetic Studies*

To determine the apparent K_m and V_{max} values of NK-104 metabolism, kinetic studies of NK-104 were conducted using ^{14}C -NK-104 at the concentration range of 2.5-170 μM in liver microsomes of male and female rat, 0.5-50 μM in monkey, and 1-100 μM in human, respectively. The incubation was carried out for 30 min as described above and the generated M-13 was estimated.

3) *Change of NK-104 Metabolism by Chemical or Immunoinhibitors*

The experiments with known chemical inhibitors of P-450 were performed at a substrate concentration of 2.5 μM and inhibitor concentrations of 300 μM SKF-525A, 5 μM α -naphthoflavone, 2 μM ketoconazole and 4 μM sulfaphenazole using human liver microsomes. ^{14}C -NK-104 was co-incubated with these CYP inhibitors before the reaction using human microsomes. For immunoinhibition experiments, human microsomes were first incubated at room temperature for 20 min with 50 μl (0.5 mg eq. IgG) of rabbit/goat antiserum or control serum. The metabolic reaction was carried out as in the liver microsome studies. The inhibitory effect was estimated from the ratio of M-13 formation in the presence

and absence of inhibitor.

4) Presumption of CYP Isoforms for NK-104 Metabolism

Using fifteen recombinant human P-450 expressing microsomes of lymphoblastoid cell lines, the human P-450 isoforms participating in the hydroxylation from NK-104 to M-13 were investigated. At concentrations of 0.5, 2.5 and 25 μ M of NK-104, the reaction mixtures (1 mg protein/ml) were incubated for 2 h with the NADPH generation system. In addition, an incubation with control microsomes (native AHH-1 cells) was performed in parallel.

5) Effect of NK-104 on the Metabolism of Tolbutamide

The inhibitory effect of NK-104 on P-450 mediated metabolism was examined using 14 C-tolbutamide at the concentration range of 40–800 μ M. NK-104 (0.5–5 μ M) was co-incubated with CYP marker substrate before the reaction. Then human liver microsomes were added to give a final concentration of 1mg protein/ml for initiating a reaction and the mixture incubated at 37°C for 1h. In the presence or absence of NK-104, CYP2C9 mediated 4-hydroxytolbutamide production was measured.

6. Analytical Methods

The determination of NK-104 was performed by column switching-HPLC as in our previous report.⁸⁾ On the other hand, total radioactivity was measured with a liquid scintillation counter (Tri-calb 1500; Packard) for 10 min after addition of scintillation cocktail (Hionic-Fluor; Packard). In *in vitro* studies, the measurement of the radioactivity of NK-104 and its metabolites was carried out by the HPLC-RLG method.⁴⁾ All these analyses were performed under light resistant conditions to prevent the degradation of NK-104 and its metabolites. In addition, the measurement of 4-hydroxytolbutamide was performed according to the thin-layer chromatography method of Ludwig, *et al.*⁷⁾ Dried TLC plates were exposed to an imaging plate and quantified by use of a bioimaging analyzer (BAS-2000; Fuji film).

7. Calculations

In protein binding studies, the sample was taken from each side of the dialysis cell and the concentration or radioactivity of the plasma side (Cp) and buffer side (Cb) was measured. The unbound fraction rate (fu) was calculated according to the following equation: $fu = Cb/Cp \times 100$

In kinetic studies, the michaelis-menten parameters of apparent K_m and V_{max} were calculated by analysis of Lineweaver-Burk reciprocal plots. The *in vitro* intrinsic metabolic clearance (V_{max}/K_m) was calculated from the K_m and V_{max} . The type of inhibition was discriminated based on visual inspections of the double reciprocal plots in the presence and absence of inhibitor. The inhibition constant (K_i) was estimated by Dixon plot analysis. Results represent the mean $\pm$ S.E. of 3–5 samples.

Results

1. Plasma Protein Binding

1) In Vitro

NK-104 was highly bound to plasma protein: the unbound fraction rate was 0.7–0.8% in male rat, 0.5–0.7% in female rat, 3.2–3.4% in mouse, 1.6–1.7% in dog, 0.5–0.6% in rabbit, 1.3% in monkey and 0.4–0.5% in human, respectively (Table I). The binding was independent of sex in rats and of concentration in all animal species. Among isolated human plasma proteins, the major NK-104 binding protein was HSA, with an unbound frac-

Table I Plasma unbound fraction of NK-104 in different species

Animal	Test condition	Time (h)	Concentration (μ g/ml)	Ratio of unbound (%)
Male rat	<i>in vitro</i>		0.1	0.8 $\pm$ 0.0
			0.3	0.8 $\pm$ 0.0
			1.0	0.7 $\pm$ 0.0
	<i>ex vivo</i>	0.5	0.504	0.7 $\pm$ 0.0
		1	0.361	0.8 $\pm$ 0.1
		2	0.195	0.8 $\pm$ 0.1
		4*	0.121	0.9
Female rat	<i>in vitro</i>		0.1	0.5 $\pm$ 0.0
			0.3	0.6 $\pm$ 0.0
			1.0	0.7 $\pm$ 0.0
Mouse	<i>in vitro</i>		0.1	3.3 $\pm$ 0.4
			0.3	3.2 $\pm$ 0.1
			1.0	3.4 $\pm$ 0.2
Rabbit	<i>in vitro</i>		0.1	0.6 $\pm$ 0.1
			0.3	0.5 $\pm$ 0.0
			1.0	0.5 $\pm$ 0.0
Dog	<i>in vitro</i>		0.1	1.6 $\pm$ 0.1
			0.3	1.7 $\pm$ 0.1
			1.0	1.7 $\pm$ 0.1
Monkey	<i>in vitro</i>		0.1	1.3 $\pm$ 0.0
			0.3	1.3 $\pm$ 0.0
			1.0	1.3 $\pm$ 0.0
Human	<i>in vitro</i>		0.1	0.5 $\pm$ 0.0
			0.3	0.4 $\pm$ 0.0
			1.0	0.4 $\pm$ 0.0
4% HSA**	<i>in vitro</i>	*	0.1	0.4
		*	0.3	0.4
		*	1.0	0.5
0.06% α_1 -AGP***	<i>in vitro</i>		0.1	5.7 $\pm$ 0.1
			0.3	5.1 $\pm$ 0.8
			1.0	5.5 $\pm$ 0.7

Each value represents the mean $\pm$ S.E.

* : n=2

**HSA : Human serum albumin

*** α_1 -AGP : Human acidic α_1 -glycoprotein

Table II Influence of different concentrations of several highly bound drugs on the protein binding of NK-104 in human plasma

Interacting Drug	Concentration ($\mu\text{g/ml}$)	Unbound ratio of NK-104 (%)	Interacting Drug	Concentration ($\mu\text{g/ml}$)	Unbound ratio of NK-104 (%)
Warfarin	0	0.4 ± 0.0	Furosemide	0.0	0.3 ± 0.0
	3	0.4 ± 0.0		0.5	0.3 ± 0.0
	15	0.4 ± 0.0		2.5	0.4 ± 0.0
Diazepam	0	0.5 ± 0.0	Ibuprofen	0	0.4 ± 0.0
	15	0.5 ± 0.0		50	0.4 ± 0.0
	75	0.5 ± 0.0		250	0.4 ± 0.0
Digitoxin	0.0	0.3 ± 0.0	Nitrendipine	0.0	0.3 ± 0.0
	0.1	0.4 ± 0.0		0.1*	0.3 ± 0.0
	0.5	0.4 ± 0.0		0.5	0.3 ± 0.0
Phenylbutazone	0	0.5 ± 0.0	Glibenclamide	0	0.4 ± 0.1
	100	0.4 ± 0.0		100	0.5 ± 0.1
	500	0.6 ± 0.0		500*	0.6 ± 0.2
Phenytoin	0	0.3 ± 0.1			
	20	0.4 ± 0.0			
	100	0.4 ± 0.0			

Each value represents the mean $\pm$ S.E. of four plasma samples.

* $n=3$

Final plasma concentration of NK-104 was $0.3 \mu\text{g/ml}$.

Table III Influence of different concentrations NK-104 on the protein binding of several highly protein bound drugs in human plasma

Interacting Drug	NK-104 ($\mu\text{g/ml}$)	Unbound ratio of interacting drug (%)	Interacting Drug	NK-104 ($\mu\text{g/ml}$)	Unbound ratio of interacting drug (%)
Warfarin	0.0	0.8 ± 0.0	Propranolol	0.0	13.9 ± 1.4
	0.3	0.8 ± 0.1		0.3	13.6 ± 1.0
	1.0	0.8 ± 0.1		1.0	13.0 ± 1.2
Diazepam	0.0	1.5 ± 0.1	Nitrendipine	0.0	7.1 ± 0.2
	0.3	1.5 ± 0.2		0.3	6.7 ± 0.1
	1.0	1.5 ± 0.1		1.0	6.8 ± 0.1
Digitoxin	0.0	2.2 ± 0.1	Glibenclamide	0.0	1.0 ± 0.0
	0.3	$2.6 \pm 0.1^*$		0.3	1.2 ± 0.2
	1.0	$2.7 \pm 0.0^{**}$		1.0	1.1 ± 0.0
Digitoxin	0.0	2.4 ± 0.1			
	0.03	2.3 ± 0.0			
	0.1	2.2 ± 0.0			

Each value represents the mean $\pm$ S.E. of four plasma samples.

^{14}C -Warfarin ($3 \mu\text{g/ml}$), ^{14}C -Diazepam ($15 \mu\text{g/ml}$), ^3H -Digitoxin ($0.1 \mu\text{g/ml}$), ^3H -Propranolol ($1 \mu\text{g/ml}$), ^{14}C -Nitrendipine ($0.1 \mu\text{g/ml}$), ^3H -Glibenclamide ($100 \mu\text{g/ml}$)

*: $P < 0.05$, **: $P < 0.01$ (Dunnet's test in comparison to $0 \mu\text{g/ml}$.)

tion rate of 0.4–0.5%. The binding of NK-104 to α_1 -AGP was also strong and was concentration independent in this experimental range.

2) Ex Vivo

After oral administration of 3 mg/kg of NK-104 to male rats, the ex vivo protein binding was compared as in the *in vitro* results, and the unbound fraction was 0.7–0.9% (**Table I**). No marked difference in the protein

binding ratio was observed at any time point after dosing.

3) Displacement of Protein Binding

The drug/drug interaction for protein binding in human plasma between NK-104 and various highly protein bound drugs was investigated and the results are shown in **Table II** and **III**. The binding of NK-104 to proteins was unaffected by warfarin, diazepam, digitoxin, phenyl-

Fig. 2 Representative HPLC pattern of radioactivity in microsome incubates after incubation with ^{14}C -NK-104

butazone, phenytoin, furosemide, ibuprofen, nitrendipine and glibenclamide. Similarly, NK-104 had no influence on the binding of ^{14}C -warfarin, ^{14}C -diazepam, ^3H -propranolol, ^3H -nitrendipine and ^3H -glibenclamide. Although the unbound fraction of ^3H -digitoxin was slightly affected at high concentrations of NK-104, no significant interaction between digitoxin and NK-104 was found at therapeutic concentrations of NK-104 (0.03–0.1 $\mu\text{g}/\text{ml}$).

2. *In Vitro* Studies of NK-104 Metabolism

1) Metabolism of ^{14}C -NK-104 in Liver Microsomes

Fig. 2 illustrates typical chromatograms derived from a reaction mixture using liver microsomes of rat, rabbit, dog, monkey, guinea pig and human. ^{14}C -NK-104 was metabolized slightly by liver microsomes of all animals

except monkey. In contrast, ^{14}C -NK-104 was markedly metabolized in monkey microsome. M-13 and unknown metabolites were mainly generated in monkey microsome 38.4% and 21.4%, respectively. On the other hand, the composition of fractional radioactivity in male and female rat, dog, rabbit, guinea pig and human microsomes consisted mostly of the parent compound, and the ratio of unchanged NK-104 was 83.4, 81.0, 98.0, 93.1, 89.8 and 78.8%, respectively. Also, the ratio of M-13 as a minor metabolite was as follows: 9.5, 17.7, 0.4, 2.0, 2.7 and 16.3%, respectively. The other metabolites, M-3 (dehydrogenerated NK-104) and M-8 (propenoic acid derivative) were negligible in all species. In addition, the metabolic patterns of NK-104 were unchanged in the human S-9 fraction compared with the microsomes (**Table IV**).

Table IV Composition of NK-104 and its metabolites after 2 hr incubation with microsomes and S-9

Species	% of composition				
	NK-104	M-13	M-3	M-8	Unknown
Male rat microsome	83.4 $\pm$ 0.8	9.5 $\pm$ 0.8	4.3 $\pm$ 0.2	0.7 $\pm$ 0.2	2.1 $\pm$ 0.3
Female rat microsome	81.0 $\pm$ 0.8	17.7 $\pm$ 0.5	0.4 $\pm$ 0.0	0.0	0.9 $\pm$ 0.3
Dog microsome	98.0 $\pm$ 0.6	0.4 $\pm$ 0.0	1.5 $\pm$ 0.5	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0
Rabbit microsome	93.1 $\pm$ 0.4	2.0 $\pm$ 0.2	0.0	0.0	5.0 $\pm$ 0.2
Guinea pig microsome	89.8 $\pm$ 0.5	2.7 $\pm$ 0.2	0.0	0.0	7.5 $\pm$ 0.5
Cynomolgus monkey microsome	39.4 $\pm$ 3.1	38.4 $\pm$ 2.3	0.7 $\pm$ 0.1	0.0	21.4 $\pm$ 1.4
Human microsome	78.8 $\pm$ 1.2	16.3 $\pm$ 0.8	0.5 $\pm$ 0.1	0.0	4.3 $\pm$ 0.6
Human S-9	90.5 $\pm$ 0.8	7.5 $\pm$ 0.8	0.1 $\pm$ 0.0	0.0	2.0 $\pm$ 0.2

Each value represents the Mean $\pm$ S.E. of four-five samples.

Table V Kinetic constants of metabolic reaction NK-104 to M-13 after incubation of ^{14}C -NK-104 with several human and animal liver microsomes

Microsomes	Lot No.	K_m μM	$V_{\max}$ pmol/min/ mg protein	$V_{\max}/K_m$ $\mu\text{l/min/mg protein}$
Male rat		323.3	319.4	0.99
Female rat		83.1	173.1	2.08
Monkey		13.6	82.4	6.06
Human	HHM-0249	32.9	63.8	1.94
	HHM-0256	77.0	133.0	1.73
	HHM-0219	47.7	64.0	1.34
	HHM-0250	22.6	48.7	2.15
	Mean	45.1	77.4	1.79
	S.E.	11.8	18.9	0.17

2) Intrinsic Metabolic Clearance

Kinetic studies of ^{14}C -NK-104 metabolism in male and female rat, male monkey and human pooled microsomes (4 lots) were done. The apparent K_m values of M-13 in male rat, female rat, monkey and human were 323, 83, 14 and 45 μM , respectively. Also, the $V_{\max}$ of M-13 was 320, 173, 82 and 77 pmol/min/mg protein, respectively. In addition, the intrinsic clearance ($V_{\max}/K_m$) of M-13 was 1.0, 2.1, 6.1 and 1.8 $\mu\text{l/min/mg protein}$, respectively (Table V).

3) Inhibition Studies of NK-104 Hydroxylation

Among the P-450 chemical inhibitor tested, SKF-525A and sulfaphenazole had strong inhibitory effects on M-13 formation. However, ketoconazole and α -naphthoflavone did not affect the metabolism of ^{14}C -NK-104. In the immunoinhibition studies, strong inhibitory effects on the metabolism of ^{14}C -NK-104 were ob-

Fig. 3 Inhibition effect of several chemical inhibitors and immuno inhibitors on the metabolism of ^{14}C -NK-104

served for human anti-CYP2C and antiserum P-450 NADPH reductase. In contrast, no inhibitory effect was observed for human anti-CYP1A1/2, anti-CYP3A4 and anti-CYP2D6 (Fig. 3).

4) Metabolism of ^{14}C -NK-104 by Various CYP Isoforms

Using fifteen recombinant human P-450 expressing microsomes, the metabolism of ^{14}C -NK-104 was investigated. The catalytic properties of each human CYP isoform for the formation of M-13 are shown in Fig. 4. These results indicated that CYP2C9 was principally responsible for the hydroxylation of NK-104 by human

Fig. 4 Formation of M-13 in microsomes from human lymphoblastoid cells expressing P-450 isoforms

Fig. 5 Inhibition of tolbutamide 4-hydroxylation by NK-104 in human liver microsomes (Dixon plot) v: nmol/min/mg protein

microsomes, with some involvement of CYP2C8. In contrast, no M-13 was produced in the presence of the other CYP isoforms up to a concentration of 25 μ M, indicating that CYP1A1, 1A2, 1B1, 2A6, 2B6, 2D6, 2E1, 3A4 and 4A11 do not have significant roles in the metabolism of NK-104. In addition, there was little difference in the production of M-13 between the CYP2C9-*arg* of wild type and CYP2C9-*cys* variant type.

5) Effect of NK-104 on the Metabolism of Tolbutamide

The inhibitory effect of NK-104 on tolbutamide 4-hydroxylation was estimated. CYP2C9-mediated tolbutamide 4-hydroxylation was not inhibited by NK-104 up to a concentration of 5 μ M. Dixon-plots for the metabolic reactions in the presence of NK-104 showed parallel lines without a focal point, demonstrating that there was no inhibition by NK-104 (Fig. 5).

Discussion

The plasma protein binding and *in vitro* metabolism of NK-104 were examined in the present study. NK-104 was highly bound to plasma protein of all animal species over a wide range of tested concentrations (more than 96%). In the *ex vivo* study, NK-104 was also highly bound to plasma protein independent of collection times after dosing. These results agreed with our previous study.⁴⁾ The erythrocyte/plasma partition coefficients for the intravenous administration of NK-104 were low in the animals, suggesting little transfer in erythrocytes. The binding of NK-104 to the isolated plasma protein was strong for HSA and α_1 -AGP. In particular, the amount of unbound NK-104 in human plasma was similar to that of HSA, suggesting that a major binding protein in plasma was HSA. Because NK-104 possesses a carboxylate moiety and quinolin structure, it is considered that these acidic or basic properties enhance pro-

tein binding. High plasma-binding ratios were also reported for lovastatin, fluvastatin, simvastatin and cerivastatin, and were more than 96%.⁹⁻¹¹⁾ On the other hand, it has been reported that pravastatin has little plasma protein binding ability compared with other statins.¹²⁾ These results could reflect their lipophilic nature.

The binding of drugs to plasma proteins has been long recognized as having an important influence on the duration and intensity of drug action. Since the unbound drug in plasma is considered to account for the pharmacological activity of the drug, the mutual displacement of highly bound drugs from the protein binding sites may result in significant drug/drug interactions. On the other hand, many drugs have been shown to bind to one of three distinct binding sites on HSA (warfarin site, diazepam site or digitoxin site).¹³⁾ Therefore, to evaluate the mutual displacement at a binding site, the competitive binding characteristics for plasma proteins of NK-104 and several highly bound drugs potentially co-administered to human were investigated. At the therapeutic plasma concentration of each drug, the plasma protein binding of NK-104 was unaffected by warfarin, diazepam, digitoxin, phenylbutazone, phenytoin, furosemide, ibuprofen, nitrendipine and glibenclamide. Similarly, there was no influence on the binding of these drugs at the therapeutic plasma concentration of NK-104. By the way, the saturation of binding to α_1 -AGP is easy to occur because of the alterations which associate with several diseases.¹⁴⁾ In particular, the displacement of drugs such as propranolol that possess the bind site for α_1 -AGP may occur due to the binding saturation. However, NK-104 had no influence on the binding of propranolol, suggesting that the mutual displacement from α_1 -AGP did not occur.

In our previous study, high bioavailability (about 80%) was observed in all laboratory animal species except for monkey.⁵⁾ To evaluate the inter-species variation of the biotransformation of NK-104, the species difference in the hydroxylation of NK-104 was investigated *in vitro*. M-13 (8-hydroxy NK-104) as a metabolite was often detected after incubation in monkey microsome. In contrast, the fractional radioactivity in liver microsomal preparations of other animal species was due mainly to unchanged NK-104, indicating that NK-104 was hardly metabolized. This knowledge could be retrospectively applied to provide a pharmacokinetic rationale for our *in vivo* findings.⁴⁾ It is suggested that the poor BA of NK-104 in monkey is due to a difference of drug metabolism. No change in the metabolic patterns of NK-104 was observed between human S-9 and microsomal reactions. It is also indicated that there was little drug metabolism *via* the phase-II metabolic pathway.

Kinetic analysis of the formation of M-13 in liver microsomes indicates that the $V_{\max}/K_m$ in male rat, female rat, monkey and human was 1.0, 2.1, 6.1 and 1.8 μ l/min/mg protein, respectively. Thus, M-13 was

formed with relatively low intrinsic clearance in all species. Although no marked difference was observed in the composition of unchanged NK-104 between male and female rats, the $V_{\max}/K_m$ of M-13 in female rats was larger than in male rats, implying a sex difference. It is well known that the hydroxylation of each parent compound is a major biotransformation pathway of fluvastatin, simvastatin, cerivastatin and atorvastatin.¹⁵⁻¹⁸⁾ In human microsomal metabolism, the $V_{\max}/K_m$ of hydroxylation with lovastatin was 100-fold that with NK-104, likewise, for simvastatin it was 50-fold, atorvastatin 8-fold and fluvastatin 30-fold.^{18,19)} Therefore, remarkable differences were observed in the intrinsic clearance of the ring hydroxylation, which was the major metabolic pathway of NK-104 and other statins. It is suggested that NK-104 with relatively low intrinsic clearance of M-13 formation was little metabolized compared with other statins.

Cytochrome P-450s are important in the metabolism of both xenobiotic and endogenous substances. They exist as a family of closely related isoenzymes, with over 270 different genes having been isolated.²⁰⁾ Recently, to prevent the drug/drug interaction of drug metabolism, much effort has been made to evaluate the intrinsic clearance and to clarify the CYP isoforms of the substrates. The inhibition of NK-104 metabolism by SKF-525A and antiserum of P-450 NADPH reductase indicates that NK-104 is metabolized *via* the microsomal electron transport system associated with P-450. Specific CYP2C9 inhibitors, such as sulfaphenazole and anti-CYP2C, strongly inhibit the hydroxylation of NK-104. In contrast, specific inhibitors of CYP3A4, CYP2D6 and CYP1A1/1A2 did not inhibit the metabolism of NK-104, indicating that P-450 isozymes except CYP2C did not play a significant role. From the metabolic studies using recombinant human P-450 expressing microsomes, it is concluded that CYP2C9 and CYP2C8 are the enzymes responsible for the formation of M-13. Also, several amino acid variants have been reported for CYP2C9.²¹⁾ However, a large difference was not observed between the CYP2C9-*arg* of wild type and CYP2C9-*cys* variant type, suggesting that CYP2C9 polymorphism was absent in the NK-104 metabolism.

As previously reported by several researchers, fluvastatin was catalyzed by CYP2C9 whereas CYP3A4 plays a major role in the metabolism of atorvastatin, simvastatin, lovastatin and cerivastatin.^{16-18,22)} Many drug/drug interactions related to CYP mediated drug metabolism involve inhibition or induction.²³⁾ The CYP3A4 subfamily is probably the most important isozyme for human drug metabolism. CYP3A4 is found universally in human and is abundant in both the liver and intestine. It has been reported that several chemical compounds and grapefruit juice inhibit the CYP3A4 mediated metabolism of statins.²⁴⁻²⁶⁾ Fukuda *et al.* isolated specific CYP3A4 inhibitors from grapefruit juice and identified the chemicals as furocoumarin derivatives.²⁷⁾

These components strongly inhibited the metabolism mediated by CYP3A4 as well as ketoconazole, suggesting new candidates for causative ingredients in grapefruit juice causing drug interaction. On the other hand, no inhibitory effect on M-13 formation occurred when NK-104 and CYP3A4 inhibitors such as ketoconazole and anti-CYP3A were incubated with human liver microsome. In addition, the low intrinsic clearance of NK-104 in *in vitro* metabolism indicates that the change in the metabolic clearance of NK-104 would be small even with co-administered drugs in clinical practice.

Transon *et al.* reported that fluvastatin caused the selective inhibition of CYP2C9.²²⁾ Also, Fisher *et al.* reported that fluvastatin inhibited the metabolism of tolbutamide with an inhibition constant (K_i) of 0.3 μM .¹⁹⁾ On the other hand, the inhibitory effect of NK-104 on CYP2C9-mediated tolbutamide 4-hydroxylation was not observed in the present study. These results indicate that the likelihood for serious metabolic drug/drug interactions is minimal with NK-104 compared with other statins.

In conclusion, the present *in vitro* results indicate that NK-104 undergoes little metabolism in human liver, in agreement with the *in vivo* findings.⁴⁾ Therefore NK-104 would be highly unlikely to undergo drug/drug interaction in clinical practice.

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