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Simultaneous determination of taxol and its metabolites in microsomal samples by a simple thin-layer chromatography radioactivity assay — inhibitory effect of NK-104, a new inhibitor of HMG-CoA reductase

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Abstract

The inhibitory effect of NK-104, a potent inhibitor of HMG-CoA reductase, on taxol metabolism was examined using radio-TLC. This method is described for in vitro measurement of taxol metabolites as an alternative to the commonly used HPLC assay. After incubation of ^{14}C-taxol with human liver microsomes, the supernatants were developed using a solvent system consisting of toluene–acetone–formic acid (60:39:1, v/v) and quantified with a bioimaging analyzer. The described method provides a valuable tool for the simultaneous determination of unchanged taxol and its major metabolites. There was no inhibitory effect of NK-104 on CYP-mediated metabolism of taxol in human liver microsomes.

Keywords: Taxol; NK-104

1. 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 causes significant reductions in serum total cholesterol, LDL cholesterol and triglyceride levels in animals and humans. NK-104 was metabolized via biotransformation pathways as follows: (1) lactonization; (2) β-oxidative degradation of the side-chain; (3) hydroxylation of the quinoline ring and (4) conjugation with β-glucuronic acid and taurine, as described in our previous reports [4,5]. In particular, lactonization was the major metabolic pathway in human metabolism and the lactonization of NK-104 is a dehydration reaction that does not participate with P450 enzymes and NK-104 lactone can be reversibly converted to NK-104. On the other hand, NK-104 was hardly metabolized by P450 and the V_{max}/K_m of M-13 (8-hydroxy NK-104) was low, its value being less than 2 μl/min/mg protein in human liver microsomes [6]. Recently, to obviate the drug–drug interaction, considerable attention has been paid to in vitro metabolism. We demonstrated that the metabolic pathway of NK-104 to M-13 was catalyzed by the human cytochrome P450 family of enzymes.

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CYP2C9 was principally responsible for the hydroxylation of NK-104 by human microsomes, with some involvement of CYP2C8 [6]. No inhibitory effect on CYP-mediated metabolism was detected on tolbutamide 4-hydroxylation (CYP2C9) in the presence of NK-104 [7]. However, there have been no reports regarding the inhibitory effect of NK-104 on CYP2C8-mediated metabolism. We selected taxol as a model substrate to investigate the inhibition of CYP2C8 by NK-104.

Taxol is a novel anticancer drug originating from the bark of the Pacific yew, *Taxus brevifolia*. This drug has been shown to have clinical efficacy in the treatment of various human malignancies, and has become especially important for the management of breast and ovarian tumors [8]. Due to the potential therapeutic importance of taxol metabolism, a number of investigations have focused on characterization of the P450 isoform involved in its metabolism [9–13]. The principal taxol biotransformation reactions of human hepatic in vitro preparations are 6α-hydroxylation of the taxane ring (6α-hydroxytaxol, 6-OH) and oxidation of the phenyl ring (3′-*p*-hydroxytaxol, 3-OH). It was recently concluded that formation of the main metabolite, 6-OH, is catalyzed by CYP2C8, and that of 3-OH is mediated by CYP3A4.

In the present study, we developed a thin-layer chromatography (TLC) method for in vitro measurement of CYP2C8 and CYP3A4 activities as an alternative to the commonly used high-performance liquid chromatography (HPLC) method. This TLC assay employs ^{14}C-taxol, a commercially available radiolabeled compound, and allows rapid and sensitive measurement of taxol metabolism. We examined the characteristics of the inhibitory effect of NK-104 using the known P-450 functional marker CYP2C8.

2. Experimental

2.1. Chemicals and reagents

NK-104 (monocalcium bis{(3*R*,5*S*,6*E*)-7-[2-cyclopropyl-4-(4-fluorophenyl)-3-quinolyl] 3,5-dihydroxy-6-hepteonate}) was synthesized by Nissan Chemical Industries Ltd. (Chiba, Japan) and the chemical structure is shown in Fig. 1. [2-Benzoyl ring-U-^{14}C]-labeled Paclitaxel (^{14}C-taxol, No. P1958-14C) was purchased from Sigma (Saint Louis, MO, USA). The specific radioactivity of labeled compound was 2.23 MBq/mg, and the radiochemical purity was more than 99% during the experimental period. 6α-Hydroxytaxol, which was used as a model substrate marker of CYP2C8, and quercetin dihydrate, which was used as a positive control substrate marker of CYP2C8, were purchased from Ultrafine Chemicals (Manchester, UK) and Wako Pure Chemical Industries (Osaka, Japan), respectively. Solvents of analytical grade were used for HPLC and TLC. The TLC plates (Silicagel $60F_{254}$, 20×20 cm) were purchased from Merck (Darmstadt, Germany). All other chemicals and solvents were purchased commercially and were of either analytical- or extra-pure grade.

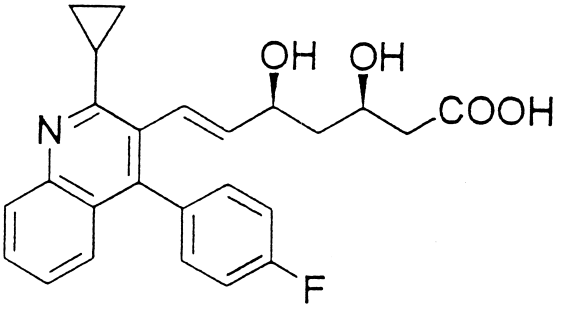

Fig. 1. Chemical structure of NK-104.

2.2. Microsomes and human cDNA expressing cytochrome P450s

Pooled human liver microsome (H-161) and recombinant microsome derived from baculovirus-infected insect cells expressing human P450 (CYP3A4 and CYP2C8) were purchased from GENTEST Co. Ltd. (Woburn, MA, USA) and were stored at −80°C until use. The NADPH-regenerating system (β-NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase) was obtained commercially.

2.3. Incubation conditions

Using pooled human liver microsomes, the in vitro metabolism of ^{14}C-taxol was investigated. The incubation mixture was prepared as follows: ^{14}C-taxol,

0.1 *M* sodium phosphate buffer, pH 7.4, and liver microsomes were added to a tube and preincubated at 37°C for 5 min. Then the components of the NADPH regenerating system were added individually to the incubation mixture at a final concentration of 1 mg protein/ml or 40 pmol P450/ml to initiate the metabolic reaction. After incubation at 37°C for 15 min, the reaction was quenched by adding the methanol solution. Total radioactivity was measured using a portion of reaction-terminated samples with a liquid scintillation counter (Tri-carb 1500, Packard) for 10 min after addition of scintillation cocktail (Hionic-Fluor, Packard). The reaction-terminated samples were centrifuged at 6000 *g* for 3 min and aliquots of the supernatant were evaporated to dryness, and the residue was dissolved in 15 μl of methanol or 0.4 ml of the HPLC mobile phase, respectively. In this study, incubation was performed using polypropylene tubes, to prevent the binding of taxol and its metabolites [14].

2.4. Chromatographic separation

2.4.1. Thin-layer chromatography

Aliquots of about 2 μl of the supernatant were spotted onto TLC plates and developed with toluene–acetone–formic acid (60:39:1, v/v) to 12 cm in a horizontal TLC chamber that was saturated with solvent vapor. The TLC plates were dried and then exposed for 12 h to a phosphor imaging plate (IP) and quantified using a bioimaging analyzer (BAS-2000, Fuji Film). The IP scanning conditions were as follows: gradation, 256; resolution, 200; latitude, 4 and sensitivity, 10 000. The reaction rates were calculated from the ratio of labeled metabolite to the total radioactivity. The radioactive metabolites were positively identified by comparison of the R_f value with that of authentic unlabeled standard.

2.4.2. HPLC instrumentation and conditions

The HPLC conditions were taken from the previous report by Huizing et al. [15]. The HPLC system (L-6000 series, Hitachi) was equipped with a UV detector, a reversed-phase column (Inertosil ODS; 150×4.6 mm; ϕ, 5 μm; GL Science) and an autosampler (AS-4000, Hitachi) with a mobile phase consisting of acetonitrile–methanol–0.02 *M* ammonium acetate buffer (pH 5, 4:5:1, v/v). The chromatographic signals were monitored at 230 nm for discrimination of taxol and its metabolites (total run time of 40 min). The flow-rate of the mobile phase was 1 ml/min. Measurement of the radioactivity of taxol and its metabolites was carried out by the HPLC–radioluminographic method [16,17]. The HPLC eluate was fractionated into the wells of microplates at 20-s intervals for 32 min using a fraction collector (Liquid Handler 222XL, Gilson). The plates, polystyrene flat-bottomed microplates (Beta48, Toyobo Engineering Ltd., Osaka, Japan), were allowed to stand for 12 h at room temperature, to allow the solvent to evaporate. The samples were overlaid with Diafoil plastic film and placed in contact with an IP for 12 h. The radioactivity recorded on the IP was quantified using a BAS-2000 bio-imaging analyzer. The scanning conditions of the IP and calculation of the reaction rate were carried out in the same manner as in the TLC method.

2.4.3. Method validation

For comparison of TLC and the HPLC assay, samples were run as duplicates on TLC and HPLC systems ($n=26$). The results of both assays were compared using linear regression analysis. The intra-plate and inter-plate precision of the TLC assay were determined by analyzing four different samples ($n=4$), four times on the same day, and daily three times.

2.5. Kinetic studies

To determine the apparent K_m and V_{max} values of taxol metabolism, kinetic studies of NK-104 were conducted using ^{14}C-taxol in the concentration range of 1–50 μ*M*, using CYP2C8-expressing microsomes or human microsomes. The incubation was carried out as described above and the generated metabolites were estimated. The apparent V_{max} and K_m values were calculated from the Lineweaver-Burk plots.

2.6. Chemical inhibition

Chemical inhibition of CYP2C8 metabolism was performed at a concentration of 5 μ*M* ^{14}C-taxol and an inhibitor concentration of 50 μ*M* quercetin, 5 μ*M* of NK-104 using human liver microsomes. In addition, NK-104 was preincubated for 0 or 5 min in separate incubations prior to initiation of the reaction

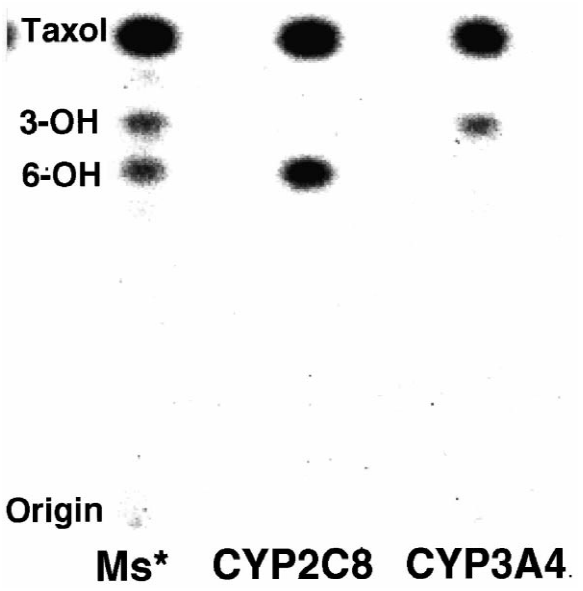

Fig. 2. TLC chromatogram of taxol and its metabolites in hepatic microsome and P450-expressing microsome. *Ms, microsome; 3-OH, 3-hydroxytaxol and 6-OH, 6α-hydroxytaxol.

by the addition of ^{14}C-taxol. The inhibitory effect was estimated from the ratio of metabolite formation in the presence and absence of inhibitors. The inhibition experiment with NK-104 was performed with a range of 0.5–10 μ*M*, to generate a Dixon plot using ^{14}C-taxol in the concentration range of 2.5–50 μ*M*. NK-104 was co-incubated with ^{14}C-taxol before the reaction. In the presence or absence of NK-104, CYP2C8-mediated 6-OH and CYP3A4-mediated 3-OH production was measured. The type of inhibition was discriminated based on visual inspection of the double reciprocal plots in the presence and absence of inhibitor. The inhibition constant (K_i) was estimated by Dixon-plot analysis.

3. Results

3.1. In vitro metabolism of ^{14}C-taxol

Figs. 2 and 3 illustrate typical TLC and HPLC chromatograms derived from reaction mixtures using human liver microsomes and CYP2C8- or CYP3A4-expressing microsomes, respectively. Using standard silica gel TLC plates, good separation of unchanged taxol and its metabolites (6-OH and 3-OH) was achieved using a solvent system consisting of toluene–acetone–formic acid (60:39:1, v/v). The R_f values of taxol, 6-OH and 3-OH were about 0.33, 0.20 and 0.25, respectively. The unchanged taxol and its metabolites were also well separated by HPLC. The retention times for metabolites 3-OH, 6-OH and unchanged taxol were about 11, 15 and 24 min, respectively. The recovery of the radioactive HPLC eluates into flat-bottomed microplates was 99.7%.

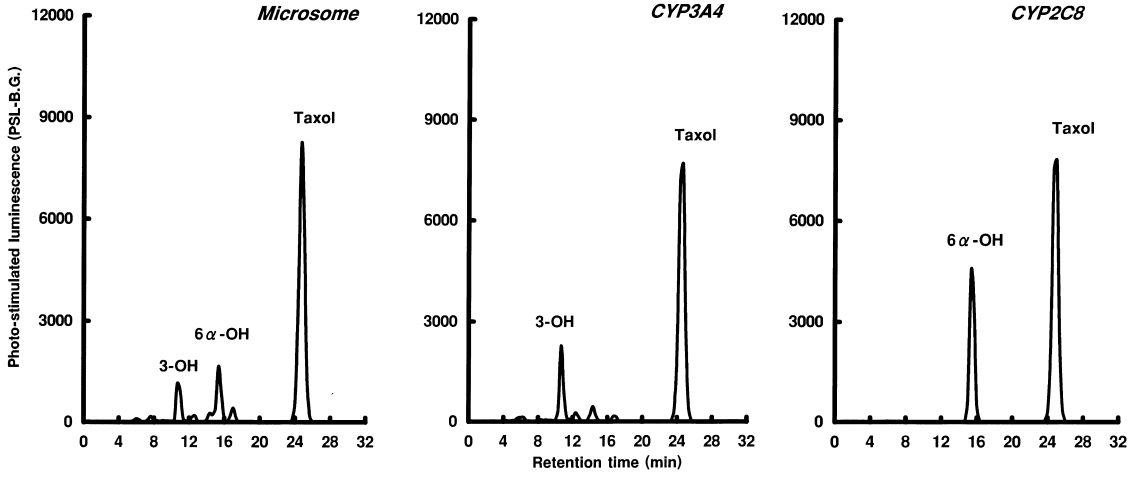

Fig. 3. HPLC–radiochromatogram of taxol and its metabolites in hepatic microsomes and P450-expressing microsomes.

3.2. Method validation

To ensure the identity of measurements, a direct comparison of TLC and HPLC results was carried out using 26 individual samples of human liver microsomes or CYP2C8-expressing microsomes. Linearity of TLC and HPLC assays was evaluated over concentration ranges to 50 μ*M* of unchanged taxol, to 3 μ*M* of 6-OH and to 2.5 μ*M* of 3-OH, respectively (Fig. 4). The correlation coefficients (R^2) of unchanged taxol and both metabolites, between both methods, were more than 0.997. Intra- and inter-plate variabilities are summarized in Tables 1 and 2. In human microsomes, the inter-plate C.V. values within the concentration range were less than 1.61% for unchanged taxol, 1.18% for 6-OH and 6.01% for 3-OH, respectively. Also, the intra-plate C.V. values and were less than 6.39% for all analytes.

Table 1
Inter-day variability of the TLC assay[a]

Compound	Mean concentration of taxol and metabolites (μ*M*)	SD	C.V. (%)
Unchanged taxol	0.552	0.009	1.61
	3.042	0.016	0.51
	7.820	0.039	0.50
	40.617	0.025	0.06
6α-OH	0.133	0.001	0.80
	0.696	0.008	1.18
	0.870	0.010	1.14
	0.817	0.000	0.00
3-OH	0.104	0.006	6.01
	0.439	0.012	2.82
	0.587	0.006	1.02
	0.559	0.000	0.00

[a] Four different samples were analyzed by TLC four times on the same day.

3.3. Intrinsic metabolic clearance

Kinetic studies of ^{14}C-taxol metabolism in human liver microsomes and in CYP2C8-expressing microsomes were carried out (Table 3). The apparent K_m values for 6-OH and 3-OH formation in human liver microsomes were 27.4 and 28.8 μ*M*, respectively. The V_{max} values were 0.36 and 0.24 nmol/min/mg protein, respectively. The intrinsic clearance (V_{max}/K_m) values were 13 and 8 μl/min/mg protein, respectively. On the other hand, the apparent K_m, V_{max} and V_{max}/K_m of 6-OH formation in CYP2C8-expressing microsomes were 4.3 μ*M*, 0.15 nmol/min/mg protein and 34 μl/min/mg protein, respectively.

3.4. Effects of NK-104 on metabolism of taxol

Among the P450 chemical inhibitors tested, quer-

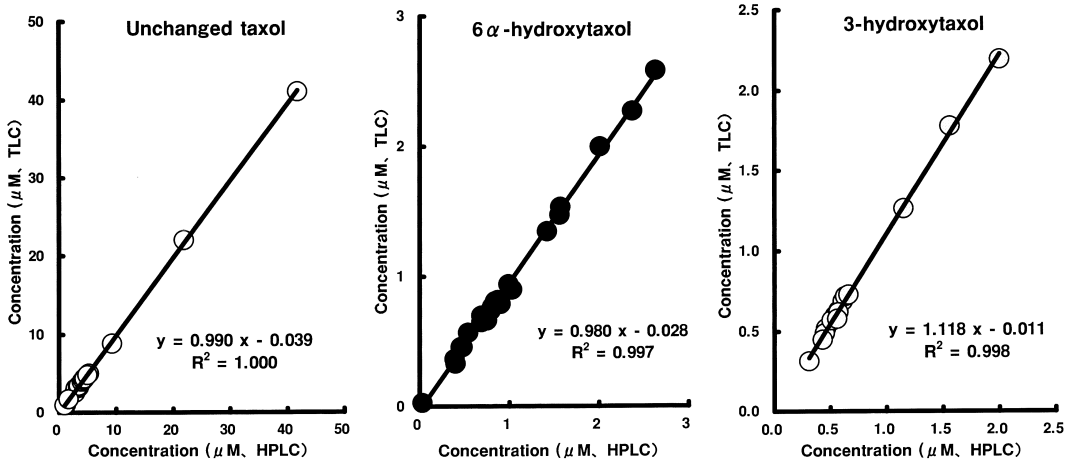

Fig. 4. Correlation between TLC and HPLC methods for assays of taxol and its metabolites.

Table 2
Intra-plate variability of the TLC assay[a]

Compound	Mean concentration of taxol and metabolites (μM)	SD	C.V. (%)
Unchanged taxol	0.533	0.032	5.99
	3.027	0.061	2.03
	7.770	0.055	0.71
	40.488	0.116	0.29
6α-OH	0.134	0.004	3.28
	0.667	0.038	5.68
	0.867	0.024	2.72
	0.839	0.022	2.56
3-OH	0.102	0.007	6.39
	0.427	0.015	3.40
	0.571	0.027	4.72
	0.563	0.006	1.10

[a] Four different samples (each $n=4$) were analyzed by TLC three times.

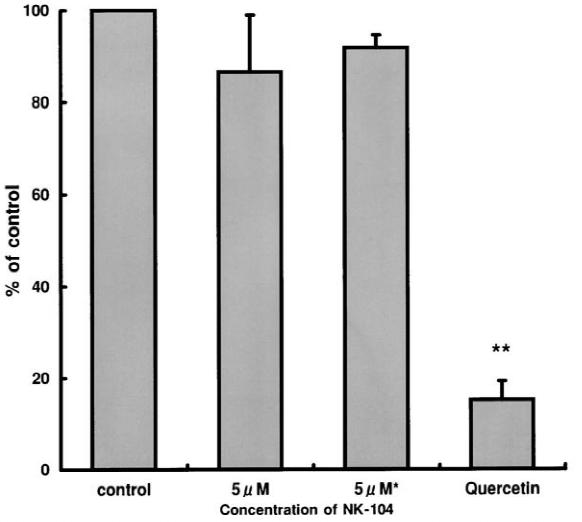

Fig. 5. Inhibitory effect of NK-104 on taxol 6α-hydroxylation after incubation with human hepatic microsomes (taxol, 5 μM). *, 5 min pre-incubation; **, significantly different from control ($P<0.001$). Each data point represents the mean±SD of three points.

cetin showed the strongest inhibitory effect on taxol 6α-hydroxytaxol formation in human liver microsomes. On the other hand, NK-104 did not inhibit the metabolism of taxol, and no marked difference was noted in the inhibition of taxol metabolism after preincubation of NK-104 (Fig. 5).

The inhibitory effects of NK-104 on taxol 6α-hydroxylation and 3-hydroxylation were estimated in Dixon-plot studies. CYP2C8-mediated taxol 6α-hydroxylation and CYP3A4-mediated taxol 3-hydroxylation were not inhibited by NK-104 up to a concentration of 10 μM in human liver microsomes. Dixon plots for both 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. 6).

4. Discussion

Cytochrome P450s 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 isolated to date [18]. Recently, to prevent drug–drug interactions of drug metabolism, a great deal of effort has been made to evaluate the intrinsic clearance and to clarify the CYP isoforms of the substrates. In view of the widespread clinical use of NK-104 and the importance of drug–drug interactions, we set out to clarify the inhibitory effect of NK-104 on taxol metabolism. Previous in vitro and in vivo studies indicated that 6-OH is the major taxol metabolite formed in

Table 3
Kinetic constants of metabolic reactions of taxol after incubation with human microsomes or CYP2C8-expressing microsomes

	Metabolic pathway	K_m μM	V_{max} nmol/min/mg protein	V_{max}/K_m μl/min/mg protein
Microsomes	6α-Hydroxylation	27.4	0.359	13.10
	3-Hydroxylation	28.8	0.239	8.30
CYP2C8	6α-Hydroxylation	4.3	0.147	34.19

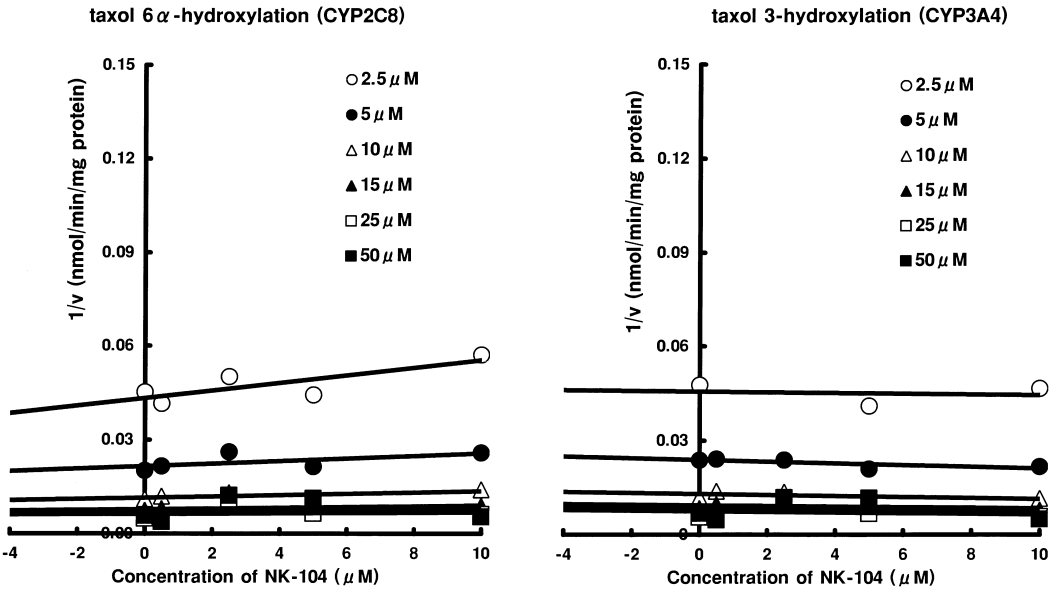

Fig. 6. Inhibition of taxol 6α-hydroxylation and 3-hydroxylation by NK-104 in human hepatic microsomes (Dixon plots).

humans and also that 6-OH is catalyzed by CYP2C8 [11].

In the present study, we developed a method for the simultaneous determination of taxol and its metabolites by TLC as an alternative to the commonly used HPLC assay. The assay conditions of the TLC method were compared to HPLC results. Few additional radioactive metabolites were detected either by HPLC or TLC. There was no detectable metabolite formation in the absence of NADPH. Moreover, the kinetic constants K_m and V_{max} of taxol metabolism measured were almost the same as those in the previous reports in the present study [9,10]. It was concluded, therefore, that the TLC assay yielded results that were nearly identical to those of conventional HPLC analysis and, thus, provided valid results regarding taxol hydroxylation activities. In addition, the TLC method proved to be robust and eliminated the need for time-consuming quantification using calibration curves and equilibration of the analytical system, which is necessary if HPLC is used together with UV detection. In this study, the HPLC conditions were taken from a previous report [15]. It was reported that the lower limit of quantitation of this method was 10 ng/ml for unchanged taxol and its metabolites. On the other hand, the limit of detection of the imaging plate was reported previously and was about 0.35 Bq [17]. Therefore, the sensitivity of this method is much higher than non-radioactive assays. Using horizontal TLC development, up to 16 samples can be run on a single 20×20 cm TLC plate. It provided a simple, sensitive and reliable technique for determining the radioactivity of many samples simultaneously. Due to its flexibility, this TLC approach is probably also applicable for other P450 test reactions.

In kinetic studies, 6-OH and 3-OH formations were the major metabolic pathways in human microsomes. No marked differences were noted in the apparent K_m values of both metabolic reactions; however, the V_{max}/K_m of 6-OH formation was larger than that for 3-OH formation. These results suggest that the predominant pathway of taxol metabolism is via 6α-hydroxylation by CYP2C8 in human microsomes.

In inhibition studies, no marked differences were observed in inhibitory effects at different incubation times (0 and 5 min). These results indicate that the role of NK-104 or its metabolites does not depend on its inhibitory effect on taxol metabolism. Moreover, no inhibitory effects of NK-104 were observed on CYP2C8-mediated taxol 6α-hydroxylation or CYP3A4-mediated taxol 3-hydroxylation following Dixon-plot analysis. Quercetin and retionic acid have already been shown to be potent competitive inhibitors of CYP2C8-mediated 6-OH formation.

Other inhibitors of 6-OH formation interestingly include drugs that are generally known to be substrates for CYP3A4, verapamil, testosterone, nifedipine and ketoconazole. Moreover, inhibitors of 3-OH formation include drugs that are typical inhibitors of CYP3A4 [12]. These reports suggest that the inhibitory effect of NK-104 on P450 metabolism is not mediated by its effect on CYP2C8 or CYP3A4. Moreover, we reported previously that there was no evidence of inhibition of tolbutamide metabolism (CYP2C9) in the presence of NK-104 [6].

The present in vitro results indicate that NK-104 undergoes little inhibition in the human liver. In conclusion, NK-104 would be unlikely to show significant drug–drug interactions in clinical use.

Acknowledgements

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