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Full-length article

CYP3A4 mediated *in vitro* metabolism of vinflunine in human liver microsomes[1]

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Key words

vinflunine; metabolism; CYP3A4; liver microsomes

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Abstract

Aim: To study the metabolism of vinflunine and the effects of selective cytochrome P-450 (CYP450) inhibitors on the metabolism of vinflunine in human liver microsomes. **Methods:** Individual selective CYP450 inhibitors were used to investigate their effects on the metabolism of vinflunine and the principal CYP450 isoform involved in the formation of metabolites M_1 and M_2 in human liver microsomes. **Results:** Vinflunine was rapidly metabolized to 2 metabolites: M_1 and M_2 in human liver microsomes. M_1 and M_2 were tentatively presumed to be the N-oxide metabolite or hydroxylated metabolite and epoxide metabolite of vinflunine, respectively. Ketoconazole uncompetitively inhibited the formation of M_1, and competitively inhibited the formation of M_2, while α-naphthoflavone, sulfaphenazole, diethyl dithiocarbamate, tranylcypromine and quinidine had little or no inhibitory effect on the formation of M_1 and M_2. **Conclusion:** Vinflunine is rapidly metabolized in human liver microsomes, and CYP3A4 is the major human CYP450 involved in the metabolism of vinflunine.

Introduction

Vinca alkaloids, including the natural products vinblastine (VBL) and vincristine (VCR) and the semisynthetic derivatives vindesine (VND) and vinorelbine (VRL), are antimitotic drugs that are widely and successfully used in the treatment of cancer. Their interactions with tubulin, the major component of microtubules in the mitotic spindle, and the subsequent arrest of cells in mitosis are generally accepted as key events in their mechanisms of action[1,2]. Although VBL and VCR have reasonable potency in clinics, there are considerable toxic side effects associated with them. Semisynthetic development of the Vinca alkaloids has produced a successful second-generation compound, VRL, which has shown improved efficacy and reduced toxicity[3]. It is effective in the treatment of non-small cell lung cancer, metastatic breast cancer and ovarian cancer, and it has shown promise in the management of lymphomas, esophageal cancer and prostatic carcinomas[4–6]. Now, it has been further modified through super-acidic chemistry to generate new and more active derivatives. The process of production involves the insertion of 2 fluorine atoms at the 20' position and reduction of the 3'4' double bond to produce 20',20'-difluoro-3'4'-dihydrovinorelbine, known as vinflunine (Figure 1)[3].

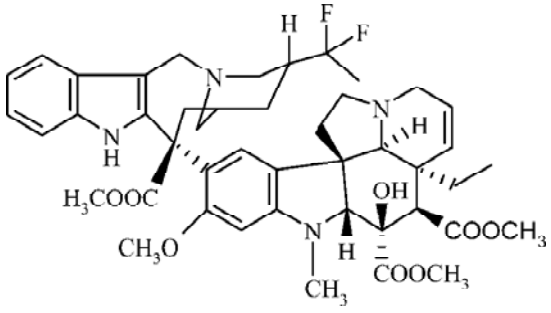

Figure 1. Structure of vinflunine.

As a new vinca alkaloid, vinflunine exerted markedly superior effects on antitumor activities against a panel of 13 murine and human tumor models compared to the parent compound, VRL[7–9]. *In vitro* investigations have confirmed the mitotic-arresting and tubulin-interacting properties of vinflunine shared by other Vinca alkaloids[10,11]. However, differences in terms of the inhibitory effects of vinflunine on

microtubules dynamics and its tubulin-binding affinities have been identified, which appear to distinguish it from the other Vinca alkaloids[12]. Vinflunine induced smaller spirals with a shorter relaxation time, which might be associated with reduced neurotoxicity[13]. Furthermore, an *in vivo* study has suggested that vinflunine mediates its antitumor activity at least in part via an antivascular mechanism, even at subcytotoxic doses[14]. Although vinflunine appeared to participate in P-glycoprotein-mediated drug resistance mechanisms, it proved only a weak substrate for this protein and a far less potent inducer of resistance than VRL[15]. Vinflunine is presently in phase III experimentation for treatment of bladder cancer and non-small-cell lung cancer[16].

Vinca alkaloids are rapidly and extensively converted by human hepatocytes to a number of unidentified biotransformation products[17]. In addition, it is reported that CYP3A catalytic activities made a major contribution to the overall metabolism of VBL and VND in human liver microsomes. However, the chemical structures of these metabolites remain unknown[18,19]. It has also been reported that VRL is metabolized to 3 metabolites, 4-*O*-deacetylvinorelbine, 20' hydroxyvinorelbine and VRL 6'-oxide, in human blood[20]. Five metabolites, 15,16-epoxyvinorelbine, 11'-hydroxyvinorelbine, 19'-hydroxyvinorelbine, 15,16-epoxy-10'-hydroxyvinorelbine and 10'-hydroxyvinorelbine, are identified in rats[21]. Also, it is reported that CYP3A4 is mainly responsible for the metabolism of VRL in human liver microsomes[22,23].

Compared to the extensive literatures on pharmacodynamic investigations, very little information is available on the pharmacokinetics of vinflunine. The purpose of these experiments was to investigate the metabolism of vinflunine in human liver microsomes *in vitro*, and to identify the major cytochrome P-450 (CYP450) isoform involved in the metabolism of vinflunine.

Materials and methods

Chemicals Vinflunine was supplied by Qilu Pharmaceutical Co, Ltd (Ji-nan, China). The internal standard VCR was a gift from Organic Chemistry Laboratories of China Pharmaceutical University (Nanjing, China). Glucose-6-phosphate dehydrogenase (G-6-PDH, Type V), α-naphthoflavone (α-Naph), sulfaphenazole (Sul), quinidine (Qui), diethyl dithiocarbamate (DDC) and tranylcypromine (Tra) were purchased from Sigma Chemical Co (St Louis, MO, USA). Ketoconazole (Ket) was kindly provided by Nanjing Second Pharmaceutical Factory. α-Nicotinamide adenine dinucleotide phosphate (NADP) and G-6-P were purchased from Shanghai Lizhudongfeng Biotechnological Co (Shanghai, China). All other supplies were of the highest grade available from standard commercial sources.

Tissue samples and preparation of liver microsomes The human liver used in the present study was obtained from Jinling Hospital (Nanjing, China), and the study was approved by the Ethics Committee of Jinling Hospital. The human liver microsomes were prepared by differential centrifugation[24]. The microsomal protein concentration was determined by the method of Lowry.

Incubation and sample preparation The incubation conditions of the experiment were established and controlled to provide a reproducible and linear rate of the metabolite. A typical incubation mixture consisted of potassium phosphate buffer (PH 7.4) 100 mmol/L, an NADPH generating system ($MgCl_2$ 5 mmol/L, G-6-P 10 mmol/L, NADP 1 mmol/L, G-6-PDH 1 kU/L), vinflunine 10 μmol/L, and microsomal protein 1 g/L, in a final volume of 1 mL[17]. The reaction was initiated by the addition of the NADPH generating system. After incubation at 37 °C for 1 h, the reaction was terminated by adding 200 μL of NaOH 1 mmol/L. Vinflunine and its metabolites, M_1, M_2, in the incubation mixture were determined by the LC mass solution method. After adding 1 μg/mL VCR as the internal standard, the reaction mixtures were extracted with 5 mL of ethyl acetate and centrifuged at 2100×*g* for 10 min. The organic fraction evaporated to dryness using an evaporator at 50 °C. The residue was reconstituted in 100 μL of the mobile phase. After centrifugation at 9000×*g* for 10 min, a 10 μL aliquot of the solution was injected into the chromatographic system.

The HPLC system consisted of a DGU-20A3 degasser (Shimadzu corporation, Kyoto, Japan), Shimadzu 20AD pumps (Shimadzu corporation, Kyoto, Japan), a high pressure mixer, a CTO-20A column oven (Shimadzu corporation, Kyoto, Japan) and a Shimadzu SIL-20AC autosampler (Shimadzu corporation, Kyoto,Japan). A Shimadzu 2010 liquid chromatograph-mass spectrometer (Shimadzu corporation, Kyoto,Japan) equipped with an electrospray ionization (ESI) probe, QoQ system (Q-array-octapole-quadrupole mass analyzer; Shimadzu corporation, Kyoto, Japan) was used in the study. The analysis was carried out on an ODS column (Shim-pack, 5 μm, 2.1mm×250 mm ID; Shimadzu, Japan). The mobile phase was acetonitrile, 1 mmol/L ammonium acetate (35:65, *v/v*), and the column temperature was maintained at 35 °C. A constant mobile phase flow-rate of 0.2 mL/min was employed throughout the analyses. Mass spectrometric conditions were optimized to obtain maximum sensitivity. The final ESI conditions used were as follows: curve dissolution line voltage was fixed as that in Tuning and the probe high voltage was set at 4.5 kV, Q-array voltage of DC -35 V

and RF 150 V. Mass spectra were obtained at a dwell time of 0.2 s in selected ion monitor (SIM) mode and 1 s in scan mode. Liquid nitrogen (99.995%, from Gas Supplier Center of Nanjing University, Nanjing, China) was used as the nebulizing gas at 4.5 L/min. LCMS Solution Version 3.2 (Shimadzu corporation, Kyoto, Japan) worked on Windows 2000. Vacuum in the mass detector was obtained by a Turbomolecular pump (Pfeiffer Vacuum GmbH, Asslar, Germany). Vinflunine in the incubation mixture were determined by mass analysis[25]. The metabolites were quantitated by peak area ratio.

Inhibition study The effects of various selective CYP450 inhibitors on the formation of metabolites M_1 and M_2 in human liver microsomes were investigated. The inhibitors studied were α-Naph (CYP1A2), Qui (CYP2D6), DDC (CYP2E), Sul (CYP2C9), Tra (CYP2C19) and Ket (CYP3A). The concentration of vinflunine was 10 μmol/L, and the concentration range of inhibitors was 2.5–20 μmol/L for Qui, 12.5–100 μmol/L for α-Naph, DDC, Tra and Sul, and 0.5–5 μmol/L for Ket.

Study of inhibition type After identifying the major metabolic enzyme of vinflunine *in vitro*, we incubated vinflunine in human liver microsomes in the absence and presence of Ket for 30 min, and then used a graphical method for analyzing enzyme data to identify the types of inhibition of Ket on the formation of M_1 and M_2. The concentration range of vinflunine was 2.5–40 μmol/L and Ket was 0.25–5 μmol/L.

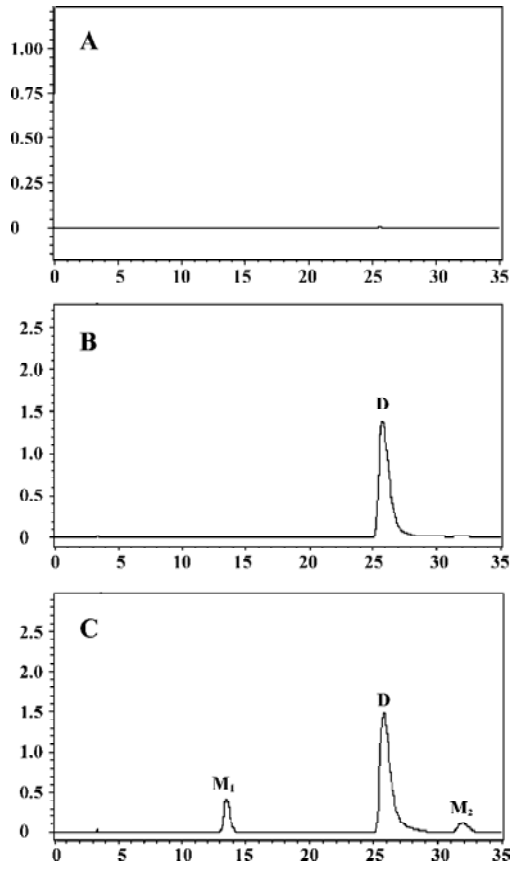

Figure 2. HPLC chromatograms of (A) Blank human liver microsomes; (B) Vinflunine standard in blank human liver microsomes; (C) Incubation of vinflunine with human liver microsomes.

Results

Microsomal incubation of vinflunine in the absence of the NADPH-generating system resulted in a single peak of parent vinflunine. Vinflunine metabolism by human liver microsomes was found to be an NADPH-dependent process. For the optimization of both reaction time and protein concentration, M_1 and M_2 formations were tested and the condition was chosen within the linear rang of these 2 reactions. M_1 and M_2 formed linearly with the reaction time less than 45 min and protein concentration up to 2 mg/mL. The substrate concentrations were determined based on the Km values for both M_1 and M_2 which were estimated by the Michaelis-Menten saturation curve in preliminary experiments. Following the incubation of vinflunine with human liver microsomes and the NADPH-generating system, vinflunine was rapidly metabolized, and 2 metabolites (M_1, M_2) were isolated in the incubation mixture (Figure 2). Direct HPLC/MS analysis using an electrospray ionization interface under positive ion mode resolved 2 drug-related compounds in the incubations (Figure 3). M_1 exhibited the protonated molecular ion $[M+H]^+$ at m/z 833, and M_2 exhibited the protonated molecular ion $[M+H]^+$ at m/z 815. Thus, the molecular weight of M_1 and M_2 were 832 and 814, respectively. According to mass spectra analysis and the metabolic pathway of VRL[18,19], we can tentatively presume that M_1 was the N-oxide metabolite or hydroxylated metabolite, and M_2 was the epoxide metabolite of vinflunine, although this needs further identification. The proposed metabolic pathway of vinflunine is presented in Figure 4.

Ket, the specific inhibitor of CYP3A, could inhibit the formation of M_1 and M_2 while other inhibitors had no significant inhibitory effects on their formation (Figuge 5). The Lineweaver-Burk plot of vinflunine metabolism activity in human liver microsomes in the absence or presence of Ket is presented in Figure 6. Ket uncompetitively inhibited the formation of M_1 and competitively inhibited the formation of M_2, resulting in a lower rate of vinflunine metabolism in human liver microsomes (Figure 7).

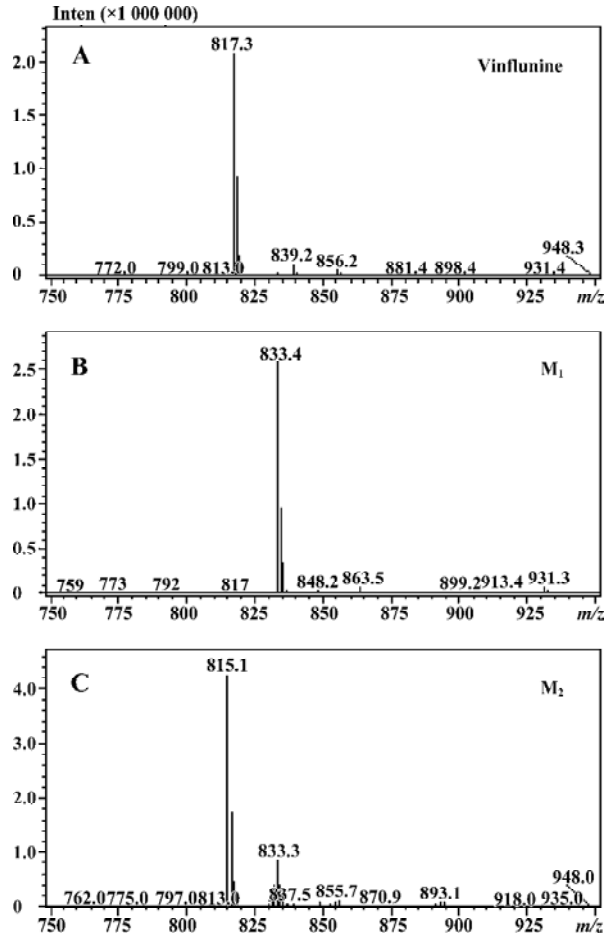

Figure 3. LC-MS spectra of vinflunine and its metabolites in human liver microsomes.

Discussion

Biotransformation of vinflunine in human liver microsomes is NADPH dependent; this suggests that liver microsome CYP450 was involved in the metabolism of vinflunine. After incubation with human microsomes, vinflunine was rapidly metabolized to 2 metabolites, M_1 and M_2. Direct HPLC/MS analysis using an electrospray ionization interface under positive ion mode resolved 2 drug-related compounds in the incubations. The molecular weight of M_1 (832) is 16 more than that of its parent drug (816). Vinflunine, 20'-difluoro-3'4'-dihydrovinorelbine, is similar to VRL in chemical structure, while 20'-hydroxyvinorelbine and VRL N-oxide was reported to be the metabolites of VRL in humans[20]. So we can prospect that M_1 was vinflunine N-oxide metabolite or hydroxylated metabolite. The molecular weight of M_2 (814) is 2 less than that of vinflunine. According to the metabolic pathway of VRL in rats[21], M_2 was prospected to be epoxy vinflunine.

Double reciprocal plots indicated that Ket, the specific inhibitor of CYP3A, could inhibit the formation of M_1 by an uncompetitive mechanism, while M_2 could inhibit formation by a competitive mechanism. The results of inhibition study suggest that CYP3A4 is a major CYP450 isoform involved in the metabolism of vinflunine, which is consistent with reports that CYP3A4 was the main CYP450 enzyme involved in the VRL metabolism[22,23]. CYP3A4 represents the major CYP450 isozyme in the human liver (about 30%)[26]. Fifty percent of marketed drugs belonging to various therapeutic areas (including antidepressants, HIV antivirals, calcium

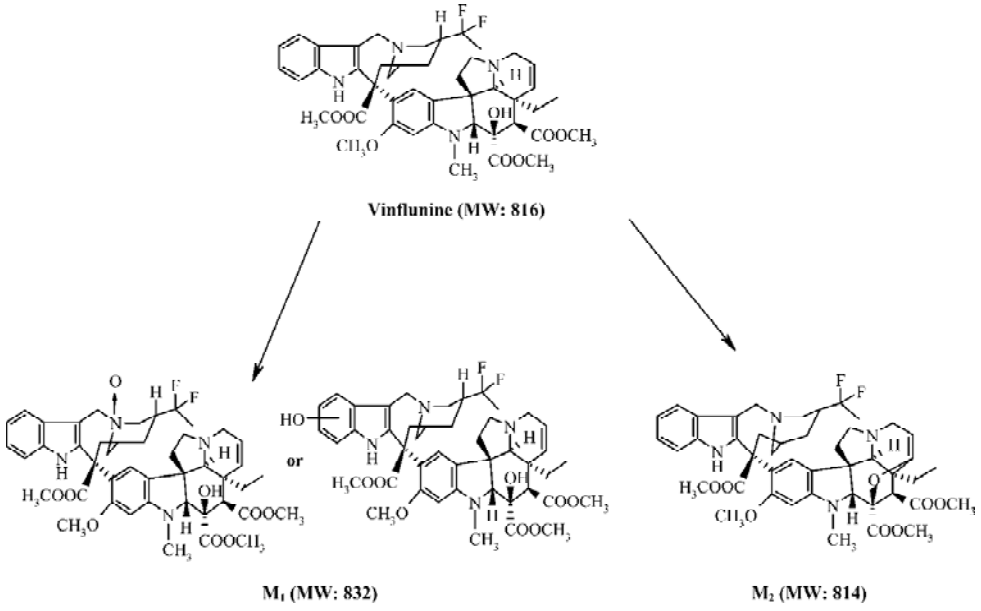

Figure 4. Proposed metabolic pathway of vinflunine in human liver microsomes.

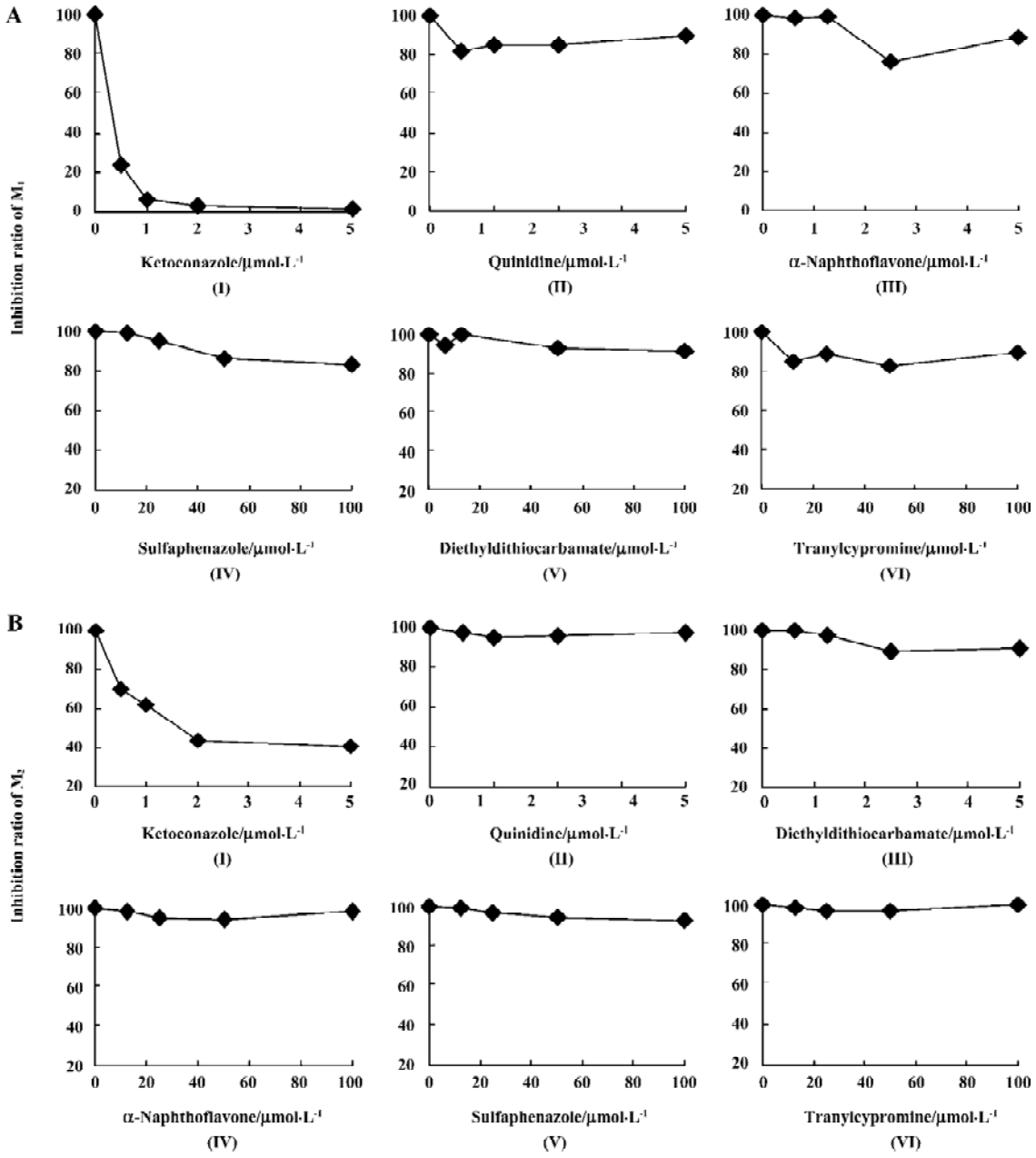

Figure 5. (A) Effects of CYP450 selective inhibitors on the formation of metabolite M_1 of vinflunine (10 μmol/L) in human liver microsomes. Effects of each inhibitor were compared with the control values determined by the incubation of vinflunine alone and expressed as the percentage of control values (100%). Each point represents the mean of duplicate determination. (B) Effects of CYP450 selective inhibitors on the formation of metabolite M_2 of vinflunine (10 μmol/L) in human liver microsomes. Effects of each inhibitor were compared with the control values determined by the incubation of vinflunine alone and expressed as the percentage of control values (100%). Each point represents the mean of duplicate determination.

channel blockers, anticancer agents) are metabolized by the CYP3A family and especially CYP3A4. As a consequence, some drug-drug interactions might occur in clinical practice and precisely for anticancer therapy as several drugs may be in combination with vinflunine, such as docetaxel, tamoxifen, and etoposide. However, we have not yet examined the effect of vinflunine on the metabolism of those anticancer drugs. Because of the narrow therapeutic range of these drugs, further studies are needed to investigate the effect of vinflunine on the metabolism of anticancer drugs that are

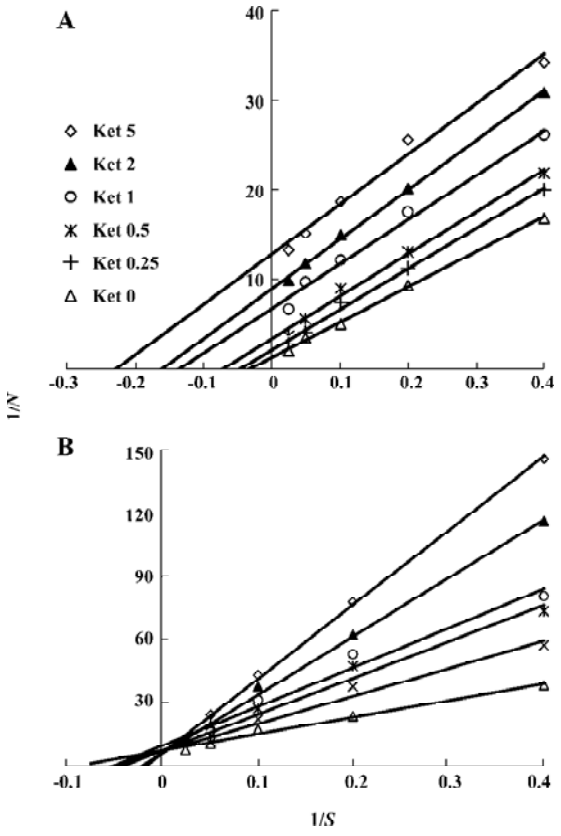

Figure 6. (A) Effect of Ket on the formation of M_1 in human liver microsomes. Each data point represents the mean of duplicate determination. V, reaction velocity; S, substrate concentration. (B) Effect of Ket on the formation of M_2 in human liver microsomes. Each data point represents the mean of duplicate determination. V, reaction velocity; S, substrate concentration.

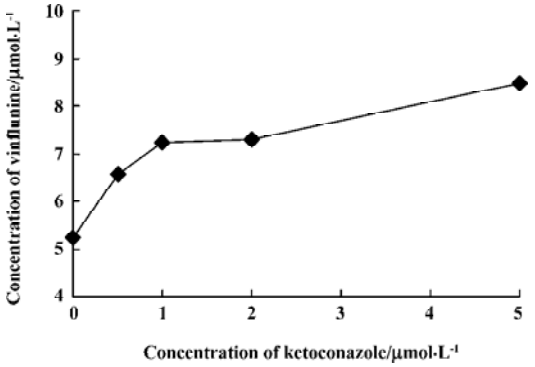

Figure 7. Effect of Ket on the metabolism of vinflunine (10 μmol/L) in human liver microsomes. Each point represents the mean of duplicate determination.

likely to be co-administered with vinflunine. In addition, large interindividual variations of CYP3A activity in human liver microsomes lead to a variable metabolism of most CYP3A substrates[26]. It was reported that the VRL metabolism varied approximately 10-fold between individuals[23]. As the structure and metabolism of vinflunine is similar to VRL, interindividual variability of the vinflunine metabolism needs to be investigated. In conclusion, the present study indicates that the vinflunine metabolism may be affected by the drugs that have an inhibitory or inductive effect on CYP3A4, because CYP3A4 plays a major role in the metabolism of vinflunine in human liver microsomes.

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