CYP3A4 IS MAINLY RESPONSIBLE FOR THE METABOLISM OF A NEW VINCA ALKALOID, VINORELBINE, IN HUMAN LIVER MICROSONES

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ABSTRACT:

The metabolism of vinorelbine, a new anticancer agent belonging to the vinca alkaloid family, was investigated in human liver microsomes. Vinorelbine biotransformation consisted of one saturable and one nonsaturable process, and the $K_m$ and $V_{max}$ values for the saturable process were 1.90 $\mu$M and 25.3 pmol/min/mg of protein, respectively. Several studies, including metabolism by cytochrome P450 (CYP) enzymes in a cDNA expression system and inhibition by specific antibodies and chemical inhibitors, showed that the main CYP enzyme involved in vinorelbine metabolism was CYP3A4. Also, the effects of vinorelbine on each of the CYP activities in human liver microsomes were investigated. High concentrations (100 $\mu$M) of vinorelbine inhibited CYP3A4 activity (testosterone 6β-hydroxylation activity) by 45.2%. However, the inhibitory effects of vinorelbine on the other CYP activities were minimal. The 50% inhibitory concentration (IC$_{50}$) of vinorelbine for testosterone 6β-hydroxylase was estimated to be 155 $\mu$M. The plasma concentration in patients is expected to be much lower than this value. These results indicate that vinorelbine metabolism is expected to be modulated by the drugs that are able to inhibit or induce CYP3A activity.
underlying mechanism of the interaction between vincristine and itraconazole is unknown (Bohme et al., 1995). Acute pulmonary reactions have been reported with vinorelbine and other anticancer vinca alkaloids used in conjunction with mitomycin (Konits et al., 1982; Luedke et al., 1985; Raderer et al., 1996). However, there are no published reports of pharmacokinetic drug-drug interactions involving vinorelbine. Therefore, the purpose of this study was to investigate the major enzymes involved in the biotransformation of vinorelbine and possible vinorelbine-drug interactions using human liver microsomes.

Materials and Methods

Chemicals. Vinorelbine tartrate (3′,4′-dihydro-4′-deoxy-C′-norvincaleukoblastine [R(R*),R*]-2,3-dihydroxbutaneiodate (1:2) (salt)) was supplied by Pierre Fabre Medicament (Castres, Cedex, France). [3H]Vinorelbine (93 GBq mmol) was purchased from Commissariat a l’energie Atomique (Gif-sur-Yvette, France) and was more than 98% pure as ascertained by high performance liquid chromatography (HPLC). Ketocazole was supplied by Janssen Pharmaceutica (Beerse, Belgium). Itraconazole was supplied by Janssen-Kyowa Co. (Tokyo, Japan). Other chemicals were obtained from the following sources: β-NADP⁺, glucose 6-phosphate (G-6-P) and G-6-P dehydrogenase from Oriental Yeast Co. (Tokyo, Japan); fluconazole from Pfizer Pharmaceutica (Beerse, Belgium). Itraconazole was supplied by Janssen-Kyowa Co. (Tokyo, Japan). Other chemicals were obtained from the following sources: β-NADP⁺, glucose 6-phosphate (G-6-P) and G-6-P dehydrogenase from Oriental Yeast Co. (Tokyo, Japan); fluconazole from Pfizer Pharmaceutica Inc. (Tokyo, Japan); 4-acyethylaminophenol from Tokyo Chemical Industries (Tokyo, Japan); bufuralol from Gentest Co. (Woburn, MA); vinblastine, vincristine, and vindesine from Shionogi & Co (Osaka, Japan); S-mephenytoin, 4′-hydroxy-phenytoin, and 6′-hydroxytestosterone from Sumitomo Chemical Co. (Osaka, Japan); tranylcypromine, sulfaphenazole, troleandomycin, and chlorozacon from Sigma Chemical Co. (St. Louis, MO); furafylline, 4-hydroxytolbutamide, chlorpropamide, 1′-hydroxybufuralol, 6-hydroxychloroxazone, pentoxifylline, and corticosterone from Research Biochemicals International (Natick, MA); and quinidine, diethyldithiocarbamate, phenacetin, caffeine, phenobarbital, tobutamide, and testosterone from Wako Pure Chemical Industries (Osaka, Japan). Other chemicals were of the highest grade commercially available. HPLC-grade methanol, acetonitrile, hexane, chloroform, dichloromethane, and tetrahydrofuran were used (Kanto Chemical Co., Tokyo, Japan). Other chemicals were of the highest grade commercially available.

Incubation Conditions. Vinorelbine [0.5 μM, 46 kBq/ml] was incubated with liver microsomes (1 mg of protein/ml of final protein concentration) in phosphate buffer (100 mM, pH 7.4) at 37°C. Reactions were initiated by the addition of NADPH-generating system (0.8 mM β-NADP⁺, 8 mM G-6-P, 1 unit/ml G-6-P dehydrogenase, and 6 mM MgCl₂) for up to 2 h. When the reaction was terminated at the specified time points, aliquots of the reaction mixtures (200 μl) were removed and placed in other tubes, and the volume was adjusted to 400 μl by the addition of an equal volume of ice-cold methanol. The samples were centrifuged at 14,020 g for 10 min, and the supernatant was filtered before carrying out HPLC analysis.

For metabolism by the recombinant CYP expression system, [3H]Vinorelbine (0.5 μM, 46 kBq/ml) or vinorelbine (0.5 μM) was incubated with the NADPH-generating system in phosphate buffer at 37°C. Reactions were initiated by the addition of microsomes of specific CYP cDNA-transfected human B-lymphoblastoid (1 mg of protein/ml), baculovirus-infected insect cells (100 pmol of CYP/ml), or S. cerevisiae AH22 cells (125 pmol of CYP/ml) microsomes at 37°C. For CYP2A6 and CYP2C9 of B-lymphoblastoid microsomes, 100 mM Tris-HCl buffer (pH 7.4) was used instead of 100 mM phosphate buffer (pH 7.4) according to the instructions supplied with the product. The recovery of total counts after the protein-precipitation procedure and after elution from the HPLC following injection of vinorelbine metabolism sample was calculated to be >95%.

Kineties in Human Liver Microsomes. Preliminary results indicated that the rate of metabolism of vinorelbine was linear at 37°C for an incubation time up to 30 min and for a microsomal protein concentration up to 1 mg/ml at a vinorelbine concentration of 0.5 μM. Accordingly, the kinetics study was performed at 37°C with an incubation time of 30 min at a micromolar protein concentration of 1 mg/ml at a vinorelbine concentration of 0.5–500 μM. The kinetic data for vinorelbine metabolism were fitted using the nonlinear least-squares regression program MULTI (Yamaoka et al., 1981) in which each data point was given a weight of 1/v².

\[ v = V_{\text{max}} \cdot S / (K_m + S) + C_{\text{in}} \cdot S \]

where \( V_{\text{max}} \) is the maximal velocity of vinorelbine metabolism, \( K_m \) is the Michaelis-Menten constant, \( S \) is the initial vinorelbine concentration, and \( C_{\text{in}} \) is the intrinsic metabolic clearance for the nonsaturable process. Fitting was evaluated by Akaike’s information criterion (Yamaoka et al., 1978). The results from three experiments are analyzed.

Inhibition of Vinorelbine Metabolism. In immunoinhibition experiments, microsomes were preincubated with anti-rat CYP enzyme polyclonal anti-serum (125 μl/ml) or an anti-human CYP antibody (2.5 mg of IgG/ml) at room temperature for 30 min, followed by the addition of [3H]Vinorelbine (0.5 μM, 46 kBq/ml). The amounts of the antibody or antisera that can inhibit almost 50% of the typical CYP activity based on product information were used. The reaction was initiated by the addition of the NADPH-generating system at 37°C for 30 min.

In the chemical inhibition experiments, incubations contained [3H]Vinorelbine (25 μM, 46 kBq/ml), inhibitors, microsomes (1 mg of protein/ml), and the NADPH-generating system at 37°C in a final volume of 200 μl. In the case of the mechanism-based inhibitors, furafylline, diethylidithiocarbamate, or troleandomycin, the mixture of microsomes and inhibitor was preincubated in the presence of the NADPH-generating system at 37°C for 30 min, and the reaction was initiated by addition of [3H]Vinorelbine. The concentration of inhibitors was 25 μM. In the experiments involving the inhibition of triazole antifungal drugs, [3H]Vinorelbine (5 μM, 46 kBq/ml) was incubated with microsomes (1 mg of protein/ml) in the presence of different concentrations of itraconazole (0.2–2 μM), ketoconazole (0.005–0.2 μM), and fluconazole (5–200 μM). The mean values were calculated from the duplicate experiments.

HPLC Condition for Vinorelbine and Metabolites. Vinorelbine and its metabolites were separated by an HPLC system (Beckman, Fullerton, CA), and detection of the tritiated compounds was performed using a radioactive flow detector (171, Beckman). Reversed phase chromatography was carried out.
FIG. 2. Radiochromatograms of vinorelbine metabolism in human liver microsomes and recombinant CYP3A4.

Vinorelbine (0.5 μM) was incubated using human liver microsomes (0.2 mg of protein) and recombinant CYP3A4 (15 pmol of CYP) for 30 min at 37°C with and without the NADPH-generating system in a final volume of 0.2 ml.

using a Develosil ODS-HG-5 (150 × 4.6 mm, 5 μm; Nomura Chemical, Aichi, Japan), and the mobile phase consisted of 50 mM ammonium acetate buffer (pH 4.5) in methanol (50:50, v/v) at a flow rate of 1 ml/min. The recovery in each HPLC analysis was over 90%.

Effects of Vinorelbine on the CYP Enzymes Specific Activities. The effect of various concentrations of vinorelbine (0.01, 0.1, 1.0, and 100 μM) on CYP activities was examined by two different incubation methods. The preincubation method can estimate metabolism-dependent inhibition, such as suicidal inhibition, and the simultaneous incubation method estimates metabolism-independent inhibition, such as competitive inhibition. The following activities were measured for each type of CYP enzyme: phenacetin O-deethyl- 

- Eadie-Hofstee plot of the metabolism of vinorelbine in human liver microsomes.

- Liver microsomes (0.2 mg of protein) were incubated for 30 min at 37°C with vinorelbine (0.5–500 μM) in the presence of the NADPH-generating system in a final volume of 0.2 ml. The points and solid line represent the observed data and fitted curve, respectively.

Results

Vinorelbine Metabolism in Human Liver Microsome. Microsomal incubation of vinorelbine in the absence of the NADPH-generating system resulted in a single peak of parent vinorelbine. Figure 2 shows a typical radiochromatogram obtained from an incubation containing 0.5 μM [3H]vinorelbine, NADPH-generating system, and human liver microsomes at 30 min. Vinorelbine metabolism by human liver microsomes was found to be an NADPH-dependent process. In addition, this metabolism was dependent on protein concentration up to 2 mg/ml and at 0.5 μM, the remaining vinorelbine decreased linearly up to 30 min (data not shown). The velocity of vinorelbine metabolism was calculated to be 4.37 ± 0.83 pmol/mg of protein/min (mean ± S.D., n = 3).

The kinetics of vinorelbine metabolism were investigated in human liver microsomes over the concentration range of 0.5 to 500 μM. There were many metabolite peaks in the chromatogram (Fig. 2). In these experiments, structural identification of these metabolites was difficult, because there was very little of each metabolite. Consequently, the metabolic rates were analyzed using the biotransformation rate of vinorelbine itself. Eadie-Hofstee plots for vinorelbine metabolism are shown in Fig. 3. We analyzed these models using a model with one saturable and one nonsaturable component, because the Eadie-Hofstee plot for the metabolism of vinorelbine showed that multiple components were involved. Data fitting was attempted using the following three models: 1) one saturable component, 2) one saturable and one nonsaturable component (equation), and 3) two saturable components.

Identification of CYP Enzymes in Vinorelbine Metabolism. Several approaches were used to identify the CYP enzyme responsible
for the metabolism of vinorelbine. First, we compared the velocity of vinorelbine metabolism using a recombinant CYP enzyme expression system. The velocity of vinorelbine metabolism by CYP3A4 microsomes of lymphoblast cells was 60.8 fmol/min/pmol of CYP. The profile of vinorelbine metabolites produced by the CYP3A4 expression system was almost the same profile as that produced by human liver microsomes (Fig. 2). CYP1A2, CYP2D6, and CYP2E1 microsomes (0.0373, 0.154, and 0.0175 fmol/min/pmol of CYP, respectively) exhibited very weak metabolic activity, 1/300 that of CYP3A4. The other recombinant CYP enzymes (CYP1A1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, and CYP2C19) and the control microsomes of lymphoblast cells did not metabolize vinorelbine at all. Also, vinorelbine metabolism by a recombinant CYP enzyme expression system from another source (S. cerevisiae AH22 cells) was similar (data not shown). The velocity of vinorelbine metabolism by CYP3A4 and CYP3A5 microsomes of baculovirus-infected insect cells was 343 and 72.8 fmol/min/pmol of CYP, respectively. In addition, the vinorelbine metabolic activity was not enhanced by cytochrome b$_5$ coexpression.

Second, we examined the inhibitory effect of the antiserum or antibody of anti-CYP enzymes on the metabolism of vinorelbine. The anti-rat CYP3A2 serum and antihuman CYP3A4/5 antibody inhibited vinorelbine metabolism by about 50% compared with the control. However, the anti-rat CYP1A1 serum and antihuman CYP2C2 antibody did not inhibit the metabolism of vinorelbine (Fig. 4).

Third, we examined the inhibitory effect of the selective inhibitor for CYP enzymes and antifungal azole derivatives on the metabolism of vinorelbine. Troleandomycin, a selective inhibitor for CYP3A4, markedly inhibited the metabolism of vinorelbine (Fig. 5). However, other selective inhibitors of CYP enzymes (furafylline, sulfaphenazole, quinidine, tranylcyromine, and diethyldithiocarbamate) had little effect on vinorelbine metabolism (Fig. 5). On the other hand, ketoconazole, itraconazole, and fluconazole also inhibited vinorelbine metabolism in a concentration-dependent manner. The IC$_{50}$ value of ketoconazole, itraconazole, and fluconazole for vinorelbine metabolism was 0.0361, 0.550, and 7.45 $\mu$M, respectively. There was no clear differential inhibition of the metabolites in terms of either the chemical inhibitors or antibodies, except for CYP3A antibody, troleandomycin, ketoconazole, itraconazole, and fluconazole.

**Effect of Vinorelbine on Individual CYP Activities.** The effect of vinorelbine on CYP enzyme-selective activity was examined in human liver microsomes, and the results are shown in Fig. 6. These activities were determined in human liver microsomes preincubated (or untreated) with vinorelbine and the NADPH-generating system. When human liver microsomes were preincubated with vinorelbine (100 $\mu$M) and NADPH-generating system, the activity of CYP2D6 and CYP3A4 fell to 85.1 ± 2.5 and 36.0 ± 2.9% of control, respectively. In the case of simultaneous addition of substrate and vinorelbine (100 $\mu$M), the activity of CYP2C8/9, CYP2C19, CYP2D6, and CYP3A4 fell to 76.4 ± 2.5, 86.5 ± 0.8, 77.9 ± 3.5, and 45.2 ± 5.1% of control, respectively. However, the effect of vinorelbine on other
CYP enzymes was scarcely affected by the two different incubation methods.

Figure 7 shows the effects of vinca alkaloids on testosterone 6β-hydroxylase activity in human liver microsomes. Testosterone 6β-hydroxylase activity fell to 13.8 ± 2.0, 41.2 ± 3.2, 70.5 ± 4.9, and 43.4 ± 6.5% of control, respectively, when vinorelbine, vinblastine, vincristine, and vindesine (500 μM each) were added to untreated human liver microsomes. The IC₅₀ value of vinorelbine for testosterone 6β-hydroxylase was 155 μM, which was lower than that of vinblastine (384 μM) and vindesine (409 μM).

Discussion

We have investigated the contribution of CYP enzymes to vinorelbine metabolism and the effects of vinorelbine on CYP activities in human liver microsomes to assess the potential for drug-drug interactions between vinorelbine and other drugs. Vinorelbine was metabolized in human liver microsomes in the presence of the NADPH-generating system and small amounts of polar metabolites were formed (Fig. 2). After incubation with human hepatocytes, vinorelbine was metabolized to two metabolites (Zhou et al., 1994). Also, Sahmoun et al. (1990) and Lacarelle et al. (1991) have reported that vinorelbine was metabolized to at least three metabolites after incubation with human liver microsomes. There is little structural information on these metabolites, and their identity remains unclear. In this study, the identification of these metabolites was difficult because of the small amount of metabolites available, although we tried to analyze the vinorelbine metabolites using liquid chromatography-mass spectrometry. Some vinorelbine metabolites were estimated, the hydroxylation, oxidation or demethylation of catharanthine and vindorine moieties, but the position was unclear. In addition, 17-deacetylvinorelbine, which is a minor metabolite in urine but not detected
Liver microsomes (0.5 mg/ml) were incubated for 30 min at 37°C with testosterone (250 μM) and various concentrations of vinca alkaloids in the presence of the NADPH-generating system in a final volume 0.2 ml. The formation of 6β-hydroxytestosterone was analyzed by HPLC as described under Materials and Methods. Activities are expressed as a percentage relative to the control experiments. The control activity was 2860 pmol/min/mg of protein. Values are the mean ± S.D. of three experiments.

in serum in human (Jehl et al., 1991), was not observed in this study (data not shown).

This metabolism was analyzed kinetically by the biotransformation of vinorelbine, which consisted of one saturable and one nonsaturable process, with \( K_m \) and \( V_{\text{max}} \) values for a saturable process of 1.90 μM and 25.3 pmol/min/mg of protein, and the clearance for a nonsaturable process was 3.39 μl/min/mg of protein (Fig. 3). The intrinsic clearance \( (V_{\text{max}}/K_m) \) of the saturable process was 13.3 μl/min/mg of protein, and this value was 4-fold higher than the value for the clearance for the nonsaturable process. Thus, the saturable clearance of vinorelbine makes a relatively higher contribution to the clearance than the nonsaturable process in vivo, because the peak serum concentrations of the unbound form of vinorelbine, between 105 and 236 ng/ml (i.e., 0.13 and 0.30 μM, Jehl et al., 1991; Urien et al., 1993), was lower than the \( K_m \) value for the saturable process. On the other hand, the \( K_m \) value for vinblastine and vindesine metabolism in human liver microsomes had been reported to be 6.82 (Zhou-Pan et al., 1993) and 24.7 μM (Zhou et al., 1993), respectively. Therefore, the process involved in vinorelbine metabolism has a higher affinity and a lower capacity than that of vinblastine or vindesine.

Three approaches have been used to identify the CYP enzymes responsible for vinorelbine metabolism: 1) metabolism by cDNA-expressing human CYP enzymes; 2) immunochemical inhibition (Fig. 4); and 3) chemical inhibition (Fig. 5). CYP3A4 exhibited the highest activity, which was over 10-fold higher than the activity of other CYP enzymes. The vinorelbine metabolite activity in liver microsomes was 4 pmol/min/mg of protein, which can be converted to 30 fmol/min/pmol of CYP3A using the amount of CYP and the CYP3A content (Shimada et al., 1994). This value was almost comparable with the activity in the CYP3A4 expression system. In addition, the vinorelbine metabolism was catalyzed by CYP3A4 and CYP3A5 in microsomes from baculovirus-infected insect cells, but the turnover of vinorelbine metabolism by CYP3A5 was lower than that by CYP3A4. Anti-rat CYP3A2 serum, anti-human CYP3A4/5 antibody, and troleandomycin significantly inhibited vinorelbine metabolism. However, other antibodies and chemical inhibitors, expect for troleandomycin, did not inhibit vinorelbine metabolism. These results suggest that the major CYP enzyme involved in vinorelbine metabolism is CYP3A4.

Following chemical and immunochemical inhibition, and a correlation analysis, vinblastine and vindesine metabolisms were also found to be mediated by the same CYP enzyme, CYP3A (Zhou-Pan et al., 1993; Zhou et al., 1993).

There are many reports of drug-drug interactions involving CYP3A4 inhibitors with CYP3A4 substrates. It has been reported that antifungal azole drugs, itraconazole, ketoconazole, and fluconazole, inhibited CYP3A4 activity (Bertz and Granneman, 1997). Also, Bohme et al. (1995) reported evidence of neurotoxicity during coadministration of itraconazole with vincristine. Coadministration of antifungal azole drugs induced drug-drug interactions involving the metabolism of cyclosporin in a clinical situation (Keogh et al., 1995). The IC₅₀ values of ketoconazole and itraconazole for cyclosporin metabolism in human liver microsomes were 0.24 and 2.20 μM, respectively (Back and Tjia, 1991). In this study, itraconazole, ketoconazole, and fluconazole inhibited vinorelbine metabolism in a concentration-dependent manner.

We investigated the possibility that vinorelbine affects CYP-dependent metabolism of other drugs by two different methods. These involve preincubation and incubation with vinorelbine and human liver microsomes in the presence of the NADPH-generating system. The metabolism-based inhibitors, such as troleandomycin, furafylline, and sorivudine, irreversibly bind to the enzyme and reduce both the activity and amount of the target enzymes (Ito et al., 1998). The metabolism-based inhibitors were incubated simultaneously with target enzymes, inhibitors, and coenzymes of target enzymes before the addition of substrates. On the other hand, the reversible (competitive) inhibitors were incubated simultaneously with target enzyme, inhibitors, coenzyme, and substrates. Although CYP3A4 activity was clearly inhibited by vinorelbine, no difference in the effect of vinorelbine on CYP activity was detected between the two experiments. This indicates that vinorelbine may act as a reversible inhibitor for CYP3A4. At 100 μM, vinorelbine inhibited CYP3A4 activity (Fig. 7). Ritonavir, an HIV protease inhibitor, and ketoconazole have a very strong inhibitory effect on CYP3A4 activity at lower concentrations (>0.5 μM).

The effect on CYP3A4 activity involving testosterone 6β-hydroxylation has been compared with the vinca alkaloids vinorelbine, vinblastine, vincristine, and vindesine. The IC₅₀ values of vinorelbine, vinblastine, and vindesine on CYP3A4 activity were 155, 384, and 409 μM, respectively (Fig. 7). Also, IC₅₀ value of vincristine on the activity was more than 500 μM. Thus vinorelbine is a stronger inhibitor of CYP3A4 than are other vinca alkaloids in systems in vitro. However, it should be emphasized that this IC₅₀ value of vinorelbine is much higher than the maximum total plasma concentration of vinorelbine (1.5 μM) seen in the patients given a dose of 30 mg/m² (Jehl et al., 1991). Even if all of the vinorelbine at 25 mg/m² was accumulated in the liver, the maximum concentration in the liver can be calculated to be approximately 35 μM. Even in this case, inhibition of the testosterone 6β-hydroxylation activity was 21%. However, vinorelbine was rapidly eliminated from plasma 1 h after i.v. administration (Jehl et al., 1991; Tosó and Lindley, 1995), and vinorelbine may have little potential to affect the metabolism of CYP3A4 substrates, if these are administered following i.v. administration of vinorelbine.

In this study, we only determined the IC₅₀ value of vinorelbine for testosterone 6β-hydroxylase activity as a typical CYP3A4 substrate. It is well known that CYP3A4 catalyzes not only the metabolism of many anticancer drugs such as docetaxel, tamoxifen, and etoposide but also dihydropropyridine calcium antagonists, benzodiazepines, and other drugs (Rendic and Di Carlo, 1997). However, we have not yet examined the effect of vinorelbine on the metabolism of those anti-
cancer drugs. Because of the narrow therapeutic range of many of these drugs, further studies are needed to investigate the effect of vinorelbine on the metabolism of anticancer drugs that are likely to be coadministered with vinorelbine. To predict drug-drug interaction more precisely, it is necessary to determine the $K$ value of vinorelbine on CYP3A4 activity for each drug.

In conclusion, the present study indicates that vinorelbine 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 vinorelbine in human liver microsomes.

References


