IDENTIFICATION AND CHARACTERIZATION OF POTENT CYP3A4 INHIBITORS IN SCHISANDRA FRUIT EXTRACT

Hiroshi Iwata, Yasuhiro Tezuka, Shigetoshi Kadota, Akira Hiratsuka, and Tadashi Watabe

Division of Natural Products Chemistry, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, Toyama, Japan (H.I., Y.T., S.K.); Division of Analysis and Metabolism, Kashima Laboratory, Mitsubishi Chemical Safety Institute Ltd., Ibaraki, Japan (H.I.); Department of Drug Metabolism and Molecular Toxicology, Tokyo University of Pharmacy and Life Science, Tokyo, Japan (A.H., T.W.); and Department of Molecular Toxicology, Nihon Pharmaceutical University, Saitama, Japan (T.W.)

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ABSTRACT:

Schisandra fruit, a Schisandraceae family herb, is used as a component in Kampo medicines (developed from Chinese medicines, but established in Japan). It can act as a sedative and antitussive, improve hepatic function, and give a general tonic effect. An extract of Schisandra fruit has been shown to have a potent inhibitory effect on human liver microsomal erythromycin N-demethylation activity mediated by cytochrome P450 3A4 (CYP3A4). The present study was conducted to identify Schisandra fruit components having inhibitory effects on CYP3A4 by surveying the effect on human liver microsomal erythromycin N-demethylation activity. Known components of Schisandra fruit, gomisin B, C, G, and N and γ-shizandrin, showed inhibitory effects on N-demethylation activity. Among these components, gomisin C displayed the most potent and competitive inhibitory effect, with a Ki value of 0.049 μM. Furthermore, the inhibitory effect of gomisin C was stronger than that of ketoconazole (Ki = 0.070 μM), a known potent CYP3A4 inhibitor. Gomisin C, however, inhibited CYP1A2, CYP2C9, CYP2C19, and CYP2D6-dependent activities only to a limited extent (IC50 values > 10 μM). Moreover, gomisin C inactivated human liver microsomal erythromycin N-demethylation activity in a time- and concentration-dependent manner. The inactivation kinetic parameters, Kinet and K, were 0.092 min-1 and 0.389 μM, respectively. The human liver microsomal erythromycin N-demethylation activity inactivated by gomisin C did not recover on dialysis of the microsomes. Spectral scanning of CYP3A4 with gomisin C yielded an absorbance at 455 nm, suggesting that gomisin C inactivated the cytochrome P450 via the formation of a metabolite intermediate complex. This pattern is consistent with the metabolism of the methylenedioxy substituent in gomisin C. These results indicate that gomisin C is a mechanism-based inhibitor that not only competitively inhibits but irreversibly inactivates CYP3A4.

Several drugs used in combination, rather than singly, are commonly employed in modern medical treatment regimens. Concomitant use of herbal medicines with synthetic drugs has recently gained considerable attention as useful supplements, or even as a potentially effective treatment, particularly in reducing the adverse effect of synthetic drugs. The combined use of several drugs may produce interactions based on the pharmacological effects of the individual drugs. Such an interaction may result in a serious adverse effect(s), as seen in the following cases: arrhythmia from concomitant use of ketoconazole or erythromycin with terfenadine (Honik et al., 1992, 1993); hematopoiesis suppression in the bone marrow induced by concomitant use of a carcinostatic fluorouracil derivative, like 5-fluorouracil, with sorivudine (Pharmaceutical Affairs Bureau, 1994; Okuda et al., 1999). Ketoconazole and erythromycin have been shown to inhibit cytochrome P450 3A4 (CYP3A4), involved in the metabolism of terfenadine (Jurima-Romet et al., 1994). Sorivudine has recently been shown to irreversibly inhibit dehydroprymidine dehydrogenase, involved in the major metabolic reaction of 5-fluorouracil (Ogura et al., 1998). In addition to adverse reactions associated with a combination of synthetic drugs, drug-natural product interactions have also been observed. One such well documented example relates to the ingestion of grapefruit juice by patients taking certain medications. Consumption of grapefruit juice has been shown to increase the plasma concentration of drugs, such as calcium antagonists (Bailey et al., 1993ab; Lundahl et al., 1997), cyclosporine (Ducharme et al., 1993), midazolam (Kupferschmidt et al., 1995), and saquinavir (Kupferschmidt et al., 1998). Drug interactions have also been reported with some herbal medicines or supplements, such as St. John’s wort (Hypericum perforatum) (Johne et al., 1999; Barone et al., 2000; Yue et al., 2000) and garlic (Allium sativum) (Piscitelli et al., 2002). Under these circumstances, not only drug interactions between synthetic drugs but also those seen with food and herbal medicines have attracted much attention.

Cytochrome P450 (P450) is a representative enzyme involved in phase I metabolic reactions of drugs. Mostly, P450 catalyzes the initial reaction resulting in an increase in polarity of the substrate...
molecule. Thus, P450 is regarded as the rate-limiting enzyme for the elimination of drugs. Among the members of the P450 family, CYP3A4 is the most important isozyme and is involved in the majority of the P450-mediated reactions (Guengerich, 1996). In recent years, in vitro systems using human liver microsomes or recombinant P450 enzymes have been established as the evaluation tool for potential inhibitory effects of drugs on P450 enzyme activities (Ito et al., 1998; Kanamitsu et al., 2000). Accordingly, in vitro evaluation systems are now widely used in screening procedures to exclude synthetic drug candidates with a potent P450-inhibitory effect. However, interactions between drugs and natural products (e.g., St. John’s wort, grapefruit juice) are mostly unavoidable before a serious adverse event occurs in a clinical situation. Natural products, like herbal medicines, consist of thousands of components that are not necessarily identified as having inhibitory effects on P450. This is probably a major reason for the difficulty in avoiding serious events associated with the consumption of natural products. Furthermore, the ingredients in herbal medicine may differ qualitatively and quantitatively, depending upon the cultivation conditions, harvest time, and plant storage conditions. To predict interactions between drugs and herbal medicines, the first priority is to identify at least the major components having inhibitory effects on P450 activities and then to determine their inhibition mechanism and inhibition kinetics.

We have screened the P450-inhibitory effects of herbal medicines currently used in Japan and completed the evaluation of 78 herbal extracts. During the screening, we have found that Schisandra fruit extract shows a potent inhibitory effect on CYP3A4 activity (Iwata et al., 2004). The results indicate that Schisandra fruit contains at least one component having an inhibitory effect on CYP3A4 activity. Schisandra fruit is an herbal medicine prepared from mature fruit of the plant, Schisandra chinensis Baillon, of the Schisandraceae family. Schisandra fruit is widely used in Japan as a component of Kampo medicines, such as sho-seiryu-to, seihai-to, ninjin-eiyou-to, etc. In the United States, Schisandra fruit is used as a component of dietary supplement products. Therefore, due to its widespread use, Schisandra fruit is highly likely to be used in combination with various synthetic drugs.

The present study was conducted to identify CYP3A4-inhibiting components in Schisandra fruit extract by using an in vitro human liver microsomal system. We have identified a component having a CYP3A4-inhibiting effect comparable with that of ketonazole. In addition, we have shown that the component irreversibly inactivates CYP3A4.

**Materials and Methods**

**Chemicals.** Schisandra fruit extract powder and gomisin A, B, C, G, and N standards were kindly provided by Tsumura Ltd. (Tokyo, Japan). Schisandra fruit extract was prepared by immersing the herb in distilled water and heating it at 95°C, then passing it through a filter. The filtrate was evaporated under reduced pressure and then spray-dried to give the powder. [N-methyl-14C]Erythromycin (2.035 GBq/mmol; radiochemical purity >99%) was purchased from American Radiolabeled Chemicals (St. Louis, MO). Schizandrin, erythromycin, ketonazole, tolbutamide, and testosterone were purchased from Wako Pure Chemicals (Osaka, Japan). 7-Ethoxresorufin and resorufin were purchased from Sigma-Aldrich (St. Louis, MO). 4-Hydroxytolbutamide, (S)-mephénytoin, 4'-hydroxymephénytoin, bufuralol, 1'-hydroxybufuralol, and 6β-hydroxytestosterone were purchased from Daiichi Pure Chemicals (Tokyo, Japan). NADP+, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Oriental Yeast (Tokyo, Japan). All other reagents and solvents were analytical grade or chromatographic grade.

**Enzymes.** Human liver microsomes, which were prepared from 16 individuals (male, 11; female, 5), were obtained from XenoTech LLC (Kansas City, KS) and stored at −80°C until use. Human small intestinal (jejunum) microsomes, prepared from 10 individuals (male), were obtained from Tissue Transformation Technologies (Edison, NJ). Microsomes from baculovirus-infected insect cells coexpressing CYP3A4, NADPH-P450 oxidoreductase, and cytochrome b5 were purchased from BD Gentest (Woburn, MA).

**Preparation of Water-, Methanol-, Ethyl Acetate-, and Diethyl Ether-Soluble Fractions from Schisandra Fruit Extract.** The powder of Schisandra fruit extract (1 g) was mixed with 30 ml of water, and the mixture was vigorously shaken for 1 h at room temperature. The mixture was centrifuged at 1600g for 10 min. and then the supernatant was separated. One microliter of water-soluble fraction was equivalent to 0.033 mg of Schisandra fruit extract powder. The extracts with methanol, ethyl acetate, and diethyl ether from Schisandra fruit extract were prepared as described previously (Iwata et al., 2004). One microliter of the methanol-, ethyl acetate-, and diethyl ether-soluble fractions was equivalent to 0.33 mg of Schisandra fruit extract powder.

**P450 Marker Substrate Assays.** The N-demethylation of erythromycin, a specific substrate for CYP3A4, was determined by quantifying the amount of formaldehyde product as described previously with minor modification (Riley and Howbrook, 1998). Reaction mixture (0.5 ml) containing 100 μM [N-methyl-14C]erythromycin, 100 mM potassium phosphate (pH 7.4), 50 μM EDTA, 0.4 mg/ml human liver microsomal protein, and an NADPH-generating system (NADPH-gs) (0.5 mM NADP+; 5 mM MgCl2; 5 mM glucose 6-phosphate, and 1 U/ml glucose-6-phosphate dehydrogenase) was equilibrated at 37°C for 5 min. The incubation was performed at 37°C for 10 min.

Human liver microsomal 7-ethoxresorufin O-deethylation, tolbutamide 4-hydroxylation, (S)-mephénytoin 4'-hydroxylation, bufuralol 1'-hydroxylation, and testosterone 6β-hydroxylation activities were used as selective markers for CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4, respectively. All assays were performed in a final volume of 1 ml containing 100 mM potassium phosphate (pH 7.4), 50 μM EDTA, 0.4 mg/ml microsomal protein, and NADPH-gs, incubated at 37°C for an appropriate time [7-ethoxresorufin, 10 min; tolbutamide, 60 min; (S)-mephénytoin, 90 min; bufuralol, 20 min; testosterone, 10 min]. 7-Ethoxyresorufin, tolbutamide, (S)-mephénytoin, bufuralol, and testosterone were used at a final concentration of 0.5, 500, 40, 10, and 100 μM, respectively. The quantifications of each metabolite were performed with an L-7000 series high-performance liquid chromatography (HPLC) apparatus (Hitachi, Ltd., Tokyo, Japan). The extraction and HPLC analyses of each metabolite were carried out according to the methods previously described (Meier et al., 1985; Kronbach et al., 1987; Chen et al., 1993; Leclercq et al., 1996; Iwata et al., 1998).

**Enzyme Inhibition Experiments.** To calculate the IC50 value of Schisandra fruit extract toward human liver and small intestinal (jejenum), microsomal erythromycin N-demethylation activity, each methanol-, ethyl acetate-, or diethyl ether-soluble fraction was added to the reaction mixture at a concentration range of 0 to 1.65 mg of extract powder per ml. In addition, to investigate the effect of preincubation, human liver microsomal erythromycin N-demethylation activity was conducted with or without preincubation for 10 min in the presence of NADPH-gs. The IC50 values for the fractions were determined by nonlinear regression analysis using WinNonlin Ver.3.1 pharmacokinetic analysis software (Pharsight, Mountain View, CA).

To compare the inhibitory effects of six components (schizandrin and gomisin A, B, C, G, and N) from Schisandra fruit extract toward five different human P450 isoforms (CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4), marker assays for each P450 isoform were conducted in the presence of various concentrations of each component. All components were dissolved in methanol to give a final concentration of 1.0% v/v solvent. All incubations were performed in duplicate. The IC50 value for each component was determined by nonlinear regression analysis using WinNonlin Ver.3.1.

To determine the IC50 value for CYP3A4 inhibition on human liver microsomal erythromycin N-demethylation activity, the compounds (0, 0.1, 0.2, and 0.4 μM) were added to the reaction mixture with different concentrations of erythromycin (25, 50, 100, and 200 μM). Dixon plots were used to determine the IC50 value and the inhibitory type. All incubations were performed in duplicate.

**Fractionation of Schisandra Fruit Extract.** The diethyl ether-soluble fraction of Schisandra fruit extract (5 μl, equivalent to 1.65 mg of Schisandra fruit extract powder) was subjected to HPLC. HPLC analysis was performed with a Mightysil RP-18 column (4.6 × 250 mm; Kanto Chemical Co., Inc., Tokyo, Japan) using a model L-7000 system (Hitachi, Ltd.). A gradient
condition of solvent A (water) and solvent B (acetonitrile) was programmed as follows: 0 min, 10% (B); 50 min, 90% (B); 55 min, 90% (B) (condition 1). The flow rate was set at 1 ml/min and column temperature was set at 40°C. The eluate was monitored by the absorption at 254 nm, and fractions were collected every minute. An aliquot of each fraction (1 ml) was transferred to a 13 × 100 mm glass test tube, and the solvent was evaporated under a stream of nitrogen.

Human liver microsomal erythromycin N-demethylation activity was tested as described above. The fractions A [retention time (Rt), 30–35 min] and B (Rt, 45–50 min), eluted on HPLC under condition 1, were collected. The eluates in fractions A and B were evaporated to dryness under a stream of nitrogen, and each residue was dissolved in methanol. An aliquot of each methanol solution was subjected to further purification using different HPLC conditions. The fractions A and B were eluted on HPLC under 50% acetonitrile isocratically (condition 2) and 65% acetonitrile isocratically (condition 3), respectively. All other HPLC conditions were identical to those described previously. Each peak fraction was collected in a glass test tube, and the eluate was evaporated to dryness under a stream of nitrogen. The peak fraction of each residue on erythromycin N-demethylation activity using human liver microsomes according to the method described above.

Isolation of the CYP3A4-Inhibitory Components from Schisandra Fruit Extract. The diethyl ether-soluble fraction of Schisandra fruit extract was repeatedly analyzed by HPLC with the mobile phase of condition 1 as described above. Aliquots of fractions A and B were collected separately. The eluates in both fractions were freeze-dried, and each residue was dissolved in a small volume of methanol. Fractions A and B were repeatedly subjected to HPLC using the mobile phase of condition 2 and condition 3, respectively. A total of five peak fractions showing CYP3A4-inhibitory effects were identified. Three of the peak fractions (Rt 22.3 min, Peak A4; Rt 27.6 min, Peak A8; Rt 29.0 min, Peak A9) were collected from fraction A, and the other two (Rt 20.2 min, Peak B5; Rt 21.6 min, Peak B6) were collected from fraction B. After removing the solvent by freeze-drying, the five inhibitory compounds were dissolved in a small volume of methanol and subjected to structural analysis.

MS, NMR, and Optical Rotation Analysis of Inhibitory Components. Mass spectra and high-resolution electron impact-mass spectrometry (EI-MS) were obtained on a JEOL JMS-700T (ionization voltage, 40 eV; accelerating voltage, 5.0 kV) mass spectrometer (JEOL, Tokyo, Japan) using a direct inlet system. 1H NMR spectra were determined using a JEOL JNM-LA400 spectrometer in CDCl3 with tetramethylsilane as an internal standard. Chemical shifts were recorded as δ values. Optical rotations were measured on a JASCO DIP-4 automatic polarimeter (JASCO Co., Tokyo, Japan) at 25°C.

Mechanism-Based Inhibition Experiments. Microsomes were preincubated at 37°C for 5, 10, 20, and 30 min for the inactivation of CYP3A4 activity with various concentrations of gomisin C in the presence of NADPH-gs. After preincubation, erythromycin N-demethylation activity was measured according to the method described in the previous section. All incubations were performed in duplicate.

Kinetic parameters of the inactivation process were calculated according to the method of Waley (1985) and Silverman (1988). The initial rate constant for inactivation (k inact, min−1) at each concentration of gomisin C was estimated from the initial slope (0–10 min) of the linear regression line of the “percent-age of control activity” versus “preincubation time” profile plotted on a semilogarithmic scale. Thus, k inact = k inact I/(K I + I), where I is the initial inhibitor concentration. The k inact is the maximum rate constant of the inactivation, and K I is the inhibitor concentration required for a half-maximal inactivation. The values of k inact and K I were determined by the double-reciprocal plotting of the values of k obs and I.

Dialysis Experiments. Pooled human liver microsomes (4 mg protein/ml) were incubated for 20 min at 37°C with NADPH-gs and ketoconazole (1 μM) as negative control of irreversible inhibitor, gomisin B (5 μM), or gomisin C (5 μM) in 100 mM potassium phosphate buffer (pH 7.4) containing 50 μM EDTA. Subsequently, the samples were transferred to a Slide-A-Lyzer minidialysis unit with a molecular weight cutoff of 10,000 (Pierce Chemicals, Rockford, IL). Dialysis was performed at 4°C for 16 h in 2 liters of water. The samples were assayed for erythromycin N-demethylation activity as described above, before and after dialysis.

Optical Difference Spectroscopy. Optical difference spectra were measured at 37°C with a U-3310 dual-beam spectrophotometer (Hitachi High-Technologies Co., Tokyo, Japan) using 1-cm cuvettes. One milliliter of microsomal suspensions (500 pmol/ml of CYP3A4) in 66 mM Tris-HCl buffer (pH 7.4) was added to each cuvette. To the reference cuvette was added water and to the sample cuvette was added NADPH (1 mM). After adjustment of the baseline, a solution of gomisin C in methanol was added to each cuvette. The final concentration of gomisin C was 1 μM. A difference spectrum was scanned between 400 and 500 nm after incubation for 3 min.

Results

Inhibition of Microsomal Erythromycin N-Demethylation by Schisandra Fruit Extract. Inhibition of microsomal erythromycin N-demethylation activity was tested by the addition of water-, methanol-, ethyl acetate-, and diethyl ether-soluble fractions of the Schisandra fruit extract. These water and organic solvent-soluble fractions of Schisandra fruit extract showed dose-dependent inhibitory effects on CYP3A4 activity. Similar IC50 values were detected in water-, methanol-, ethyl acetate-, and diethyl ether-soluble fractions (111.9, 127.1, 123.7, and 122.8 μg/ml, respectively; Table 1). Although the IC50 value of the methanol-soluble fraction was 127.1 μg/ml, it was markedly reduced by 40.5 μg/ml after preincubation with microsomes in the presence of NADPH-gs, suggesting that a mechanism-based inhibitory component was present in Schisandra fruit extract. In addition, the methanol-soluble fraction of Schisandra fruit extract showed dose-dependent inhibitory effects on erythromycin N-demethylation activity in human intestinal microsomes, and the IC50 value (104.9 μg/ml) was similar to that in human liver microsomes.

Isolation of CYP3A4-Inhibitory Components from Schisandra Fruit. A typical HPLC chromatogram of the diethyl ether-soluble fraction of Schisandra fruit extract is shown in Fig. 1A. The HPLC eluent was fractionated every minute, and the inhibitory effect on CYP3A4 was investigated for each fraction. The fractions of 35 to 40 min (fraction A) and 45 to 50 min (fraction B) showed strong inhibition of CYP3A4 activity (Fig. 1B). Fractions A and B were collected, and both samples were further separated using different HPLC conditions (described under Materials and Methods). As shown in Fig. 2, A and B, each of the main peaks, separately isolated, showed inhibition to its corresponding peak fraction.

Each sample was isolated as a single peak on the HPLC chromatogram monitored at 254 nm, using HPLC methods described under Materials and Methods. Peaks A4, A8, A9, B5, and B6 on HPLC analysis were obtained at about 200, 150, 400, 500, and 1000 μg, respectively.

Analysis of the Chemical Structure of CYP3A4-Inhibitory Components. Isolated compounds A4, A8, and A9, obtained from fraction A, and B5 and B6, from fraction B, were subjected to structural analysis by high-resolution EI-MS, 1H NMR spectrum, and optical rotation. Their physical data are shown below.

Peak A4: [α]D 215° (CHCl3), high-resolution EI-MS 536.2089 (calculated for C30H32O9 (M+) 536.2047), 1H NMR (CDCl3) δ 7.36–
7.46 (5H, m, H-phenyl), 6.77 (1H, s, H-4), 6.69 (1H, s, H-11), 6.02 and 6.00 (each 1H, d, J = 1.5 Hz, OCH2O), 5.90 (1H, s, H-6), 3.98, 3.81, 3.40, and 3.15 (each 3H, s, OCH3 x4), 2.31 (2H, m, H2-9), 2.14 (1H, m, H-8), 1.34 (3H, s, H3-7), 1.20 (3H, d, J = 7.0 Hz, H3-8). These spectral data coincide with those of gomisin G standard. The retention time of peak A4 was the same as that determined with the gomisin G standard under the same HPLC conditions. From these results, it was confirmed that this component is gomisin G.

Peak A8: [α]D = −185.6° (CHCl3), high-resolution EI-MS 536.2079 (calculated for C30H32O9 (M+)) 536.2046), 1H NMR (CDCl3) 7.31–7.51 (5H, m, H-phenyl), 6.81 (1H, s, H-4), 6.56 (1H, s, H-11), 5.81 and 5.78 (each 1H, d, J = 1.5 Hz, OCH2O), 5.65 (1H, s, H-6), 3.94, 3.89, 3.57, and 3.31 (3H, s, OCH3 x4), 2.29 (2H, m, H2-9), 1.65 (1H, m, H-8), 1.38 (3H, s, H3-7), 1.18 (3H, d, J = 7.0 Hz, H3-8). These spectral data coincide with those of gomisin C standard. The retention time of peak A8 was the same as that determined with the gomisin C standard under the same HPLC conditions. From these results, it was confirmed that this component is gomisin C.

Peak A9: [α]D = −41.2° (CHCl3), high-resolution EI-MS 400.1887 (calculated for C28H34O9 (M+)) 400.1886), 1H NMR (CDCl3) δ 6.55 (1H, s, H-4), 6.48 (1H, s, H-11), 5.95 (2H, s, OCH2O), 3.89, 3.88, 3.83, and 3.54 (each 3H, s, OCH3 x4), 2.43 (2H, m, H2-9), 2.23 (2H, m, H2-6), 1.88 (1H, m, H-7), 1.80 (1H, m, H-8), 0.99 (3H, d, J = 7.0 Hz, H3-7), 0.73 (3H, d, J = 7.0 Hz, H3-8). These spectral data coincide with those of γ-schizandrin standard. The retention time of peak B5 was the same as that determined with the γ-schizandrin standard under the same HPLC conditions. From these results, it was confirmed that this component is γ-schizandrin.

Peaks B5: [α]D = +0.02° (CHCl3), high-resolution EI-MS 400.1897 (calculated for C32H36O9 (M+)) 400.1888), 1H NMR (CDCl3) δ 6.54 (1H, s, H-4), 6.48 (1H, s, H-11), 5.95 (2H, s, OCH2O), 3.89, 3.88, 3.83, and 3.54 (each 3H, s, OCH3 x4), 2.43 (2H, m, H2-9), 2.23 (2H, m, H2-6), 1.88 (1H, m, H-7), 1.80 (1H, m, H-8), 0.99 (3H, d, J = 7.0 Hz, H3-7), 0.73 (3H, d, J = 7.0 Hz, H3-8). These spectral data coincide with those of gomisin B standard. The retention time of peak A9 was the same as that determined with the gomisin B standard under the same HPLC conditions. From these results, it was confirmed that this component is gomisin B.

Peak B6: [α]D = −41.2° (CHCl3), high-resolution EI-MS 400.1887 (calculated for C32H36O9 (M+)) 400.1886), 1H NMR (CDCl3) δ 6.55 (1H, s, H-4), 6.48 (1H, s, H-11), 5.95 (2H, s, OCH2O), 3.89, 3.88, 3.83, and 3.54 (each 3H, s, OCH3 x4), 2.57 (2H, m, H2-6), 2.12 (2H, m, H2-9), 1.89 (1H, m, H-7), 1.79 (1H, m, H-8), 0.97 (3H, d, J = 7.0 Hz, H3-7), 0.73 (3H, d, J = 7.0 Hz, H3-8). These spectral data coincide with those of gomisin N standard. The retention time of peak B5 was the same as that determined with the gomisin N standard under the same HPLC conditions. From these results, it was confirmed that this component is gomisin N.

FIG. 1. HPLC chromatogram of diethyl ether-soluble components of Schisandra fruit extract and the inhibition of human liver microsomal CYP3A4 activity by each fraction eluted from HPLC. A diethyl ether-soluble fraction of Schisandra fruit extract (1.65 mg equivalent to the powder) was injected into HPLC (condition 1 as described under Materials and Methods) (A). The eluent was monitored at 254 nm, and samples were collected every minute. In the presence of each fraction, microsomal erythromycin N-demethylation activity was determined (B). The concentration of microsomal protein was 0.4 mg/ml and the concentration of erythromycin was 100 μM. Microsomes were incubated for 10 min at 37°C in the presence of an NADPH-gs and 0.1 mM EDTA. Inhibition data are expressed as means of duplicate experiments.
firmed that this component is gomisin N. The structural formulas of the five inhibitory components of CYP3A4 and two main components of Schisandra fruit are shown in Fig. 3.

**Inhibitory Effects of Components from Schisandra Fruit on Various Human P450 Activities.** Inhibitory effects of six components (schizandrin and gomisins A, B, C, G, and N) from Schisandra fruit extract toward five different major human P450 isoforms were investigated to clarify the selectivity of inhibition. Gomisins B, C, and G strongly inhibited microsomal erythromycin N-demethylation catalyzed by CYP3A4, and the IC₅₀ values were 0.399, 0.254, and 1.02 μM, respectively (Table 2). The IC₅₀ values for the erythromycin N-demethylation reaction by gomisins A and N and schizandrin were 10.1, 9.67, and >100 μM, respectively (Table 2). Inhibitory effect of the 6β-hydroxylation of testosterone, catalyzed by CYP3A4, was similar to that of the erythromycin N-demethylation reaction. The IC₅₀ value of gomisin A to CYP2C9 activity was 57.7 μM; IC₅₀ values to all the other P450 isoforms were all greater than the highest concentration used (10 μM for gomisins B, C, G, and N; 100 μM for schizandrin). The IC₅₀ value of gomisin A and N to CYP2C19 activity were 10.4 and 5.08 μM, respectively; IC₅₀ values to all other P450 isoforms were greater than the highest concentration used. All components displayed only slight inhibitory effects toward CYP1A2 and CYP2D6 activities (Table 2). Dixon plots yielded the Kᵢ values for gomisin B and C of 0.131 and 0.049 μM, respectively (Fig. 4, A and B). The Kᵢ value of ketoconazole, a typical CYP3A4 inhibitor, was simultaneously determined (0.070 μM; Fig. 4C).

**Inactivation of Human Liver Microsomal CYP3A4 Activity by Gomisin C.** Human liver microsomal erythromycin N-demethylation activity was inactivated by gomisin C in a time- and concentration-dependent manner in the presence of NADPH-gs (Table 2). Inactivation was observed in the absence of NADPH-gs (data not shown). The loss of activity followed pseudofirst-order kinetics. Linear regression analysis of the time course data were used to determine the kₐᵢ values at various concentrations of gomisin C (Fig. 5). The kᵢ values were determined from double-reciprocal plots of kₐᵢ values and inactivator concentration. The kᵢ and Kᵢ values were 0.092 min⁻¹ and 0.399 μM, respectively.

**Reversibility of Inactivation by Gomisin C on Microsomal CYP3A4 Activity.** To determine whether the inactivation effect by gomisins B and C was reversible, gomisins B and C were preincubated at 37°C for 20 min with human liver microsomes and NADPH-gs. The samples were then transferred to a mini-dialysis unit and dialyzed at 4°C for 16 h. In a simultaneous experiment, ketoconazole, a known reversible inhibitor, was used as a control. As indicated in Fig. 6, recovery of CYP3A4 activity was not observed with gomisin C after dialysis, whereas complete recovery was observed with ketoconazole. A partial recovery of CYP3A4 activity was observed with gomisin B after dialysis.

**Spectral Complex Formation.** Spectral difference scanning of CYP3A4 upon incubation of gomisin C yielded an absorbance at 455 nm, along with development of a shoulder peak at 430 nm (Fig. 7).

**Discussion**

In the present study, we isolated gomisins B, C, G, and N and γ-shizandrin, as CYP3A4-inhibiting components from a diethyl ether-soluble fraction of Schisandra fruit extract and identified their structures by spectral analysis. These Schisandra fruit components were found to be lignans having a basic structure of dibenzo[cyclooctadiene and known components of Schisandra fruit (Taguchi and Ikeya, 1975, 1977; Ikeya et al., 1982). The ether-soluble components of Schisandra fruit extract were isolated and identified as gomisins B, C, G, and N.
fruit extract showed an IC₅₀ value of 122.8 μg/ml in the CYP3A4-mediated reaction, which was comparable to those (111.9 and 127.1 μg/ml) observed with water and methanol fractions. These results indicated that almost all components having CYP3A4-inhibiting activities in water and methanol fractions were soluble in diethyl ether. In addition, the methanol-soluble fraction of Schisandra fruit extract also inhibited the erythromycin N-demethylation activity in human intestinal microsomes, and the IC₅₀ value (104.9 μg/ml) was similar to that in human liver microsomes. CYP3A4 is also the major isomorph in the small intestine (Zhang et al., 1999). Since Schisandra fruit is administered orally in the clinical situation, components of the extract could also inhibit CYP3A4 in the small intestine.

To examine the potency of the lignans in Schisandra fruit extract to inhibit CYP3A4, preparations of six components (schizandrin and gomisins A, B, C, G, and N) were examined for the inhibitory effect on microsomal erythromycin CYP3A4-mediated reactions. Six components of the extract (schizandrin and gomisins A, B, C, G, and N) were examined for their inhibitory effects on CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4. More specifically, schizandrin and gomisins A, B, C, G, and N were examined for their inhibitory effect on CYP1A2, CYP2C9, CYP2C19, and CYP2D6 activities. As a result, gomisins B, C, and G inhibited CYP1A2, CYP2C9, CYP2C19, and CYP2D6 only to a limited extent (IC₅₀ > 10 μM). Thus, the inhibitory effects of these gomisins were specifically observed in CYP3A4-mediated reactions. Schizandrin showed only a slight inhibitory effect on any of the CYP1A2, CYP2C9, CYP2C19, and CYP2D6 activities (IC₅₀ > 100 μM). Therefore, it is unlikely that schizandrin will have a dramatic effect on P450 activity. Gomisins A and N inhibited not only CYP3A4 but also CYP2C9, although the inhibitory effects were rather weak (IC₅₀ = 10.4 and 5.08 μM, respectively).

The IC₅₀ values of the methanol-soluble fraction of Schisandra fruit extract were examined for their inhibitory effects on P450 isoforms other than CYP3A4. More specifically, schizandrin and gomisins A, B, C, G, and N were examined for their inhibitory effect on CYP1A2, CYP2C9, CYP2C19, and CYP2D6 activities. As a result, gomisins B, C, and G inhibited CYP1A2, CYP2C9, CYP2C19, and CYP2D6 only to a limited extent (IC₅₀ > 10 μM). Thus, the inhibitory effects of these gomisins were specifically observed in CYP3A4-mediated reactions. Schizandrin showed only a slight inhibitory effect on any of the CYP1A2, CYP2C9, CYP2C19, and CYP2D6 activities (IC₅₀ > 100 μM). Therefore, it is unlikely that schizandrin will have a dramatic effect on P450 activity. Gomisins A and N inhibited not only CYP3A4 but also CYP2C19, although the inhibitory effects were rather weak (IC₅₀ = 10.4 and 5.08 μM, respectively).

Dixon plot analysis was performed with the infection data of erythromycin N-demethylation activity mediated by gomisins B and C to clarify their mode of action. Both gomisins B and C showed the typical pattern of competitive inhibition with a Kᵢ value of 0.131 or 0.049 μM, respectively. Interestingly, the Kᵢ value of gomisin C is lower than that of the well known potent CYP3A4 competitive inhibitor, ketoconazole (0.070 μM). This result indicates that gomisin C is indeed a potent CYP3A4 inhibitor.

The IC₅₀ value of the methanol-soluble fraction of Schisandra fruit extract in the CYP3A4-mediated reaction decreased when preincu-
bated for 10 min at 37°C in the presence of an NADPH-gs (IC50 = 40.5 μM) and gomisin B, gomisin C, and ketoconazole (0, 0.1, 0.2, or 0.4 μM) were used. Each point represents the means of duplicate experiments.

The IC50 value decreased to about one-third of that estimated in the reactions without any preincubation. These results indicate that Schisandra fruit extract contains a mechanism-based inhibitor. In the P450-mediated metabolism of methylenedioxyphenyl compounds, a carbene metabolite is formed as a reactive intermediate, which readily reacts with P450 to form a stable complex or the so-called metabolite-intermediate (MI) complex. The formation of the MI complex has been reported to play a critical role in the inhibition mechanism of P450 by methylenedioxyphenyl compounds (Wilkinson et al., 1984; Murray and Reidy, 1990; Murray, 1997). It is also known that such MI complexes exhibit a characteristic absorbance peak at 455 nm, with a secondary peak at 430 nm, and are referred to as type 3 binding spectra. Since a characteristic spectrum change with CYP3A4 was observed in gomisin C, gomisin C exerts its inhibitory effect on CYP3A4 via the same mechanism as that postulated for other methylenedioxyphenyl compounds. Preincubation of human liver microsomes with gomisin C in the presence of NADPH-gs caused a decrease in erythromycin N-demethylation activity in the microsomes in a manner dependent on the incubation time. The k_{inact} and K_i values were calculated to be 0.092 min^{-1} and 0.399 μM, respectively. These values are comparable with those reported for GF-I-1 (k_{inact} = 0.05 min^{-1} and K_i = 0.31 μM) and GF-I-4 (k_{inact} = 0.06 min^{-1} and K_i = 0.30 μM).

FIG. 4. Dixon plots of gomisin B (A), gomisin C (B), and ketoconazole (C) on human liver microsomal erythromycin N-demethylation activity. Microsomal enzyme activity was determined under the same conditions as described in Fig. 1. Different concentrations of erythromycin (25, 50, 100, or 200 μM) and gomisin B, gomisin C, and ketoconazole (0.1, 0.2, or 0.4 μM) were used. Each point represents the means of duplicate experiments.

FIG. 5. Inactivation of human liver CYP3A4 activity by gomisin C. Human liver microsomes (0.4 mg protein/ml) were preincubated with 1% methanol (vehicle control) or varying concentrations of gomisin C (0, 0.1, 0.2, and 0.5 μM) and an NADPH-gs at 37°C for 0, 10, 20, and 30 min in 100 mM potassium phosphate buffer (pH 7.4) containing 0.05 mM EDTA. Erythromycin was added after the incubation and microsomal erythromycin N-demethylation activity was determined under the same conditions as described in Fig. 1. A, a plot of the log of percentage of control activity versus preincubation time. Each point represents the mean of duplicate experiments. B, a plot of the half-life of enzyme inactivation versus the inverse of the gomisin C concentration.

FIG. 6. Effect of dialysis on the inactivation of human liver microsomal CYP3A4 activity by gomisin B, gomisin C, and ketoconazole. Human liver microsomes (4 mg protein/ml) were preincubated with gomisin B, gomisin C, or ketoconazole (KCZ) and an NADPH-gs at 37°C for 20 min in 100 mM potassium phosphate buffer (pH 7.4) containing 0.05 mM EDTA. A 0.5-ml aliquot was transferred to a Slide-A-Lyzer mini-dialysis unit and dialyzed at 4°C for 16 h. Microsomal enzyme activity was determined under the same conditions as described in Fig. 1 before and after dialysis. Data are expressed as means of duplicate experiments.
FIG. 7. Optical difference spectra formed on incubation of gomisin C (1 μM) with human CYP3A4 (500 pmol/mL) coexpressed with NADPH-P450 oxidoreductase and cytochrome b₅. Incubation conditions were as described under Materials and Methods.

0.05 min⁻¹ and Kᵢ = 0.13 μM), grapefruit juice components have a potent CYP3A4-inhibitory effect (Tassaneeyakul et al., 2000). Moreover, no recovery was observed in erythromycin N-demethylation activity on dialysis of microsomes inactivated by gomisin C, suggesting that gomisin C inactivated CYP3A4 by forming an M₁ complex with CYP3A4.

In conclusion, we identified the components in Schisandra fruit extract, gomisins B and C, as potent inhibitors of CYP3A4. The inhibitory effects of these components were comparable with that of ketoconazole. In addition, we clearly showed that gomisin C is a mechanism-based inhibitor of CYP3A4. From these results, it is possible that concomitant use of Schisandra fruit with a drug that is metabolized by CYP3A4 results in interaction via inhibition of CYP3A4. To determine whether Schisandra fruit induces a drug interaction in clinical situations, further studies on absorption and metabolic stability of gomisins B and C are needed.

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References


Address correspondence to: Yasuhiro Tezuka, Division of Natural Products Chemistry, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama, 930-0194, Japan. E-mail: tezuka@ims.toyama-med.ac.jp