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Brief Communication

Isolation of CYP3A4 Inhibitors from the Black Cohosh (*Cimicifuga racemosa*)

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Recent investigation on drug interaction has shown that some foods and herbal medicines increase the oral availability of a variety of CYP3A4 substrates, which is caused by the reduction of CYP3A4 in intestinal epithelium. During the course of our investigation on CYP3A4 interaction, we found that the commercially available dietary supplement made from black cohosh (*Cimicifuga racemosa*) showed CYP3A4 inhibition. Black cohosh has been used for the treatment of menopausal and post-menopausal symptoms as a dietary supplement. Bioassay-guided isolation from the supplement afforded six active principles, which were identified as cycloartanoid triterpene glycosides.

Keywords: black cohosh – *Cimicifuga racemosa* – CYP3A4 – cytochrome P450 – inhibition – triterpene glycoside

Introduction

The rhizomes of *Cimicifuga* species (Ranunculaceae) are used as Traditional Chinese Medicines. Among *Cimicifuga* species, *C. heracleifolia, C. dahurica* and *C. foetida* are listed in the Chinese Pharmacopoeia and used as an anti-inflammatory, antipyretic and analgesic remedy (1). *Cimicifuga racemosa*, commonly known as black cohosh, is a herb used among Native Americans to treat a variety of ailments, including diarrhea, sore throat and rheumatism. It is best known for its health benefit in treating menopausal disorders (2). It is used therapeutically to reduce the frequency and intensity of hot flushes (3), and in the USA the extract of *C. racemosa* is available for sale as a dietary supplement used for the treatment of menopausal and post-menopausal symptoms (4). Cytochrome P450 (CYP) enzymes belong to heme-containing monooxygenases and constitute three families, CYP1, CYP2 and CYP3 (5), and these enzymes are recognized to be responsible for drug metabolism, carcinogenesis and degradation of xenobiotics. Among a family of CYP enzymes, CYP3A4 is the most abundant enzyme in human liver microsomes and intestinal epithelium; ~30% of the total CYP was suggested to be CYP3A4 (6), and >50% of clinically used drugs are oxidized by CYP3A4 (7,8). It is widely known that concomitant oral administration of several foods and herbs affects drug metabolism in humans by inhibiting CYP3A4 activity. Among them, the inhibition by grapefruit juice has been well studied and it is reported that concomitant intake of the juice alters the pharmacokinetics of various drugs, including cyclosporin (9,10), midazolam (11), dihydropyridine-type calcium channel blockers (12) and triazolam (13). In the course of our study on CYP inhibitors from foods, we have reported the isolation and structural elucidation of CYP inhibitors from grapefruit (*Citrus paradisii*) juice (14–16), white pepper, *Piper nigrum* (17,18) and the strawberry fruit, *Fragaria ananassa* (19). Recently, we found that the commercially available black cohosh, *C. racemosa* Nutt, showed CYP3A4 inhibition. Here we report the isolation, structural identification and CYP inhibitory activity of constituents from black cohosh.

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Methods

General Methods

Nuclear magnetic resonance (NMR) spectra were recorded on a JEOL GSX-500 NMR spectrometer in pyridine-d_5. Mass spectra were measured on a JEOL SX-102 mass spectrometer.

Isolation of Triterpene Glycosides 1–6 from *C. racemosa*

Commercially available black cohosh, *C. racemosa* Nutt, was generously provided by from Tokiwa Phytochemical Co., Ltd. (Japan). The powder of black cohosh (20 g) was suspended in water and extracted with ethyl acetate (EtOAc). The organic layer was then partitioned between hexane and 90% methanol (MeOH)-H_2O. The polar fraction (6.1 g), which exhibited significant CYP3A4 inhibition, 44% at 5 mg/ml, was subjected to silica gel chromatography with EtOAc/MeOH followed by octadecyl silica gel (ODS) chromatography with MeOH/H_2O and then acetonitrile (CH_3CN)/H_2O to afford a potent CYP3A4 inhibitory fraction. The purification by ODS high-performance liquid chromatography (HPLC) with CH_3CN/H_2O gave six triterpene glycosides **1–6**. On the basis of ^{1}H NMR spectra in C_5D_5N and fast atom bombardment mass spectrometry (FABMS), the six triterpene glycosides were identified as cimiracemoside H (**1**, 2.0 mg, 0.01% yield) (19), 26-deoxyactein (**2**, 1.1 mg, 0.0055%) (20,21), 23-*O*-acetylshengmanol 3-*O*-β-D-xylopyranoside (**3**, 1.9 mg, 0.0095%) (22), actaeaepoxide 3-*O*-β-D-xylopyranoside (**4**, 1.1 mg, 0.0055%) (23), 25-*O*-acetylcimigenol 3-*O*-α-L-arabinopyranoside (**5**, 5.9 mg, 0.029%) (24) and 25-*O*-acetylcimigenol 3-*O*-β-D-xylopyranoside (**6**, 4.2 mg, 0.021%) (25,26) (Figure 1).

Assay of CYP3A4 Inhibition

CYP3A4 activity was based on nifedipine oxidation. Various amounts (0–10 μM, final concentration) of samples in 1 μl of dimethylsulfoxide (DMSO) were added to 192 μl of solution containing 100 mM phosphate buffer (pH 7.4) containing 50 μM nifedipine (Wako Pure Chemical Industries, Ltd, Osaka, Japan), 5 mM glucose-6-phosphate (Oriental Yeast Co., Ltd, Tokyo, Japan), 0.5 mM β-$NADP^+$ (Oriental Yeast Co., Ltd), 0.5 mM $MgCl_2$ and 4.3 μg/ml glucose-6-phosphate dehydrogenase (Oriental Yeast Co., Ltd) and incubated at 37°C for 5 min. CYP3A4 (Gentest Co., Woburn, MA) was also pre-incubated in 7 μl of the buffer at 37°C for 5 min and added to the sample solution. After the incubation at 37°C for 1 h, the reaction was quenched by the addition of 100 μl of MeOH. After adding 3.7 μg of 6-methoxycarbonyl-5-methyl-7-(2-nitrophenyl)-4,7-dihydrofuro[3,4-b]pyridin-1-(3*H*)-one in 1 μl of DMSO as an internal standard, the reaction mixture was extracted with 1 ml of ether, and the ether layer was evaporated. The residue was dissolved in 100 μl of MeOH, and an aliquot (20 μl) was analyzed by reverse-phase HPLC (column, TSK-gel ODS-120T, 4.6 mm i.d. × 150 mm; mobile phase, 64% MeOH-H_2O; flow rate, 1.0 ml/min; detection, UV 254 nm); retention times: 2.9 min for the internal standard, 4.0 min for the nifedipine metabolite (nifedipine pyridine) and 5.5 min for nifedipine. The IC_{50} value, the concentration required for 50% inhibition of CYP3A4 activity, was calculated from the data of duplicate measurements.

Results

Bioassay-guided Isolation of Triterpene Glycosides 1–6 from *C. racemosa*

The CYP inhibition was tested using human CYP3A4 as shown in Methods. Commercially available supplement of *C. racemosa* was extracted with EtOAc, and the organic layer was then partitioned between hexane and 90% MeOH-H_2O. The polar fraction exhibited significant CYP inhibition. Forty-four percent inhibition at 5 mg/ml was as potent as that by ketoconazole, 58% at 5 μg/ml. Bioassay-guided fractionation afforded six triterpene glycosides **1–6**. On the basis of their ^{1}H NMR spectra in C_5D_5N and FABMS, they were identified as cimiracemoside H (**1**, 0.01% yield) (20), 26-deoxyactein (**2**, 0.0055%) (21,22),

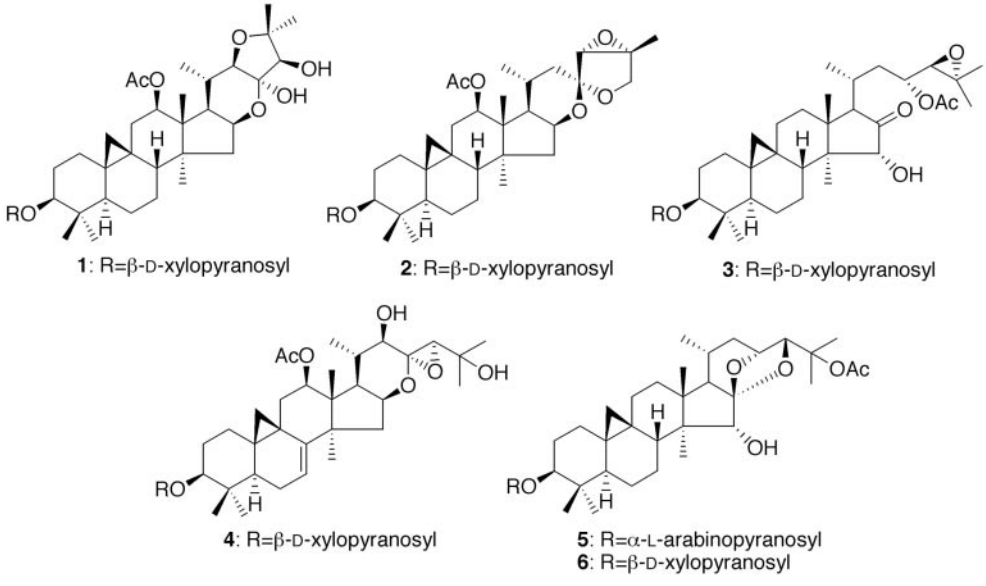

Figure 1. Structures of CYP inhibitors isolated from *C. racemosa*

Table 1. CYP3A4 inhibition of *C. racemosa* metabolites

Compound	IC_{50} (mM)
1	0.48
2	0.10
3	0.11
4	7.78
5	1.4
6	0.83
Ketoconazole[a]	0.00011

[a]A typical CYP3A4 inhibitor.

23-*O*-acetylshengmanol 3-*O*-β-D-xylopyranoside (**3**, 0.095%) (23), actaeaepoxide 3-*O*-β-D-xylopyranoside (**4**, 0.0055%) (24), 25-*O*-acetylcimigenol 3-*O*-α-L-arabinopyranoside (**5**, 0.0029%) (25) and 25-*O*-acetylcimigenol 3-*O*-β-D-xylopyranoside (**6**, 0.0021%) (26,27). The CYP inhibitory activities of **1–6** are shown in Table 1, which indicates moderate IC_{50} values.

Discussion

Bailey *et al*. demonstrated that the blood concentration of antihypertensive drugs such as nifedipine was kept at a high level with grapefruit juice co-administered to mask the taste of alcohol. Since that time there has been a great deal of interest in food–drug interactions because of the clinical risks associated with changes in the bioavailability or metabolic rate of clinically administered drugs. In spite of the increasing use of herbal remedies and nutraceuticals, information on the drug interaction that occurs with them including foodstuffs, is still insufficient to understand the clinical risks.

We have studied drug interactions with foodstuffs such as grapefruit juice and pepper, isolating several potent CYP3A4 inhibitors in each material. The fact that a foodstuff contains more than one inhibitor suggests that the clinical effect of drug interaction with herbs and foodstuffs could be better understood by studying the mixture of inhibitors and/or an extract of these.

During our study on CYP3A4 inhibitors contained in food, we found that the supplement of *C. racemosa* exhibited potent inhibition. The polar fraction from the extract showed 44% inhibition at 5 mg/ml, which was as potent as the inhibition produced by ketoconazole, 58% at 5 μg/ml. We clarified the main constituents, cognate triterpene glycosides, as the CYP3A4 inhibitory principles. To date, highly potent CYP3A4 inhibitors in food have been reported, e.g. paradisins A and B (IC_{50}, 0.07 and 0.07 μM) from grapefruit juice (15), dipiperamide A (IC_{50}, 0.18 μM) from white pepper (17) and gomisin C (IC_{50}, 0.254 μM) from Schisandra fruit (29). Although the IC_{50} value of each isolate was moderate, the high content of a series of cognates in the supplement could result in the exhibition of significant CYP inhibition. Care should be taken to avoid concomitant intake of black cohosh with any kind of medication because of the possibility of increasing the bioavailable concentration of drugs in the blood by the down-regulation suppression of CYP3A4. Medical practitioners therefore should pay more attention in order to avoid any trouble with diet containing black cohosh.

The IC_{50}, 0.027 mg/ml, of the extract of black cohosh was very low in spite of the weak activities of the isolated compounds. The IC_{50} value indicates that 40 mg of the black cohosh extract could be estimated to inhibit the metabolism of roughly half a dose of nifedipine for 1 day.

Evaluation of the IC_{50} of both constituents and the extract may be useful for clinical study of drug interactions of herbs and foodstuffs predicted to be a clinical risk, especially for the study of the mechanism of action and the pharmacokinetics.

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