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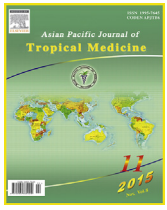

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In vitro inhibitory effects of plumbagin, the promising antimalarial candidate, on human cytochrome P450 enzymes

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ABSTRACT

Objective: To investigate the propensity of plumbagin to inhibit the three isoforms of human cytochrome P450 (CYP), *i.e.*, CYP1A2, CYP2C19, and CYP3A4 using human liver microsomes *in vitro*.

Methods: Inhibitory effects of plumbagin on the three human CYP isoforms were investigated using pooled human liver microsomes. Phenacetin *O*-deethylation, omeprazole hydroxylation and nifedipine oxidation were used as selective substrates for CYP1A2, CYP2C19 and CYP3A4 activities, respectively. Concentrations of paracetamol, 5-hydroxyomeprazole, and oxidized nifedipine were determined in microsomal incubation mixture using high-performance liquid chromatography.

Results: Plumbagin showed significant inhibitory effects on all CYP isoforms, but with the most potent activity on CYP2C19-mediated omeprazole hydroxylation. The IC_{50} (concentration that inhibits enzyme activity by 50%) values of plumbagin and nootkatone (selective inhibitor) for CYP2C19 were (0.78 ± 0.01) and (27.31 ± 0.66) μM, respectively. The inhibitory activities on CYP1A2-mediated phenacetin *O*-deethylation and CYP3A4-mediated nifedipine oxidation were moderate. The IC_{50} values of plumbagin and α-naphthoflavone (selective inhibitor) for CYP1A2 were (1.39 ± 0.01) and (0.02 ± 0.36) μM, respectively. The corresponding IC_{50} values of plumbagin and ketoconazole (selective inhibitor) for CYP3A4 were (2.37 ± 0.10) and (0.18 ± 0.06) μM, respectively.

Conclusions: Clinical relevance of the interference of human drug metabolizing enzymes should be aware of for further development scheme of plumbagin as antimalarial drug when used in combination with other antimalarial drugs which are metabolized by these CYP isoforms.

1. Introduction

Malaria is widespread in tropical and subtropical regions. The resistance of *Plasmodium falciparum* (*P. falciparum*) to first-line antimalarial drugs has resulted in resurgence in treatment failures [1]. Since antimalarial drug resistance compromises the effective treatment of the disease, there is a pressing need for ongoing drug discovery research that provides effective and affordable antimalarial agents. Natural products including medicinal plants may offer cheap alternative treatment opportunities for malaria patients.

Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) is the major constituent in several plants including those in *Plumbaginaceae, Droseraceae, Ancestrocladaceae*, and *Dioncophyllaceae* families. It is a yellow naphthoquinone pigment which occurs in the plant roots [2]. This compound has been shown to display a wide effect of pharmacological activities such as activities against malaria, leishmaniasis, chagas disease, viral infections, and cancers [2–6]. The ethanol extract of *Plumbago indica* Linn. was demonstrated promising antimalarial activity on chloroquine-resistant (K1) and chloroquine-sensitive (3D7) *P. falciparum* clones with median

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(range) IC_{50} of 3.0 (2.7–3.1) and 6.2 (6.2–7.3) μg/mL [7]. Furthermore, we have recently demonstrated the antimalarial activity of plumbagin both *in vitro* and *in vivo* [8]. The aim of the present study was to further investigate the propensity of plumbagin to inhibit the three isoforms of human cytochrome P450 (CYP), *i.e.*, CYP1A2, CYP2C19 and CYP3A4 using human liver microsomes *in vitro*. The CYP enzyme system plays crucial roles in the metabolism of xenobiotics and endogenous substances and thus, has a significant impact on the occurrence of drug–drug interactions particularly metabolic drug interaction [9]. Interference of hepatic drug metabolizing enzyme(s) of one drug by the co-administered drug may result in unexpectedly high plasma concentration of the affected drug and severe adverse effect or toxicity [10].

2. Materials and methods

2.1. Chemicals

The authentic plumbagin (purity 98.2%) was obtained from Apin chemicals Co. Ltd (OX, UK). Phenacetin, paracetamol, caffeine, omeprazole, 5-hydroxyomeprazole, nifedipine, oxidized nifedipine, ketoconazole, nootkatone, α-naphthoflavone, and diazepam were purchased from Sigma–Aldrich (St. Louis, MO, USA). 1, 4-naphthoquinone was purchased from Wako Pure Chemical Industries, Co. Ltd. (Osaka, Japan). β-nicotinamide adenine dinucleotide phosphate (reduced form) tetrasodium salt (NADPH) was purchased from Merck KGaA (Darmstadt, Germany). Pooled human liver microsomes (from 50 donors) were obtained from Gibco BRL Life Technologies (Grand Island, NY, USA).

2.2. CYP inhibition

Inhibitory effects of plumbagin on CYP1A2, CYP2C19 and CYP3A4 activities were investigated *in vitro* using pooled human liver microsomes in a total volume of 500 μL of 0.1 M sodium phosphate buffer (pH 7.4). The concentration range of each substrate used was approximately equal to its *K*m (Michaelis constant) value. Each experiment was repeated four times.

2.2.1. HPLC system

Analysis of concentrations of each CYP-mediated metabolite was performed using the validated high-performance liquid chromatography (HPLC) method. The HPLC system consisted of TSP HPLC with P4000 solvent delivery system, equipped with an AS3000 auto sampler, UV1000 detector, SN4000 controller (Thermo Finnigan, San Jose, CA, USA), and Chrome Quest software (version 4.0). The HPLC column used was a Thermo Hypersil Gold C-18 reversed phase column (210 mm × 4.6 mm, 5 μm particle size). Quality control (QC) samples were run in duplicate in each analytical batch at low, medium, and high concentrations. Criteria for acceptability were four out of six of the QC analyses to lie inside (100 ± 15)% of the nominal values.

2.2.2. Analytical assay validation

The precision of the assay methods based on intraday repeatability was determined by analyzing five series concentrations of paracetamol, 5-hydroxyomeprazole, or oxidized nifedipine in phosphate buffer. The repeatability between days was established using the same concentration range of the three compounds, but the analysis was performed on three consecutive days. Results are expressed as relative standard deviation (% RSD) of replicate measurements as follow:

$$\%\ \mathrm{RSD} = \frac{\text{Standard deviation}}{\text{Mean}} \times 100$$

The accuracy of the analytical methods was determined by comparing the measured concentration of paracetamol, 5-hydroxyomeprazole, and oxidized nifedipine in phosphate buffer at each concentration level (n = 5) to the true concentration in three replicates within one day and on three consecutive days. Accuracy was reported as percentage bias calculated from the equation: % Bias = [(Measure value − True value)/True value] × 100.

Sensitivity of the analytical methods was obtained by the determination the limit of quantification (LOQ). The LOQ was determined based on signal-to-noise approach by comparing measured signals from samples with known lowest concentrations of the test compounds (paracetamol, 5-hydroxyomeprazole, and oxidized nifedipine) and by establishing the minimum concentrations at a typical signal-to-noise ratio is 10:1.

2.2.3. CYP inhibition

The inhibitory effect of plumbagin on CYP1A2-mediated phenacetin *O*-deethylation was performed using α-naphthoflavone as a selective inhibitor [11]. In brief, the reaction mixture was pre-incubated (at 37 °C, 5 min) with human liver microsomes (0.3 mg/mL, 100 μL), 20 μM phenacetin, and plumbagin (0–10 μM). The reaction was initiated with the addition of 1 mM NADPH. Following an incubation (at 37 °C) for 60 min, the reaction was stopped by the addition of 500 μL of cold acetonitrile. The internal standard caffeine (500 μM, 50 μL) was added and the incubation mixture was cooled on ice for 5 min and centrifuged at 12 000 × *g* for 15 min. The supernatant was transferred to an autosampling vial and an aliquot of 20 μL was injected onto the HPLC column. The concentrations of paracetamol (metabolite) were measured by HPLC with UV detection (240 nm) [11]. The gradient mobile phase consisted of a mixture of (A) acetonitrile and (B) distilled water; the initial ratio of mobile phase components (A:B) was 90:10 at a flow rate of 1 mL/min. The calibration curve was plotted using high ratio of paracetamol to caffeine on the ordinate, and concentrations of paracetamol (1–50 μM) on the abscissa.

The inhibitory effect of plumbagin on CYP2C19-mediated omeprazole hydroxylation was performed using nootkatone as a selective inhibitor [12]. In brief, the reaction mixture was pre-incubated (at 37 °C for 5 min), with human liver microsomes (0.5 mg/mL, 100 μL), 10 μM omeprazole, and plumbagin (0–200 μM). The reaction was initiated with the addition of 1 mM NADPH. Following an incubation (at 37 °C for 60 min), the reaction was stopped by the addition of 500 μL of cold acetonitrile. The internal standard 1,4-naphthoquinone (10 μM, 50 μL) was added and the incubation mixture was cooled on ice for 5 min and centrifuged at 12 000 × *g* for 15 min. The supernatant (800 μL) was transferred to an eppendorf tube and evaporated using speed vacuum concentrator (FTS System, Stone Ridge, NY, USA).The dried residue was reconstituted with 100 μL of a mixture of acetonitrile and water (50%:50%, v:v) and 10 μL injected onto the HPLC column. The concentrations

of the metabolite 5-hydroxyomeprazole were measured by HPLC with UV detection (302 nm) [12]. The gradient mobile phase consisted of a mixture of (A) acetonitrile and (B) distilled water; the initial ratio of mobile phase components (A:B) was 10:90 at a flow rate of 1 mL/min. The calibration curve was plotted using high ratio of 5-hydroxyomeprazole to 1,4-naphthoquinone on the ordinate, and concentrations of 5-hydroxyomeprazole (40–2 500 nM) on the abscissa.

The inhibitory effect of plumbagin on CYP3A4-mediated nifedipine oxidation was performed using ketoconazole as a selective inhibitor [13]. In brief, the reaction mixture was pre-incubated (at 37 °C, 5 min), with human microsomes (0.3 mg/mL, 100 μL), 40 μM nifedipine, and plumbagin (0–20 μM). The reaction was initiated with the addition of 1 mM NADPH. Following an incubation (at 37 °C for 40 min), the reaction was stopped by the addition of 500 μL of cold acetonitrile. The internal standard diazepam (80 ng/mL, 50 μL) was added and the incubation mixture was cooled on ice for 5 min and centrifuged at 12 000 × *g* for 15 min. The supernatant was transferred to an autosampling vial and an aliquot of 20 μL was injected onto the HPLC column. The concentrations of oxidized nifedipine (metabolite) were measured by HPLC with UV detection (270 nm) [13–15]. The gradient mobile phase consisted of a mixture of (A) methanol and (B) distilled water; the initial ratio of mobile phase components (A:B) was 55:45 at a flow rate of 1 mL/min. The calibration curve was plotted using high ratio of oxidized nifedipine to diazepam on the ordinate, and concentrations of oxidized nifedipine (1–25 μM) on the abscissa.

2.3. Data analysis

IC_{50} (concentrations causing 50% inhibition of enzyme activity) values were calculated from a logdose–response curve plotted using the Calcusyn™ version 1.1 (BioSoft, Cambridge, UK). Data are presented as mean ± SD of the four experiments.

3. Results

3.1. Analytical assay validation

The analytical methods for determination of paracetamol, 5-hydroxyomeprazole, and oxidized nifedipine used in the study was found to be sensitive and accurate. The linearity of all calibration curves were demonstrated with coefficient (r^2) of greater than 0.995.

CYP1A2-mediated metabolism: The LOQ of paracetamol at a signal-to-noise ratio ≥10 was 1 μM. The precision (intra- and inter-) of analytical method (% RSD) was <6.3% and the accuracy (intra- and inter-) of the method was <±10% (Table 1).

CYP2C19-mediated metabolism: The LOQ of 5-hydroxyomeprazole at a signal-to-noise ratio ≥10 was 40 nM. The precision (intra- and inter-) of analytical method (% RSD) was <6.22% and the accuracy (intra- and inter-) of the method was <±7% (Table 2).

CYP3A4-mediated metabolism: The LOQ of oxidized nifedipine at a signal-to-noise ratio ≥10 was 1 μM. The precision (intra- and inter-) of analytical method (% RSD) was <7.05% and the accuracy (intra- and inter-) of the method was <±6% (Table 3).

Table 1
Intra- and inter-assay accuracy and precision of the analytical method for determination of paracetamol (n = 3 for each concentration).

Quantity of paracetamol spiked (μM)	Accuracy (% Bias)		Precision (% RSD)	
	Intra-assay	Inter-assay	Intra-assay	Inter-assay
1	−4.63	−9.18	1.32	6.21
5	−8.10	−4.44	6.30	6.28
10	3.17	−1.92	2.85	5.73
25	4.46	3.54	3.01	5.70
50	−1.13	−0.78	0.86	1.23

Table 2
Intra- and inter-assay accuracy and precision of the analytical method for determination of 5-hydroxyomeprazole (n = 3 for each concentration).

Quantity of 5-hydroxy omeprazole spiked (nM)	Accuracy (% Bias)		Precision (% RSD)	
	Intra-assay	Inter-assay	Intra-assay	Inter-assay
40	3.21	3.23	1.46	2.69
200	−5.86	−6.43	2.03	1.80
800	0.89	2.26	3.93	4.64
1 600	3.25	3.52	4.63	2.16
2 500	4.45	4.06	4.08	6.22

Table 3
Intra- and inter-assay accuracy and precision of the analytical method for determination of oxidized nifedipine (n = 3 for each concentration).

Quantity of oxidized nifedipine spiked (μM)	Accuracy (% Bias)		Precision (% RSD)	
	Intra-assay	Inter-assay	Intra-assay	Inter-assay
1.0	−0.94	2.38	1.17	7.05
2.5	2.64	5.20	3.60	0.92
5.0	−1.41	−2.73	5.39	3.54
12.5	0.07	−1.19	4.51	3.23
25.0	0.01	0.35	0.93	0.72

3.2. CYPs inhibition

The inhibitory effects of plumbagin and positive controls on the activities of three major human CYP isoforms, *i.e.*, CYP1A2, CYP2C19 and CYP3A4 are shown in Figure 1A–C and the IC_{50} values are presented in Table 4. Plumbagin clearly inhibited CYP1A2-mediated phenacetin *O*-deethylation, CYP2C19-mediated omeprazole hydroxylation and CYP3A4-mediated nifedipine oxidation in concentration-dependent manners. Among the three CYP isoforms, the inhibitory activity was most potent for CYP2C19, of which its potency was about 35-fold of the selective inhibitor nootkatone (mean IC_{50} values of 0.78 μM *vs.* 27.31 μM). The inhibitory activities on CYP1A2 and CYP3A4 were moderate (about 13- to 69-fold lower than the selective inhibitors).

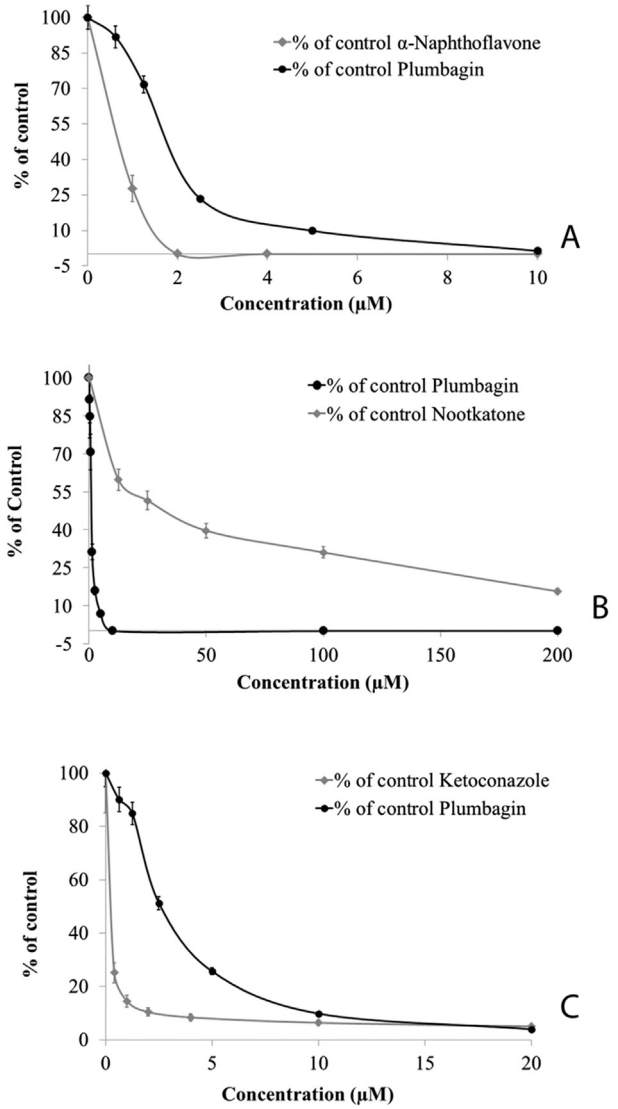

Figure 1. Inhibitory activities of plumbagin and selective inhibitors on (A) CYP1A2-mediated phenacetin *O*-deethylation, (B) CYP2C19-mediated omeprazole hydroxylation, and (C) CYP3A4-mediated nifedipine oxidation (n = 4 for each experiment).

Table 4

IC_{50} values (μM) of plumbagin and selective inhibitors on CYP1A2-mediated phenacetin *O*-deethylation, CYP2C19-mediated omeprazole hydroxylation, and CYP3A4-mediated nifedipine oxidation (n = 4 for each experiment).

Compound	CYP1A2	CYP2C19	CYP3A4
α-naphthoflavone (selective inhibitor for CYP1A2)	0.02 ± 0.36	–	–
Nootkatone (selective inhibitor for CYP2C19)	–	27.31 ± 0.66	–
Ketoconazole (selective inhibitor for CYP3A4)	–	–	0.18 ± 0.06
Plumbagin	1.39 ± 0.01	0.78 ± 0.01	2.37 ± 0.10

4. Discussion

Interactions between phytochemicals in herbal medicines and CYP are now well recognized because of their potential clinical and toxicological implications. These phytochemicals could act as substrates, inhibitors or inducers of the CYP isoforms, which can lead to pharmacokinetic interactions with the co-administered drugs metabolized by the same CYP isoform [9,16]. Our results provide evidence for the inhibitory effect of plumbagin on the three major hepatic CYP isoforms, *i.e.*, CYP1A2, CYP2C19, and CYP3A4. CYP1A2, CYP2C and CYP3A are expressed in human liver at approximately 13, 20 and 30% of total CYP, respectively [17]. Specific inhibitors recommended by the US FDA were used as reference compounds for the inhibitory activity on each CYP. For CYP2C19 however, since there has been no recommended selective inhibitor, nootakone was used as a reference compound as its inhibitory activity was shown to be selective toward CYP2C19 [12]. Certain extent of variations in inhibitory activities of the selective inhibitors were observed compared with that previously been reported. The discrepancy could be due mainly to the choices of substrates used.

Among the three CYP isoforms under investigation, the inhibitory activity of plumbagin on CYP2C19 was most evident, with potency of about 35-fold of the selective inhibitor nootkatone. CYP2C19 is a major metabolizing enzyme of several clinically important drugs such as proton-pump inhibitors like omeprazole and lanzoprazole, anti-epileptic-like mephenytoin, diazepam, antidepressants, the antiplatelet drug clopidogrel, the antifungal voriconazole and selective serotonin reuptake inhibitors like citalopram [18]. Previous investigations in man have shown that CYP2C19 activity is susceptible to induction by herbs and natural products, *e.g.*, St John's wort, Ginko biloba, and the Chinese herbal mixture Yin Zhi Huang [19–21]. Nevertheless, there has been no clear evidence on the inhibitory effect of herbal remedies on CYP2C19. Although inhibitory activity on CYP3A4 was moderate (mean IC_{50} = 2.37 μM), the clinical relevance of such interaction should not be overlooked as this CYP isoform is involved in the metabolism of 50% of all pharmaceuticals [22]. CYP1A2 is known to play a major role in the metabolism of pre-carcinogens and inhibitory effect of plumbagin to this CYP isoform may contribute only minor interaction with the co-administered drugs [23]. Several other factors are necessary to be considered for definitive conclusion on the clinical relevant metabolic drug interactions. These include comparative disposition of the individual constituents responsible for inhibition, as well as the locations of the affected CYP (intestine, liver, *etc*.) [24]. Until further clinical investigations in healthy subjects are confirmed, the potential of this compound for use in treatment of malaria infection may be limited.

The study demonstrated the propensity of plumbagin to interfere with the three human hepatic CYP isoforms, *i.e.*, CYP1A2, CYP2C19, and CYP3A4. The inhibitory potency was highest on CYP2C19. Concurrent administration of plumbagin (as pure compound or as the extract of *Plumbago indica* Linn.) may result in highly toxic plasma concentrations of the co-administered drugs that are metabolized by these CYP

isoforms. Clinical relevance of the interference of human drug metabolizing enzymes should be aware of for further development scheme of plumbagin as antimalarial drug when used co-administration with other antimalarial drugs which are metabolized by CYP1A2, 2C19 and 3A4, *i.e.*, quinine, mefloquine and chloroquine.

Conflict of interest statement

We declare that we have no conflict of interest.

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