Short Communication

Acetylshikonin is a Novel Non-selective Cytochrome P450 Inhibitor

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/bdd.2101

Running title: Acetylshikonin as non-selective P450 inhibitor

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ABBREVIATIONS:

P450, cytochrome P450; HLM, human liver microsome; LC-MS/MS, liquid chromatography-tandem mass spectrometry; SKF-525A, 2-diethylaminoethyl-2,2-diphenylvalerate-HCl; SRM, selected reaction monitoring

Abstract

Acetylshikonin is biologically active compound with anti-cancer and anti-inflammatory activity, which is isolated from the root of *Lithospermum erythrorhizoma*. We have recently discovered a inhibitory effect of acetylshikonin against CYP2J2 activity. Based on this result, we expanded our study to evaluate the inhibitory effects of acetylshikonin against nine different cytochrome P450 (P450) isoforms in human liver microsomes (HLMs) using substrate cocktails incubation assay. Acetylshikonin showed strong inhibitory effect against all P450s tested with IC_{50} values of 1.4–4.0 μM. Pre-incubation of acetylshikonin with HLMs and NADPH did not alter inhibition potency, indicating that acetylshikonin is not a mechanism-based inhibitor. SKF-525A, a widely used non-specific P450 inhibitor, had no inhibitory activity against CYP1A2, 2A6, 2E1, and 2J2 while it showed inhibitory effect against CYP2B6, CYP2C19, and 2D6 with IC_{50} values of 2.5, 3.6, and 0.5 μM, respectively. Our findings indicate that acetylshikonin may be a novel general P450 inhibitor, which could replace SKF-525A.

Key words: acetylshikonin; cytochrome P450; inhibitor; SKF-525A

Introduction

Identification of the specific cytochrome P450 (P450) enzymes responsible for the biotransformation of drugs is important in drug discovery [1]. This information is essential for understanding the drug-drug interaction potential and the inter-individual variability in drug metabolism and pharmacokinetics. Appropriate inhibitors of the various P450 enzymes are essential for conducting these *in vitro* studies [2].

SKF-525A (2-diethylaminoethyl-2,2-diphenylvalerate-HCl) is one of the most widely used non-selective P450 inhibitor, and has been used to determine whether a drug is metabolized by P450 enzymes [3, 4]. SKF-525A has inhibitory effects against CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A, but not against CYP1A2, CYP2A6, or CYP2E1 (the IC_{50} values for these three are > 50 μM) [4-6]. Another useful non-specific P450 inhibitor is 1-aminobenzotriazole (ABT), which is thought to inactivate P450 enzymes following complex formation with metabolic intermediates (MI) [2]. ABT inhibits the activities of most P450s; however, it does not inhibit CYP2C9-mediated diclofenac 4'-hydroxylation [4, 6]. In addition, the inhibitory activities of SKF-525A and ABT against CYP2J2, which is responsible for the metabolism of drugs including albendazole, astemizole, ebastine, and terfenadine [7], have not been reported. These results collectively demonstrate that using ABT and SKF-525A as non-specific P450 inhibitors is not optimal.

Acetylshikonin (CAS 54984-93-9) is a bioactive compound derived from the roots of *Lithospermum erythorhizon* and has been shown to have anti-cancer and anti-inflammatory activities [8, 9]. It also showed strong noncompetitive inhibitory effects against CYP2J2-mediated astemizole *O*-demethylation [10]. In the present study, we investigated the inhibitory effects of acetylshikonin against ten major P450 isoforms (CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 2J2, and 3A) in order to evaluate the possibility of using it as a nonselective general P450 inhibitor in phenotyping studies. In addition, we compared the

P450 inhibitory potential of acetylshikonin with that of the currently used non-specific P450 inhibitors SKF-525A and thelephoric acid.

Materials and Methods

Chemicals and Reagents. Acetylshikonin (> 98.0%) and thelephoric acid (95.8%) were gifts from the National Development Institute of Korean Medicine (Daegu, Korea). Astemizole, dextrorphan, midazolam, omeprazole, tolbutamide, *O*-desmethylastemizole, 1'-hydroxymidazolam, hydroxyomeprazole, and hydroxytolbutamide were purchased from Toronto Research Chemicals (North York, Canada). Glucose-6-phosphate (G6P), glucose-6-phosphate dehydrogenase (G6PDH), nicotinamide adenine dinucleotide phosphate ($NADP^+$), SKF-525A, shikonin (CAS 517-89-5), acetaminophen, amodiaquine, bupropion, chlorzoxazone, coumarin, dextromethorphan, phenacetin, *N*-desethylamodiaquine, hydroxybupropion, hydroxychlorzoxazone, hydroxycoumarin, uridine 5'-diphosphoglucuronic acid (UDPGA), mebendazole (MBZ, internal standard (IS)), and terfenadine (TFD, IS) were obtained from Sigma-Aldrich (St. Louis, MO). Solvents were of high-performance liquid chromatography (HPLC) grade, and all other reagents and chemicals were of analytical grade (≥ 98%, Fisher Scientific Co., Pittsburgh, PA). Pooled human liver microsomes (HLMs, H0630) were purchased from XenoTech (Lenexa, KS).

Microsomal Incubation

Reversible inhibition study: All incubations were performed in triplicate, and the data are presented as average values. The inhibitory effect of acetylshikonin, shikonin, and SKF-525A against CYP2J2-mediated astemizole *O*-demethylase activity was evaluated using pooled HLMs. In brief, the incubation reaction mixtures contained 0.25 mg/ml HLMs, astemizole (1 μM) and inhibitor (0.5 – 50 μM) in 0.1 mM phosphate buffer (pH 7.4), and

were pre-incubated for 5 min at 37°C. The reaction was initiated by the addition of NADPH-generating system (containing 1.3 mM $NADP^+$, 3.3 mM G6P, 3.3 mM $MgCl_2$, and 500 unit/ml G6PDH). The final volume of the incubation mixture was 100 μl. After a 15 min incubation period, the reactions were stopped by adding ice-cold acetonitrile containing 15 ng/ml terfenadine as the internal standard (IS). After centrifugation, aliquots (1 μl) were injected into a liquid chromatography-tandem mass spectrometry system (LC-MS/MS) as described previously [11].

To evaluate the inhibitory activity of acetylshikonin, shikonin, and SKF-525A against nine other P450 isoforms, namely CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A, a previously-developed substrate cocktail method was used [12, 13]. The substrate concentration, the selected reaction monitoring (SRM) transitions, and the collision energies determined for each metabolite are listed in Table 1. Following a 15-min incubation of HLMs (0.25 mg/ml) in the presence or absence of the inhibitor, the reaction was terminated and the mixtures were centrifuged. Aliquots of the supernatants were analyzed by LC-MS/MS as described previously [12, 13], with some modifications. The inhibitory effect of acetylshikonin was also evaluated for CYP3A-mediated testosterone and nifedipine metabolism as previously described [14].

Time-dependent inhibition study: Time-dependent inhibition (TDI) study was measured using an IC_{50} shift method. The HLMs (0.25 mg/ml) were pre-incubated with the inhibitor (acetylshikonin or thelephoric acid) at five different concentrations (0.5–50 μM) in the presence of an NADPH-generating system for 30 min. Thelephoric acid, a known non-specific and time-dependent P450 inhibitor [5], was used as a positive control. The reaction was initiated by adding P450 substrate, samples were further incubated for 15 min, and then

the reaction was terminated by the addition of 100 μl ice-cold acetonitrile containing IS. After centrifugation, aliquots of the supernatants were analyzed by LC-MS/MS.

Data Analysis. IC_{50} values were determined using the WinNonlin software (version 2.1, Pharsight, Mountain View, CA).

Results and Discussion

In this study, we evaluated the inhibitory potential of acetylshikonin against ten P450 isoforms (CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 2J2, and 3A) and compared them with those of SKF-525A [3] and thelephoric acid [5], known non-specific P450 inhibitors. SKF-525A, which is the most commonly used general P450 inhibitor in reaction phenotyping studies, showed strong inhibitory effects against CYP2B6, CYP2C19, and CYP2D6, with IC_{50} values of 2.5, 3.6, and 0.5 μM, respectively, whereas it had no inhibitory effects against CYP1A2, CYP2A6, CYP2E1, or CYP2J2 (IC_{50} > 50 μM) (Table 1). In a previous study, SKF-525A was also reported to weakly inhibit CYP1A2, CYP2A6, and CYP2E1 with an IC_{50} in the range 50–1,100 μM [5, 6]. Similar to the previous results [5], thelephoric acid, which is isolated from *Polyozellus multiplex*, moderately inhibited CYP2C8-mediated amodiaquine *O*-deethylation and CYP2C9-mediated tolbutamide hydroxylation, with IC_{50} values of 6.9 and 11.4 μM, respectively. Thelephoric acid showed strong inhibitory activities against CYP1A2, CYP2A6, CYP2B6, CYP2C19, CYP2D6, CYP2E1, and CYP3A (IC_{50} < 5 μM) (Table 2).

However, acetylshikonin exhibited strong inhibitory activities against all P450 enzymes tested, with IC_{50} values < 5.0 μM. These findings showed that the compound had similar inhibitory potential against all ten P450 enzymes in the range 1.4–4.0 μM (Table 2), and its inhibitory effects were stronger than those of thelephoric acid, a well-documented non-specific P450 inhibitor (2.0 μM ≤ IC_{50} ≤ 11.4 μM), and the commonly used SKF-525A. Acetylshikonin also inhibited CYP3A-mediated testosterone and nifedipine metabolism with IC_{50} values of 5.2 and 3.0 μM, respectively, indicating that it inhibits CYP3A activity in a substrate-independent manner. These experiments demonstrated that acetylshikonin is a more potent non-specific inhibitor of these ten P450 isoforms than are SKF-525A and thelephoric acid in HLMs. Similar to acetylshikonin, shikonin, a structural analogue of acetylshikonin, showed strong inhibitory effects against ten P450 isoforms (IC_{50} < 5.2 μM, Table 2).

We next investigated whether the presence of NADPH led to shifts in the IC_{50} (Table 2). The inhibitory potential of acetylshikonin against the ten P450 isoforms in the presence of an NADPH-generating system (0.7 μM ≤ IC_{50} ≤ 1.8 μM) was similar to that in untreated HLMs (0.9 μM ≤ IC_{50} ≤ 3.3 μM), suggesting that acetylshikonin is not a time-dependent inhibitor. Acetylshikonin (at 5 μM) inhibited all P450 isoforms tested by more than 60% (Fig. 1), whereas SKF-525A did not inhibit any of its target isoforms (CYP1A2, CYP2A6, CYP2C9, and CYP2E1) by more than 10%. In a previous study, acetylshikonin inhibited CYP2J2-mediated astemizole *O*-demethylase activity in a noncompetitive manner [10]. Therefore, acetylshikonin is speculated to inhibit noncompetitively other nine P450 enzymes.

In conclusion, we evaluated the inhibitory potential of acetylshikonin against P450 isoforms. Acetylshikonin strongly inhibited the activities of ten P450 isoforms in a NADPH-independent manner. Our results suggest that acetylshikonin can be used instead of SKF-525A or thelephoric acid as a novel non-specific P450 inhibitor in reaction phenotyping studies using HLMs.

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Footnotes

This study was supported by the Bio-Synergy Research Project [Grants NRF-2014M3A9C4066459] and the National Research Foundation of Korea, Ministry of Education [NRF-2016R1D1A1A09916782], Republic of Korea.

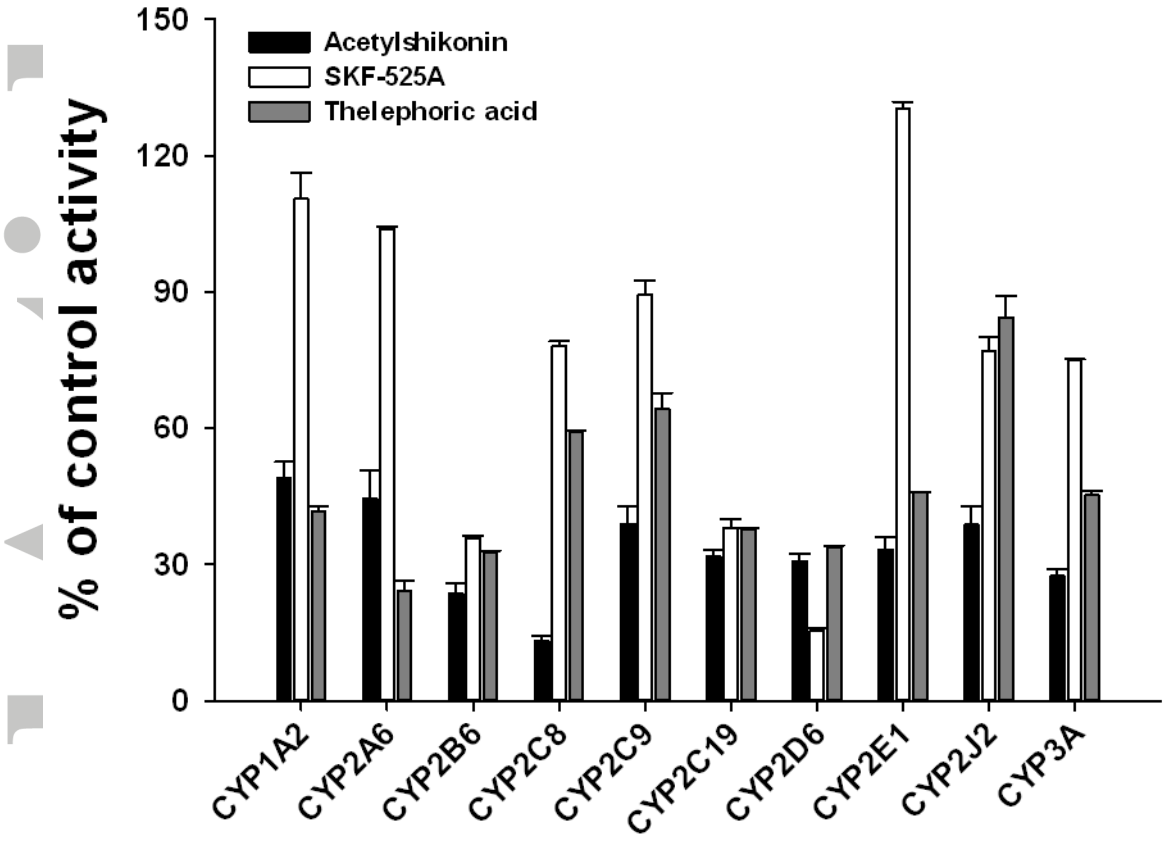

Fig. 1. Inhibitory effects of 5 μM acetylshikonin (Black), SKF-525A (White), and thelephoric acid (Gray) on the enzyme activities of ten P450 isoforms in pooled human liver microsomes (0.25 mg/ml, Xenotech H0630). The corresponding control activities of phenacetin *O*-deethylation, coumarin 7-hydroxylation, bupropion 4-hydroxylation, amodiaquine *N*-deethylation, tolbutamide 4-hydroxylation, omeprazole 5-hydroxylation, dextrorphan *O*-demethylation, chlorzoxazone 6-hydroxylation, astemizole *O*-demethylation, and midazolam 1'-hydroxylation were 188, 57.0, 4.4, 41.7, 120, 137.5, 53.2, 282.4, 26.5, and 298.5 pmol/min/mg protein, respectively. The data are shown as the average of triplicate determinations (n=3).

Table 1. SRM parameters for the major metabolites of ten cytochrome P450s' probe substrates used in all assays

P450 enzyme	Substrate	Concentration (μM)	Metabolite	Transition (*m/z*)	Collision energy (eV)
1A2	Phenacetin	100	Acetaminophen	152>110	25
2A6	Coumarin	5	7-Hydroxycoumarin	163>107	35
2B6	Bupropion	50	Hydroxybupropion	256>238	20
2C8	Amodiaquine	1	*N*-Desethylamodiaquine	328>283	17
2C9	Tolbutamide	100	4-Hydroxytolbutamide	287>89	60
2C19	Omeprazole	20	5-Hydroxyomeprazole	362>214	10
2D6	Dextromethorphan	5	Dextrorphan	258>157	35
2E1	Chlorzoxazone	50	6-Hydroxychlorzoxazone	184>120*	25
2J2	Astemizole	1	*O*-Desmethylastemizole	445>204	35
3A	Midazolam	5	1'-Hydroxymidazolam	342>203	25
	Testosterone	2	6β-Hydroxytestosterone	305>269	30

	Nifedipine	2	Dehydronifedipine	345>284	30
IS	Terfenadine	15 ng/mL	-	472>436	25

* negative ionization mode

Table 2. Inhibitory potential of acetylshikonin, shikonin, SKF-525A, and thelephoric acid against the activities of ten cytochrome P450 isoforms in human liver microsomes.

P450 enzyme	Marker activity	IC_{50} (μM)[a]				
		Acetylshikonin		Shikonin	SKF-525A	Thelephoric acid
		Reversible inhibition	Time-dependent inhibition	Reversible inhibition	Reversible inhibition	Time-dependent inhibition
1A2	Phenacetin *O*-deethylase	4.0	4.9	3.0	> 50	3.5
2A6	Coumarin 7-hydroxylase	3.8	4.6	2.5	> 50	2.0
2B6	Bupropion 4-hydroxylase	2.0	2.1	2.3	2.5	2.3
2C8	Amodiaquine *N*-deethylase	1.4	2.3	1.7	26.6	6.9
2C9	Tolbutamide 4-hydroxylase	3.3	3.4	2.8	24.4	11.4
2C19	Omeprazole 5-hydroxylase	2.5	3.9	2.2	3.6	2.8
2D6	Dextromethorphan *O*-demethylase	2.5	3.7	2.5	0.5	2.6
2E1	Chlorzoxazone 6-hydroxylase	2.7	4.6	4.5	> 50	4.0
2J2	Astemizole *O*-demethylase	3.3	5.1	5.2	> 50	3.4
3A	Midazolam 1'-hydroxylase	2.3	3.6	4.1	23.4	4.5

[a]Values are presented as average of triplicate determination (*n*=3).