Sophora flavescens (Ku-Shen) as a booster for antiretroviral therapy through cytochrome P450 3A4 inhibition

ZX Lin *, CT Che, SS Lee, RCY Chan, PSP Ip, JM Yang

KEY MESSAGES

1. *Sophora flavescens* extract dose-dependently inhibited human hepatic CYP3A4 activity. The ethyl acetate (EA) fraction containing prenylated flavonoids was most effective.
2. *Sophora* EA fraction slightly inhibited the efflux of indinavir from the basolateral to apical side in a Caco-2 cell monolayer model.
3. Unlike ritonavir, coadministration of *Sophora* EA fraction did not enhance plasma indinavir concentration.
4. Treatment with *Sophora* total extract significantly decreased plasma exposure of indinavir, associated with intestinal and hepatic P-gp induction, and upregulation of CYP3A activity.
5. Patients prescribed indinavir should be cautioned about intake of *S flavescens* extract or *Sophora*-derived products.

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[1] **ZX Lin**, [1,4] **CT Che**, [2,3] **SS Lee**, [2] **RCY Chan**, [1] **PSP Ip**, [1] **JM Yang**

The Chinese University of Hong Kong:
[1] *School of Chinese Medicine, School of Biomedical Science*
[2] *Department of Microbiology*
[3] *Stanley Ho Centre for Emerging Infectious Diseases*
[4] *Department of Medicinal Chemistry and Pharmacognosy, University of Illinois at Chicago, Chicago, USA*

* Principal applicant and corresponding author: linzx@cuhk.edu.hk

Introduction

Combination pharmacotherapy for the treatment of human immunodeficiency virus (HIV) is effective for viral load reduction and clinical success.[1] Nonetheless, HIV drugs are limited by their low bioavailability, limited central nervous system penetration, and undesirable side effects. One factor resulting in low bioavailability is the drug's susceptibility to metabolism by cytochrome P450 (CYP), in particular CYP3A4, a predominant CYP subfamily in human liver. Thus, concurrent use of CYP inhibitors (eg ritonavir) and a protease inhibitor (eg lopinavir) is the preferred highly active antiretroviral regimen.[1] The enzyme inhibitor acts as a 'bioavailability booster' by inhibiting CYP3A4 that metabolises the protease inhibitor drug molecules. We hypothesised that Chinese herbs that possess CYP3A4 inhibitory activity may potentiate (boost) the therapeutic effects of anti-HIV drugs. In our preliminary study, *Sophora flavescens* was the most effective in CYP3A4 inhibition among 50 herbs tested.[2] To establish biological evidence to support the use of *Sophora flavescens* as an adjuvant (booster) in the treatment of HIV, an *in vitro* CYP3A4 inhibition assay was established. Using a probe substrate method, the CYP3A4-inhibitory effects of *Sophora* extract and its ingredients were evaluated. This study also determined the effect of *Sophora* on P-gp in a Caco-2 cell monolayer model. In addition, an animal system was used to measure the *in vivo* effect of *S flavescens* on the oral pharmacokinetics of indinavir, a typical protease inhibitor in anti-HIV therapy. The possible involvement of P-gp and CYP3A in this interaction was examined by measuring their intestinal and hepatic mRNA/protein level and enzyme activity.

Methods

This study was conducted from November 2009 to October 2011. Dried root of *Sophora flavescens* was extracted by heating with 70% ethanol. The extract was filtered, concentrated by rotary evaporation, and freeze-dried to obtain a powder that contained approximately 11.1% oxymatrine. Sequential liquid extraction with ethyl acetate (EA) and butanol was performed to produce EA-, butanol- and water-soluble fractions. *Sophora* EA fraction was further separated using an open-pressure column embedded with Sephadex packing and preparative HPLC to produce nine compounds. The purity of isolated compounds was higher than 97% as determined by HPLC, and their identity was established by MS and NMR analysis.

CYP3A4 activity was determined by 6β-hydroxylation of testosterone. Briefly, a reaction mixture containing testosterone, potassium phosphate buffer, pooled human liver microsomes, an NADPH-generating system, and various concentrations of crude extracts, fractions or pure compounds was incubated at 37°C for 30

minutes. After sample extraction with EA, 6β-hydroxytestosterone was analysed using a HPLC method.

The effect of *Sophora* EA fraction on intestinal dual transport of indinavir was determined in Caco-2 cell monolayers grown in Transwell inserts for 21 days. Transfer buffer containing indinavir or indinavir plus *Sophora* EA fraction was added to the apical or basolateral chamber. Aliquots were taken from the basolateral or apical chamber at 20, 40, 60, 90, 120, 180 minutes.

Rats were orally gavaged with indinavir (40 mg/kg), and an hour later with 1.5% Tween 80 (vehicle), ritonavir (10 mg/kg), Marine capsule (45 mg/kg of oxymatrine equivalent), *Sophora* extract (0.158 or 0.63 g/kg), or *Sophora* EA fraction (82 mg/kg, 0.164 g/kg or 0.328 g/kg), respectively. All treatments were given twice a day for 7 days to reach a steady state and mimic the combined use of a protease inhibitor and ritonavir in humans. On day 8, blood samples were collected at 0.25, 0.5, 1, 1.5, 2, 3, 4, and 5 hours after dosing by orbital bleeding under anaesthetic with isoflurane. Plasma was obtained by centrifugation. After alkalisation and extraction with EA, indinavir was separated by a C_{18} column and determined by APCI-MS using midazolam as an internal standard (Fig).[3]

The amounts of mRNA that encoded CYP3A1, CYP3A2, and P-gp (mdr1a and mdr1b) in the intestine and liver were quantified by real-time PCR. Total RNA was extracted using chloroform and isopropanol, and then converted to cDNA using a high-capacity cDNA reverse transcription kit. Taqman assays using specific primers were performed for the quantification of mRNA in an ABI step-one real time PCR system. The relative mRNA levels were calculated by the $2^{-\Delta\Delta CT}$ method.[3]

Intestinal brush border membrane, intestinal mucosa homogenate and liver microsomes were used for protein analysis. Protein content of CYP3A and P-gp was quantified by western-blotting using specific antibodies.[3]

Hepatic CYP3A activity was measured by a luminescent assay (P450-Glo) according to the manufacturer's instructions (Promega, WI, USA).[3]

Results

Sophora flavescens extract caused dose-dependent inhibition of human hepatic CYP3A4 activity. The EA fraction was most effective in inhibiting CYP3A4, followed by butanol fraction and water fraction. Nine CYP3A4-inhibitory flavonoids were further isolated from the EA fraction (Table 1). Sophoraflavanone G was the most potent inhibitor, with an IC_{50} of 4.83 μg/mL. *Sophora* alkaloids did not show any significant effect. *Sophora* EA fraction also slightly inhibited the efflux of indinavir from the basolateral to apical side in the Caco-2 cell

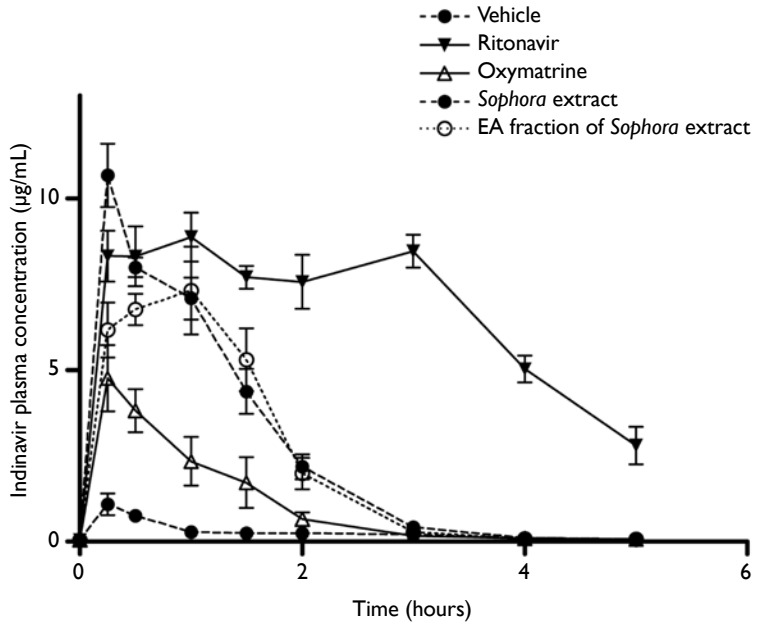

FIG. Plasma concentrations of indinavir: animals received oral administration of indinavir (40 mg/kg) together with 2% Tween 80 (vehicle), ritonavir (10 mg/kg), oxymatrine (45 mg/kg), 70% ethanol extract of *Sophora flavescens* (0.63 g/kg), or ethyl acetate (EA) fraction of the *Sophora* extract (82 mg/kg) twice a day for 7 days, respectively. Values are expressed as mean±SEM (n=6)

TABLE 1. Inhibitory effects of *Sophora* extract and fractions, the major alkaloid components, and the isolated flavonoids on CYP3A4 activity in pooled human liver microsomes

Parameter	Mean
Inhibitory effects (IC_{50}) [μg/mL]	
Total extract	51.2
Water fraction	>3000
Butanol fraction	47.2
Ethyl acetate fraction	5.2
Major alkaloid components (μM)	
Matrine	>500
Oxymatrine	>500
Sophoridine	>500
Sophocarpine	>500
Ketoconazole	0.126
Isolated flavonoids on CYP3A4 activity in pooled human liver microsomes (IC_{50}) [μg/mL]	
Sophoraflavanone G	4.8
Kushenol I/N	25.4
Kuraridin	10.5
2'-Methoxykurarinone	19.3
Leachianone A	5.5
Kushenol A	33.9
Kushenol X	8.43
Kushenol L	7.33
Xanthohumol	13.1

TABLE 2. *In vitro* and *in vivo* effects of *Sophora flavescens* and ritonavir (a positive booster) on CYP3A4 and P-gp, and the impact on plasma exposure of indinavir

Effect	*Sophora* extract	Ethyl acetate fraction	Oxymatrine	Ritonavir
In vitro CYP3A4 activity	Inhibition (IC_{50}=51.2 μg/mL)	Inhibition (IC_{50}=5.2 μg/mL)	No effect	Inhibition (previously reported)
P-gp mediated indinavir efflux	-	Slight inhibition	-	Inhibition (previously reported)
Plasma indinavir concentration	Decrease (up to 83% in AUC)	No effect	Decrease (up to 61% in AUC)	Increase (2.5-fold in AUC)
In vivo CYP3A activity	Upregulation	No effect	No effect	Inhibition
CYP3A expression	Induction	-	-	-
P-gp expression	Induction	-	-	-

monolayer model, indicating that it might increase intestinal absorption of indinavir.

In a rat model, co-administration (7 days) with ritonavir (a potent CYP3A4 inhibitor) significantly increased plasma exposure of indinavir, which was associated with inhibition of hepatic CYP3A activity (Table 2). Nonetheless, unlike ritonavir, *Sophora* EA fraction did not enhance plasma indinavir concentration. Co-administration with *Sophora* total extract markedly decreased plasma exposure of indinavir with a reduction in AUC and C_{max}, values. Oxymatrine was likely one of components responsible for this herb-drug interaction. The decreased indinavir exposure by *S flavescens* was, at least partly, attributed to the intestinal and hepatic P-gp induction, and upregulation of CYP3A activity.

Discussion

In the treatment of HIV infection, it is common practice to boost the protease inhibitor effect using a CYP3A4 inhibitor. The combined use of herbs and prescribed drugs can potentially attenuate drug efficacy and/or enhance toxicity. Prolonged co-administration of health products, such as St John's wort, leads to a decrease in plasma indinavir concentration.[4]

To determine the potential of *Sophora flavescens* as a booster for antiretroviral therapy, the present study established an experimental system that combined an *in vitro* CYP3A4 inhibition assay and *in vivo* pharmacokinetic study. *In vitro* findings implied the boosting potential of *Sophora* EA fraction, but *in vivo*, indinavir showed no enhancing effect on plasma levels in rats, and CYP3A activity was also not affected by treatment with an EA fraction.

Sophora EA fraction contains a mixture of prenylated flavonoids. Sophoraflavanone G was the most effective isolated compound in inhibiting CYP3A4, but it was still much less potent than ritonavir. Thus, the *in vivo* study used a high dose of EA fraction (maximum: 0.656 g/kg/day), which was comparable with ritonavir in terms of *in vitro* CYP3A4 inhibition. As such, the inability of EA fraction to boost indinavir is not a matter of dosage. The concentrations of *Sophora* flavonoids were quite low after oral administration of EA fraction. The discrepancy between *in vitro* and *in vivo* study in terms of EA fraction could be explained by the low oral bioavailability of the flavonoid components.

Co-administration of *Sophora* extract and indinavir (7 days) significantly decreased the plasma concentrations of indinavir. Moreover, co-administration with Marine capsule also decreased the plasma indinavir concentrations, with 61% reduction in $AUC_{0-\infty}$. Although there may be other ingredients present in the extract, oxymatrine is definitely a component in *S flavascens* and associated with the pharmacokinetic interaction with indinavir.

The bioavailability of indinavir is largely limited by efflux through intestinal P-gp and first-pass metabolism by CYP3A.[5] *Sophora* treatment could induce mRNA expression of CYP3A1 in the small intestine and liver. As *Sophora* alkaloids could induce CYP3A4 mRNA expression via activation of the pregane X receptor, the elevated CYP3A1 mRNA levels might also be caused by activation of this CYP3A-transcriptional regulator. Although CYP3A protein expression did not change, hepatic CYP3A activity increased after *Sophora* treatment. The up-regulation of CYP3A activity enhanced indinavir metabolism and hence facilitated drug elimination, which can, at least partially, explain the decreased plasma exposure of indinavir. Meanwhile, *Sophora* treatment dose-dependently increased mRNA level and protein expression of P-gp in the intestine and liver. Since intestinal and hepatic CYP3A and P-gp can be concurrently induced through the activation of common transcription factors—pregane X receptor and the constitutive androstane receptor, it is speculated that *Sophora* alkaloids may also induce P-gp. By extruding the substrate (indinavir)

during absorption or interplaying with CYP3A to further enhance intestinal first-pass metabolism, the induction of intestinal P-gp by *S flavascens* could lead to decreased plasma indinavir. It is evident that P-gp and CYP3A cannot fully account for the entire process of indinavir absorption and metabolism, and there must be other mediators that remain to be identified.

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