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The Potential of *Sutherlandia frutescens* for Herb-Drug Interaction

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ABSTRACT

In Africa, *Sutherlandia frutescens* is a popular medicinal herb widely consumed by people living with human immunodeficiency virus/AIDS. Concomitant use with antiretroviral drugs has generated concerns of herb-drug interaction (HDI). This study investigated the inhibitory effects of the crude extracts of *S. frutescens* on the major cytochrome P450 isozymes with the use of pooled human liver microsomes. Its effect on the metabolic clearance of midazolam using cryopreserved hepatocytes was also monitored. The potential of *S. frutescens* to inhibit human ATP-binding cassette transporters (P-gp and BCRP) and the human organic anion transporting polypeptide (OATP1B1 and OATP1B3) activity was assessed using cell lines overexpressing the transporter proteins. *S. frutescens* showed inhibitory potency for CYP1A2 (IC_{50} = 41.0 μg/ml), CYP2A6 (IC_{50} = 160 μg/ml), CYP2B6 (IC_{50} = 20.0 μg/ml), CYP2C8 (IC_{50} = 22.4 μg/ml), CYP2C9 (IC_{50} = 23.0 μg/ml), CYP2C19 (IC_{50} = 35.9 μg/ml), and CYP3A4/5 (IC_{50} = 17.5 μg/ml [with midazolam1′-hydroxylation]; IC_{50} = 28.3 μg/ml [with testosterone 6β-hydroxylation]). Time-dependent (irreversible) inhibition by *S. frutescens* was observed for CYP3A4/5 (K_I = 296 μg/ml, k_{inact} = 0.063 min^{-1}) under the conditions of this study. *S. frutescens* also delays the production of midazolam metabolites in the hepatocytes, decreasing its clearance by 40%. Furthermore, *S. frutescens* inhibited P-gp (IC_{50} = 324.8 μg/ml), OATP1B1 (IC_{50} = 10.4 μg/ml), and OATP1B3 (IC_{50} = 6.6 μg/ml). The result indicates the potential for HDI between *S. frutescens* and the substrates of the affected enzymes, if sufficient in vivo concentration of the extract is attained.

Introduction

Although the use of medicinal herbs, a principal component of traditional medicine, predates the emergence of human immunodeficiency virus (HIV)/AIDS, herbal products as immune boosters and for symptomatic management of AIDS have been popularized in Africa (Namuddu et al., 2011). The high burden of HIV/AIDS has attracted various policy designs to accommodate a holistic approach to management. Although the crux of the various HIV/AIDS policies has been the introduction of free antiretroviral drugs (ARVs), it is believed that the past controversy over HIV/AIDS and the official promotion of herbal medicine for its management have contributed to the persistently high rate of consumption of medicinal herbs by persons living with HIV/AIDS in South Africa, despite the availability and/or consumption of ARVs (Morris, 2002; Mills et al., 2005; Malangu, 2007). As a popular and culturally attached practice, an estimated 80–85% of Africans consult traditional health practitioners (THP) for the management of various physical ailments and social disharmony (UNAIDS, 2006). For the majority of this group of individuals, THPs are the first point of call for health services.

Studies have shown that the majority of South Africans consult THPs, especially for the treatment of sexually transmitted infections, such as gonorrhea, syphilis, and assumed HIV/AIDS (Peltzer, 2001, 2003). Although some patients with HIV/AIDS consume medicinal herbs ab initio, it has been reported that a number of them resort to herbal medicine in response to perceived adverse reactions to ARVs (Peltzer and Mngqundaniso, 2008). The number of patients with HIV/AIDS who consume herbal medicine alone or concomitantly with ARVs is often underestimated. In a cross-sectional study performed by Peltzer and colleagues (2008), up to 90% of respondents who were taking herbal therapies for HIV infection did not disclose this to their health care providers. This, therefore, calls for more research to establish the therapeutic benefit and safety of such practices.

Widely known as cancer bush because of its traditional use in the management of cancer, *Sutherlandia frutescens* (Sutherlandia) is one of the most reputable herbal remedies consumed among persons living with HIV/AIDS in South Africa, to boost immunity and enhance general well-being (Gericke, 2001). It is referred to variously in local languages as insiswa, kankerbos, kankerbossie, lerumolamadi, mukakana, phetola, and unwele (Gericke et al., 2001). Its decoction is used in the treatment of open wounds, fever, chicken pox,

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ABBREVIATIONS: ARV, antiretroviral drug; BCRP, breast cancer resistance protein; FBS, fetal bovine serum; HDI, herb-drug interaction; HIV, human immunodeficiency virus; HLM, human liver microsomes; K_I, inhibition constant (time-dependent inhibition); K_{inact}, maximal rate of enzyme inactivation (time-dependent inhibition); Ko143, (3*S*,6*S*,12a*S*)-1,2,3,4,6,7,12,12a-octahydro-9-methoxy-6-(2-methylpropyl)-1,4-dioxo-pyrazino[1′,2′:1,6]pyrido[3,4-*b*]indole-3-propanoic acid 1,1-dimethylethyl ester; LC-MS, liquid chromatography–mass spectrometry; LLC-PK1, porcine kidney cell line; MDCKII, Madin-Darby canine kidney II cells; OATP, organic anion transport polypeptide; P450, cytochrome P450; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine-2; THP, traditional health practitioner.

TABLE 1

Probe substrates, microsomal protein concentrations, and incubation times

Enzyme	Probe Substrate	Microsome Concentration	Probe Substrate Concentration	Literature K_m Value	Internal Standard
		mg protein/ml		μM	
CYP1A2	Phenacetin	0.2	10	9.0[1], 14[2], 31[3], 54[4]	[$^2H_4$]Acetaminophen
CYP2A6	Coumarin	0.2	2.5	0.5	[$^{13}C_6$]7-Hydroxy coumarin
CYP2B6	Bupropion	0.1	25	76[4], 89[5],130[6]	[$^2H_6$]Hydroxybupropion
CYP2C8	Paclitaxel	0.2	10	4.0[7], 15[8]	Warfarin
CYP2C9	Diclofenac	0.1	5	3.4[9], 9.0[10]	[$^{13}C_6$]4′-Hydroxy diclofenac
CYP2C19	*S*-Mephenytoin	0.5	30	51[12], 42[13], 31[14]	[$^2H_3$]4′-Hydroxy mephenytoin
CYP2D6	Bufuralol	0.2	5	13[15], 44[16]	[$^2H_9$]1′-Hydroxybufuralol
CYP2E1	Chlorzoxazone	0.5	10	22-49[17]	[$^2H_3$]4′-Hydroxy mephenytoin
CYP3A4/5	Midazolam	0.1	1	3.3, 2.5-5.6[18]	[$^2H_4$]1′-Hydroxymidazolam
CYP3A4/5	Testosterone	0.2	30	50-60[19], 51[20]	[$^2H_3$]6β-Hydroxy testosterone

[1]Tassaneeyakul et al., 1993.
[2]Brøsen et al., 1993.
[3]Venkatakrishnan et al., 1998a.
[4]Rodrigues et al., 1997.
[5]Faucette et al., 2000.
[6]Hesse et al., 2000.
[7]Li et al., 2003.
[8]Rahman et al., 1994.
[9]Cresteil et al., 1994.
[10]Transon et al., 1996.
[11]Bort et al., 1999.
[12]Coller et al., 1999.
[13]Venkatakrishnan et al., 1998b.
[14]Schmider et al., 1996.
[15]Boobis et al., 1985.
[16]Yamazaki et al., 1994.
[17]Peter et al., 1990.
[18]Ghosal et al., 1996.
[19]Draper et al., 1998.
[20]Kenworthy et al., 2001.

gastrointestinal cramps, rheumatism, heartburn, hemorrhoids, diabetes, inflammation, and eye infections among indigenous South Africans (Van Wyk and Albrecht, 2008). It has been investigated for many of the anecdotal claims. Many of such studies have suggested its potential for beneficial effects in HIV/AIDS (Harnett et al., 2005), cancer (Stander et al., 2007, 2009; Skerman et al., 2011), diabetes (Chadwick et al., 2007; Mackenzie et al., 2009, 2012), inflammation (Ojewole, 2004), microbial infection (Katerere and Eloff, 2005), stress (Prevoo et al., 2004), and convulsion (Ojewole, 2008). Commercial twice-daily Sutherlandia capsules containing 300 mg of powdered dried leaves are also available in the herbal medicines shops in South Africa (Van Wyk and Albrecht, 2008).

Studies have identified various phytochemical constituents of Sutherlandia to include L-canavanine, GABA, and D-pinitol. A recent study by the South Africa Medical Research Council has suggested that Sutherlandia is free of obvious toxicities (Seier et al., 2002). Products and formulations of Sutherlandia are in different stages of clinical trials (clinicaltrials.gov). A search on the US patents showed various patented products containing Sutherlandia as synergistic HIV/AIDS and/or immune disease remedy or supportive therapy (Rangel and Angel, 2009).

Although the consumption of Sutherlandia among patients with HIV/AIDS, most of whom are receiving ARV therapy, is popular, the safety of such concomitant administration with orthodox medicine has not been demonstrated. This is necessary to ascertain the presence or absence of the potential for herb-drug interaction (HDI). HDI may be potentially deleterious in persons living with HIV/AIDS because of the high number of medications used in ARV therapy and other comorbidities. There is no information available in the literature to address this concern. A recent study on the in vitro effects of Sutherlandia on Caco-2 cell line suggests its potential to influence the absorption of atanavir (Müller et al., 2012). Therefore, the aim of the current study was to investigate the potential of the crude extracts of Sutherlandia to inhibit nine major cytochrome P450 (P450) isozymes with use of human liver microsomes (HLMs), two efflux and two uptake proteins using cell lines expressing the transporter proteins, and CYP3A4-mediated midazolam clearance in human hepatocytes.

Materials and Methods

Plant Materials

Fresh leaves of Sutherlandia were sourced from two South African THPs who identified the plant as unwele in local Xhosa language. The collected samples were identified with the help of experts in the Compton Herbarium, South African National Biodiversity Institute, Cape Town, and Voucher specimens were prepared and housed at the Division of Pharmacology, University of Stellenbosch. Information on the mode of use, dose, and specific HIV/AIDS-related indications were obtained and documented through semi-structured interview.

Ethical approval was obtained from the University of Stellenbosch Health Research Ethics Committee.

Chemical Compounds

The necessary chemical compounds were obtained as indicated: 1′-hydroxybufuralol maleate, 1′-hydroxymidazolam, 4′-hydroxymephenytoin, 6β-hydroxytestosterone, bufuralol hydrochloride, and midazolam from Ultrafine Chemicals (Pty) Ltd (Manchester, UK); 6-hydroxychlorzoxazone, 7-hydroxycoumarin, acetaminophen, bupropion, chlorzoxazone, coumarin, furafylline, NADPH, estradiol-17β-D-glucuronide (sodium salt), paclitaxel, tranylcypromine, troleandomycin (TAO), warfarin, rifamycin, thioconazole, digoxin, cyclosporine A, and testosterone from Sigma-Aldrich (Pty) Ltd. (St. Louis, MO); dimethyl sulfoxide (DMSO), di-potassium hydrogen phosphate (K_2HPO_4), acetonitrile, formic acid and methanol from Merck Chemicals (Pty) Ltd (Darmstadt, Germany); [$^{13}C_6$]4′-hydroxydiclofenac, [$^{13}C_6$]7-hydroxycoumarin, [$^2H_3$]4′-hydroxymephenytoin,

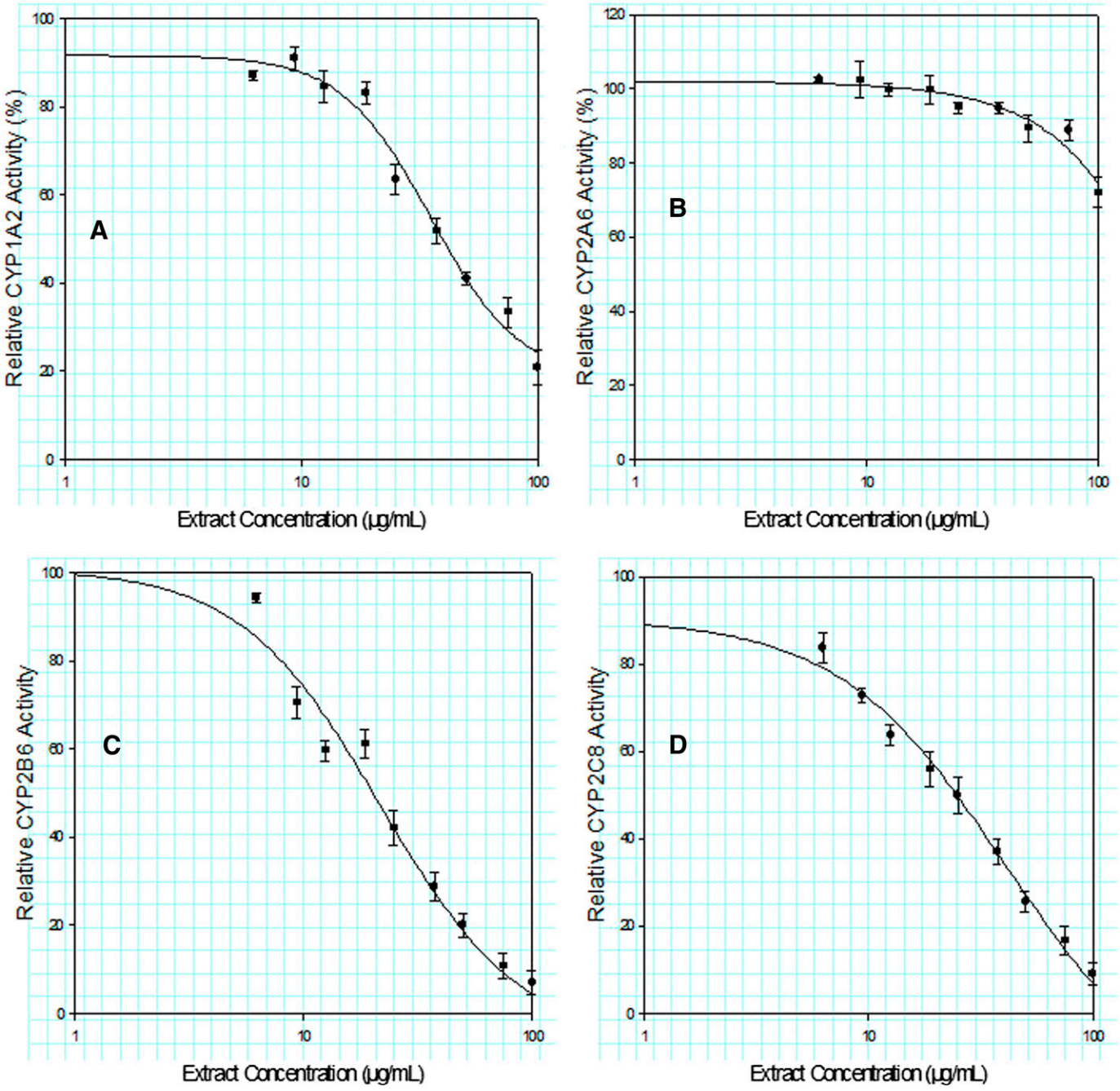

Fig. 1. Effect of Sutherlandia on CYP1A2-mediated phenacetin deethylation (A), CYP2A6-mediated coumarin 7-hydroxylation (B), CYP2B6-mediated bupropion hydroxylation (C), and CYP2C8-mediated paclitaxel 6α-hydroxylation in pooled human liver microsomes (D).

diclofenac, and paroxetine from Novartis Pharma AG (Basel, Switzerland); hydroxybupropion, [$^2H_6$]hydroxybupropion, and [$^2H_9$]1′-hydroxybufuralol from BD Biosciences (Pty) Ltd (San Jose, CA); 6α-hydroxypaclitaxel from Gentest BD Biosciences (Woburn, MA); [$^2H_3$]6β-hydroxytestosterone, [$^2H_4$]1′-hydroxymidazolam from Cerilliant Chemicals (Pty) Ltd (Texas); phenacetin, magnesium chloride hexahydrate, ticlopidine hydrochloride, and potassium dihydrogen phosphate (KH_2PO_4) from Fluka Chemicals (Pty) Ltd (Buchs, Switzerland); 4′-hydroxydiclofenac from Calbiochem (Pty) Ltd (La Jolla, CA); (3*S*,6*S*,12*aS*)-1,2,3,4,6,7,12,12*a*-octahydro-9-methoxy-6-(2-methylpropyl)-1,4-dioxopyrazino[1′,2′:1,6]pyrido[3,4-*b*]indole-3-propanoic acid 1,1-dimethylethyl ester (Ko143) from Tocris Bioscience (Pty) Ltd (Ellisville, MO); 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine-2 (PhIP) and its radiolabeled from (1.85 MBq/nmol, radiochemical purity > 99%) from Toronto Research Chemicals (Pty) Ltd (North York, Toronto, ON, Canada); radiolabeled [^{3}H]digoxin (1.103 MBq/nmol, radiochemical purity > 97%) and [^{3}H] estradiol-17β-D-glucuronide (1.72 MBq/nmol, radiochemical purity > 97%); from PerkinElmer Radiochemicals (Pty) Ltd (Waltham, MA); and zosuquidar trihydrochloride from Chembiotek (Pty) Ltd (Kolkata, India).

Assay Enzymes and Cells

Pooled mixed-gender HLMs prepared from 50 individual donors with total P450 and cytochrome b_5 content of 290 and 790 pmol/mg protein, respectively, was obtained from Gentest BD Biosciences (Woburn, MA). The Catalytic activities of enzymes were provided by the manufacturer. Pooled, mixed-gender cryopreserved hepatocytes prepared from 20 donors were obtained from Celsis In Vitro Technologies (Pty) Ltd (Baltimore, MD),

TABLE 2

The potency of inhibitory effects of Sutherlandia on P450 expressed in IC_{50} values

P450 Enzyme	Probe Reaction	IC_{50} Value
		μg/ml
CYP1A2	Phenacetin *O*-deethylation	41.0 ± 1.2
CYP2A6	Coumarin 7-hydroxylation	160 ± 2.1
CYP2B6	Bupropion hydroxylation	20.1 ± 1.7
CYP2C8	Paclitaxel 6α-hydroxylation	22.4 ± 1.3
CYP2C9	Diclofenac 4′-hydroxylation	23.0 ± 1.5
CYP2C19	*S*-Mephenytoin 4′-hydroxylation	35.9 ± 1.0
CYP2D6	Bufuralol 1′-hydroxylation	n.i.o.
CYP2E1	Chlorzoxazone 6-hydroxylation	n.i.o.
CYP3A4/5	Midazolam 1′-hydroxylation	17.5 ± 1.3
CYP3A4/5	Testosterone 6β-hydroxylation	28.3 ± 1.3

n.i.o., no inhibition observed.

stored in a liquid nitrogen tank (until use), and thawed according to supplier instructions before use.

Extraction of Plant Material

The air-dried and powdered leaves of Sutherlandia were extracted with water/methanol (40/60, 50 mg/ml) in a round bottom flask. After the initial constant stirring for 2 hours, the mixture was allowed to extract for 24 hours, decanted, and centrifuged (20,000 rpm, 5 minutes). The supernatant was filtered (0.45μl; Whatman International LTD, Maidstone, UK) and dried using a vacuum rotary evaporator and freeze drying. The dried extract of Sutherlandia was reconstituted in methanol and stored at −20°C for further use.

Incubation in HLM

Thawed HLMs were diluted with potassium phosphate buffer (50 mM; pH = 7.4) and incubated in 96-well plate format. Graded concentrations of Sutherlandia were prepared in methanol, such that the addition of 1 μl to 200μl incubation mixture yielded a final extract concentration of 0.39, 0.78, 1.56, 3.13, 6.25, 12.5, 25, 50, and 100μg/ml. Appropriate P450 substrate (Table 1) was added, and the mixture was preincubated at 37°C for 10 minutes with use of an IS89 96-well plate incubator (Wesbart, Leimuiden, The Netherlands). Metabolic reactions were initiated by adding NADPH (1 mM) and magnesium chloride (5 mM) solution and were terminated after 20 minutes through the addition of formic acid (10 μl; 50%). All incubations were performed in duplicate. Control incubations contained P450-specific inhibitor and incubations without inhibitors. The final methanol concentration in the incubations was 0.5% (v/v). Probe substrate concentrations used were less or equal to published K_m values.

Incubations in HLMs for Assessment of Time-Dependent Inhibition

For the assessment of time-dependent inhibition of CYP3A4 activity, all incubation steps were performed in a 96-well plate format at 37°C with use of a liquid handling workstation epMotion 5075VAC (Eppendorf, Hamburg, Germany). Sutherlandia (3.13, 6.25, 12.5, 25, 50, and 100 μg/ml) and positive control (5 μM troleandomycin) were added to phosphate buffer. After the addition of HLM (0.5 mg/ml protein) and 10 minute thermal equilibration, the reactions were initiated by adding NADPH (1 mM). The samples were preincubated for 0, 4, 8, 16, 32, and 48 minutes, respectively. All incubations were done in duplicate. After the preincubation period, 20 μl of the microsomal incubations (corresponding to 0.025 mg/ml protein) were diluted 20-fold with buffer containing the probe substrate and NADPH (to minimize competitive inhibition by the test compounds) and incubated with midazolam (20 μM) for 6 minutes at 37°C. Potassium phosphate buffer (1 mM) was used as negative control. Reactions were terminated by the addition of 20 μl of formic acid (50%).

Quantitative analysis of Metabolites

Liquid chromatography–mass spectrometry (LC-MS) methods were developed to simultaneously analyze the metabolites of bupropion, paclitaxel, diclofenac, and bufuralol (group A); phenacetin, coumarin, midazolam, and testosterone (group B); and *S*-mephenytoin with chlorzoxazone (group C) with the appropriate internal standard (Table 1). Total separation and elution of the analytes were achieved within 10 minutes retention time, using the Luna Phenyl-Hexyl (3 μm, inner dimensions 50 × 1 mm; Phenomenex, Torrance, CA) column (30°C), in a dual mobile phase of water and acetonitrile (each containing 1% v/v formic acid), with a gradient (group A) and isocratic (group B and C; 70:30) flow set at 60 μl/min.

Before chromatographic analysis, samples were pooled according to the groups and subjected to solid-phase extraction using an OASIS HLB 96-well plate 30 μm (30 mg) Elution plate (Waters, Milford, CT). This was performed by sequential washing with 1 ml each of water and water-methanol (95/5; v/v), followed by two-time elution with 1 ml of methanol. The elutes were dried using the 96-well Micro-DS96 evaporator (Porvair Sciences Ltd., Shepperton, UK) at 37°C and reconstituted in 100 μl of 10% acetonitrile containing 0.1% formic acid for LC-MS analysis. Enzyme activity was measured in terms of the production of the P450-specific metabolite. Relative activity (100%) was defined in terms of metabolite production in the absence of inhibitor. The enzyme inhibition parameter (IC_{50}) was calculated by using the kinetic equation for sigmoid curves (Eq. 1), where x = concentration, y = relative enzyme activity, and s = slope factor.

$$y = \frac{100\%}{1 + \left(\frac{x}{IC_{50}}\right)^{s}} \quad (1)$$

Calculation of Kinetic Parameters for Time-Dependent Inhibition

The enzyme activity in the presence of each inhibitor concentration was determined relative to activity at time, $t = 0$. The resulting data pairs of relative enzyme activities and preincubation times were fitted with an Excel template in a least square approach to the exponential function (Eq. 2), where y = relative activity, x = preincubation time, a = optimal starting value for relative activity, and b = optimal negative rate of inactivation for a given inhibitor concentration.

$$y = a \cdot e^{-b.x} \quad (2)$$

The rate of CYP3A4 inactivation in the absence of test substance was subtracted from all other inactivation rates. The resulting values, along with the corresponding inhibitor concentrations, were used in a second least square fit to calculate kinetic parameters by linear regression to the Michaelis-Menten–like equation (Eq. 3), where y = inactivation rate constant, x = inhibitor concentration, K_I = inhibitor concentration that produces half-maximal inhibition; and k_{inact} = maximal inactivation rate constant using the Enzyme Kinetic SigmaPlot, version 12.1.

$$y = \frac{k_{inact} \times x}{K_I + x} \quad (3)$$

The Effects of Sutherlandia on the Clearance of Midazolam in Human Hepatocytes

Incubation Procedure. The cryopreserved hepatocytes were thawed, and the cells were suspended in HepatoZYME buffer. The viability of the suspended hepatocytes was determined at the beginning and end of incubation (in the presence and absence of Sutherlandia) with use of a Guava EasyCyte Mini system using the ViaCount assay according to the instructions of the supplier (Guava Technologies, Hayward, CA). After cell counting, the cell density was adjusted with HepatoZYME to ~1.3 million viable cells/ml. The incubation procedure involved the coincubation of 1 μM midazolam and 100 μg/ml Sutherlandia in 1 ml of hepatocyte mixture at 37°C under a humidified atmosphere of 95% air and 5% CO_2 in a Heraeus incubator/Cytoperm. Aliquots of the incubation mixtures were taken for LC-MS analysis at 0, 1, 2, 4, and 6 hours of incubation. The metabolic reactions were stopped by the addition of 2 volumes of acetonitrile containing 0.3 μM *d*4-hydroxymidazolam as internal standard. Samples were kept at −20°C until analysis. Control incubation to monitor

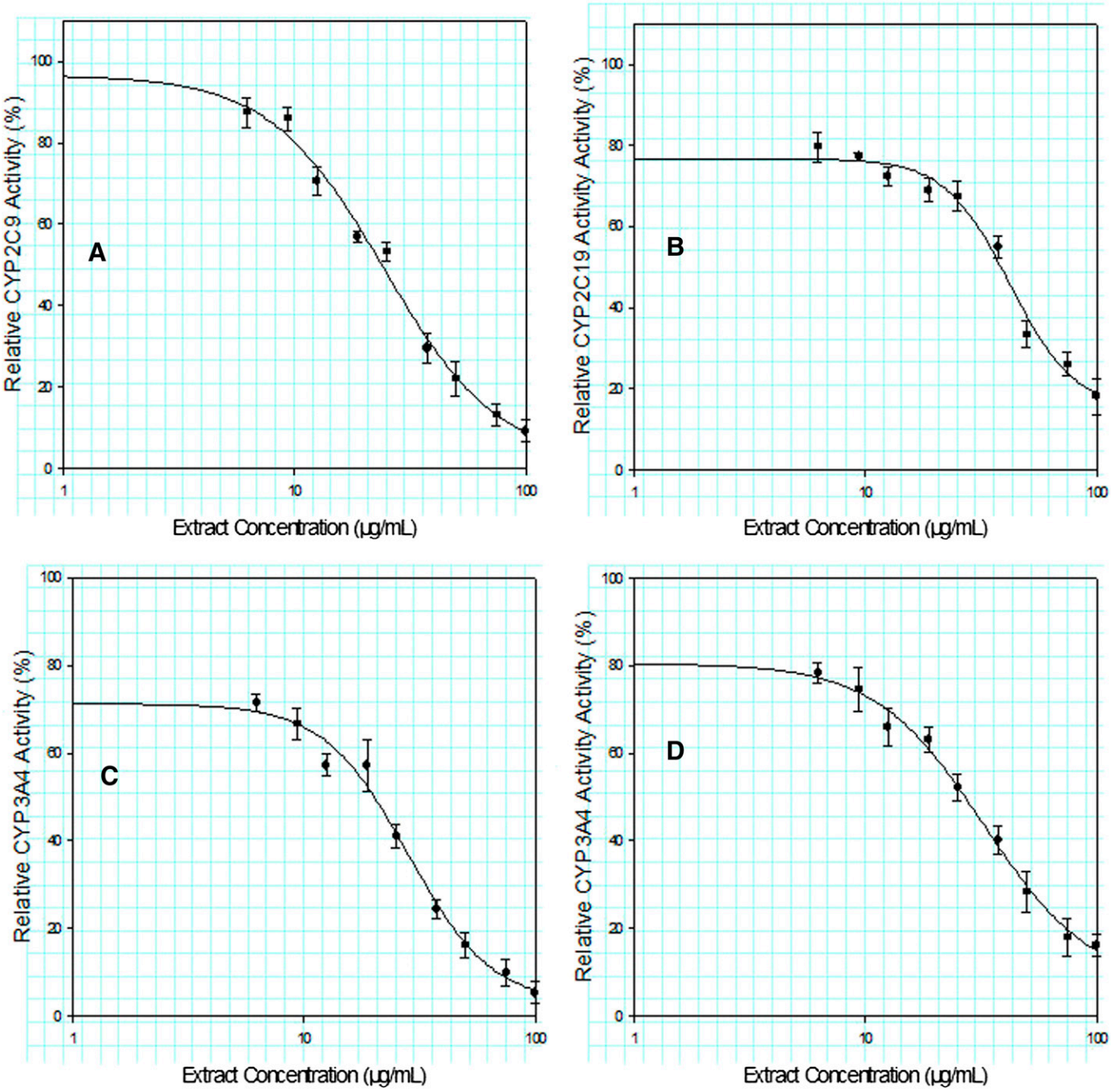

Fig. 2. Effect of Sutherlandia on CYP2C9-mediated diclofenac 4′-hydroxylation (A), CYP2C19-mediated *S*-mephenytoin 4′-hydroxylation (B), CYP3A4/5-mediated midazolam 1′-hydroxylation (C), and CYP3A4/5-mediated testosterone 6β-hydroxylation in pooled human liver microsomes (D).

the stability of midazolam in cell-free HepatoZYME was performed. The rate of metabolism of midazolam in the presence and absence of Sutherlandia was compared.

Clearance calculations. Initial half-life ($t_{1/2}$) and elimination rate constants (lambda = $\ln 2/t_{1/2}$) of MDZ in hepatocyte incubates were calculated by log-linear regression of MDZ concentrations profiled against time with use of data from the sampling points of the 1 μM MDZ incubates.

The intrinsic clearance in vitro ($CL_{int,in\ vitro}$) was calculated from lambda and the cell density in the respective incubation (number of viable hepatocytes per milliliter at time zero) and scaled up to the intrinsic clearance in vivo ($CL_{int,in\ vivo}$) with use of the human liver mass (25.7 g/kg body mass) and the hepatocellularity (number of hepatocytes per gram of liver = 99 million cells/g liver):

$$CL_{int,in\ vitro} = \text{Lambda} / \text{cell density}$$

$$CL_{int,in\ vivo} = CL_{int,in\ vitro} \times \text{liver mass} \times \text{heptocellularity}$$

From CL_{int} in vivo and the hepatic blood flow [Q (20.7 ml/min)/kg body mass], the hepatic metabolic blood clearance ($CL_{h,b}$) was predicted using the well-stirred model as follows:

$$CL_{h,b} = (CL_{int,in\ vivo} \times Q) / (CL_{int,in\ vivo} + Q)$$

No corrections were made for the free fractions in vitro or in vivo (i.e., they were assumed to be identical) (Davies and Morris, 1993).

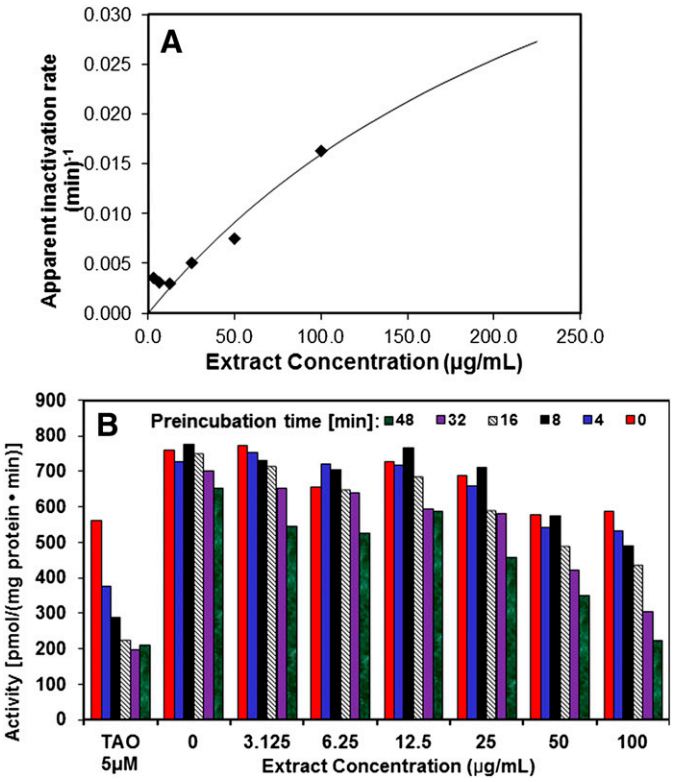

Fig. 3. Hyperbolic inactivation plot (A) and the effect of preincubation time (B), Sutherlandia concentration, and the positive control troleandomycin (TAO) on the enzymatic activity of CYP3A4/5.

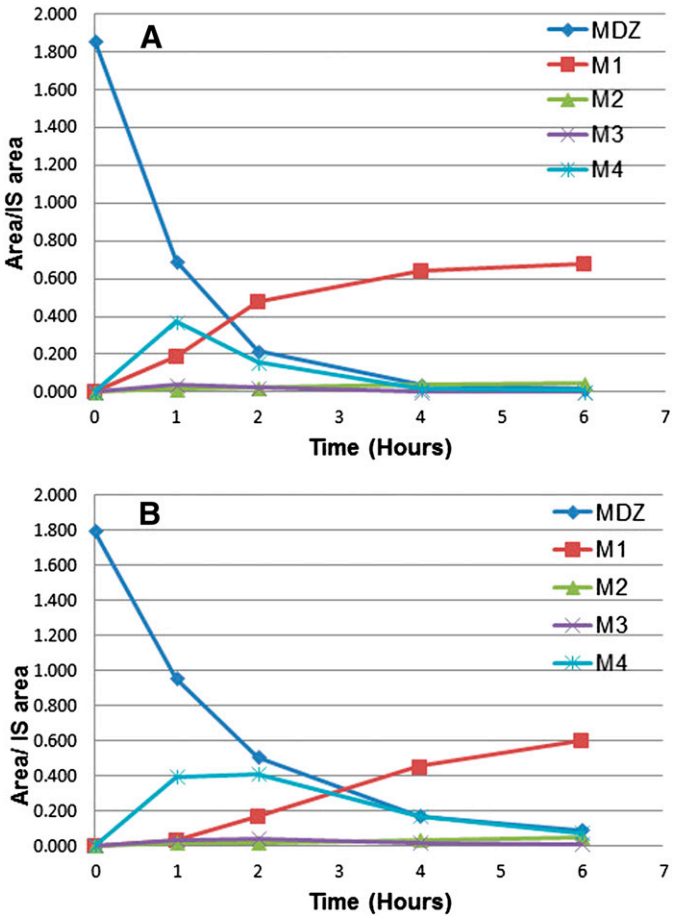

Fig. 4. Time course over 6 hours showing the disappearance of midazolam and the formation of metabolites M1, M2, M3, and M4 in human hepatocyte incubations in the absence (A) and presence (B) of Sutherlandia.

Determination of the Effects of Sutherlandia on P-gp and BCRP

Sutherlandia was soluble up to a final concentration of 700 μg/ml in P-gp uptake buffer and 200 μg/ml in BCRP uptake buffer. LLC-PK1 cells stably transfected with human P-gp were grown and maintained in Medium 199 supplemented with 10% fetal-bovine serum (FBS), 50 ng/μl gentamycin, and 100 ng/μl hygromycin B at 37°C under an atmosphere of 5% CO_2. MDCKII cells stably transfected with human BCRP were grown and maintained in DMEM supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin at 37°C under an atmosphere of 5% CO_2. Cells (~0.6 × 10^5 cells per well for LLC-PK1-P-gp and MDCKII-BCRP transfectants) were seeded into Falcon clear-bottom 96-well plates (Becton Dickinson) with 200 μl of culture medium, and the assay was performed after 24 hours. Graded concentrations of Sutherlandia were prepared in HBSS containing 12.5 mM HEPES adjusted with HCl to pH of 7.4 (for P-gp) and OPTI-MEM containing 12.5 mM HEPES adjusted to pH of 7.8 (for BCRP). The assay procedure was begun by the aspiration and replacing the culture medium with preincubation uptake buffer system containing Sutherlandia or the specific inhibitors as positive control (10 μM cyclosporine A for P-gp and 1 μM Ko143 for BCRP). Plates were subsequently incubated at 37°C for 10 minutes. Transporter-mediated uptake was initiated by the addition of the radiolabeled probe substrates (1 μM digoxin for P-gp and 1 μM PhIP for BCRP). The uptake reaction was terminated after 40 minutes by removing the incubation solution. The cells were then washed twice with ice-cold phosphate-buffered saline buffer, and monolayer integrity was assessed optically. This was followed by the dissolution of the cells by the addition on 0.2 N NaOH (200 μl per well) and incubation for 20 minutes at 37°C. Dissolved cells were then transferred to scintillation vials containing scintillation cocktail, and uptake substrates were measured using a scintillation counter. All uptake studies were performed in triplicate in an incubator without shaking. Methanol/DMSO was always below 1% v/v of the total volume, and all solutions used in cell growth were maintained at the appropriate pH.

Determination of the Effects of Sutherlandia on OATP1B1 and 1B3

Human embryonic kidney 293 cells stably transfected with human OATP1B1 were grown and maintained in DMEM supplemented with 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin, and 50 ng/μl hygromycin B at 37°C under an atmosphere of 5% CO_2. Human embryonic kidney 293 cells stably transfected with human OATP1B3 were grown and maintained in DMEM supplemented with 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin, and 0.8 mg/ml G418 at 37°C under an atmosphere of 5% CO_2. Cells (~0.2 × 10^6 cells per well) for overexpressing OATP1B1 and OATP1B3 were seeded in precoated (poly-L-lysine, poly-L-ornithine, 0.1 mg/ml) clear-bottom 96-well plates (Corning Product No. 734-1795; Corning Incorporated Life Sciences (Pty) Ltd, Tewksbury, MA) with 200 μl of culture medium. Graded concentrations of Sutherlandia were prepared in HBSS containing 12.5 mM HEPES adjusted with HCl to pH of 7.4. Uptake studies, performed 72 hours after seeding, was started by aspirating and replacing the culture medium with the final incubation buffer system containing Sutherlandia or the positive control inhibitor (20 μM rifamycin and 10 μM atorvastatin) The uptake reactions were terminated after 5 minutes by removing the incubation solution. Subsequently, the wells were washed twice with ice-cold phosphate-buffered saline buffer, and monolayer integrity was assessed optically. Radioactive samples were analyzed using liquid scintillation counting in a similar procedure as described above. All uptake studies were performed in triplicate in an incubator without shaking.

Drug Uptake Clearance Calculations

Probe substrate uptake clearance (nl/min/mg protein) by the stably transfected cells expressing the transporter proteins was determined from the specific amount of radiolabeled probe substrate inside the cells divided by the concentration in the incubation medium and normalized to the incubation time and the mean protein concentration measured in test wells.

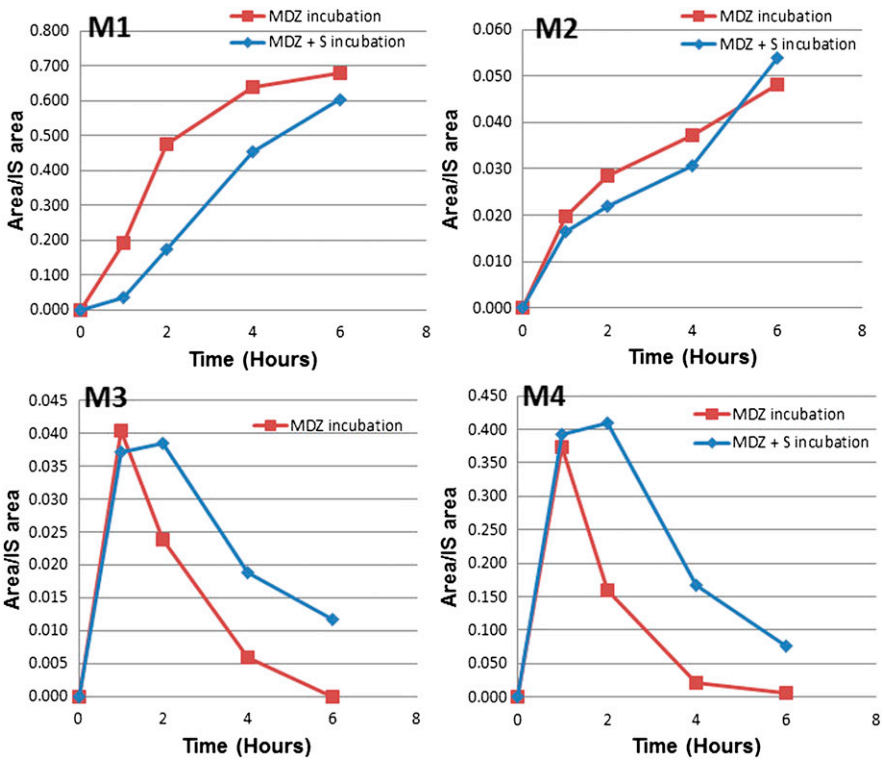

Fig. 5. Time course showing the influence of Sutherlandia on the formation of metabolites M1, M2, M3, and M4.

Absolute transporter uptake data were converted into relative inhibition values by defining membrane permeability of the probe substrate in the absence of inhibitor as 0% inhibition while the positive control exerted 100% transporter inhibition. Relative inhibition (%) was profiled against inhibitor concentration, and the IC_{50} was calculated using the nonlinear-regression method using the Enzyme Kinetic SigmaPlot, version 12.1.

The IC_{50} values were calculated using the following equation:

$$y = y0 + \frac{a \cdot I^n}{IC_{50}{}^n + I^n} \quad (4)$$

where, n is the slope factor (Hill coefficient), $y0$ is the relative baseline inhibition, and a is the maximal transporter inhibition (%).

Results

Influence of Sutherlandia on P450s

Sutherlandia showed a concentration-dependent inhibition of CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, and CYP3A4/5. The extract concentration profiled against relative enzyme activity is shown in Fig. 1, and Table 2 shows the IC_{50} values. Little or no competitive inhibition of CYP2D6 and CYP2E1 was observed at Sutherlandia concentrations up to 100 μg/ml.

Sutherlandia-Induced Time-Dependent Inhibition of CYP3A4

Time-dependent (irreversible) inhibition of CYP3A4/5 by graded concentration of Sutherlandia was observed with a $K_I = 296$ μg/ml and $k_{inact} = 0.063$ min^{-1} under the conditions of this study. The influence of preincubation time, Sutherlandia concentration, and the positive control troleandomycin on the enzymatic activity of CYP3A4/5 is shown in Figs. 2 and 3.

Influence of Sutherlandia on Midazolam Clearance in Human Hepatocytes

During the incubation with MDZ, the viability of the hepatocytes (percentage viable cells relative to total cells) decreased from 78.3% at time zero to 42.7% without test inhibitor and 45.6% with Sutherlandia. Thus, no cell degradation was attributable to the presence of Sutherlandia.

Midazolam was also found to be stable in the HepatoZYME media and the absence of hepatocytes as determined after 6 hours of

TABLE 3

The intrinsic clearance of midazolam in hepatocytes in the presence and absence of Sutherlandia

Hepatocytes	MDZ	MDZ + Sutherlandia
Half-life (h)[a]	0.72	1.18
CL_{int} (μl/min/million cells)[b]	16	10
$CL_{int,scaled}$ (ml/min/kg)[c]	40.98	24.98
$CL_{h,b}$ (hepatic blood clearance) (ml/min/kg body weight)[d,e]	14	11

[a] Half-lives were evaluated by linear extrapolation.
[b] $(\ln 2/T_{1/2})$/mio viable cells × 1000.
[c] $(CL_{int}/1000)$ × (cells/g liver) × (gliver/kg body weight).
[d] $CL_h = (Q_h \times CL_{int,scaled})/(Qh + CL_{int,scaled})$, where Q_h is hepatic blood flow (well-stirred liver model).
[e] Q_h (ml/min/kg body weight): 55 (rat); 30.9 (dog); 43.6 (monkey); and 20.7 (human).

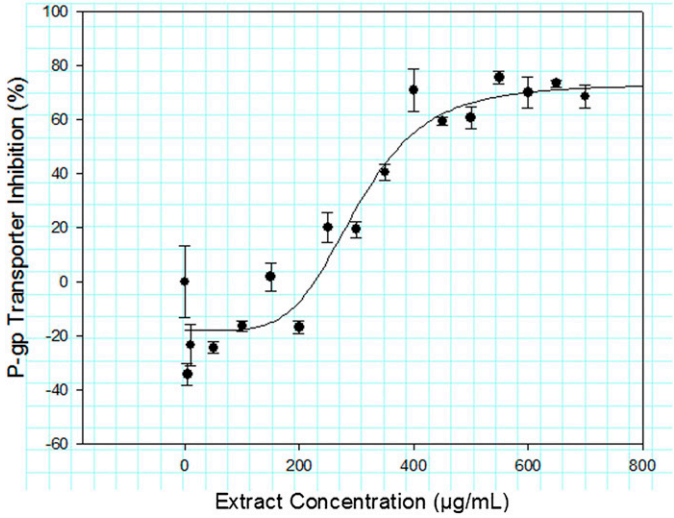

Fig. 6. Effect of varying Sutherlandia on digoxin uptake by P-gp expressing LLC-PK1 cells. The line represents the best fit of the data to the Eq. 4 (R^2 = 0.88), which are means of triplicate determinations, and the bars represent the mean ± SD.

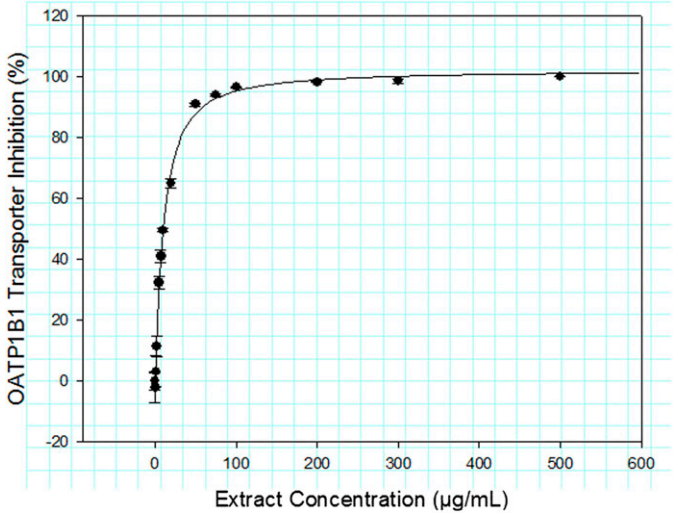

Fig. 7. Effect of varying Sutherlandia on estradiol-17β-D-glucuronide uptake by OATP1B1 expressing human embryonic kidney (HEK) 293 cells. The line represents the best fit of the data to the Eq. 4 (R^2 = 0.99), which are means of triplicate determinations, and the bars represent the mean ± SD.

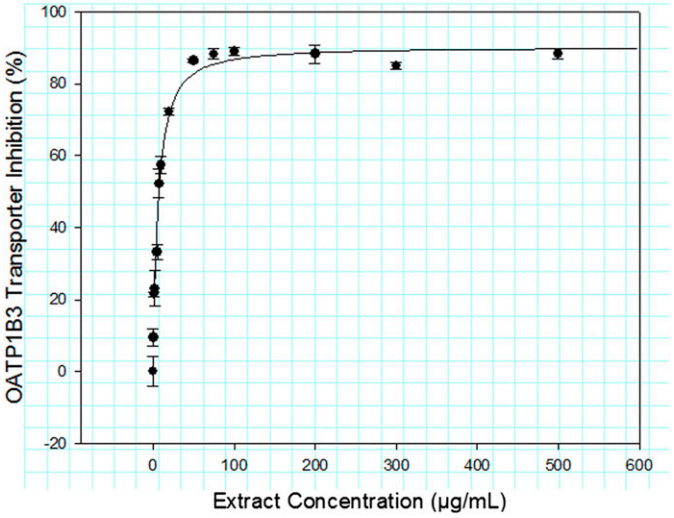

Fig. 8. Effect of varying Sutherlandia on estradiol-17β-D-glucuronide uptake by OATP1B3 expressing HEK293 cells. The line represents the best fit of the data to the Eq. 4 (R^2 = 0.98), which are means of triplicate determinations, and the bars represent the mean ± SD.

incubation. Thus, degradation in the presence of hepatocytes is attributable to the cellular activities.

Influence of Sutherlandia on the Formation of Midazolam Metabolites

After the analysis of an aliquot of human hepatocyte incubate with midazolam (t = 6 hours) by LC-MS/MS, four metabolites were detected and were assigned as M1, M2, M3, and M4. With use of a combination of accurate mass measurement, elemental composition, and MS/MS analysis, the four metabolites were identified as a combination of oxygenation and glucuronidation (M1), direct glucuronidation (M2), and a single oxygenation of MDZ (M3 and M4). These metabolites are consistent with previously published data on the metabolism of midazolam (Kronbach et al., 1989). The influence of Sutherlandia on the formation of the metabolites is shown in Figs. 4 and 5.

Intrinsic Metabolic Clearance from Hepatocytes

The intrinsic metabolic clearance of midazolam in the presence and absence of Sutherlandia was measured in human hepatocytes (Table 3). Extrapolation to hepatic blood clearance was performed using the well-stirred liver model. Sutherlandia reduced the clearance of midazolam by 40%.

TABLE 4

Summary of results

Transporter	Probe Substrate	IC_{50} Value[a,b]	K_i[c]	Maximum Inhibition[b,d]
		µg/ml		*%*
P-gp	Digoxin	324.8	324.3	69.5 ± 7.7
BCRP	PhIP	Not observed[e]	Not applicable	Not observed[e]
OATP1B1	Estradiol-17β-D-glucuronide	10.4 ± 0.6	8.2	101.4 ± 0.6
OATP1B3	Estradiol-17β-D-glucuronide	6.6 ± 0.9	5.6	85.3 ± 5.4

[a] Sutherlandia concentration estimated to inhibit transporter activity by 50%.
[b] Data used for curve fitting are mean ± S.D. (n = 3).
[c] Calculated with $K_i = IC_{50}/(1 + S/K_m)$, where S is the substrate concentration of the probe substrate and K_m is the transporter affinity of the probe substrate according to Michaelis-Menten.
[d] Maximal observed inhibition with respect to positive control.
[e] No inhibition of BCRP was observed up to the maximum Sutherlandia concentration of 200 μg/ml.

TABLE 5

Interpretation of the in vitro findings

Variable	Value
Part used in traditional practice	Leaves
Usual human dose (single; mg)	300
Extraction yield (% w/w)	28.5
Estimated extract per dose (mg)	85.5
Putative GIT concentration (μg/ml)[a]	340
In vitro IC_{50} range (μg/ml)	6.6–324
Potential for clinically significant HDI[b]	Yes

[a] Estimated gastrointestinal (GIT) fluid = 250 ml, with the assumption that the 60% methanol-soluble extracts are extracted in the GIT.

[b] This refers to HDI in the GIT with possible effects on drug absorption.

Influence of Sutherlandia on Transport Proteins

Sutherlandia exerted inhibitory activity on the transport activities of P-gp, OATP1B1, and OATP1B3. At the concentration investigated (up to 200 μg/ml), Sutherlandia exerted no inhibitory activity on the activity of BCRP. The effect of increasing concentration of Sutherlandia on the activity of the transport proteins is shown in Figs. 6, 7, and 8. An overall summary is provided in Table 4.

Discussion

With the increasing popularity of traditional medicine in Africa and the attention that it is receiving from government and policy makers, scientific validation of herbal safety is paramount. For Sutherlandia, an anti-HIV regimen with a high propensity for concurrent administration with ARVs, the potential for clinically significant HDI would be of concern. In this study, Sutherlandia was obtained from THPs. This is considered to be a representative source reflecting actual practice of patients' patronage. Other than a few open shops where traditional medical products are obtained, the majority of South Africans, for cultural and traditional reasons, consult THPs for herbal medications.

On the basis of the information obtained from the THPs, Sutherlandia is often taken as aqueous decoction. Commercial Sutherlandia is consumed whole as dried and ground leaves in capsules or suspension, which exposes the body to all of its constituents. Thus, the choice of 60% methanol for extraction (which produced 28.5% w/w extraction yield) was necessary to extract most phytoconstituents, both hydrophilic and hydrophobic composition.

The use of HLM is well accepted to assess the potential of new chemical entities and drug candidates for drug interactions. This technology has also been widely used to assess the HDI potentials. The results from such studies provide an indication for clinically significant interactions. Results from in vitro metabolic studies have been extrapolated for in vivo correlation with a high degree of predictability (Umehara and Camenisch, 2012). The usual dose of commercial Sutherlandia capsules is 300 mg (of powdered dried leaves), taken twice daily. With just the consideration of a single dose of 300 mg, a gastrointestinal concentration 50 times the IC_{50} values for most of the tested enzymes and transport proteins is achievable (Table 5). This could have deleterious effects on intestinal P-gp and P450, potentially affecting drug absorption. Viral protease inhibitors, calcium channel receptor blockers, various chemotherapeutic agents, for example, undergo significant presystemic intestinal metabolism. The inhibition of the intestinal P450 and P-gp may alter the pharmacokinetic profile of such drugs, with the potential of exceeding safety margins. This will be more important for drugs with a narrow therapeutic window.

This inhibitory influence will be more pronounced on repeated Sutherlandia consumption, as shown with the time-dependent inhibition of P450. In reality, herbal remedies are consumed repetitively over a long period. As shown from this study, a continuous suppression of enzymatic activity may occur. This may precipitate drug accumulation and toxicity.

There is no information on the extent of absorption after Sutherlandia administration. However, with claims of its systemic activity on immune boosting, cancer treatment among others, its phytochemicals are expected to be absorbed for efficacy. Because OATP1B1 and OATP1B3 are located at the sinusoidal membrane of the liver, the inhibitory effects on the OTA1B1 and OTP1B3 will be important if there is absorption. In addition to this, absorbed phytoconstituents of Sutherlandia may inhibit the activity of P450s and transport proteins in the liver and other organs, with consequent alteration of the pharmacokinetic profile of coadministered drugs.

Compared with microsomes, results from in vitro use of cryopreserved hepatocytes are closer to in vivo conditions because of the complete expression of the cell matrix and the presence of other enzymes (Fasinu et al., 2012). Thus, the reduction of midazolam clearance by 40% by Sutherlandia, as shown with the in vitro–in vivo correlation is significant and may reflect a stronger likelihood in vivo. With the ongoing policy making focusing on the integration of traditional and orthodox medicine in Africa, it is important to understand the potential for HDI and the necessary caution required in concomitant herb-drug administration.

However, a few factors may limit the conclusions derivable from this study. Sutherlandia was obtained from traditional healers, just as their clients do. The phytochemical composition of herbs is known to vary depending on the place and time of harvesting. In addition, methanol and water will extract most phytochemicals. This may not be the same with intestinal fluids. The extracts that may be systemically available in significant quantities are also not known. Although in vitro HDI studies provide the indication for in vivo relevance, in vivo human studies are the ultimate proof of clinically significant HDI.

In conclusion, Sutherlandia may inhibit the metabolic clearance of comedications metabolized by CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP3A4/5, P-gp, OATP1B1, and OATP1B3 if sufficient in vivo concentration is achieved. CYP3A4/5 inhibition may be amplified by administration of multiple doses. Sutherlandia is not expected to inhibit human CYP2D6, CYP2E1, or BCRP. Thus, there is the potential for pharmacokinetic interaction if Sutherlandia is coadministered with the substrates of these enzymes and transport proteins. Extreme caution should therefore be taken, especially in using Sutherlandia in HIV-infected patients treated with ARVs.

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Authorship Contributions

Participated in research design: Fasinu, Gutmann, Schiller, James, Bouic, Rosenkranz.

Conducted experiments: Fasinu.

Contributed new reagents or analytical tools: Gutmann, Schiller, James, Rosenkranz.

Performed data analysis: Fasinu, Gutmann, Schiller, James.

Wrote or contributed to the writing of the manuscript: Fasinu, Gutmann, Schiller, James, Bouic, Rosenkranz.

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