The potential of Sutherlandia frutescens for herb-drug interaction

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The influence of Sutherlandia on drug metabolism

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List of Abbreviations

- **BCRP:** Breast cancer resistance protein
- **CYP:** Cytochrome P450
- **HLM:** Human liver microsomes
- **HDI:** Herb-drug interaction
- **KI:** Inhibition constant (time-dependent inhibition)
- **Kinact:** Maximal rate of enzyme inactivation (time-dependent inhibition)
- **Ko143:** (3S,6S,12aS)-1,2,3,4,6,7,12,12a-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
- **LLC-PK1:** Porcine kidney cell line
- **MDCKII:** Madin-Darby canine kidney II cells
- **OATP:** Organic anion transport polypeptide
- **PhIP:** 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine-2
**Abstract**

*Sutherlandia frutescens* (ST) is a popular medicinal herb widely consumed in Africa by people living with HIV/AIDS. Concomitant use with antiretroviral drugs has generated concerns of herb-drug interaction. This study investigated the inhibitory effects of the crude extracts of ST on the major cytochrome P450 isozymes employing pooled human liver microsomes. Its effect on the metabolic clearance of midazolam using cryopreserved hepatocytes was also monitored. The potential of ST to inhibit human ATP-binding cassette (ABC) 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. ST showed inhibitory potency for CYP1A2 (*IC*<sub>50</sub> = 41.0 µg/mL), CYP2A6 (*IC*<sub>50</sub> = 160 µg/mL), CYP2B6 (*IC*<sub>50</sub> = 20.0 µg/mL), CYP2C8 (*IC*<sub>50</sub> = 22.4 µg/mL), CYP2C9 (*IC*<sub>50</sub> = 23.0 µg/mL), CYP2C19 (*IC*<sub>50</sub> = 35.9 µg/mL) and CYP3A4/5 (*IC*<sub>50</sub> = 17.5 µg/mL [with midazolam 1'-hydroxylation]; *IC*<sub>50</sub> = 28.3 µg/mL [with testosterone 6β-hydroxylation]). Time-dependent (irreversible) inhibition by ST was observed for CYP3A4/5 (*KI* = 296 µg/mL, *kinact* = 0.063 min<sup>-1</sup>) under the conditions of this study. ST also delays the production of midazolam metabolites in the hepatocytes, decreasing its clearance by 40%. Further, ST inhibited P-gp (*IC*<sub>50</sub> = 324.8 µg/mL); OATP1B1 (*IC*<sub>50</sub> = 10.4 µg/mL, and of OATP1B3 (*IC*<sub>50</sub> = 6.6 µg/mL). The result indicates the potential for HDI between ST and the substrates of the affected enzymes, if sufficient *in vivo* concentration of ST is attained.
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Introduction

Although the use of medicinal herb, a principal component of traditional medicine, predates the emergence of 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 manage the scourge. While the crux of the various HIV/AIDS policies has been the introduction of free antiretroviral drugs (ARV), 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 people living with HIV/AIDS in South Africa despite the availability and/or consumption of ARVs (Moris, 2002; Mills et al., 2004; Malangu, 2005). 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 (STIs) such as gonorrhoea, syphilis, and assumed HIV/AIDS (Peltzer, 2001; 2003). While some HIV/AIDS patients 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 HIV/AIDS patients who consume herbal medicine alone or concomitantly with ARVs is often under-estimated. In a cross-sectional study carried out by Peltzer and colleagues (2008), up to 90% of respondents who were taking herbal therapies for HIV 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 people living with HIV/AIDS in South Africa to boost immunity and enhance general well-being (Gericke, 2002). It is referred to variously in local languages as insiswa, kankerbos, kankerbossie, lerumo-lamadi, mukakana, phetola and unwele (Gericke et al., 2001). Its decoction is employed in the treatment of open wounds, fever, chicken pox, gastrointestinal cramps, rheumatism, heartburn, haemorrhoids, 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), diabetis (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 300mg 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).

While the consumption of Sutherlandia among HIV/AIDS patients, most of who are on ARV, 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 people living with HIV/AIDS because of the high number of medications used in ARV therapy and other co-morbidity. 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). The aim of the current study was therefore to investigate the potential of the crude extracts of *Sutherlandia* to inhibit major nine cytochrome *P450* (CYP) isozymes employing human liver microsomes (HLM), 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, dosage 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, cyclosporin A and testosterone from Sigma-Aldrich (Pty) Ltd (St. Louis, USA); dimethylsulfoxide (DMSO), di-potassium hydrogen phosphate (K₂HPO₄), acetonitrile, formic acid and methanol from Merck Chemicals (Pty) Ltd (Darmstadt, Germany); [¹³C₆] 4’ hydroxydiclofenac, [¹³C₆] 7 hydroxycoumarin, [²H₃] 4’-hydroxymephenytoin, diclofenac and paroxetine from Novartis Pharma AG (Basel, Switzerland); hydroxybupropion, [³H₆] hydroxybupropion and [³H₆] 1’-hydroxybufuralol from BD Biosciences (Pty) Ltd (San Jose, USA); 6α-hydroxypaclitaxel from Gentest BD Biosciences (Woburn, USA); [²H₃] 6β-hydroxytestosterone, [²H₄] 1’-hydroxymidazolam from Cerilliant Chemicals (Pty) Ltd (Texas, USA); phenacetin, magnesium chloride hexahydrate, ticlopidine hydrochloride and
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potassium dihydrogen phosphate (KH$_2$PO$_4$) from Fluka Chemicals (Pty) Ltd (Buchs, Switzerland); 4'-hydroxydiclofenac from Calbiochem (Pty) Ltd (La Jolla, USA), (3S,6S,12aS)-1,2,3,4,6,7,12,12a-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, MI); 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine-2 (PhilP) and its radio-labeled from (1.85 MBq/nmol, radiochemical purity >99%) from Toronto Research Chemicals (Pty) Ltd (North York, Canada); radio-labeled [³H]Digoxin (1.103 MBq/nmol, radiochemical purity >97%) and [³H]estradiol-17β-D-glucuronide (1.72 MBq/nmol, radiochemical purity >97% from Perkin Elmer Radiochemicals (Pty) Ltd (Waltham, MA, USA); zosuquidar trihydrochloride (LY335979) from Chembiotek (Pty) Ltd (Kolkata, India).

**Assay enzymes and cells**

Pooled mixed gender HLM prepared from 50 individual donors with total CYP and cytochrome b$_5$ content of 290 pmol/mg protein and 790 pmol/mg protein respectively was obtained from Gentest BD Biosciences (Woburn, USA). 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, USA), 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, 50mg/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, 5min). The supernatant was filtered (0.45µL; Whatman International LTD, Maidstone, England) and dried using a vacuum rotary evaporator and freeze drying. The dried extract of Sutherlandia,
henceforth referred to as ST was reconstituted in methanol and stored at -20°C for further use.

**Incubation in HLM**

Thawed HLM were diluted with potassium phosphate buffer (50mM; pH = 7.4) and incubated in 96-well plate format. Graded concentrations of ST were prepared in methanol such that the addition of 1µL 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 respectively. Appropriate CYP substrate (table 1) was added and the mixture pre-incubated at 37°C for 10 minutes using an IS89 96-well plate incubator (Wesbart, Leimuiden, The Netherlands). Metabolic reactions were initiated by adding NADPH (1mM) and magnesium chloride (5mM) solution, and terminated after 20 minutes through the addition of formic acid (10 µL; 50%). All incubations were performed in duplicate. Control incubations contain CYP-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 Km values.

**Incubations in human liver microsomes 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 using a liquid handling workstation epMotion 5075VAC (Eppendorf, Hamburg, Germany). ST (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 (0.5mg/mL protein) and 10 min thermal equilibration, the reactions were initiated by adding NADPH (1mM). The samples were preincubated for 0, 4, 8, 16, 32 and 48 min 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 (in order to minimize competitive inhibition by the test compounds) and incubated with midazolam (20µM) for 6 minutes at
37°C. Potassium phosphate buffer (1mM) was used as negative control. Reactions were terminated by the addition of 20 µL formic acid (50%).

**Quantitative analysis of metabolites**

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 x 1 mm (Phenomenex, Torrance, USA)) column (30°C), in a dual mobile phase of water and acetonitrile (each containing 1% /v, formic acid), with a gradient (group A) and isocratic (group B and C, 70:30) flow set at 60µL/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, USA). 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 employing 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 CYP-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 employing the kinetic equation for sigmoid curves (Equation 1) where $x =$ concentration; $y =$ relative enzyme activity; and $s =$ slope factor.

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

*Equation 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 (Equation 2) where \( y = \) relative activity; \( x = \) preincubation time; \( a = \) optimal starting value for relative activity; \( b = \) optimal negative rate of inactivation for a given inhibitor concentration

\[
y = a \cdot e^{-b \cdot x}
\]

Equation 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 (Equation 3) where \( y = \) inactivation rate constant; \( x = \) inhibitor concentration; \( K_I = \) inhibitor concentration that produces half maximal inhibition; \( k_{\text{inact}} = \) maximal inactivation rate constant employing the Enzyme Kinetic SigmaPlot Version 12.1.

\[
y = \frac{k_{\text{inact}} \cdot x}{K_I + x}
\]

Equation 3

The effects of ST 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 ST by a Guava EasyCyte Mini system using the ViaCount assay according to the instructions of the supplier (Guava Technologies). After
cell counting, the cell density was adjusted with HepatoZYME to approximately 1.3 million viable cells/mL. The incubation procedure involved the coincubation of 1 μM midazolam and 100 μg/mL ST in 1mL of hepatocyte mixture at 37°C under a humidified atmosphere of 95% air and 5% CO2 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 d4-hydroxymidazolam as internal standard. Samples were kept at -20°C until analysis. Control incubation to monitor the stability of midazolam in cell-free HepatoZYME was performed. The rate of metabolism of midazolam in the presence and absence of ST was compared.

**Clearance calculations**

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

The intrinsic clearance *in vitro* ($CL_{in \text{ vitro}}$) was calculated from lambda and the cell density in the respective incubation (number of viable hepatocytes per mL at time zero) and scaled up to the intrinsic clearance *in vivo* ($CL_{in \text{ vivo}}$) using 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):

$$CL_{in \text{ vitro}} = \frac{\lambda}{\text{cell density}}$$

$$CL_{in \text{ vivo}} = CL_{in \text{ vitro}} \times \text{ liver mass } \times \text{ hepatocellularity}$$

From $CL_{in \text{ vivo}}$ 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:

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

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).
**Determination of the effects of Sutherlandia on P-gp and BCRP**

ST 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% FBS, 50 ng/µL Gentamycin and 100 ng/µL Hygromycin B at 37°C under an atmosphere of 5% CO₂. 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% CO₂. Cells (~0.6 x 10⁵ 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 h. Graded concentrations of ST were prepared in HBSS containing 12.5 mM HEPES adjusted with HCL to pH 7.4 (for P-gp) and OPTIMEM containing 12.5mM HEPES adjusted to pH7.8 (for BCRP). The assay procedure was begun by the aspiration and replacing the culture medium with pre-incubation uptake buffer system containing ST or the specific inhibitors as positive control (10 µM cyclosporin A for P-gp and 1µM Ko143 for BCRP). Plates were subsequently incubated at 37°C for 10 min. Transporter-mediated uptake was initiated by the addition of the radiolabeled probe substrates (1µM digoxin for P-gp and 1µM 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 PBS buffer and monolayer integrity was assessed optically. This is 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**

HEK293 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% CO₂. HEK293 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% CO₂. Cells (~0.2 x 10⁶ cells per well) for overexpressing OATP1B1 and OATP1B3 were seeded into pre-coated (poly-L-lysine, poly-L-ornitin, 0.1 mg/mL) clear bottom 96 well plates (Corning Product No 734-1795) with 200 µL of culture medium. Graded concentrations of ST were prepared in HBSS containing 12.5 mM HEPES adjusted with HCL to pH 7.4. Uptake studies, performed 72 h after seeding was started by aspirating and replacing the culture medium with the final incubation buffer system containing ST 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 PBS buffer and monolayer integrity was assessed optically. Radioactive samples were analyzed by 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.
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 $IC_{50}$ calculated using the nonlinear-regression method employing the Enzyme Kinetic SigmaPlot Version 12.1.

The $IC_{50}$ values (inhibitor concentration that causes 50% inhibition of the maximal drug effect) were calculated using the following equation:

$$y = y_0 + \frac{a \cdot I^n}{IC50^n + I^n}$$  \hspace{1cm} \text{Equation 4}

Where, $n$ is the slope factor (Hill coefficient), $y_0$ is the relative baseline inhibition and $a$ is the maximal transporter inhibition (%).
Results

Influence of ST on CYPs

ST 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 Figure 1 and Table 2 shows the $IC_{50}$ values. Little or no competitive inhibition of CYP2D6 and CYP2E1 was observed at ST concentrations up to 100 µg/mL.

Sutherlandia-induced time-dependent inhibition of CYP3A4

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

Influence of Sutherlandia on midazolam clearance in human hepatocytes

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

Midazolam was also found to be stable in the HepatoZYME media and the absence of hepatocytes as determined after 6 hours of incubation. Thus, degradation in the presence of hepatocytes is attributable to the cellular activities.

Influence of Sutherlandia on the formation of midazolam metabolites

Following the analysis of an aliquot of human hepatocyte incubate with midazolam ($t = 6h$) by LC-MS/MS, four metabolites were detected and were assigned as M1, M2, M3 and M4. Using a combination of accurate mass measurement, elemental composition and MS/MS analysis, the 4 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 ST on the formation of the metabolites is illustrated in Figures 4 and 5.

**Intrinsic metabolic clearance from hepatocytes**

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

**Influence of Sutherlandia on transport proteins**

ST exerted inhibitory activity on the transport activities of P-gp, OATP1B1 and OATP1B3. Within the concentration investigated (up to 200 µg/mL), ST exerted no inhibitory activity on the activity of BCRP. The effect of increasing concentration of ST on the activity of the transport proteins is illustrated in Figures 6-9. An overall summary is provided in Table 4.
Discussion

With the increasing popularity of traditional medicine in Africa and the attention 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 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 THP for herbal medications.

Based on the information obtained from the THPs, Sutherlandia is often taken as aqueous decoction. Commercial Sutherlandia are 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% extraction yield) was necessary in order to extract most phyto-constituents, 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 300mg (of powdered dried leaves), taken twice daily. With just the consideration of a single dose of 300mg, a GIT concentration 50 times the IC50 values for most of the tested enzymes and transport proteins is achievable (Table 5). This could have deleterious effects on intestinal P-gp and CYP, potentially affecting drug absorption. Viral protease inhibitors, calcium channel receptor blockers, various chemotherapeutic agents, for example, undergo significant pre-systemic intestinal metabolism. The inhibition of the intestinal CYP 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 narrow therapeutic window.

This inhibitory influence will be more pronounced on repeated Sutherlandia consumption as shown with the time-dependent inhibition of CYP. In reality, herbal remedies are consumed repetitively over a long period of time. 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. Since 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 phyto-constituents of Sutherlandia may inhibit the activity of CYPs and transport proteins in the liver and other organs with consequent alteration of the pharmacokinetic profile of co-administered drugs.

Compared to microsomes, results from \textit{in vitro} use of cryopreserved hepatocytes are closer to \textit{in vivo} conditions due to 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 \textit{in vitro-in vivo} correlation is significant and may reflect a stronger likelihood \textit{in vivo}.

With the on-going policy making focussing 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.

While *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 co-medications 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 co-administered with the substrates of these enzymes and transport proteins. Extreme caution should therefore be taken especially in using Sutherlandia in HIV patients treated with antiretrovirals.
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Acknowledgement

Ms Nomsisi Stefans and Nombuso Keme for providing the herbal materials; the Drug-Drug Interaction (DDI) and Biotransformation (BT) Laboratories of the Drug Metabolism and Pharmacokinetics (DMPK), Novartis Institute for Biomedical Research (NIBR) for providing reagents and laboratory support; as well as the Diversity and Inclusion Department of NIBR, Basel, Switzerland.
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Authorship Contributions

Participated in research design – James, Gutmann, Schiller, Bouic, Rosenkranz, Fasinu

Conducted experiments - Fasinu

Contributed new reagents or analytical tools – Gutmann, Rosenkranz, Schiller and James

Performed data analysis – Fasinu, Schiller, Gutmann, James

Wrote or contributed to the writing of the manuscript – Fasinu, Gutmann, Schiller, James, Bouic, Rosenkranz
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References


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Bufuralol hydroxylation by cytochrome P450 2D6 and 1A2 enzymes in human liver
Footnotes

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Figure Legends

Figure 1: Effect of ST on a) CYP1A2-mediated phenacetin deethylation b) CYP2A6-mediated coumarin 7-hydroxylation c) CYP2B6-mediated bupropion hydroxylation and d) CYP2C8-mediated paclitaxel 6alpha-hydroxylation in pooled human liver microsomes.

Figure 2: Effect of ST on a) CYP2C9-mediated diclofenac 4'-hydroxylation b) CYP2C19-mediated S-mephenytoin 4'-hydroxylation c) CYP3A4/5-mediated midazolam 1'-hydroxylation and d) CYP3A4/5-mediated testosterone 6beta-hydroxylation in pooled human liver microsomes.

Figure 3: a) Hyperbolic inactivation plot and b) the effect of preincubation time, ST concentration and the positive control troleandomycin (TAO) on the enzymatic activity of CYP3A4/5

Figure 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 a) in the absence and b) presence of ST.

Figure 5: Time course showing the influence of ST on the formation of metabolites M1, M2, M3 and M4.

Figure 6: Effect of varying ST on Digoxin uptake by P-gp expressing LLC-PK1 cells The line represents the “best fit” of the data to the equation 4 (R2 = 0.88) which are averages of triplicate determinations and the bars represent the standard deviations.

Figure 7: Effect of varying ST on Estradiol-17β-D-glucuronide uptake by OATP1B1 expressing HEK293 cells. The line represents the “best fit” of the data to the equation 4 (R2 = 0.99) which are averages of triplicate determinations and the bars represent the standard deviations.

Figure 8: Effect of varying ST on Estradiol-17β-D-glucuronide uptake by OATP1B3 expressing HEK293 cells. The line represents the “best fit” of the data to the equation 4 (R2 = 0.98) which are averages of triplicate determinations and the bars represent the standard deviations.
**Table 1: Probe substrates, microsomal protein concentrations and incubation times**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Probe substrate</th>
<th>Microsome concentration (mg protein/mL)</th>
<th>Probe substrate concentration (µM)</th>
<th>Literature</th>
<th>Internal standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>Phenacetin</td>
<td>0.2</td>
<td>10</td>
<td>9.0, 14, 31,</td>
<td>$^{[14]H_4}$ acetaminophen 54</td>
</tr>
<tr>
<td>CYP2A6</td>
<td>Coumarin</td>
<td>0.2</td>
<td>2.5</td>
<td>0.5</td>
<td>$^{[13]C_6}$7-hydroxy-coumarin</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>Bupropion</td>
<td>0.1</td>
<td>25</td>
<td>76, 89, 130,</td>
<td>$^{[14]H_6}$ hydroxybupropion</td>
</tr>
<tr>
<td>CYP2C8</td>
<td>Paclitaxel</td>
<td>0.2</td>
<td>10</td>
<td>4.0, 15, 4</td>
<td>warfarin</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Diclofenac</td>
<td>0.1</td>
<td>5</td>
<td>3.4, 9.0 10</td>
<td>$^{[13]C_6}$ 4'-hydroxy-diclofenac</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>S-mephenytoin</td>
<td>0.5</td>
<td>30</td>
<td>51, 42, 31,31,</td>
<td>$^{[14]H_6}$ 4'-hydroxy-mephenytoin</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>Bufuralol</td>
<td>0.2</td>
<td>5</td>
<td>13, 44, 4, 16</td>
<td>$^{[14]H_6}$ 1'-hydroxybufuralol</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>Chlorzoxazone</td>
<td>0.5</td>
<td>10</td>
<td>22-49, 17</td>
<td>$^{[14]H_6}$ 4'-hydroxy-mephenytoin</td>
</tr>
<tr>
<td>CYP3A4/5</td>
<td>Midazolam</td>
<td>0.1</td>
<td>1</td>
<td>3.3, 2.5-5.6</td>
<td>$^{[14]H_6}$ 1'-hydroxymidazolam</td>
</tr>
<tr>
<td>CYP3A4/5</td>
<td>Testosterone</td>
<td>0.2</td>
<td>30</td>
<td>50-60, 51, 52</td>
<td>$^{[14]H_6}$ 6β-hydroxy-testosterone</td>
</tr>
</tbody>
</table>

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

**Table 2: The potency of inhibitory effects of ST on CYP expressed in IC_{50} values**

<table>
<thead>
<tr>
<th>IC_{50} (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
</tr>
<tr>
<td>CYP2A6</td>
</tr>
<tr>
<td>CYP2B6</td>
</tr>
<tr>
<td>CYP2C8</td>
</tr>
<tr>
<td>CYP2C9</td>
</tr>
<tr>
<td>CYP2C19</td>
</tr>
<tr>
<td>CYP2D6</td>
</tr>
<tr>
<td>CYP2E1</td>
</tr>
<tr>
<td>CYP3A4/5</td>
</tr>
<tr>
<td>CYP3A4/5</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th>CYP enzyme</th>
<th>Probe reaction</th>
<th>IC&lt;sub&gt;50 &lt;/sub&gt; value (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>phenacetin O-deethylation</td>
<td>41.0 ± 1.2</td>
</tr>
<tr>
<td>CYP2A6</td>
<td>coumarin 7-hydroxylation</td>
<td>160 ± 2.1</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>bupropion hydroxylation</td>
<td>20.1 ± 1.7</td>
</tr>
<tr>
<td>CYP2C8</td>
<td>paclitaxel 6α-hydroxylation</td>
<td>22.4 ± 1.3</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>diclofenac 4′-hydroxylation</td>
<td>23.0 ± 1.5</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>S-mephenytoin 4′-hydroxylation</td>
<td>35.9 ± 1.0</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>bufuralol 1′-hydroxylation</td>
<td>n.i.o.</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>chlorzoxazone 6-hydroxylation</td>
<td>n.i.o.</td>
</tr>
<tr>
<td>CYP3A4/5</td>
<td>midazolam 1′-hydroxylation</td>
<td>17.5 ± 1.3</td>
</tr>
<tr>
<td>CYP3A4/5</td>
<td>testosterone 6β-hydroxylation</td>
<td>28.3 ± 1.3</td>
</tr>
</tbody>
</table>

n.i.o. = no inhibition observed
Table 3: The intrinsic clearance of midazolam in hepatocytes in the presence and absence of ST

<table>
<thead>
<tr>
<th></th>
<th>Hepatocytes</th>
<th>MDZ</th>
<th>MDZ + ST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Half-life (h)(^1)</td>
<td>0.72</td>
<td>1.18</td>
<td></td>
</tr>
<tr>
<td>CL(_{\text{int}}) ((\mu\text{L/min/million cells}))(^2)</td>
<td>16</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>CL(_{\text{int, scaled}}) (mL/min/kg)(^3)</td>
<td>40.98</td>
<td>24.98</td>
<td></td>
</tr>
<tr>
<td>CL(_{\text{h,b}}) (hepatic blood clearance) (mL/min/kg body weight)(^4,5)</td>
<td>14</td>
<td>11</td>
<td></td>
</tr>
</tbody>
</table>

1) Half-lives were evaluated by linear extrapolation
2) (ln2/T1/2) / mio viable cells x 1000
3) (CL\(_{\text{int}}\) / 1000) * (cells / g liver) * (g liver / kg body weight)
4) CL\(_{\text{h}}\) = (Qh * CL\(_{\text{int, scaled}}\)) / (Qh + CL\(_{\text{int, scaled}}\)), Qh: hepatic blood flow; (well-stirred liver model)
5) Qh (mL/min/kg body weight): 55 (rat); 30.9 (dog); 43.6 (monkey); 20.7 (human)
### Table 4: Summary of results

<table>
<thead>
<tr>
<th>Transporter</th>
<th>Probe substrate</th>
<th>IC_{50} value^{a,b} (µg/mL)</th>
<th>Ki(^c) (µg/mL)</th>
<th>Max. inhibition(^{b,d}) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-gp</td>
<td>Digoxin</td>
<td>324.8</td>
<td>324.3</td>
<td>69.5 ± 7.7</td>
</tr>
<tr>
<td>BCRP</td>
<td>PhIP</td>
<td>not observed(^e)</td>
<td>not applicable</td>
<td>not observed(^e)</td>
</tr>
<tr>
<td>OATP1B1</td>
<td>Estradiol-17β-D-glucuronide</td>
<td>10.4 ± 0.6</td>
<td>8.2</td>
<td>101.4 ± 0.6</td>
</tr>
<tr>
<td>OATP1B3</td>
<td>Estradiol-17β-D-glucuronide</td>
<td>6.6 ± 0.9</td>
<td>5.6</td>
<td>85.3 ± 5.4</td>
</tr>
</tbody>
</table>

| a) ST concentration estimated to inhibit transporter activity by 50% |
| b) Data used for curve fitting are mean ± SD of N = 3 |
| c) Calculated with Ki=IC_{50}/(1+S/Km), where S is the substrate concentration of the probe substrate and Km 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

<table>
<thead>
<tr>
<th>Part used in traditional practice</th>
<th>Leaves</th>
</tr>
</thead>
<tbody>
<tr>
<td>Usual human dose (single; mg)</td>
<td>300</td>
</tr>
<tr>
<td>Extraction yield (% w/w)</td>
<td>28.5</td>
</tr>
<tr>
<td>Estimated extract per dose (mg)</td>
<td>85.5</td>
</tr>
<tr>
<td>Putative GIT concentration (µg/mL)</td>
<td>340</td>
</tr>
<tr>
<td><em>In vitro IC</em>&lt;sub&gt;50&lt;/sub&gt; range (µg/mL)</td>
<td>6.6-324</td>
</tr>
<tr>
<td>Potential for clinically significant HDI&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Yes</td>
</tr>
</tbody>
</table>

<sup>1</sup>Estimated GIT fluid = 250mL, with the assumption that the 60% methanol-soluble extracts are extracted in the GIT

<sup>2</sup>This refers to HDI in the GIT with possible effects on drug absorption
Figure 1

A) Relative CYP1A2 Activity (%)

Extract Concentration (μg/mL)

B) Relative CYP2A6 Activity (%)

Extract Concentration (μg/mL)

C) Relative CYP2B6 Activity (%)

Extract Concentration (μg/mL)

D) Relative CYP2C8 Activity (%)

Extract Concentration (μg/mL)
Figure 2

A. Relative CYP2C9 activity (%)

B. Relative CYP2C19 activity (%)

C. Relative CYP3A4 activity (%)

D. Relative CYP3A4 activity (%)

Extract Concentration (μg/mL)
Figure 6

![Graph showing P-gp Transporter Inhibition (%) vs. Extract Concentration (µg/mL)]
Figure 8

OATP1B3 Transporter Inhibition (%)

Extract Concentration (μg/mL)