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Research Paper

The interaction potential of herbal medicinal products: a luminescence-based screening platform assessing effects on cytochrome P450 and its use with devil's claw (*Harpagophyti radix*) preparations

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Abstract

Objectives Potential interactions between herbal medicinal products and the cytochrome (CYP) P450 system are an important safety concern. We set out to develop a screening panel for assessing such interactions and use it to evaluate the interaction potential of devil's claw.
Methods The panel consisted of luminescence-based inhibition assays for CYP1A2, 2C9, 2C19, 2D6 and 3A4, and a reporter gene (luciferase) assay for pregnane X receptor (PXR) activation and CYP3A4 induction. Caftaric acid and chlorogenic acid, two compounds with strong fluorescence quenching properties, were used to demonstrate the assay's resistance to interference. We tested 10 commercial devil's claw preparations as well as harpagoside and harpagide, two important constituents of devil's claw.
Key findings Five preparations were found to weakly inhibit CYP3A4 (IC50 124.2–327.6 μg/ml) and five were found to weakly activate PXR (EC50 10.21–169.3 μg/ml). Harpagoside and harpagide did not inhibit CYP3A4. In agreement with published data, bergamottin, a natural product known to interact with CYP3A4, was shown to inhibit CYP3A4 with an IC50 of 13.63 μM and activate PXR with an EC50 of 6.7 μM.
Conclusions Devil's claw preparations are unlikely to have a clinically relevant effect on CYP function. The assay panel proved effective in screening devil's claw preparations, demonstrating its suitability for use with plant extracts. It showed superior sensitivity and resistance to interference.
Keywords CYP interactions; cytochrome P450 enzymes; *Harpagophytum procumbens*; (herbal) drug safety; PXR (pregnane X receptor)
Abbreviations CYP, cytochrome P450 enzymes; HMPs, Herbal medicinal products; PXR, pregnane X receptor; RXR, retinoid X receptor

Introduction

Recent changes to the European legal framework for herbal medicinal products (HMPs) have made in-vitro safety testing mandatory before a product can be marketed.[1] According to European directive 2004/24/EC,[1] manufacturers who fail to both register their products and provide a full dossier on quality, safety and traditional use by 30 April 2011 will have their products removed from the market. One of the key safety concerns with HMPs is their interactions with conventional medicines and a major avenue for such interactions is the cytochrome (CYP) P450 system.[2,3] Here we report the development of a comprehensive panel for evaluating the interaction potential of HMPs and its use in characterising the interaction potential of commercially available devil's claw preparations.

Previously, we used a fluorescence-based assay for enzymatic activity to study the effect of HMPs on the CYP P450 system, and established the suitability of such in-vitro assay systems for this purpose.[4] However, a major drawback with fluorescence is that HMPs often exhibit strong intrinsic fluorescence or quenching, which can limit the range of concentrations that can be tested.[4,5] This problem can be circumvented by using second-generation luminescence-based assays, which are easier, more versatile, more sensitive and less susceptible to interference (e.g. intrinsic fluorescence or fluorescence quenching). These novel

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assay systems use luciferin derivatives, which cannot be utilised by luciferase, but are substrates for specific CYP enzymes. The CYPs convert these substrates to free luciferin, which can be detected in a separate step with a luciferase assay.[6]

Direct effects on enzymatic activity are only one aspect of how HMP can interact with the CYP enzymes. HMP can also contain compounds capable of acting on intracellular nuclear receptors, thereby altering CYP expression levels (CYP induction). The pregnane X receptor (PXR) is a key regulator of this process.[7] In response to ligand binding, PXR forms a heterodimer with the retinoid X receptor (RXR).[7,8] The complex, known as the xenobiotic responsive transcription factor, is responsible for the regulation of several enzymes involved in drug metabolism. The PXR–RXR complex is thought to activate CYP3A4 expression through the xenobiotic responsive enhancer module (XREM).[8]

For this reason our screening panel also includes a gene reporter assay capable of quantifying the increase in CYP3A4 expression in response to PXR activation.[8] This assay uses a modified HepG2 cell line (HepG2/hPXR/3A4-luciferase), which over-expresses PXR and contains a luciferase gene placed under the control of the CYP3A4 promoter and the XREM enhancer. Expression of luciferase from this construct is regulated by PXR in exactly the same way as the CYP3A4 gene. Consequently, changes in CYP3A4 expression due to PXR activation can be detected by a luciferase luminescence assay, instead of a real-time polymerase chain reaction (RT-PCR).[9]

Devil's claw root (*Harpagophytum procumbens* D.C., Pedalliacea) is a popular treatment for rheumatic diseases and for lower back pain in Africa and Europe.[10,11] Published data suggests it has anti-inflammatory and analgesic effects.[12,13] It contains a number of iridoid glycosides such as harpagoside, harpagide, procumbide and 8-coumaroyl-harpagide.[13] The iridoids have been discussed as key bioactive constituents.[13] Other constituents include phenylethanol derivatives such as verbacoside and isoacetoside, as well as oligosaccharides.[11,13,14]

Devil's claw is one of the key species controlled by the traditional herbal medicines directive in the UK.[15] It is a licensed medicine in many European countries, including Switzerland and Germany. However, there is only a single published report concerning its interactions with the CYP P450 enzymes. Unger and Frank (2004)[16] used a liquid chromatography/mass spectrometry readout to measure the inhibitory activity of a devil's claw root extract on several major CYP isoforms (1A2, 2C8, 2C9, 2C19, 2D6 and 3A4) and reported mild-to-negligible inhibition.

Because of this, the effectiveness of our screening panel was tested by using devil's claw as a model HMP. The CYP3A4 inhibitory activity and PXR activation of 10 devil's claw extracts representative of the European market was evaluated. The iridoid glycosides, harpagoside and harpagide, two well-known components of devil's claw preparations, were also tested on CYP3A4. Furthermore, one of the extracts that has documented high levels of anti-inflammatory activity (Dr B. Feistl, Finzelberg, personal communication) was investigated in more detail by assaying for inhibition on CYP1A2, 2C9, 2C19 and 2D6.

Materials and Methods

Extracts

Seven devil's claw extracts (A–G) were kindly provided by Finzelberg, Germany and another two extracts (H, I) by Bioforce Switzerland. Extract J was purchased from the Organic Pharmacy, UK. Details of the extracts used in this study are listed in Table 1. Extracts A–H were supplied as dried powder and dissolved in a mixture of ethanol/water or water as described in Table 1. Extracts I and J were supplied as tinctures.

Chemicals

Glucose-6-phosphate disodium hydrate salt (G6P), glucose-6-phosphate dehydrogenase (G6PDH), beta-nicotinamide adenine dinucleotide phosphate ($NADP^+$), magnesium chloride hexahydrate, sodium citrate tribasic hydrate, ketoconazole, tranylcypromine, furafylline, quinidine hydrochloride monohydrate, sulphenazole, L-sulforaphane, clotrimazole, rifampicin HPLC grade, trypsin-EDTA, chlorogenic acid and trizma base were purchased from Sigma-Aldrich, Poole, Dorset (UK). Potassium dihydrogen orthophosphate and dipotassium hydrogen orthophosphate 3-hydrate were obtained from VWR International Ltd (UK). Organic solvents were of HPLC grade and were obtained from Sigma-Aldrich, Poole, Dorset (UK).

Hanks' balanced salt solution, foetal bovine serum (FBS), non-essential amino acids, geneticin (G418) and Dulbecco's Modified Eagle Medium (DMEM) with and without phenol red were obtained from Invitrogen (Paisley, UK). Lipid-depleted and charcoal-stripped FBS was purchased from Biosera (UK).

The CYP substrates: luciferin-6′-pentafluoro-benzyl ether (luciferin-PFBE), 6′-deoxyluciferin (luciferin-H), luciferin-6′-methyl ether (luciferin-ME) and ethylene glycol ester of 6′-deoxyluciferin (luciferin-H EGE) were purchased from Promega (Southampton, UK). P450-glo luciferin detection reagent and steady-glo reagent, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] was obtained from Promega (Southampton, UK). Harpagide, harpagoside, caftaric acid and bergamottin were purchased from Phytolab GmbH & Co. KG (Vestenbergsgreuth, Germany).

The CYP supersomes 3A4 (with cytochrome b5), 1A2 (with cytochrome b5), 2C9, 2C19 and 2D6*1 were purchased from Gentest (Woburn).

Analytical data

The concentration of total dry mass (g/100 g) for extracts I and J was determined according to the standard German Homöopathisches Arzneibuch protocol (HAB, 2005).[17] The extracts were oven dried at 150°C for 2 h and the residue was weighed. The weight of the residue was then divided by the volume of extract dried to determine the density.

For extracts A–G the analytical determination of the harpagoside content was performed according to the European Pharmacopeia protocol for devil's claw roots[18] by Finzelberg (Germany) and for the remaining extracts by Bioforce (Switzerland). Briefly, the extracts were analysed

Table 1 Devil's claw extracts assessed for their inhibitory effects on CYP3A4 (IC50 – median concentration to inhibit CYP3A4 by 50%) and for PXR activation with the HepG2/hPXR/3A4-luciferase cells (EC50 – median concentration to activate PXR by 50%)

Extract	Supplier	EtOH content (%)	Dry mass content (mg/ml)	Batch no.	Harpagoside content (%)	IC50 value (μg/ml) CYP3A4 enzyme	EC50 value (μg/ml) for PXR activation
A	Finzelberg, Germany	0	50	UB 2008–152	1.94	327.6 (249.1–430.9)	n/a at max. conc.: 305 μg/ml:13.05% ± 1.65
B	Finzelberg, Germany	0	50	UB 2008–153	2.20	246.4 (193.8–313.2)	n/a at max .conc.: 305 μg/ml 55.13% ± 0.64%
C	Finzelberg, Germany	70	50	UB 2008–154	2.17	NI – 111.11	n/a at max .conc.; 305 μg/ml 31.04% ± 0.75% activation
D	Finzelberg, Germany	70	50	UB 2008–155	2.07	NI – 111.11	85.07(62.19–116.4)
E	Finzelberg, Germany	70	50	UB 2008–156	5.18	124.2 (97.55–158.2)	10.21(7.58–13.74)
F	Finzelberg, Germany	70	50	UB 2008–157	14	268.3 (212.7–338.3)	63.07(48.08–82.74)
G	Finzelberg, Germany	70	50	UB 2008–158	24	230.8 (182.1–292.4)	66.7(43.23–102.9)
H	Bioforce Switzerland	70	50	B01244	1.7	NI – 111.1	169.3(132.4–216.6)
I	Bioforce Switzerland	67	5.8	024710	0.194	NI – 193	n/a at max. conc.; 5.8 μg/ml 3.6% activation ± 0.90
J	The Organic Pharmacy, London, UK	60	5.3	23472H	0.168	At max. conc.; 106 μg/ml: 62% CYP3A4 activity, i.e. 38% inhibition	n/a at max. conc.; 5.3 μg/ml 4.40% activation ± 1.39

The 95% confidence band is added in brackets. All extracts are roots extracts of *Harpagophytum procumbens* D.C., with the exception that extracts H and I also contain *Harpagophytum Zeyheri* L. Decne. NI, no inhibition up to maximum concentration indicated (μg/ml); n/a, EC50 could not be calculated from the data, activation (±standard error) at maximum concentration tested is provided.

by HPLC using an octadecylsilyl silica stationary phase and methanol:water (50 : 50) as the mobile phase, with detection by absorbance at 278 nm.[18]

CYP inhibition assay

The luminescence-based CYP inhibition assay was conducted using the Promega P450-Glo assay kit, according to the following protocol based on the manufacturer's instructions.[19] The NADPH regenerating solution contained the following: 2 mg/ml $NADP^+$, 2 mg/ml G6P, 1.33 mg/ml magnesium chloride hexahydrate and 2% (v/v) G6PDH solution (40 U/ml in 5 mM sodium citrate). For CYP3A4 it also contained 400 mM potassium phosphate pH 7.4 (phosphate buffer).

The enzyme substrate mix was prepared as follows: for CYP1A2: 0.042 pmol/μl enzyme, 400 mM phosphate buffer and 400 μM luciferin-ME. For CYP2C9: 0.042 pmol/μl enzyme, 100 mM phosphate buffer and 400 μM luciferin-H. For CYP2C19: 0.021 pmol/μl enzyme, 200 mM phosphate buffer, 40 μM luciferin-H EGE. For CYP2D6: 0.021 pmol/μl enzyme, 400 mM phosphate buffer, 120 μM luciferin-ME EGE. For CYP3A4: 0.084 pmol/μl enzyme, 200 μM luciferin-PFBE.

All extracts were serially diluted in water containing an equal amount of ethanol as the extract itself and then diluted 1 : 2 with water. The reference inhibitors were dissolved and serially diluted in acetonitrile and then diluted 1 : 2 with water. The concentration of solvent in the final reaction mixture was 2% or less.

In a white 96-well plate (Greiner, Bio-One Ltd, Gloucestershire, UK), 2 μl of test compound were added to 36 μl of NADPH regenerating solution followed by 12 μl of enzyme substrate mix, pre-warmed to 37°C. The plate was incubated at 37°C for 10 min (CYP3A4 and CYP1A2), 30 min (CYP2C9 and CYP2D6) or 20 min (CYP2C19). The reaction was stopped by adding 50 μl of P450-glo luciferin detection reagent (the detection reagent was pre-warmed to room temperature –25°C) and the plate was allowed to stand for 20 min at 25°C prior to reading luminescence using the FLUOstar Optima plate reader (BMG, Labtech Ltd). Each sample and control was assayed in duplicate per experiment and three independent experiments were conducted.

In the positive control, the test compound was omitted and no solvent was added. In the solvent control, the test compound was replaced with a solution containing the same amount of solvent. For the blank, the enzyme substrate mix was added after adding luciferin detection reagent.

The luminescence quenching control was prepared in the same way as the positive control, but after the reaction was completed and the luminescence measured, the test compound was added, then the luminescence was monitored for a further 30 min. The intrinsic luminescence control was prepared in the same way as the blank except the test compound was omitted. After reading luminescence the test compound was added and the luminescence was measured for 30 min. None of the compounds and extracts tested showed any quenching or intrinsic luminescence, so these controls were omitted in later experiments. The absence of quenching and intrinsic luminescence also means that the extracts did not inhibit luciferase, since in the quenching control the luciferase

reaction was carried out in the presence of the test compound. The enzyme activity was calculated according to equation 1,

$$\%\ \text{enzyme activity} = \left[\frac{(T-\bar{b})}{(\bar{P}-\bar{b})}\right]\times 100 \qquad (1)$$

where T is the test compound luminescence, b is the blank and P is the positive control.

Cell culture

The HepG2/hPXR/3A4-luciferase cell line was a generous gift from Prof Rahmani (Laboratoire de Toxicologie Cellulaire, Moleculaire et Genomique INRA, France).[8] HepG2/hPXR/3A4-luciferase cells were routinely cultured in DMEM supplemented with 10% FBS, 1% non-essential amino acids and 800 μg/ml geneticin (G418, invitrogen) and discarded after 10 passages. The cells were kept in a humidified 37°C incubator with 5% CO_2.

CYP3A4/PXR activation assay

HepG2/hPXR/3A4-luciferase cells were seeded in sterile white 96-well plates (Greiner, Bio-One Ltd, Gloucestershire, UK) at a density of 30 000 cells per well in 100 μl of growth medium and left to attach for 24 h. The medium was then removed and replaced with assay medium (phenol red-free DMEM, 10% charcoal/dextran-stripped and lipid-depleted FBS). After 24 h the medium was removed and replaced with 100 μl assay medium containing the appropriate concentration of test compound or extract. Following another 24-h incubation, 100 μl of Steady-glo luciferase assay reagent was added before placing the plate in a shaker for 5 min. Luminescence was measured using the FLUOstar Optima plate reader (BMG, Labtech Ltd).

Rifampicin, clotrimazole, L-sulforaphane, chlorogenic acid, caftaric acid and all the dried extracts were dissolved in DMSO and diluted to the appropriate concentration in assay medium. Bergamottin, harpagide and harpagoside were dissolved in methanol instead of DMSO. Ethanolic tinctures were diluted to the appropriate concentration straight into the assay medium. Solvent concentration was kept below 0.1%. Rifampicin 10 μM was used as the positive control. The negative control contained the appropriate amount of solvent but no test compound. Each sample and control was assayed in duplicate per experiment and three independent experiments were carried out. The percentage of PXR activation and hence CYP3A4 induction was measured according to equation 2:

$$\%\ \text{activation}\ (\text{luminescence relative to positive control}) = \left[\frac{(T-\bar{n})}{(\bar{P}-\bar{n})}\right]\times 100 \qquad (2)$$

where T is the test substance luminescence, P is the positive control luminescence (rifampicin 10 μM) and n is the negative control.

Cell viability assay

Cell viability was determined using the MTS assay kit, according to the manufacturer's instructions.[20] Cells were seeded and treated with assay medium as above. After 24 h of exposure to the test compound the viability was estimated by adding 20 μl per well of MTS solution and incubating at 37°C for 4 h prior to reading absorbance at 510 nm (Labsystems Multiscan, VWR International – UK).

Regression analysis

Calculation of IC50 and EC50 values, for the CYP inhibition and PXR activation results was carried out by non-linear regression analysis with Graph Pad Prism v.4.03 (Graphpad Software, USA) using a four-parameter Hill model as described in Modarai *et al*. (2007).[4] The software was also used to estimate confidence intervals for the regression models. The graphs were plotted with Sigma Plot v 11 (Systat Software Inc, USA).

Results

CYP enzyme inhibition

The validity and accuracy of the luminescence-based CYP activity was verified by measuring the potency of the following isoform-specific reference inhibitors: furafylline (1A2), quinidine (2D6), ketoconazole (3A4), sulphenazole (2C9) and tranylcypromine (2C19).[21–27] Half-maximal inhibitory concentrations (IC50) were estimated using non-linear regression analysis (four-parameter Hill model). The results were in good agreement with the literature (Table 2), except for tranylcypromine, which had an IC50 approximately three-fold lower than the published value. The reason for the discrepancy is not known.

To further validate the assay, bergamottin, a natural product inhibitor, was also assayed for CYP3A4 inhibition. Bergamottin was found to inhibit CYP3A4 with an IC50 value of 13.63 μM (95% confidence limits of 10.98–16.92 μM) (Figures 1 and 2a), which is in agreement with the literature values: He *et al*. (1998) [28] report 75% inhibition of CYP3A4 by 10 μM bergamottin (IC50 between 10 and 100 μM), while Girennavar *et al*. (2006) [29] report an IC50 of 6.78 ± 0.09 μM.

The caffeic acid derivatives chlorogenic acid and caftaric acid exhibit strong fluorescence quenching, limiting the concentration that can be tested with the fluorescence-based assay to approximately 4.65 μM.[30] In contrast, with the luminescence assay no quenching and or CYP inhibition was observed up to 300 μM(60-fold higher).

Using this assay system we analysed the CYP3A4 inhibitory activity of 10 devil's claw preparations. Our controls for intrinsic luminescence and luminescence quenching showed that the extracts did not interfere with the assay readout. Extracts A, B, E, F and G mildly inhibited CYP3A4, with IC50 values ranging from 124 to 327 μg/ml (Table 1, Figure 3). Negligible inhibition (i.e. zero inhibition up to a concentration of at least 100 μg/ml) was seen with extracts C, D, H and I (Table 1). Extract J inhibited CYP3A4 by 38% at 106 μg/ml, but higher concentrations could not be tested due to the sensitivity of the CYP3A4 to ethanol (Table 1). The IC50 values did not differ appreciably between different extracts. The IC50 of the most potent extract (E) was only 2.5 times lower than that of extract A, which was the least effective.

Table 2 Half-maximal inhibitory concentrations (IC50) values (μM) and the upper and lower 95% confidence limits (depicted in brackets) for the reference inhibitors ketoconazole, furafylline, quinidine, tranylcypromine and sulfaphenazole (results are duplicates of three independent experiments)

CYP	Inhibitor	Fluorescence assay[4]	Luminescence assay	Literature values (non-luminogenic)	Literature values (luminogenic)[6]
3A4	Ketoconazole	0.122 (0.104–0.143)	0.111 (0.010–0.125)	0.01–0.13[21,22]	0.1
1A2	Furafylline	1.68 (1.36–2.08)	1.60 (1.34–1.90)	1.3–1.65[21–23]	0.4
2D6	Quinidine	0.00771 (0.00692–0.00859)	0.0108 (0.00911–0.129)	0.009–0.18[21,24]	0.008
2C19	Tranylcypromine	9.45 (8.05–11.1)	3.03 (2.58–3.57)	8.9–11[25–27]	Not available
2C9	Sulfaphenazole	N/A	0.101 (0.0849–0.120)	0.06–1.3[21,23]	0.2

Literature values from non-luminogenic and luminogenic systems are also shown for comparison.

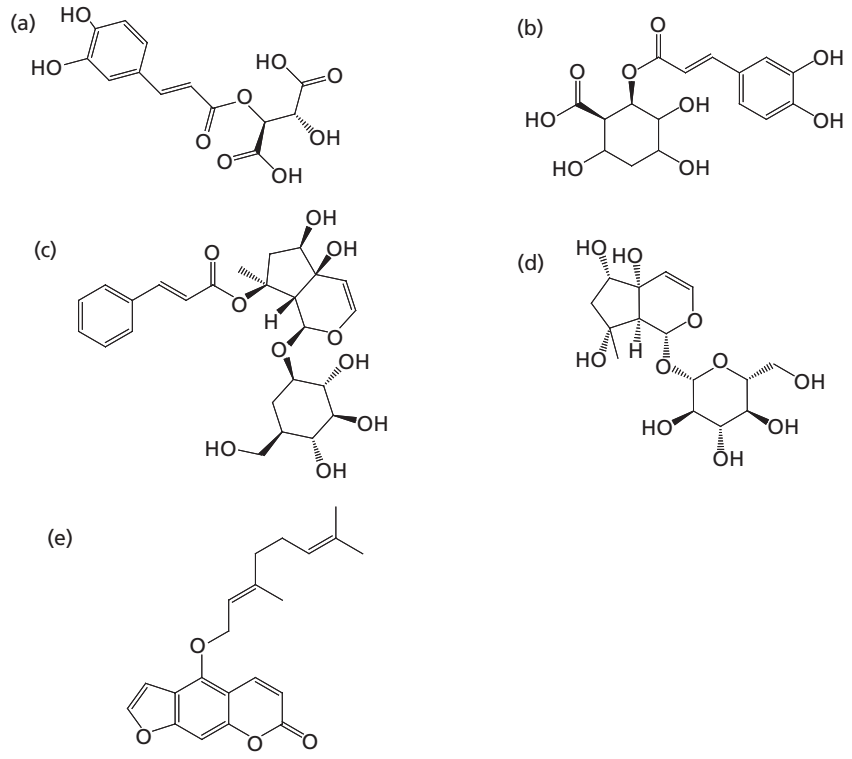

Figure 1 Chemical structures of the compounds assessed for CYP3A4 inhibitory activity. (a) caftaric acid, (b) chlorogenic acid (c) harpagoside, (d) harpagide and (e) bergamottin.

The relationship between harpagoside content and inhibitory activity was also studied. Unlike the IC50 values, the harpagoside content varied considerably between extracts. It ranged from 24% for extracts G and H to 0.168% for extract J (Table 1). The harpagoside content did not appear to correlate with CYP3A4 inhibitory activity. For example, extract G had a 12-fold higher harpagoside content than extract B, yet the IC50 values for both extracts were nearly the same (230.8 and 246.4 μg/ml, respectively). Also, extracts E and G differed five-fold in their harpagoside content but their IC50 values differed less than 2-fold (Table 1). When harpagoside and the related iridoid glycoside harpagide were assayed for inhibition on CYP3A4, no effect was seen up to a concentration of 400 μM (Figure 1).

For extract G, the investigation was further extended to include additional CYP isoforms. At concentrations below 10 μg/ml, extract G had negligible inhibitory activity on isoforms 1A2, 2C19 and 2D6 (Figure 4 and Table 3), whereas at 10 μg/ml extract G inhibited CYP2C9, by approximately 40%. Overall, CYP2C9 proved to be the most susceptible to inhibition by extract G, with an IC50 of 32.37 μg/ml and 2D6 was the most resistant (IC50: 595.6 μg/ml). The results are in

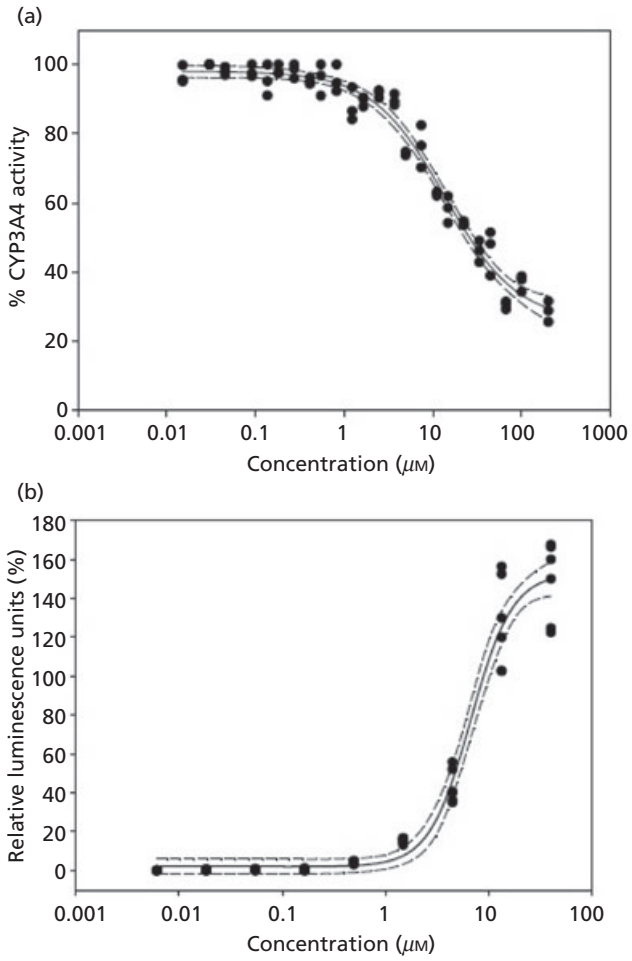

Figure 2 (a) Inhibition of CYP3A4 by bergamottin: regression models for the effects of bergamottin on purified CYP3A4. (b) Activation of PXR by bergamottin in HepG2 cells: regression models for the effect of bergamottin on HepG2/hPXR/3A4-luciferase cells. Results are duplicates from at least three independent experiments. The solid line represents the line of best fit obtained with a Hill model regression and the dashed lines the upper and lower 95% confidence limits. Results are expressed as relative luminescence units (%), where the signal from 10 μM rifampicin equals 100%.

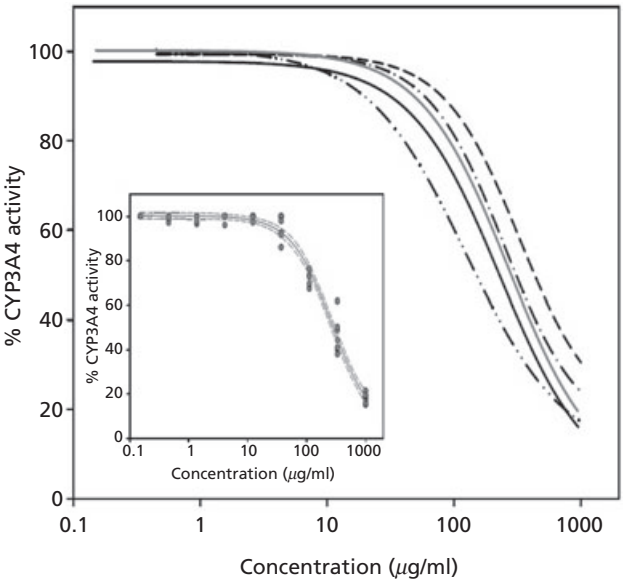

Figure 3 Inhibition of CYP3A4 by devil's claw extracts: regression models for the effects of devil's claw extracts on purified CYP3A4. Extract A (dashed), B (dot-dash), E (dash-dot-dot) F (grey) and G (black). Results are duplicates from two or more independent experiments. The lines of best fit were obtained with a Hill model regression. As a representative example of the variation, in the insert, the data points and 95% confidence limits (dashed lines) are shown for extract F.

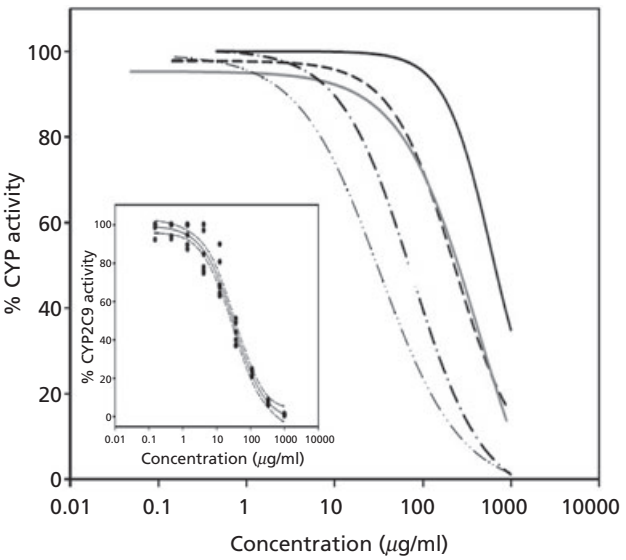

Figure 4 Inhibition of the major CYP isoforms by extract G: Hill regression models for the effects of devil's claw extract G on CYP 1A2 (grey), 2C19 (dash-dot), 2C9 (dash-dot-dot), 2D6 (black) and 3A4 (dash). Results are duplicates from at least three independent experiments. As a representative example of the experimental variation, in the insert, data points for CYP2C9 and 95% confidence limits (dashed lines) are shown.

excellent agreement with previous work by Unger and Frank,[16] who tested a single root extract (from Bioforce) on the major CYP isoforms and found inhibition in the range 100–1000 μg/ml. Their actual values for 1A2, 3A4, 2C19 and 2D6 were 997 μg/ml ± 23, 335 μg/ml ± 14, 155 μg/ml ± 9 and 1044 μg/ml ± 80 μg/ml, respectively. A comparison with the results in Table 3 shows that the pattern is remarkably similar, especially when one takes into account the differences in the values for the reference inhibitors. For example, Unger and Frank report a 4.3-fold higher value for devil's claw on CYP 2C19 and a 3.7-fold higher value for tranylcypromine (8.6 ± 0.4 μM).

Cell viability assay

The loss of cell viability was assessed with the MTS assay.[20] Only concentrations of test agent (devil's claw extracts or pure compounds) for which the cell viability remained at 100% (i.e. no statistically significant difference from the control) were used in the reporter gene assay. To avoid interference, the solvent content in the assay medium was kept below 0.1%.

Table 3 Half-maximal inhibitory effects (IC50 values) of devil's claw extract G on CYPs: 1A2, 3A4, 2C19 and 2D6

CYP	IC50 value (μg/ml)	IC50 (μg/ml) from Unger and Frank[16]
1A2	439.4 (209.9–919.8)	997 ± 23
3A4	230.8 (182.1–292.4)	335 ± 14
2C19	75.81 (62.11–92.53)	155 ± 9
2D6	595.6 (324–1095)	1044 ± 80
2C9	32.37 (25.89–40.47)	121 ± 8

95% upper and lower confidence limits are depicted in brackets. Values (mean ± standard deviation of triplicate determinations) obtained by Unger and Frank[16] are also shown for comparison.

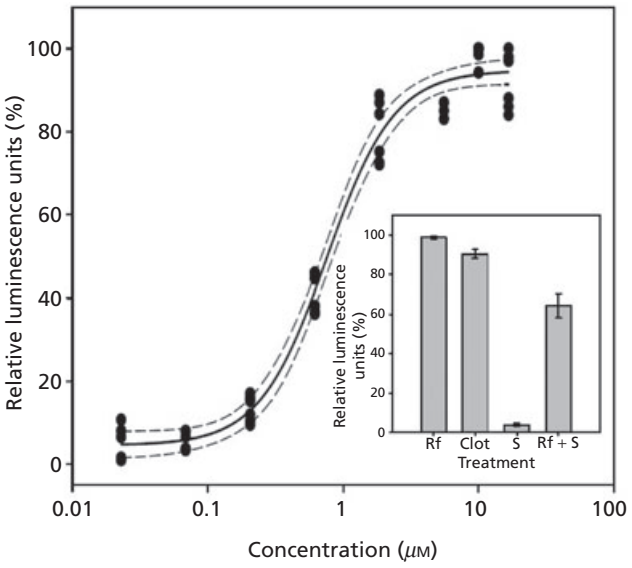

Figure 5 Activation of PXR by rifampicin in HepG2 cells: regression models for the effects of rifampicin on HepG2/hPXR/3A4-luciferase cells. Results are duplicates from at least three independent experiments. The solid line represents the line of best fit obtained with a Hill model regression and the dashed lines the upper and lower 95% confidence limits. Results are expressed as relative luminescence units (%), where the signal from 10 μM rifampicin equals 100%. In the insert, the effect are shown of treatment with different agents, 10 μM rifampicin (rif), 10 μM clotrimazole (clot), 20 μM SFN (S) and 10 μM rifampicin plus 20 μM SFN (rif+S).

CYP3A4 induction via PXR activation

Rifampicin, a potent PXR activator, was used as the positive control in the HepG2/hpxr/3A4-luciferase assay (Figure 5). The EC50 of rifampicin in this assay was determined to be 0.76 μM (95% confidence limits 0.67–0.86 μM). This is slightly lower than the reported value of 1.8 ± 0.2 μM.[7] Treating the cells with another PXR activator, clotrimazole (10 μM), gave a luminescence value (signal) that was equal to 90.4% (±2.26 standard error) of that obtained with 10 μM rifampicin. The addition of 20 μM L-sulforaphane (SFN), an inhibitor of CYP3A4 expression, reduced the signal from 10 μM rifampicin to 64.5% (±6.1 standard error) of the value obtained without SFN (Figure 5).

In addition to inhibiting CYP3A4 enzyme activity, bergamottin has been reported to induce CYP3A4 expression, so it was chosen as a natural product positive control.[31] As expected, bergamottin strongly activated PXR, with an EC50 of 6.70 μM (95% confidence intervals: 5.73–7.82 μM) (Figure 2b). At high concentrations of bergamottin, the luminescence signal was higher than the one obtained with 10 μM rifampicin and thus the relative luminescence value was greater than 100% (Figure 2b).

Of the 10 devil's claw extracts assessed for PXR activation only five (D, E, F, G and H) weakly activated PXR (Table 1 and Figure 6), with EC50 values ranging from 10 to 169.3 μg/ml. Extract E (EC50:10.21 μg/ml and 95% confidence interval 7.58–13.74 μg/ml) was the most potent activator. In contrast, extracts A, B and C produced negligible activation. At the maximum concentration tested (305 μg/ml), compared to 10 μM rifampicin, extracts A, B and C produced activations of 13.05% (±1.65), 55.13% (±0.64) and 31.04% (±0.75), respectively (Table 1). Extracts I and J could only be tested up to concentrations of 5.8 μg/ml (3.6% activation ± 0.90) and 5.3 μg/ml (4.40% activation ± 1.39), respectively, due to their high ethanol content.

Discussion

Using extracts derived from a commonly used botanical drug, we have evaluated a panel of luminescence-based assays aimed at characterising the potential of HMPs for interacting with the CYP P450 enzymes. The panel consisted of assays for the enzymatic activity of the major CYP isoforms and a reporter gene assay for determining the induction of CYP3A4 expression via PXR activation. The luminescent activity assay provided accurate results with both a series of reference inhibitors and bergamottin, a natural product known to inhibit CYPs. Plant extracts often contain factors that can interfere with fluorescence readouts but do not affect luminescence. We demonstrated this by showing that luminescence is not affected by two caffeic acid derivatives with known fluorescence quenching properties, even at relatively high concentrations (Figure 1). These results suggest that compounds with strong fluorescence quenching properties do not necessarily affect luminescent assays. Therefore luminescent assays may help overcome the frequently encountered problem of fluorescence quenching from phytochemicals. Luminescent light produced by luciferase lies in the 510–670 nm range, with a maximum at 560 nm, but light absorption by phytochemicals typically occurs at lower wavelengths.[5] Consequently it is less likely that phytochemicals would exhibit luminescence quenching or intrinsic luminescence. In contrast, fluorescence can be quenched by a larger range compounds, since quenching can occur when there is an overlap with either the absorption or the emission spectra of the fluorophore. None of the extracts tested in this study showed any sign of quenching or intrinsic luminescence. Luminescence has additional

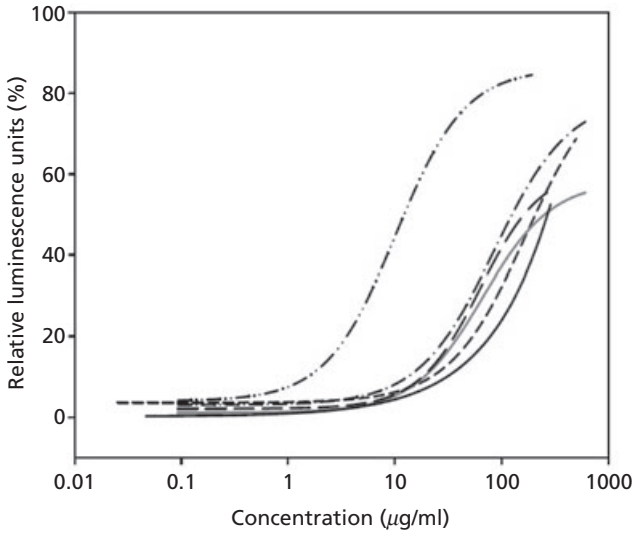

Figure 6 Activation of PXR by devil's claw in HepG2 cells: regression models for the effects of devil's claw extracts B (black), D (dash-dot), E (dash-dot-dot), F (long dash), G (grey) and H (short dash) on HepG2/hPXR/3A4-luciferase cells. Results are duplicates from at least three independent experiments. The solid line represents the line of best fit obtained with a Hill model regression. Results are expressed as relative luminescence units (%), where the signal from 10 μM rifampicin equals 100%.

advantages, such as higher sensitivity, with a higher signal-to-noise ratio (over 10 times higher in our study), no sensitivity to light bleaching and greater suitability for high-throughput screening assays.

The gene reporter assay had been validated in a previous study.[8] In our study, as expected, it showed a strong response to rifampicin, clotrimazole and bergamottin (which is also a PXR activator) (Figure 5). Furthermore the response from 10 μM rifampicin was strongly antagonised by L-sulforaphane, a potent and specific PXR antagonist,[32] clearly showing that the effect is indeed PXR dependent (Figures 2b and 5). The gene reporter assay is advantageous over real-time PCR, which is commonly employed for studying CYP gene induction, as it is less laborious, less time-consuming and has higher throughput. Overall, we have shown that the screening panel described here is very well suited to the study of plant extracts. This panel was used to study the interaction profile of 10 commercial devil's claw preparations. Extract G was tested on CYP 3A4, 2D6, 2C19 and 1A2 (Table 3, Figure 4). The results were in good agreement with values published in the literature, despite the fact that the extracts were produced using a range of extraction procedures.[16]

The remaining extracts were only tested for CYP3A4 inhibition; extracts A, B, E and F gave similar results, with IC50 values in the range 120–330 μg/ml (Table 1, Figure 3), while extracts C, D, H and I showed negligible activity. The concentration range for the ethanolic extract J was limited by the low concentration and high ethanol content, but it inhibited CYP3A4 by ~38% at a concentration of 100 μg/ml, suggesting that it has a similar potency to the more active extracts (A, B, E, F and G).

The bioavailability of devil's claw is not well studied. According to one report, a patient reached a maximum harpagoside blood concentration of 15 ng/ml after being dosed orally with a 600 mg devil's claw tablet containing 50 mg harpagoside.[33] Harpagoside makes up approximately one-twelfth of the mass of this devil's claw preparation. If the bioavailability of all devil's claw components in the tablet was the same, then the total concentration of all devil's claw components in the plasma would be 12 times higher than the harpagoside concentration, i.e. 180 ng/ml. Assuming that the bioavailability of the inhibiting compounds is similar to that of harpagoside, then after a 600 mg devil's claw dose, the CYP-inhibitory activity in the plasma should be equivalent to 180 ng/ml devil's claw. The concentration at which no inhibition or induction is observed is typically ~1 log unit lower than the IC50 or EC50 value (Figures 3–6). Thus, accounting for possible differences in bioavailability and dose, it is reasonable to assume that preparations with IC50 or EC50 values above 10 μg/ml (~2 log units above the expected maximum plasma concentration) are highly unlikely to result in clinically relevant levels of CYP inhibition or induction.

It has been proposed that harpagoside may be important for the therapeutic activity of devil's claw preparations. The biological activity of the extracts was tested *in vitro* by assaying for the inhibition of biosynthesis of eicosanoid and cysteinyl-leukotriene (both inflammatory mediators; Dr B. Feistel, Finzelberg, personal communication). The activity of the Finzelberg extracts A to E did not correlate with the harpagoside content. Only extracts F and G, which were specially enriched harpagoside-containing fractions, showed increased in-vitro biological activity. Thus it is possible that compounds related to harpagoside and present in the same fraction are mainly responsible for the biological activity of devil's claw.

We also tested pure harpagoside and the related iridoid glycoside harpagide and found that they did not inhibit CYP3A4 (Figure 1). In addition we found no correlation between harpagoside content and inhibitory activity in the actual extracts (Table 1). These results suggest that enriched

fractions of devil's claw with higher biological activity are unlikely to show clinically significant CYP3A4 inhibition.

The results of the reporter gene assay were more variable. The effect of extracts A to C was negligible, but extracts D to H showed mild to moderate PXR activation. Extract E was the most active, with an EC50 of 10 μg/ml (Table 1, Figure 6). The other four extracts (D, F, G, H) had EC50 values between 63 and 169 μg/ml. Extracts I and J could not be fully tested due to their low concentration and high ethanol content. However, since no induction was observed at ~5 μg/ml it is unlikely that they would have EC50 values lower than 50 μg/ml. All of the extracts, except E, appear to have an EC50 above 10 μg/ml, which makes it unlikely that these effects will be clinically relevant. Extract E has an EC50 value of 10 μg/ml and may warrant further investigation. In general, induction effects seemed to be more prominent than inhibition effects, so it is possible that more potent or enriched preparations than the ones we tested may result in clinically relevant levels of CYP induction. It is therefore advisable for manufacturers to check their preparations thoroughly for CYP induction.[34]

Conclusions

Devil's claw preparations are unlikely to interact with conventional medications via the CYP P450 enzyme system, which is in agreement with the pharmacovigilance data, i.e. the lack of reported interactions. However some devil's claw preparations can show non-trivial levels of PXR activation and thus manufacturers should carefully assess their products for CYP induction.

The screening used in this study represents a step forward in the methodology. As far as we are aware this is the first time that such luminescent assays have been employed in studying the CYP interaction potential of HMPs. Compared with LC/MS readouts, luminescence assays are easier, faster and more suitable for automation. Compared with fluorescence, they are more sensitive, with greater signal-to-noise ratios (well over 10-fold in this case) and are less susceptible to interference. Thus luminescence is better suited to use with complex phytochemical mixtures.

The platform can be used to accurately and rapidly determine the CYP interaction profile of an HMP. It is hence a valuable tool for both manufacturers and in pre-clinical research.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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