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Identification of karanjin isolated from the Indian beech tree as a potent CYP1 enzyme inhibitor with cellular efficacy *via* screening of a natural product repository†‡

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CYP1A1 is thought to mediate carcinogenesis in oral, lung and epithelial cancers. In order to identify a CYP1A1 inhibitor from an edible plant, 394 natural products in the IIIM's natural product repository were screened, at 10 μM concentration, using CYP1A1-Sacchrosomes™ (*i.e.* microsomal enzyme isolated from recombinant baker's yeast). Twenty-seven natural products were identified that inhibited 40–97% of CYP1A1's 7-ethoxyresorufin-*O*-deethylase activity. The IC_{50} values of the 'hits', belonging to different chemical scaffolds, were determined. Their selectivity was studied against a panel of 8 CYP-Sacchrosomes™. In order to assess cellular efficacy, the 'hits' were screened for their capability to inhibit CYP enzymes expressed within live recombinant human embryonic kidney (HEK293) cells from plasmids encoding specific CYP genes (1A2, 1B1, 2C9, 2C19, 2D6, 3A4). Isopimpinellin (IN-475; IC_{50}, 20 nM) and karanjin (IN-195; IC_{50}, 30 nM) showed the most potent inhibition of CYP1A1 in human cells. Isopimpinellin is found in celery, parsnip, fruits and in the rind and pulp of limes whereas different parts of the Indian beech tree, which contain karanjin, have been used in traditional medicine. Both isopimpinellin and karanjin negate the cellular toxicity of CYP1A1-mediated benzo[*a*]pyrene. Molecular docking and molecular dynamic simulations with CYP isoforms rationalize the observed trends in the potency and selectivity of isopimpinellin and karanjin.

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Introduction

Cancer is one of the leading causes of death worldwide particularly in developing countries. Unfortunately, the number of deaths continues to rise.[1] It has been globally accepted by oncologists that cancer treatment using conventional therapeutics and surgical approaches is a challenging task; it is associated with poor survival and higher mortality rates. Therefore, chemoprevention is gaining more attention from the scientific community.[2] Chemoprevention involves the use of relatively non-toxic compounds for prevention, inhibition of carcinogenesis in normal healthy cells or even reversal of carcinogenesis. "Carcinogenesis" usually occurs through the three distinct phases of initiation, promotion, and progression.[3] These sequential cellular events may be triggered by etiological factors such as carcinogenic chemicals, radiation or infectious organisms (*e.g.* viruses) or endogenous factors such as inherited mutations, deregulated hormone levels and the immune status.[4]

The human cytochrome P450 (CYP) family of enzymes, especially CYP1A1, CYP1A2 and CYP1B1, has been implicated in the metabolism, *via* the aromatic hydrocarbon receptor (AhR)-mediated signaling pathway, of a large number of procarcinogens which are mainly polycyclic aromatic hydrocarbons such as benzo[*a*]pyrene (B[*a*]P), 7,12-dimethylbenz[*a*]-anthracene (DMBA), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 4-nitroquinoline-1-oxide, *N*-nitroso-*N*-methylurea, heterocyclic amines, and estrogen (17β-estradiol) to cytotoxic, mutagenic and carcinogenic intermediates. AhR belongs to the hormone receptor family of transcription factors and is responsible for the induction of the *CYP1* sub-family of genes at the transcriptional level. For example, CYP1A1 protein,

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induced by B[*a*]P ligand-bound AhR, is responsible for the hydroxylation of B[*a*]P to form the metabolite, benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide, which initiates the process of carcinogenesis through the formation of DNA adducts. This leads to DNA damage, chromosomal aberration, and generation of excessive reactive oxygen species (ROS) ultimately leading to chronic inflammation of lung cells.[5]

A significant amount of cumulative evidence suggests a role for the CYP1 sub-family of enzymes in carcinogenesis, particularly in oral, lung, renal, mammary, colon, bladder, skin and ovarian cancers because of their involvement in the bioactivation of a number of pro-carcinogens.[6] The expression of CYP1 enzymes is significantly higher in cancer cells in comparison with normal healthy tissues. Therefore, it has been proposed that the selective reduction in catalytic activity of these CYP1 enzymes in cancer cells would offer a window for medicinal chemists and cell biologists to develop novel chemopreventive strategies.[7] In the past two decades, more than four dozen reports have been published which have demonstrated the chemopreventive potential of a large number of synthetic compounds in the prevention of CYP1-mediated bioactivation of carcinogens.[8] Several chemopreventive natural products have been discovered which include ellagic acid, epigallocatechin gallate, resveratrol, *trans*-stilbenes,[2d,9] α-naphthoflavone (ANF), galangin, genistein,[2b,10] eupatorin,[11] quercetin,[12] luteolin,[13] bergamottin, 8-methoxypsoralen and similar furanocoumarins,[14] 7,8-dehydrorutecarpine, berberine alkaloids,[15] polyisoprenylated quinones, naphthoquinones, terpenoids and steroids[16] and many more dietary flavonoids.[17] It has also been reported that CYP1A1-mediated metabolism results in the generation of more active metabolites.[11a,18] The chemical structures of known CYP1A1 inhibitors are shown in Fig. 1.

In continuation of our interest in this area,[19] herein, we report the screening of the in-house natural product repository of compounds for CYP1A1 enzyme inhibition using CYP1A1-Sacchrosomes which consist of active microsome-bound enzymes isolated from recombinant baker's yeast.[19e]

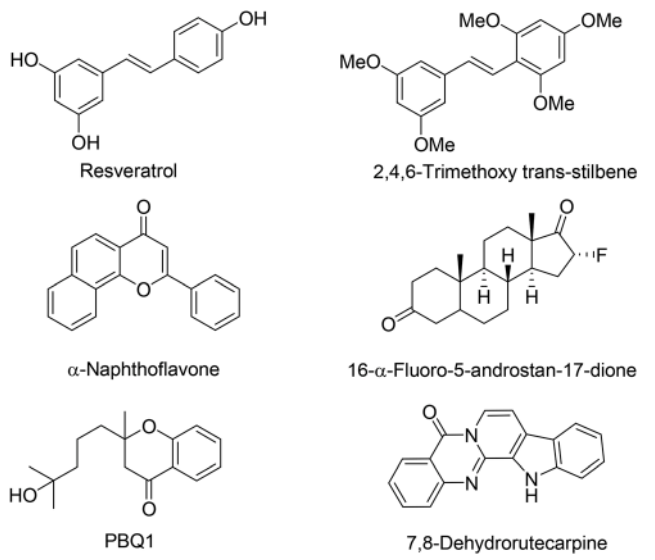

Fig. 1 Known CYP1A1 inhibitors with chemopreventive activity.

The library was also screened using live normal human cells which express CYP1A1.[19e] Both assays ascertained CYP1A1's 7-ethoxyresorufin-*O*-deethylase (EROD) activity in being able to convert the substrate 7-ethoxyresorufin (7-ER), *via* de-ethylation, to the product resorufin which is fluorescent and can be conveniently monitored using a plate reader. The two most potent 'hits' were explored for their ability to protect normal cells from the CYP1A1-mediated toxicity of B[*a*]P by blocking its metabolism to an active carcinogen. Molecular modelling studies were carried out for these two natural products in order to understand molecular interactions and selectivity patterns with the major xenobiotic CYP enzymes.

Results and discussion

In vitro screening in Sacchrosomes™

The in-house natural product repository of 394 natural products was screened for CYP1A1-enzyme inhibition activity using Sacchrosomes™ by the EROD assay. In the EROD assay,[50,51] the formation of resorufin from 7-ER *via* CYP1A1 enzyme-mediated dealkylation was measured in terms of end-point fluorescence intensity at an excitation/emission wavelength of 530/590 using 96-well microtiter plates and a fluorescence plate reader.[20] Alpha-naphthoflavone (ANF), a known CYP1A1 inhibitor, was used as a positive control.[19e] The preliminary screening using a test concentration of 10 μM identified 27 natural products displaying 40–97% CYP1A1-inhibition, as indicated by the change in fluorescence intensity due to the decrease in substrate metabolism with respect to time. Out of these 27 'hits', 8 natural products were reported earlier as CYP1A1 inhibitors *in vitro*. The remaining 19 natural products were identified as CYP1A1 enzyme inhibitors for the first time.

These 27 'hits' were categorized as per their chemical classes into alkaloids, flavonoids, coumarins, naphthoquinones and steroidal terpenoids. The name, structure and percentage inhibition of CYP1A1 for all 394 natural products at 10 μM are provided in Table S2 of the ESI.‡ Piperine (IN-102; 42% inhibition), berberine (IN-109; 48% inhibition), 6-methoxy chelerythrine (IN-229; 90% inhibition) and meridianin C (IN-333; 50% inhibition) were the alkaloids which showed CYP1A1 inhibition in the EROD assay. Among these alkaloids, piperine and berberine were previously reported as inhibitors of AhR-mediated CYP1A1 signaling.[15a,21] The chemical structures of the four alkaloids, which were identified as CYP1A1 inhibitors, are shown in Fig. 2.

Flavonoids and coumarins, for example ANF and khellin,[22] are known to exhibit CYP1A1 inhibitory activity. In our screen, cycloaudinal (IN-49; 42% inhibition), karanjin (IN-195; 97% inhibition), khellin (IN-199; 92% inhibition), hispidulin (IN-214; 86% inhibition), anisomalin (IN-226; 80% inhibition) and pongamol (IN-299; 87% inhibition) were identified as CYP1A1 inhibitors in the EROD assay. The chemical structures of these flavonoid inhibitors are shown in Fig. 3.

In the coumarin class of compounds, phellopterin (IN-309; 97% inhibition) was found to be the most potent 'hit' in

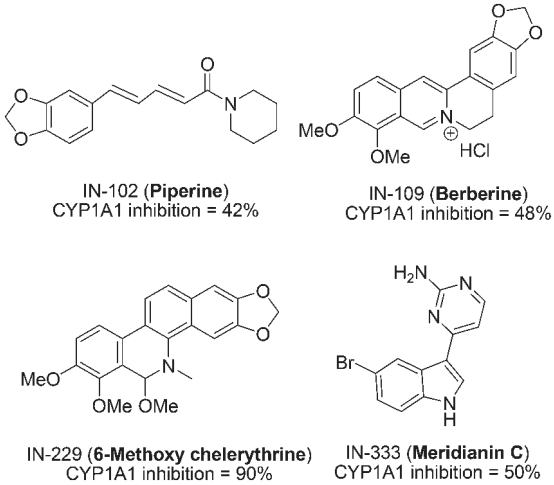

Fig. 2 Chemical structures of potent CYP1A1 inhibitor alkaloids.

the CYP1A1 screen. Meanwhile, bergapten, oxypeucedanin, sphondin, xanthotoxin, (*E*)-1-(5,7-dihydroxy-2,2,6-trimethyl-2*H*-chromen-8-yl)-3-phenylprop-2-en-1-one, imperatorin, 5,7-diallyl oxycoumarin and 8-ethoxypsoralen were observed as moderate CYP1A1 inhibitors. Besides these, heraclenol,[14a] psoralen,[14a] isopsoralen[14a] and isopimpinellin[14d] were previously reported as moderate inhibitors of CYP1A1. The chemical structures of the identified coumarins are shown in Fig. 4. Plumbagin (IN-114; 48% inhibition) and naphthazarin (IN-416; 96% inhibition) are two naphthoquinones and withaferin A (IN-04; 50% inhibition) is a steroidal CYP1A1 inhibitor identified in the screen. The chemical structures of the naphthoquinones and the steroid are shown in Fig. 5.

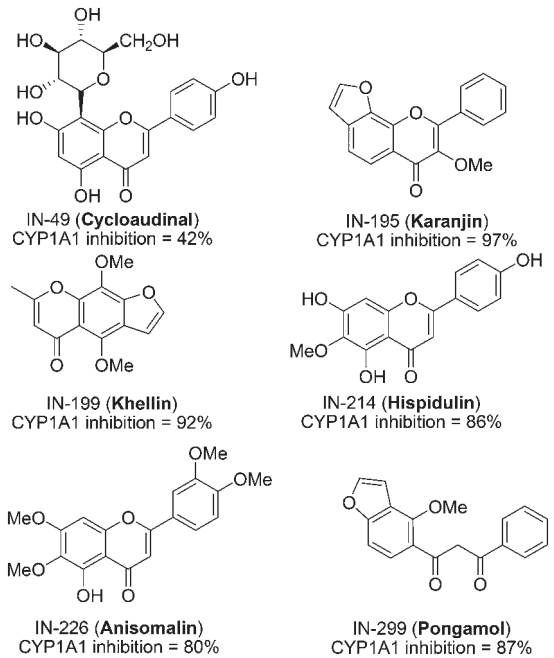

Fig. 3 Chemical structures of flavonoids identified as CYP1A1 inhibitors.

Among these, naphthazarin[23] was previously reported as a CYP1A1 inhibitor.

All obtained hits were tested at least 4 times in the EROD assay before proceeding to IC_{50} calculation. Amongst all molecules screened, it was found that karanjin (IN-195) had the highest percentage inhibition of the CYP1A1 enzyme. Its IC_{50} was determined using CYP1A1-Sacchrosomes™ again. It was found to have an IC_{50} value of 1.6 μM (Table 1).

A time-dependent inhibition study[24] of selected compounds was also performed. Compounds were incubated with enzymes for up to 45 minutes. There were no differences in inhibition as determined from the IC_{50} values after incubation for different time periods. The Lineweaver–Burk plot of karanjin is shown in section S4 of the ESI,‡ which clearly shows that karanjin is a competitive inhibitor of CYP1A1.

Selectivity profile of potent CYP1 inhibitors *versus* CYP2 and CYP3 isoforms

Cytochrome P450 enzymes (CYP1, CYP2 and CYP3) are part of the xenobiotic metabolic system in humans which is utilized to metabolize PAHs, amines, estrogens and various drugs. Selective inhibition of CYP1 family enzymes by various natural products is usually observed due to the difference in their structure, function and sequence identity. The selective inhibition behaviour of these natural product 'hits' toward various CYP enzymes on Sacchrosomes™ is detailed in Table 1.

All identified 'hits' were found to be selective for CYP1 family enzymes in comparison with the CYP2 and CYP3 family members. The lower propensity to inhibit CYP2 and CYP3 family members indicates that the identified natural 'hits' may be free from major liability of drug–drug interactions, which is associated with CYP2 and CYP3 family members such as CYP2D6, CYP2C9, CYP2C19 and CYP3A4 (as they play a significant role in the metabolism of xenobiotics). Although the CYP1 sub-family members CYP1A1 and CYP1B1 and similarly CYP1A1 and CYP1A2 share only 38% and 72% amino acid sequence identity, they are grouped together because of their characteristic metabolic propensity towards PAHs like TCDD and B[*a*]P. CYP1 sub-family members possess planar and compact structural architectures in contrast to CYP2 and CYP3 family members, which have open and wider binding cavities.

Human cell-based enzyme inhibition assays

Live recombinant human cell-based CYP enzyme inhibition assays were carried out to find out if there was any correlation between the *in vitro* data obtained using Sacchrosomes™ (isolated from baker's yeast cells) and those expressed within 'live' human cells. Furthermore, the results from the assay would reveal the cellular potential of compounds which would make them amenable for further pre-clinical studies. For the live cell assay, human kidney cells (HEK293), grown in 'suspension', were transfected individually with the respective plasmids pcDNA3.1/CYP1A1, pcDNA3.1/CYP1B1, pcDNA3.1/CYP1A2, pcDNA3.1/CYP2D6, pcDNA3.1/CYP2C9, pcDNA3.1/CYP2C19 and pcDNA3.1/CYP3A4.

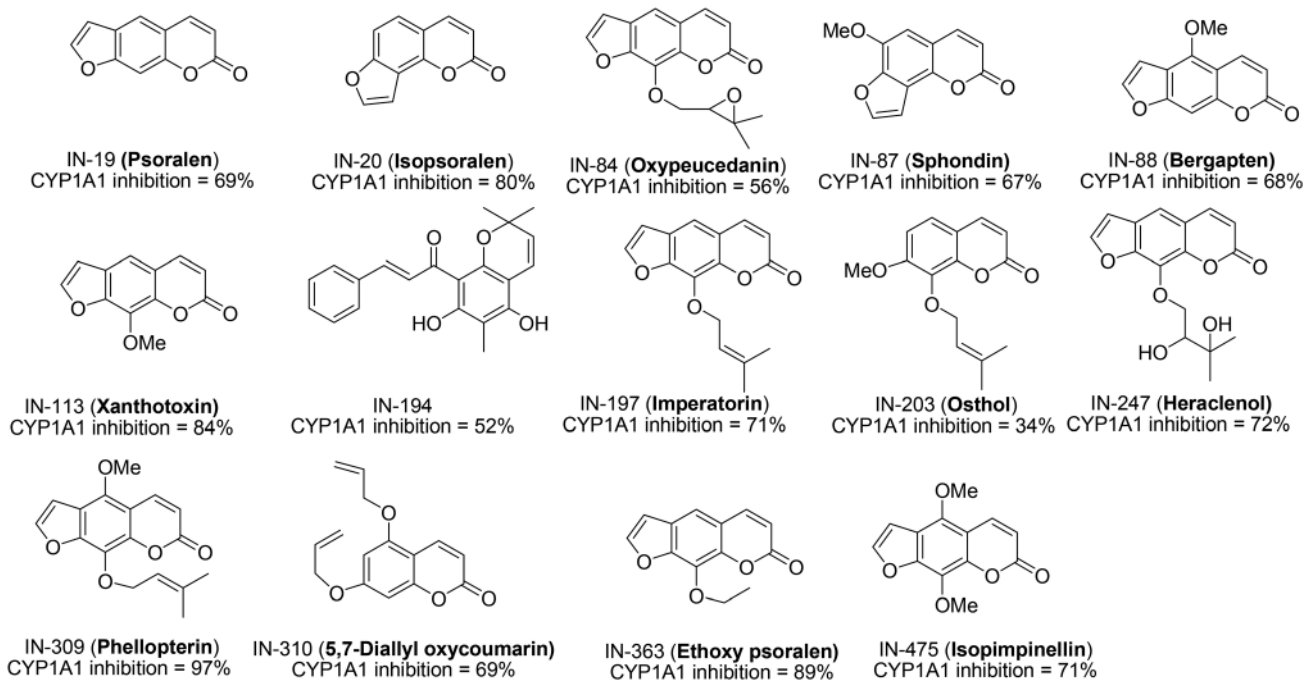

Fig. 4 Chemical structures of coumarins identified as CYP1A1 inhibitors.

The aim of using live HEK293 cells was to mimic an environment present in human hepatocytes (growing in suspension) which are preferred, over microsomal enzymes, by the pharmaceutical industry as a screening tool. The CYP-expressing HEK293 "suspension" cells are more similar to hepatocytes than microsomal enzymes with the added advantage that different "suspension" cell lines could be created *in situ*, according to the need of the experiment, which would express one or more active CYP enzymes. Recombinant plasmid-bearing cells in "suspension" allow rapid screening of chemical libraries for CYP inhibition using active enzymes lying within live cells. The expression of all CYP enzymes was validated by fluorescence-based kinetic activity assays. Assays similar to those used for microsomes were used for the recombinant CYP-expressing live cells. End-point assays were performed to determine the percentage inhibition and IC_{50} values.

Transiently transfected recombinant HEK293 cells were incubated with different concentrations of compounds for 30 min for the determination of IC_{50} values. After incubation, the activities of CYP1A1 and CYP1B1 were measured *via* the EROD assay, using 7-ethoxyresorufin (7-ER) as the substrate; for assaying the inhibition of CYP1A2, CYP2C9, and CYP2C19, 7-ethoxycoumarin (CEC) was used as the substrate whereas for CYP2D6 and CYP3A4, 7-ethoxy-methyloxy-3-cyanocoumarin (EOMCC) and dibenzylfluorescein (DBF) were the substrates. The percentage inhibition and IC_{50} of the natural products, identified in the CYP1A1-Sacchrosome assay, in a panel of CYP enzymes expressed within live human cells (HEK293 cells) are shown in Table 2.

Bergapten (IN-88), isopimpinellin (IN-475) and karanjin (IN-195) are the most potent inhibitors. The IC_{50} value for the inhibition of the human CYP1A1 enzyme expressed within human HEK-293 cells, after transfection with the plasmid pcDNA3.1/CYP1A1, was 80, 20 and 30 nM, respectively. Phellopterin (IN-309) inhibits CYP1A1 with moderate potency (IC_{50} = 580 nM).

From the alkaloid class of compounds, 6-methoxy chelerythrine (IN-229) and meridianin C (IN-333) inhibit the human CYP1A1 enzyme with IC_{50} values of 3.03 and 9.23 μM, respectively. From the coumarin class of compounds, bergapten (IN-88) and isopimpinellin (IN-475) display 192 and 319 fold selectivity for the 1A1 isoform over 1B1. However, another 'hit', phellopterin (IN-309), is a pan inhibitor of CYP1 enzymes. Flavonoids karanjin (IN-195), anisomalin (IN-226) and pongamol (IN-299) display moderate selectivity for CYP1A1 over the CYP1B1 isoform. Broadly, it is observed that most of the inhibitors are more or less equipotent in their inhibition of CYP1A1 and CYP1A2, with some of them inhibiting these two isoforms with IC_{50} values in the nanomolar range. However, they display good selectivity over the 1B1 isoform. More importantly, the 'hits' display excellent selectivity over 2D6, 2C9, 2C19 and 3A4 isoforms.

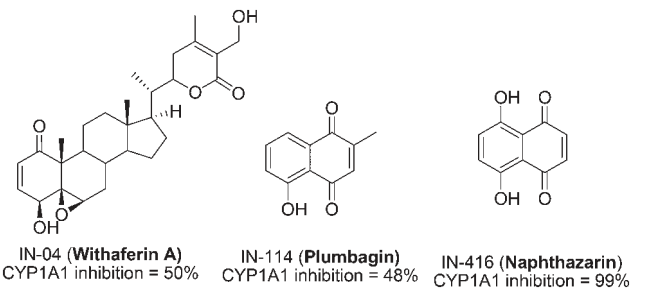

Fig. 5 Chemical structures of potent CYP1A1 natural products with miscellaneous scaffolds.

Table 1 Percentage inhibition of compounds having >50% inhibition of CYP1A1 in Sacchrosomes™ at 10 μM. Their percentage inhibition, at the same concentration, of CYP1B1, CYP1A2, CYP2D6, CYP3A4, CYP2C9 and CYP2C19is also shown

	% CYP inhibition at 10 μM[b]						
Code (compound name)	1A1	1A2	1B1	2D6	3A4	2C9	2C19
IN-88 (**Bergapten**)	68	73	20	9	3	4	5
IN-114 (**Plumbagin**)	51	55	40	10	15	11	12
IN-195 (**Karanjin**)	97	88	89	7	5	0	0
IN-199 (**Khellin**)	92	99	5	8	11	12	8
IN-226 (**Anisomalin**)	80	90	70	13	9	7	5
IN-229 (**6-Methoxy-chelerythrine**)	90	7	10	11	6	4	3
IN-299 (**Pongamol**)	85	8	12	14	9	0	3
IN-309 (**Phellopterin**)	87	96	98	20	12	3	5
IN-333 (**Meridianin C**)	52	55	7	5	10	3	2
IN-416 (**Naphthazarin**)	79	82	8	5	15	5	4
IN-475 (**Isopimpinellin**)	71	62	55	0	0	0	0
ANF[a]	97	95	96	5	6	10	9

[a] α-Naphthoflavone (ANF), a known CYP1 inhibitor, was used as a control in these studies. [b] % inhibition values are the average of three independent observations in triplicate.

Table 2 *In vitro* CYP enzyme inhibition in live human cells transfected with plasmids encoding the respective *CYP* genes

	% inhibition at 10 μM or IC_{50}/Ki in μM[a]						
Code (compound name)	1A1 % inhibition	1A1 IC_{50}	1A1 Ki	1A2 IC_{50}	1A2 Ki	1B1 IC_{50}	2D6, 2C9, 2C19 and 3A4 IC_{50}
IN-88 (**Bergapten**)	91%	0.08 ± 0.01	0.06 ± 0.01	0.09 ± 0.03	0.06 ± 0.02	15.3 ± 0.08	>10
IN-114 (**Plumbagin**)	86%	2.77 ± 0.09	2.56 ± 0.11	2.46 ± 0.09	2.36 ± 0.09	6.15 ± 0.04	>10
IN-195 (**Karanjin**)	90%	0.03 ± 0.005	0.019 ± 0.004	0.01 ± 0.003	0.006 ± 0.002	0.22 ± 0.05	>10
IN-199 (**Khellin**)	75%	4.02 ± 0.12	3.80 ± 0.11	0.04 ± 0.005	0.031 ± 0.005	34.6 ± 0.12	>10
IN-226 (**Anisomalin**)	96%	1.38 ± 0.08	1.42 ± 0.12	0.90 ± 0.06	0.78 ± 0.06	2.50 ± 0.21	>10
IN-229 (**6-Methoxy-chelerythrine**)	80%	3.03 ± 0.09	3.25 ± 0.15	8.93 ± 0.13	9.16 ± 0.18	7.70 ± 0.04	>10
IN-299 (**Pongamol**)	85%	2.23 ± 0.08	1.96 ± 0.13	3.16 ± 0.12	3.86 ± 0.22	6.04 ± 0.09	>10
IN-309 (**Phellopterin**)	90%	0.58 ± 0.03	0.62 ± 0.6	0.42 ± 0.08	0.38 ± 0.10	0.22 ± 0.002	>10
IN-333 (**Meridianin C**)	41%	9.23 ± 0.16	9.48 ± 0.22	8.72 ± 0.16	8.78 ± 0.22	0.213 ± 0.004	>10
IN-416 (**Naphthazarin**)	78%	3.82 ± 0.10	3.66 ± 0.16	2.16 ± 0.7	2.22 ± 0.55	>10	>10
IN-475 (**Isopimpinellin**)	91%	0.02 ± 0.005	0.012 ± 0.003	0.04 ± 0.004	0.028 ± 0.006	6.38 ± 0.92	>10

[a] The IC_{50}/Ki values represent the mean (± standard deviation) from three independent experiments.

From the results obtained from Sacchrosomes and human live cells, it can be concluded that the natural products show a much higher potency in live cells than in Sacchrosomes. It should be emphasized that CYP-expressing live cells mimic more closely the cells *in vivo* than the isolated microsomal enzymes. Further, upon considering the K_m of the CYP1A1 and CYP1A2 enzyme isoforms for substrate 7-ER conversion to the product in live HEK-293 cells, it was ascertained that the most active compound karanjin (IN-195) inhibits 1A1 and 1A2 CYP isoforms with Ki values of 0.019 and 0.006 μM, respectively. Similarly, isopimpinellin (IN-475) inhibits 1A1 and 1A2 isoforms with Ki values of 0.012 and 0.028 μM, respectively (Table 2).

Protection from B[*a*]P toxicity in CYP1A1-expressing normal adherent human cells

The plasmid pcDNA3.1/CYP1A1 was used along with the empty plasmid pcDNA3.1 (which contained no *CYP1A1* gene) for transfection of adherent human kidney HEK293 cells. HEK293 cells were cultured in RPMI1640 without L-glutamine (Lonza, BE12-167F) supplemented with 4 mM L-glutamine (Invitrogen, 25030024), 10% heat-inactivated foetal bovine serum (FBS; Sigma, F6178), 1% non-essential amino acids (Sigma, M7145) and 1% penicillin–streptomycin solution (Invitrogen, 15140-122). Transfected and non-transfected cells (~1 × 10^3) were seeded in a 96-well plate with different concentrations of benzo[*a*]pyrene B[*a*]P, in triplicate. Enzyme inhibition studies were carried out by co-administering at 2 × IC_{50} concentrations, in the cell culture medium, karanjin and isopimpinellin, the two most potent inhibitors of CYP1A1, and ANF (a known CYP1A1 inhibitor; data not shown). The MTT assay was performed using cells transfected with pcDNA3.1/CYP1A1 and pcDNA3.1 using protocols published earlier (Table 3).[25] Each experiment was performed independently three times. We observed that over-expression of CYP1A1 protein, from the plasmid pcDNA3.1/hCYP1A1, in adherent HEK293 cells can result in sensitivity to B[*a*]P when compared with cells that do not express CYP1A1 (Table 3). Resistance was observed by determining EC_{50} values (*i.e.* the concentration of B[*a*]P that provides half-maximal response to cell growth) by monitoring cell viability in the presence of B[*a*]P.

We then attempted to find out if the two potent CYP1A1 natural product inhibitors, karanjin and isopimpinellin, can overcome the sensitivity to B[*a*]P observed in CYP1A1-

expressing HEK293 cells. The results show that karanjin and isopimpinellin can indeed overcome CYP1A1-mediated sensitivity to B[*a*]P in adherent HEK293 cells (Table 4).

Molecular dynamic (MD) simulation of karanjin with CYP1 enzymes

The X-ray crystal structure of human CYP1A1 with ANF suggests that inhibitor interactions with the F-helix of CYP1A1/1B1/1A2 enzymes leads to narrowing of substrate binding sites.[22a] Karanjin (IN-195) interacts with heme atoms at the substrate binding site of CYP1B1 and blocks the formation of a reactive heme iron-oxo intermediate between the substrate and CYP1 family enzymes required for hydroxylation. In order to determine the rationale for the observed trend in the potency and selectivity of identified natural products, molecular modeling and molecular dynamic simulation studies were carried out with the panel of CYP enzymes. The furanoflavonoid karanjin is a compact hydrophobic molecule, which acts as a pan inhibitor of CYP1 family enzymes. It selectively inhibits CYP1 enzymes in comparison with CYP2 and CYP3 family members (like CYP3A4, CYP2D6, CY2C9 and CYP2C19).

During MD simulation, the RMSD of the protein backbone C-α atoms and individual inhibitors and RMSF in individual amino acid and ligand protein interactions were recorded with respect to time over a period of 10 ns of MD simulation. The RMSD in protein backbone C-α atoms and RMSF in individual amino acid side chain and ligand protein interactions are depicted in Fig. 6A–F. The total energy of the dynamic ligand–protein complex was found to be stable in the last 6 ns of the total simulation. Furthermore, the temperature, pressure, volume and potential energy of the complex remained constant, indicating the robustness and reliability of the MD simulation. The RMSD of the simulation converges to around 2.3 Å, which denotes the stability of the macromolecular ligand–protein complex in due course of 10 ns simulation. The RMSF in individual amino acid residues during the entire simulation is below 3.0 Å, indicating a lower degree of conformational movements in the side chains and that the complex is stable.

The final frame analysis at the ANF binding site shows that the terminal 2-phenyl ring of karanjin interacts with the heme atom and aromatic π clouds of the chromane core interacts with the F-helix Phe224 residue *via* hydrophobic π–π interactions. However, its higher potency for the CYP1A2 isoform is due to the additional polar H-bonding with the Gly316 residue at the I-helix and hydrophobic π–π interactions with the Phe226 residue in the F-helix. In the case of the CYP1B1 isoform, similar to ANF, karanjin adopts a 180° flip in orientation although other hydrophobic interactions remain similar including hydrophobic π–π interactions with Phe231 (*i.e.* the corresponding residue of Phe224 of CYP1A1) in the F helix as shown in Fig. 7A–F. However, despite the similar mechanistic and functional space, it displays a huge difference in inhibition potency towards CYP3A4, CYP2D6, CYP2C9 and CYP2C19 due to the difference in the structural architectures and functional properties of these enzymes (*i.e.* shape and amino acid residue that form edges of the cavity)[26] which ultimately leads to loss of necessary polar and hydrophobic π–π interactions with CYP2D6, CYP2C9 and CYP2C19.

Similar to karanjin (IN-195), isopimpinellin (IN-475) also inhibits the CYP1 family enzymes with greater propensity in comparison with the CYP2 and CYP3 enzyme isoforms. The final frame analysis of IN-475 suggests that it interacts with hydrophobic Phe-224 in the F helix and the phe-123 residue *via* π–π interactions. No H-bonding was observed like in ANF and karanjin (IN-195). The details of the molecular dynamic studies of isopimpinellin (IN-475) with CYP1A1 are provided in ESI‡ section S3 and molecular docking results with the CYP2 and CYP3 isoforms are provided in ESI‡ section S4. The details of molecular docking studies of IN-195 with CYP2 and CYP3 isoforms are provided in ESI‡ section S5.

Table 3 EC_{50} values of B[*a*]P after treatment of cells with a range of concentrations of B[*a*]P (0.05–100 μM)[a]

Cell line	EC_{50} of B[*a*]P
HEK293::—	14 ± 1.2
HEK293::pcDNA3.1	12.8 ± 0.9
HEK293::pcDNA3.1/CYP1A1	0.8 ± 0.1

[a] The cells used were: (a) untransfected HEK293 cells (HEK293::—), (b) HEK293 cells transfected with pcDNA3.1 (*i.e.* HEK293::pcDNA3.1, the basic plasmid which does not contain a gene insert) and (c) HEK293 cells transfected with pcDNA3.1/hCYP1A1. All values, presented in μM concentrations, represent the mean and standard deviations of three independent experiments in triplicate.

Conclusion

The screening of a natural product repository comprising diverse classes of natural products resulted in the identification of several potent inhibitors of the CYP1A1 enzyme. Bergapten (IN-88), karanjin (IN-195) and isopimpinellin (IN-475) potently inhibit this enzyme in live human cells, grown in suspension, with IC_{50} values between 20–80 nM. This indicates that these inhibitors definitely have good cellular efficacy. The three inhibitors do not inhibit CYP2 and CYP3 family isoforms indicating that they may be devoid of any drug–drug interaction liability. The observed selectivity was demonstrated using molecular modeling studies. Moreover, karanjin and isopimpinellin protect adherent CYP1A1-expressing human HEK293 cells from B[*a*]P-mediated toxicity indicating that the two natural products, isolated from edible plants, could have potential as chemopreventive agents, blocking the metabolism of pro-carcinogens to active carcinogens.

Experimental section

General experimental procedures

All chemicals were obtained from Sigma-Aldrich Company and used as received. ^{1}H, ^{13}C and DEPT NMR spectra were recorded on Bruker-Avance DPX FT-NMR 500 and 400 MHz instruments. Chemical data for protons are reported in parts per million (ppm) downfield from tetramethylsilane and are

Table 4 EC_{50} values of B[*a*]P after treatment of CYP1A1-expressing HEK29 cells with B[*a*]P and a CYP1A1 inhibitor[a]

EC_{50} of B[*a*]P in μM			
Without compound	In the presence of karanjin (IN-195)	In the presence of isopimpinellin (IN-475)	In the presence of ANF
0.8 ± 0.1	13.5 ± 1.0	13.8 ± 1.1	1.8 ± 0.2

[a] A range of concentrations of B[*a*]P (0.05–100 μM) was used, at 2 × IC_{50} values (as was determined in the human cell assay where cells were grown in suspension): karanjin, 0.06 μM; isopimpinellin, 0.04 μM; and [ANF], 0.016 μM. The cells used were HEK293 cells transfected with pcDNA3.1/hCYP1A1 (the plasmid which encodes the human *CYP1A1* gene). All values, presented in μM concentrations, represent the mean and standard deviations of three independent experiments in triplicate.

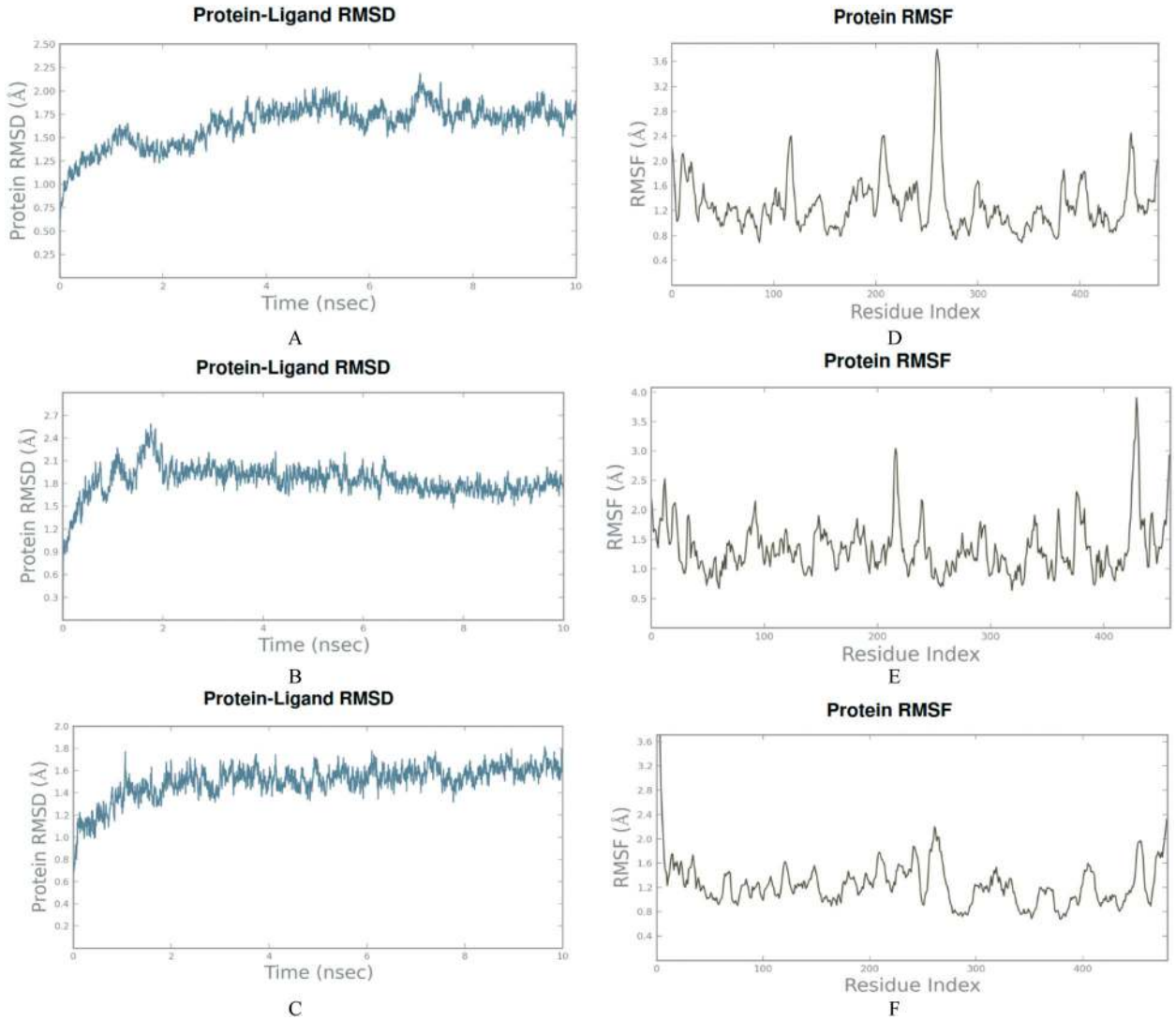

Fig. 6 MD simulation and interactions of karanjin (IN-195) with CYP enzymes. A–C: RMSD in CYP1A1, CYP1B1 and CYP1A2 enzyme C-α carbon during MD simulation, D–F: RMSF in the side chains of CYP1A1, CYP1B1 and CYP1A2 during MD simulation.

referenced to the residual proton in the NMR solvent ($CDCl_3$, 7.26 ppm; CD_3OD, 3.31 ppm). ESIMS spectra were recorded on an Agilent 1100 LC-QTOF mass spectrometer.

Natural product repository

The natural product repository (394 compounds) generated from compounds isolated from our in-house phytochemical investigation efforts on Indian medicinal plants was used for screening. In general, the compounds presented in the in-house NP repository exhibit HPLC purity >85%. The repository consisted of structures belonging to diverse chemotypes, including phenolics (flavonoids, coumarins), alkaloids, terpenoids, lignans, glycosides and polysaccharides.

Isolation of karanjin from *Pongamia pinnata*

The dried and powdered seeds (2.5 kg) of *Pongamia pinnata* were extracted with methanol (3 × 2 L) by cold percolation and the combined filtrate was concentrated to afford 1000 g

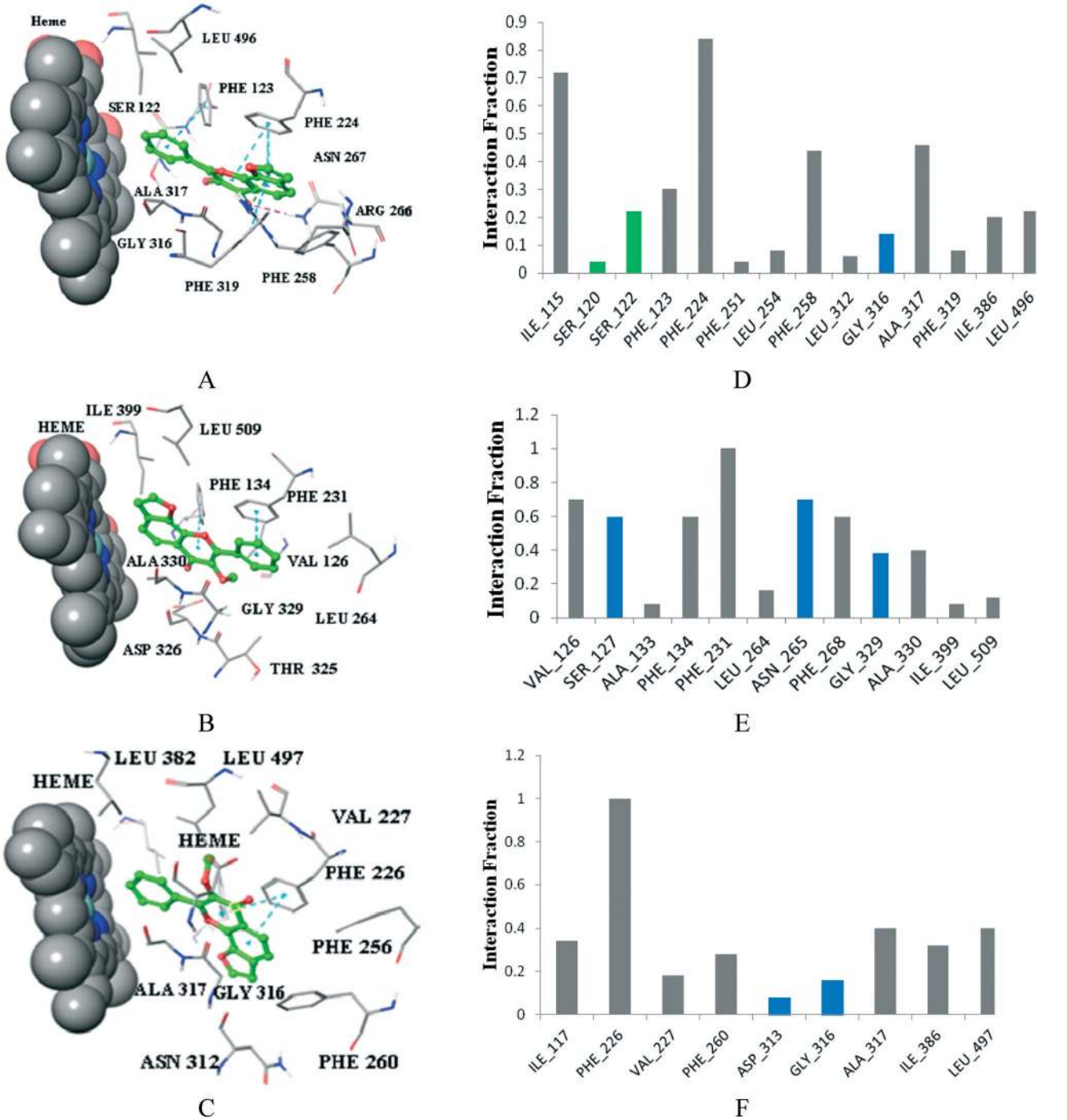

Fig. 7 Interactions of karanjin (IN-195) with CYP enzymes. A–C: 3D-depiction of karanjin (IN-195) interactions with 1A1, 1B1 and 1A2 CYP isoforms, respectively. D–F: Contact histograms showing the interactions of karanjin (IN-195) in due course of MD simulations. Grey colour represents hydrophobic, blue colour represents ionic and green colour represents H-bonding interactions.

of extract. This extract was suspended in water and sequentially fractionated with different solvents including hexane, ethyl acetate, chloroform, and *n*-butanol yielding 200, 400, 90 and 120 g extracts, respectively. The ethyl acetate fraction (400 g) was subjected to column chromatography using a step-gradient system of hexane and ethyl acetate in the range of 3 to 50%, which yielded ten major fractions. The 9th fraction produced karanjin (200 mg), which was obtained in pure form by crystallization in methanol. Karanjin (IN-195): white needle-shaped crystalline solid; HPLC: t_R = 3.7 min (100% purity); 200 mg; IR ($CHCl_3$): ν_{max} 3443, 2928, 1625, 1603, 1527, 1464, 1445, 1371, 1340, 1285, 1226 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 8.19 (d, J = 8.8 Hz, 1H, CH), 8.15 (dd, J = 7.7 Hz, 1.6 Hz, 1H, CH), 8.14 (m, 1H, CH), 7.76 (d, J = 1.7 Hz, 1H, CH), 7.56–7.54 (m, 4H, CH), 7.18 (d, J = 1.2 Hz, 1H, CH), 3.93 (s, 3H, OMe); ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm)

175.0, 158.1, 154.8, 149.9, 145.7, 141.8, 130.9, 130.6, 128.6, 128.3, 121.8, 119.6, 117.0, 110.0, 104.2, 60.2; HR-ESIMS: *m*/*z* 293.0810 $[M + H]^+$ calcd for $C_{18}H_{12}O_4 + H^+$ (293.0808).

Isolation of isopimpinellin from *Cnidium monnieri*

500 g of dried powder of *Cnidium monnieri* was extracted with 2 L of methanol for 24 h under stirring three times. The extracts were filtered, combined and evaporated to obtain a crude semisolid mass at 45 °C under reduced pressure. The enriched extract was dissolved in water (1 L) and extracted with light petroleum ether four times (500 ml each time). Further, the organic layer was combined, evaporated and purified by silica gel column chromatography using hexane : ethyl acetate with gradual increment in ethyl acetate fractions to obtain isopimpinellin (IN-475). White solid; ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 8.14 (d, J = 9.6 Hz, 1H, CH), 7.63 (d, J = 2.8 Hz, 1H, CH), 7.01 (d, J = 2.4 Hz, 1H, CH), 6.25 (d, J = 10 Hz, 1H, CH), 4.15 (s, 3H, OCH_3), 4.12 (s, 3H, OCH_3); HR-ESIMS: *m*/*z* 247.0612 $[M + H]^+$ calcd for $C_{13}H_{10}O_5 + H^+$ (247.0601).[27]

CYP screening using Sacchrosomes™

All CYP enzymes (Sacchrosomes™) used in this study were manufactured by CYP Design Ltd (Leicester, UK). This method was used to measure the percentage inhibition of CYP450 by a compound or to determine the IC_{50} value (the concentration at which 50% of the enzyme activity is inhibited) of a compound. Both percentage inhibition and IC_{50} values effectively reflect the inhibitory potential of a compound and hint at the possible effectiveness of a compound in a biological process. The percentage inhibition is determined at a particular concentration of the compound which is usually 10 μM. An assay which determines IC_{50} values includes the yeast microsomes that bear the cytochrome P450 enzymes (*i.e.* Sacchrosomes™), a chosen chemical compound in six serial dilutions in DMSO (with DMSO concentration never exceeding 0.5%), a 96-well flat-bottomed microtiter plate, and substrates such as ER (7-ethoxyresorufin), CEC (3-cyano-7-ethoxycoumarin), EOMCC (7-ethoxy-methyloxy-3-cyanocoumarin) or DBF (dibenzylfluorescein), depending on the CYP450 used in the assay. The substrates form fluorescent compounds upon CYP metabolism. A fluorescence plate reader is used to monitor the fluorescence emitted which ultimately determines IC_{50} values *via* measurement of fluorescence units at each endpoint (*i.e.* at each concentration of the compound used). As it is obvious that some of the compounds can be fluorescent, therefore the values (obtained in wells that contained only compounds and no enzyme) were subtracted from the values obtained in wells which contained enzyme + compound. The detailed procedure is provided in our previous publications[19] and the ESI‡ (section S1). All experiments were performed independently in triplicate.

CYP screening using recombinant human HEK293 cells

The adherent HEK293 cells were originally obtained from ECACC. The suspension cells were created from the adherent cells in our laboratory over-expressing particular CYP enzymes.[19] The western blot showing levels of expression of CYPs 1A1, 1B1, 1A2, 2D6, 2C9, 2C19, and 3A4 measured in recombinant HEK293 cells is shown in section S1 of the ESI.‡ For screening of potential inhibitors of these CYPs, recombinant HEK293 cells (100×10^3) expressing CYP enzymes were seeded in 50 μL volume in triplicate in black 96-well plates with a transparent bottom (Corning #3904). The test compounds either at single point concentrations (10 μM) or at various concentrations (from 1 nM to 30 μM) for determination of IC_{50} values were added in 25 μL volume to the wells followed by incubation at 37 °C with 8% CO_2 for 30 min. After incubation, a fluorogenic substrate was added at 5 μM concentration in 25 μL to the wells and the contents were mixed by shaking. The plate was read on a plate-reader (Biotek, Synergy HT) for 60 min using suitable wavelengths for emission/excitation of the fluorescent products formed. IC_{50} values were calculated using Graph-Pad Prism Software (version 6.0).

Time-dependent inhibition studies were performed under the 10-times dilution two-step incubation protocol, as has been published previously.[28] The time for pre-incubation of enzymes with compound(s) was 0, 5, 10, 20, 30 and 45 min.

Protection from B[*a*]P toxicity in CYP1A1-expressing normal adherent human cells

The plasmid pcDNA3.1/CYP1A1 was used along with the empty plasmid pcDNA3.1 (which contained no *CYP1A1* gene) for transfection of adherent human kidney HEK293 cells. HEK293 cells were cultured in RPMI1640 without L-glutamine (Lonza, BE12-167F) supplemented with 4 mM L-glutamine (Invitrogen, 25030024), 10% heat-inactivated foetal bovine serum (FBS; Sigma, F6178), 1% non-essential amino acids (Sigma, M7145) and 1% penicillin–streptomycin solution (Invitrogen, 15140-122). Transfected and non-transfected cells ($\sim 1 \times 10^3$) were seeded in a 96-well plate with different concentrations of B[*a*]P, in triplicate. Enzyme inhibition studies were carried out by co-administering the two most potent inhibitors of CYP1A1 that were identified and ANF (a known CYP1A1 inhibitor) at 1 × IC_{50} concentrations, in the cell culture medium. The MTT assay was performed using cells transfected with pcDNA3.1/CYP1A1 and pcDNA3.1 using protocols published earlier.[25] Each experiment was performed three times and statistical parameters were calculated.

Molecular modeling of identified natural product 'hits' with CYP enzymes[19]

The human CYP family enzymes are oxidoreductase enzymes involved in xenobiotic metabolism reactions mainly hydroxylation of aromatic and other substrates. Human P450 1B1 shares only 38% and 36% amino acid sequence identity with human P450s 1A1 and 1A2, respectively. The planar and compact structural architecture of CYP1 family members is quite different from that of CYP2 and CYP3 family members. The crystal structures of CYP enzymes CYP1A1 (PDB ID: 4I8V),[22a]

CYP1B1 (PDB ID: 3PMO),[29] CYP1A2 (PDB ID: 2HI4),[30] CYP2D6 (PDB ID: 4WNT),[26b] CYP2C9 (PDB ID: 1R9O)[31] CYP2C19 (PDB ID: 4GQS)[32] and CYP3A4 (PDB ID: 4NY4)[26a] were retrieved from the protein data bank and subjected to a protein preparation wizard facility under default conditions implemented in Maestro v9.0 and Impact program v5.5 (Schrodinger, Inc., New York, NY, 2009). The prepared protein was further utilized to construct a grid file by selecting a co-crystallized ligand as the centroid of the grid box. For standardization of the molecular docking procedure, co-crystallized ligands such as ANF (CYP1A1, CYP1B1 and CYP1A2), ajmalicine (CYP2D6) flurbiprofen (CYP2C9), 0XV (CYP2C19) and bromocriptine (CYP3A4) were extracted from the prepared enzyme–ligand complex and redocked to their binding site. The rest of the chemical structures were sketched, minimized and docked using GLIDE XP.[33] The ligand–protein complexes were minimized using a macromodel. In order to determine the selectivity, the corresponding binding site of CYP enzymes is flexibly aligned and analyzed with respect to CYP1A1.

Molecular dynamic simulations

The ligand–protein docked complex (.pv file) of CYP1A1, CYP1B1 and CYP1A2 obtained from XP docking (as described above) was subjected to a system builder, in which the protein inhibitor complex was solvated using explicit TIP3P water molecules in an orthorhombic box. A box having a 12 Å radius was used to define the core, and the overall complex was neutralized by adding one Cl^- counter ion for simulation. Furthermore, this complex was minimized by the steepest descent method followed by the Broyden–Fletcher–Goldfarb–Shanno algorithm with a convergence threshold of 2.0 kcal mol^{-1} and 1000 iterations. MD simulations were carried out at normal temperature and pressure (300 K and 1.01325 bar, respectively). The Nose–Hoover chain thermostat and Martyna–Tobias–Klein barostat methods were used, with the ensemble pathway comprising NVT (constant number of particles, volume, and temperature) and the isotropic coupling method. The overall model system was relaxed for 2 ns before a 10 ns simulation, and coulombic interactions were defined by a short-range cutoff radius of 9.0 Å and by a long-range smooth particle mesh Ewald tolerance of 1×10^{-9}. MD simulation was performed using a multistep protocol present in Desmond software (version 3.8) with the OPLS-2005 force field. Recording intervals of 1.2 ps and 4.8 ps were used for energy calculation and trajectory analysis.

Abbreviations

ANF	α-Naphthoflavone
B[*a*]P	Benzo[*a*]pyrene
CEC	3-Cyano-7-ethoxycoumarin
CFU per mL	Colony forming unit per mL
CYP1A1	Cytochrome P450 group enzyme 1A1
CYP1A2	Cytochrome P450 group enzyme 1A2
CYP1B1	Cytochrome P450 group enzyme 1B1
CYP2D6	Cytochrome P450 group enzyme 2D6
CYP3A4	Cytochrome P450 group enzyme 3A4
DMBA	7,12-Dimethylbenz[*a*]anthracene
DMSO	Dimethyl sulfoxide
DBF	Dibenzylfluorescein
EOMCC	7-Ethoxy-methyloxy-3-cyanocoumarin
ER	7-Ethoxyresorufin
EROD	7-Ethoxyresorufin-*O*-deethylase
hCYP	Human cytochrome P450
MIC	Minimum inhibitory concentration
MDR	Multi drug resistance
MMGB/SA	Molecular mechanics Born generalized/surface area
PAH	Polyaromatic hydrocarbons
TCDD	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin
RMSF	Root mean square fluctuation
RMSD	Root-mean-square deviation
Pgp	P-glycoprotein
S. aureus	*Staphylococcus aureus*
MDR	Multi-drug resistance
Rh123	Rhodamine 123

Conflicts of interest

The authors declare no competing interests.

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