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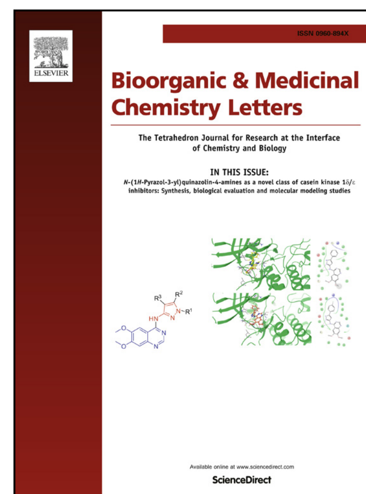

Synthesis and biological evaluation of pyrrole-based chalcones as CYP1 enzyme inhibitors, for possible prevention of cancer and overcoming cisplatin resistance

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PII: S0960-894X(17)30703-5
DOI: http://dx.doi.org/10.1016/j.bmcl.2017.07.010
Reference: BMCL 25123

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 23 May 2017
Revised Date: 1 July 2017
Accepted Date: 3 July 2017

Please cite this article as: Williams, I.S., Joshi, P., Gatchie, L., Sharma, M., Satti, N.K., Vishwakarma, R.A., Chaudhuri, B., Bharate, S.B., Synthesis and biological evaluation of pyrrole-based chalcones as CYP1 enzyme inhibitors, for possible prevention of cancer and overcoming cisplatin resistance, *Bioorganic & Medicinal Chemistry Letters* (2017), doi: http://dx.doi.org/10.1016/j.bmcl.2017.07.010

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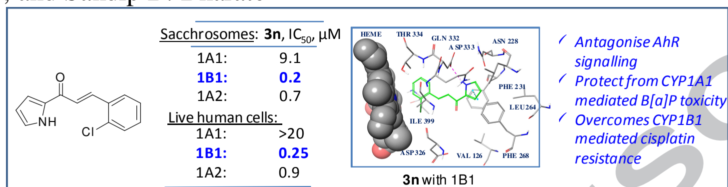

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Synthesis and biological evaluation of pyrrole-based chalcones as CYP1 enzyme inhibitors, for possible prevention of cancer and overcoming cisplatin resistance

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ARTICLE INFO

Article history:
Received
Revised
Accepted
Available online

Keywords:
Heterocyclic chalcones,
AhR antagonism,
CYP1A1,
Chemoprevention,
CYP1B1,
Cisplatin chemo-resistance.

ABSTRACT

Inhibitors of CYP1 enzymes may play vital roles in the prevention of cancer and overcoming chemo-resistance to anticancer drugs. In this letter, we report synthesis of twenty-three pyrrole based heterocyclic chalcones which were screened for inhibition of CYP1 isoforms. Compound **3n** potently inhibited CYP1B1 with an IC_{50} of ~0.2 µM in Sacchrosomes[TM] and CYP1B1-expressing live human cells. However, compound **3j** which inhibited both CYP1A1 and CYP1B1 with an IC_{50} of ~0.9 µM, using the same systems, also potently antagonized B[a]P-mediated induction of AhR signaling in yeast (IC_{50}, 1.5 µM), fully protected human cells from B[a]P toxicity and completely reversed cisplatin resistance in human cells that overexpress CYP1B1 by restoring cisplatin's cytotoxicity. Molecular modeling studies were performed to rationalize the observed potency and selectivity of enzyme inhibition by compounds **3j** and **3n**.
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Cytochrome P450 (CYP) enzymes are heme containing monooxygenases. Around 15 of them are responsible for phase I metabolism acting mainly to hydroxylate pharmaceuticals and exogenous substances, some of which may have the potential to be carcinogenic.[1] The CYP1 sub-family of enzymes consists of 1A1, 1B1 and 1A2 isoforms. The CYP1A1 isoform catalyzes hydroxylation of a large number of pro-carcinogens, such as polyaromatic hydrocarbons (PAHs), oxides, amines, and estrogens (E) converting them to cytotoxic, mutagenic and carcinogenic chemicals.[2-5] Overexpressed CYP1A1 isoform can metabolize the PAH benzo[a]pyrene [BaP] into benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide which subsequently can form a quinone intermediate which can covalently react with DNA and lead to DNA damage.[6-15] Similarly, CYP1B1 overexpression is responsible for the increased metabolism of anticancer drugs such as paclitaxel, docetaxel, doxorubicin, mitoxantrone, tamoxifen, and cisplatin in various cancer cells.[16] As a result, the cellular efficacy of cytotoxic drugs is reduced and eventually cancer cells, which overexpress CYP1B1, become resistant to a variety of chemotherapeutic agents. Recent studies have demonstrated that CYP1B1 inhibitors can overcome docetaxel resistance,[16, 17] as well as cisplatin resistance in CYP1B1-overexpressing cells.[18]

Various biochemical and cellular studies suggest that basal expression of CYP1 enzymes in healthy tissue is very low, in contrast to tissues exposed to pro-carcinogens or drugs. This selective behavior offers an opportunity to a medicinal chemist and biologist for prevention of CYP1A1-mediated lung carcinogenesis caused by B[a]P in individuals who smoke.

Several natural and synthetic compounds have been reported as potent inhibitors of CYP1 enzymes e.g. resveratrol, quercetin, and rosmarinic acid.[19-21] In continuation of our efforts in this area,[18, 22, 23] herein we report design and synthesis of 2-pyrrole based chalcones, to combat CYP1B1-mediated cisplatin resistance and CYP1A1-mediated B[a]P toxicity. A total 23 chalcones were synthesized as potential CYP1 family inhibitors using the classical Claisen-Schimdt condensation.[24-26] This reaction offers coupling of equimolar amounts of aromatic aldehydes and acetophenones in either acidic or basic conditions or vice versa. 2-Pyrrole chalcones synthesized using solution phase or solid grinding method gives moderate to good yields (24-73%). The synthetic scheme is provided in Scheme 1. All compounds were characterized by NMR, IR and HRMS analysis.

All synthesized 2-pyrrole chalcones were screened for inhibition of CYP1, CYP2 and CYP3 family enzymes using Sacchrosomes[TM] which are endoplasmic reticulum-bound CYP-reductase complexes isolated from recombinant baker's yeast, *Saccharomyces cerevisiae.* Results of CYP inhibition are depicted in Table 1. *In-vitro* CYP inhibition data suggest that all chalcones selectively inhibit CYP1 enzymes. The 2-pyrrole chalcone **3j** inhibits all three isoforms of CYP1 with almost equal potency. However, another 2-pyrrolyl chalcone derivative **3n**

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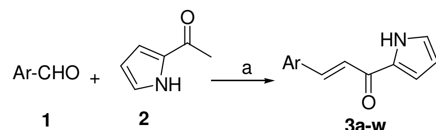
selectively inhibits CYP1B1 with an IC_{50} value of 210 nM. Based on *in-vitro* data, few key structure-activity relationship (SAR) features have been drawn for these heterocyclic chalcones. These include (a) 2-pyrrole substituted class of CYP1 inhibitors can be selective for CYP1B1 isoform (b) halogen or methoxy substitution on non-heterocyclic ring (e.g. 2-chloro substituted compound **3n**) is essential for activity (c) alkoxy substitution on the non-heterocyclic ring is critical for potency of CYP1 inhibition.



3a: Ar = Ph (3-OMe)
3b: Ar = Ph (2,6-di-OMe)
3c: Ar = Ph (2-Cl,5-NO_2)
3d: Ar = Ph (2,3-di-OMe)
3e: Ar = Ph (3,4-Methylene-dioxy)
3f: Ar = Ph (2,4-di-OMe)
3g: Ar = Cinnamyl
3h: Ar = Ph (3,5-di-OMe)
3i: Ar = Ph (2-F)
3j: Ar = Ph (2-OMe)
3k: Ar = Cinnamyl (4-OMe)
3l: Ar = Ph (4-N(CH_3)$_2$)
3m: Ar = Ph (4-OMe)
3n: Ar = Ph (2-Cl)
3o: Ar = Ph (3-Br)
3p: Ar = Ph (3-Br, 4-OMe)
3q: Ar = Furan-2-yl (5-Et)
3r: Ar = Ph (3,4.5-tri-MeO)
3s: Ar = Ph (4-OCF_3)
3t: Ar = Ph (4-COOH)
3u: Ar = Anthracen-9-yl
3v: Ar = Ph (3-Cl)
3w: Ar = Ph (4-Br)

Scheme 1. Reagents and conditions: (a) 5 equivalents NaOH/KOH with solid state grinding using mortar and pestle.

The failure of various CYP inhibitors in drug development is due to the lack of drug-like features and cellular efficacy. In order to overcome this problem, we established CYP-expressing live human cell based assays, for CYP inhibition studies.[18, 22, 23] The CYP1 inhibitors identified using Sacchrosomes^{TM} were tested for inhibition of CYP1, CYP2 and CYP3 family of enzymes in live human kidney HEK293 cells (results are shown in Table 2).[27, 28] The compound **3s**, which was inactive in Sacchrosomes, was also tested in live cells, as a negative control.

Table 2. The IC_{50} values for inhibition of CYP s expressed in live HEK293 cells by the most potent chalcones identified in assays using Sacchrosomes^{TM}.

	IC_{50} values for CYP inhibition in live HEK293 cells (μM)						
	1A1	**1B1**	**1A2**	**2D6**	**2C9**	**2C19**	**3A4**
3j	1.2±0.1	1.2±0.1	1.1± 0.1	20±0.6	>10	>10	>10
3n	>20	0.25±0.05	0.9 ± 0.2	>10	>10	>10	>10
3s	>20	>20	>20	>20	>20	>20	>20
ANF	>10	>10	nd	nd	nd	nd	nd

[a]α-naphthoflavone (ANF) was used as a control in these studies; The IC_{50} values represent mean and standard deviations from three independent experiments. nd: not determined

The compounds **3j** and **3n** bearing methoxy or chloro substitutions at 2-position inhibit all three CYP1 family enzymes in varying degrees, as it was seen with Sacchrosomes^{TM}. It was observed that the 2-pyrrole chalcone **3j** is not CYP1B1 specific since it also inhibits the 1A1 isoform with equal efficacy (IC_{50} = 1.2 μM). However, **3n** is specific to CYP1B1 inhibitor, IC_{50} = 0.25 μM compared to the CYP1A1, IC_{50} = >20 μM. Nonetheless, both **3j** and **3n** display excellent specificity (>20 fold in Sacchrosomes^{TM} and live human cells) for CYP1 family enzymes with respect to CYP2 and CYP3 family isoforms. Inhibition of CYP2/ CYP3 enzymes can be the cause of deleterious drug-drug interactions which often thwart further drug development. Sacchrosomes^{TM} and recombinant live human cell assays indicate that the 2-pyrrole chalcones **3j** and **3n** can avoid such harmful interactions.

In order to gain further insight into the experimental CYP inhibition efficacy and selectivity, molecular modeling studies

Table 1. CYP inhibition activity of pyrrole chalcones **3a-w** in Sacchrosomes^{TM}

Entry	**IC_{50} values for CYP inhibition (μM)**						
	1A1	**1B1**	**1A2**	**2D6**	**2C9**	**2C19**	**3A4**
3a	6.2±0.2	5.2± 0.1	1.5±0.1	11±0.2	>20	>20	>20
3b	10.8 ±0.3	>20	10.0±0.5	18±0.4	>20	>20	>20
3c	>20	>20	10.9±0.4	12± 0.2	>20	>20	>20
3d	5.2±0.2	>20	9.2±0.4	16± 0.3	>20	>20	>20
3e	5.8±0.2	>20	9.7±0.5	14± 0.2	>20	>20	>20
3f	11.8±0.2	>20	10.0±02	11± 0.1	>20	>20	>20
3g	>20	>20	10.5±0.3	12±0.2	>20	>20	>20
3h	6.1±0.4	>20	9.0±0.2	15±0.4	>20	>20	>20
3i	>20	2.7±0.1	2.2±0.08	16±0.4	>20	>20	>20
3j	0.9±0.07	0.9 ± 0.1	1.1±0.04	20±0.6	>20	>20	>20
3k	>20	>20	13±0.2	18 ±0.4	>20	>20	>20
3l	>20	>20	12.5±0.1	13±0.2	>20	>20	>20
3m	>20	>20	11.8±0.2	16±0.3	>20	>20	>20
3n	9.1±0.4	0.2±0.04	0.7±0.1	12±0.16	>20	>20	>20
3o	>20	13±0.4	>20	14±0.25	>20	>20	>20
3p	>20	>20	17±0.5	13±0.3	>20	>20	>20
3q	>20	9.2±0.2	2.5±0.08	17±0.4	>20	>20	>20
3r	>20	>20	15±0.4	14±0.2	>20	>20	>20
3s	>20	>20	>20	16±0.3	>20	>20	>20
3t	>20	>20	>20	18±0.4	>20	>20	>20
3u	>20	>20	>20	12±0.15	>20	>20	>20
3v	16±0.5	1.5±0.1	12±0.3	14 ±0.2	>20	>20	>20
3w	>20	8.5±0.2	13.2±0.2	18±0.4	>20	>20	>20
ANF	0.01± 0.002	0.05± 0.01	0.03± 0.01	>20	>20	>20	>20

[a]α-naphthoflavone (ANF) was used as a control in these studies; The IC_{50} values represent mean and standard deviations from three independent experiments.

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were performed with the chalcones using the 3D structures of isoforms of the CYP1, CYP2 and CYP3 sub-families. Analysis of ANF-bound CYP1A1 and CYP1B1 X-ray derived structures indicate that they share similar characteristics such as canonical helices, β-sheets, and two short helixes F' and G', which overall form the binding site for ANF. The ligand ANF interacts with the macromolecules by hydrophobic π-π interactions which involve multiple aromatic residues of CYP1A1 and CYP1B1.[29-31]

Upon analysis of molecular models, it was observed that the heteroaromatic nucleus of **3j** interacts with the CYP1A1 heme-porphyrin complex and the 2-methoxyphenyl ring interacts with the Phe-224 residue by π-π interactions. In case of CYP1B1, the pyrrole ring of **3j** interacts with the heme unit and the 2-methoxy phenyl ring interacts with the Phe-231 residue (which corresponds to Phe-224 of CYP1A1). Similarly, the most potent 2-chloro substituted analog, **3n**, interacts with CYP1B1's Phe-231 residue in its F-helix by π-π interactions. However, the ANF binding site of CYP1B1 contains the less hydrophobic Gln-332, corresponding to the highly hydrophobic Phe-319 residue of CYP1A1.This allows moderate flexibility in the substrate binding site, leading to a 180° flip in ligand orientation which is also seen in the ANF-CYP1B1 X-ray crystal structure.[30, 31] The 180° flip in orientation is stabilized by H-bonding with the Gln^{332}-Asp^{333} loop in I helix. Overall, these interactions prevent formation of a reactive heme-oxo intermediate between substrate, heme complex and the CYP1 enzyme necessary for the oxidative metabolism of a specific substrate, e.g. B[a]P, estrogen or anticancer drugs. This is shown in Figure 1. For selectivity studies, the ligand-bound 10 Å binding site of CYP2D6 and CYP3A4 isoforms were aligned to CYP1A1's binding site.

CYP2 and CYP3 family enzymes have large substrate binding cavity which is flexible enough to accommodate structurally diverse chemical scaffolds. Due to this, **3j** and **3n** interactions with heme complex become distorted and thus both compounds lose their inhibitory activity for these isoforms (images are shown in supporting information - Section S3).

The aromatic hydrocarbon receptor, AhR, a member of the hormone receptor family, is a transcription factor responsible for induction of the *CYP1* sub-family of genes. PAHs like B[a]P act as AhR ligands. Ligand-bound AhR induces transcription of *CYP1* genes. It is reported that certain compound families that function as CYP1 enzyme inhibitors can compete with PAHs for the ligand-binding site of AhR[21, 32] to act as antagonists for repression of *CYP1* gene expression. In order to study the role of the 2-pyrrole chalcones in AhR signaling, a yeast cell system was used to assess the regulation of a reporter, the enhanced green fluorescence protein (eGFP) which is controlled by a basal promoter containing a concatamer of five xenobiotic response element (XREs). A tripartite complex consisting of AhR, its co-activator ARNT and a PAH ligand (e.g. B[a]P) binds to the XRE concatamer to induce eGFP expression. The optimization of B[a]P concentration, used for induction, is provided in Table S3. In yeast, the 2-pyrrole chalcones, **3j** and **3n**, act as concentration-dependent antagonists of AhR, activated by B[a]P (as shown in Table 3), with IC_{50} values of 1.5 µM and 7.6 µM, respectively. The compound **3s**, which was inactive in Sacchrosomes and live cells, was also tested in this assay, as a negative control.

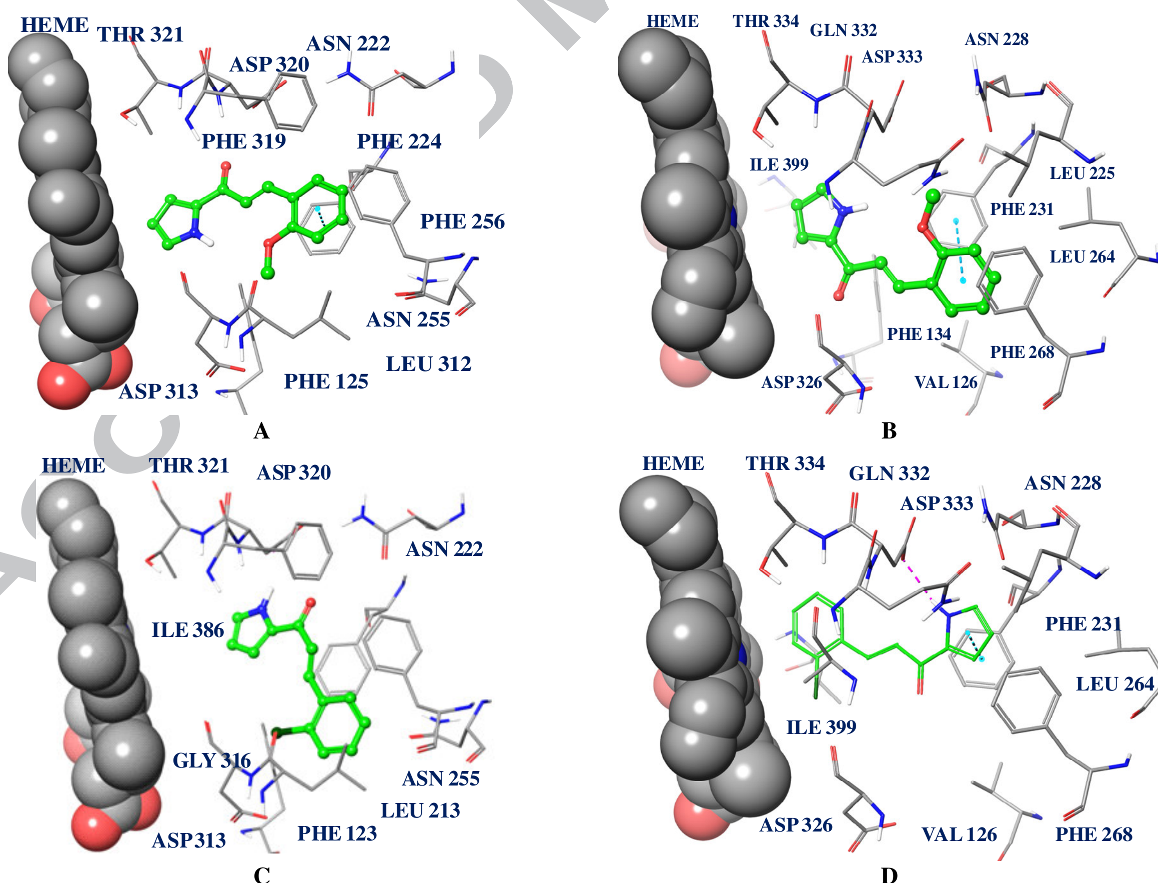

Figure 1. Interactions of **3j** with 1A1 and 1B1 (A and B) and **3n** with 1A1 and 1B1 (C and D) enzyme isoforms.

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Table 3. Concentration-dependent antagonism of AhR, relative to its activation by B[a]P at 0.75 µM, by the most potent CYP1A1 and CYP1B1 inhibitors, identified in Sacchrosomes and recombinant CYP-expressing human cells.

	AhR-ARNT complex in eGFP units at			
Entry	**1 µM**	**5 µM**	**10 µM**	**20 µM**
-	955 ± 9	955 ± 9	955 ± 9	955 ± 9
3j	600 ± 9	220 ± 5	150 ± 9	120 ± 1.2
3n	920 ± 9	770 ± 7	455 ± 5	285 ± 5
3s	955 ± 9	955 ± 9	955 ± 9	955 ± 9

All values, presented in eGFP units (excitation/emission monitored at 489/509 nm), represent the mean and standard deviations of three independent experiments.

CYP family of enzymes is part of a metabolic machinery of the cell.[4, 16, 33] Recent studies have shown a correlation between CYP1B1 expression in human cells and the metabolism of anticancer drugs such as paclitaxel, docetaxel, doxorubicin, mitoxantrone, tamoxifen and cisplatin.[16] CYP1B1-mediated metabolism leads to decrease in the cellular efficacy of cytotoxic drugs, and eventually cancer cells become resistant to these drugs. Such issues of drug metabolism were specifically been addressed by using CYP1B1-specific inhibitors.[16, 18] Cui *et al.* and Horley *et al.* have shown improvement in docetaxel and cisplatin efficacy by potent CYP1B1 inhibitors.[16, 18] Therefore, CYP1B1 inhibitors confirmed in live human cells were tested for reduction in resistance to cisplatin in adherent HEK293 cells. The introduction of *CYP1B1* gene bearing plasmid in HEK293 reduces the cytotoxicity of cisplatin by increasing the EC_{50} from 10.5 to 65 µM (shown in Table S4 of supporting information). CYP1B1 inhibitors **3j** and **3n** completely restore cytotoxicity of cisplatin at the tested concentrations, as shown in Figure 2. Due to inhibition of cisplatin metabolism, the EC_{50} of cisplatin was restored to ~10 µM. This demonstrates that CYP1B1 inhibitors have the ability to re-establish cisplatin's cytotoxicity in cells harboring the *CYP1B1* gene. The compound **3s**, which was inactive in live cells assays, does not reverse the cisplatin-resistance.

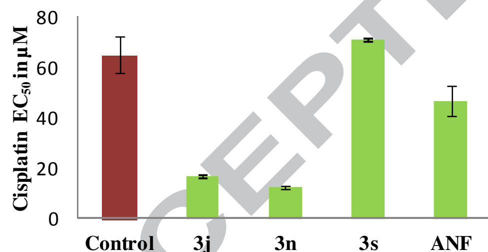

Figure 2. Overcoming cisplatin resistance by CYP1B1 inhibitors. A range of concentrations of cisplatin (0.05 µM – 100 µM) were used, in the presence of 6 x IC_{50} values of compounds **3j** and **3n**, whereas compound 3s and ANF (α-naphthoflavone) were used at 20µM concentrations. IC_{50} values had been determined in the human cell assay where cells were grown in suspension. HEK293 cells were transfected with pcDNA3.1/hCYP1B1 (the plasmid encoding the human *CYP1B1* gene). All values, presented in µM concentrations, represent the mean and standard deviations of three independent experiments.

B[a]P is a pro-carcinogen, which upon metabolism by CYP1A1 enzyme is converted into benzo[a]pyrene-6,7-dihydrodiol-9, 10 epoxide which is then able to intercalate DNA, thus producing toxicity. In the literature various CYP1A1 inhibitors have been reported to protect cells from B[a]P toxicity.[20, 32] The B[a]P EC_{50} values in untransfected cells and cells transfected with the *CYP1A1* gene-bearing plasmid are around 14 and 0.8 µM, respectively (shown in Table S5 of Supporting Information), indicating toxicity of B[a]P is mediated by expression of the *CYP1A1* gene. The CYP1A1 inhibitor **3j** was tested for chemo-preventive activity that would avert CYP1A1 mediated B[a]P toxicity in HEK293 cells. It was observed that **3j** rescued cells from B[a]P toxicity, which is shown in Figure 3. Compounds **3s** and **3n** which were inactive against CYP1A1, does not rescue cells from B[a]P toxicity.

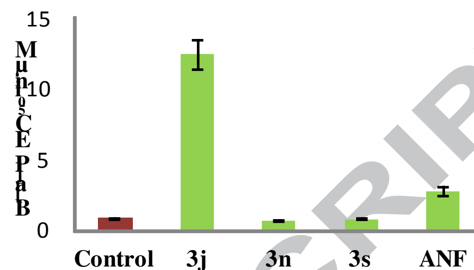

Figure 3. Protection from CYP1A1 mediated B[a]P toxicity by CYP1A1 inhibitors. A range of concentrations of B[a]P (0.05 µM – 100 µM) were used, in the presence of 8 x IC_{50} value of **3j**, whereas compounds **3n**, **3s** and ANF (α-naphthoflavone) were used at 20 µM concentrations. IC_{50} values were determined in the human cell assay where cells were grown in suspension. HEK293 cells were 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 summary, CYP1B1 structure-guided information, allowed us to develop SAR for 2-pyrrolyl chalcones as CYP1 family inhibitors. CYP1B1 selectivity can find potential for reversal of cisplatin resistance through restoration of cisplatin's toxicity in human cancer cells, while CYP1A1 inhibitory activity can find application in the prevention of cancer. Further studies, based on these findings, could lead to the discovery of novel modulators that would allow chemo-prevention and overcome chemo-resistance in cancer.

Acknowledgement:

BC would like to thank the UK Higher Education Innovation Fund (HEIF) and Research Business Innovation (RBI) directorate of De Montfort University for support. PJ acknowledges CSIR for an award of Senior Research Fellowship.

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