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Graphical Abstract

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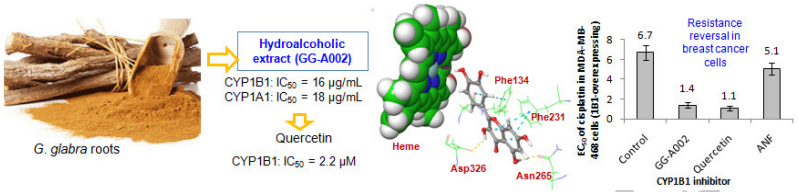

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Glycyrrhiza glabra extract and quercetin reverses cisplatin resistance in triple-negative MDA-MB-468 breast cancer cells via inhibition of cytochrome P450 1B1 enzyme

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ABSTRACT

The development of multi-drug resistance to existing anticancer drugs is one of the major challenges in cancer treatment. The over-expression of cytochrome P450 1B1 enzyme has been reported to cause resistance to cisplatin. Herein, we report the evaluation of *Glycyrrhiza glabra* (licorice) extracts and its twelve chemical constituents for inhibition of CYP1B1 (and CYP1A1) enzyme in Sacchrosomes and live human cells. The hydroalcoholic extract showed potent inhibition of CYP1B1 in both Sacchrosomes as well as in live cells with IC_{50} values of 21 and 16 µg/mL, respectively. Amongst the total of 12 constituents tested, quercetin and glabrol showed inhibition of CYP1B1 in live cell assay with IC_{50} values of 2.2 and 15 µM, respectively. Both these natural products were found to be selective inhibitors of CYP1B1, and does not inhibit CYP2 and CYP3 family of enzymes (IC_{50} > 20 µM). The hydroalcoholic extract of *G. glabra* and quercetin (**4**) showed complete reversal of cisplatin resistance in CYP1B1 overexpressing triple negative MDA-MB-468 breast cancer cells. The selective inhibition of CYP1B1 by quercetin and glabrol over CYP2 and CYP3 family of enzymes was studied by molecular modeling studies.

Chemotherapy is one of the key approaches for treatment of cancers. Chemotherapeutic drugs are used to reduce the size / progression of tumor growth; however, with most of these drugs, their eventual ability to reduce tumor growth decreases because of various resistance mechanisms. Cisplatin is an anticancer drug commonly employed in the treatment of many advanced cancers including breast cancer. Nevertheless, it develops resistance over a period of time, resulting in treatment failure.[1-3] The overexpression of human cytochrome P450 CYP1B1 has been identified as one of the important factors responsible for development of cisplatin-resistance.[4-5] Studies have demonstrated that the use of CYP1B1 inhibitors in combination with cisplatin, has resulted in reversal of this resistance.[6]

The licorice (*Glycyrrhiza glabra*) has been reported to possess many medicinal properties.[7-16] It is traditionally used for a number of aliments and is present in a wide range of herbal medicines, nutraceuticals and food supplements.[17] It has been reported that *G. glabra* display cancer chemopreventive activity via induction of nuclear factor (erythroid-derived 2)-like factor 2 (Nrf2).[18-19] Furthermore, the glycyrrhizic acid (**2**), a constituent of *G. glabra* is reported to provide protection against cisplatin-induced genotoxicity and nephrotoxicity.[20] Another constituent, isoliquiritigenin possesses chemopreventive activity through induction of a phase II enzyme, quinone reductase-1, in murine hepatoma cells.[21] Licorice extracts are also reported to prevent the *Helicobacter pylori*-initiated, salt diet-promoted gastric tumorigenesis.[22] Recently, van Breemen's group[23] have investigated the inhibitory effects of licorice and its 14 constituents against CYP2B6, CYP2C8, CYP2C9, CYP2C19 and CYP3A4 in order to understand their potential role in drug-drug interactions. However, the potential of licorice and its chemical constituents to overcome cisplatin-resistance has never been reported. Herein, we report the CYP1B1/ CYP1A1 inhibitory activity of hydroalcoholic extract of *G. glabra* and its 12 chemical constituents which is followed by studies on their ability to overcome cisplatin-resistance in breast cancer cells, and protection against toxicity mediated by a procarcinogen.

The roots of *G. glabra* were extracted with ethanol and ethanol-water to obtain an EtOH extract (GG-A003) and a hydroalcoholic extract (GG-A002). Both prepared extracts were tested for CYP1A1 and CYP1B1 inhibition in Sacchrosomes (yeast microsomes) followed by screening in live human cells. Results are shown in Table 1. It was observed that both extracts showed similar level of activity with respect to inhibition of CYP1B1 enzyme. As routine practice, the microsomes were used to study the effect of compounds on cytochrome P450 enzymes; however, results obtained from these studies may lead sometimes to false-negative inferences. Therefore, to gain a realistic picture of cytochrome P450 inhibition, we have established a live cell based assay system in human HEK293 cells which were transfected with plasmids bearing the human *CYP1B1* and *CYP1A1* genes. Both extracts GG-A002 and GG-A003 displayed CYP1B1 as well as CYP1A1 inhibitory activity in live cells. The EC_{50} values were found to be in the range of 16-20 µg/mL (Table 1).

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Table 1. CYP1A1 and CYP1B1 inhibition by *G. glabra* extracts in Sacchrosomes and in live cells[a]

Extract	Sacchrosomes IC_{50} (µg/mL)		HEK-293 cells overexpressing CYP1A1/1B1 IC_{50} (µg/mL)	
	CYP1A1	CYP1B1	CYP1A1	CYP1B1
GG-A003	31 ± 3	19 ± 1.5	16 ± 1.2	18 ± 1.5
GG-A002	34 ± 3	21 ± 1.8	20 ± 1.6	16 ± 1.6

[a]All values, presented in µg/mL concentrations, represent the mean and standard deviations of three independent experiments.

The phytochemical investigation of extracts by routine silica column chromatography using hexane to ethyl acetate to methanol gradient resulted in isolation of 12 compounds. Partially purified compounds obtained from repeated silica column chromatography were further purified by Sephadex LH20 using methanol as mobile phase (except glycyrrhizin and glycyrrhetic acid). For isolation of glycyrrhizin, the concentrated hydroalcoholic extract of *G. glabra* roots was sequentially washed with DCM and cold acetone to remove low molecular weight phenolics. By repeating this procedure, 80% pure glycyrrhizin was obtained, which was crystallized in ethanol-water (8:2) solvent. 18β-glycyrrhetic acid (**2**) was obtained from hydrolysis of glycyrrhizin (**1**) by 5% hydrochloric acid. Glycyrrhizin (**1**), glycyrrhetic acid (GA) (**2**), 4'-O-Me glabridin (**3**), quercetin (**4**), 3-hydroxyglabrol (**5**), glabrol (**6**), glabridin (**7**), formononetin (**8**), isoliquiritigenin (**9**), liquiritigenin (**10**), isoliquiritin apioside (**11**) and medicarpin (**12**) were isolated (Figure 1). All isolated compounds were characterized by comparison of ^{1}H NMR and melting point with reported values. Wherever required, ^{13}C NMR and 2D NMR data were also recorded.

All isolated compounds were tested for inhibition of CYP1A1 and CYP1B1 in Sacchrosomes at 10 µM (Table 2). Results showed that quercetin (**4**) is a potent inhibitor of CYP1B1 enzyme (67.2% inhibition at 10 µM) and glabrol (**6**) is a promising inhibitor of CYP1A1 enzyme (67.2% inhibition at 10 µM). Based on the significant inhibition showed by these two compounds, their IC_{50} values were determined in Sacchrosomes and are shown in Table 3.

Further, the CYP inhibitory activity was evaluated in live human cells. Compound **4** (quercetin) was found to be the most potent CYP1B1 inhibitor with IC_{50} value of 2.2 µM. Glabrol (**6**) was found to be a moderate inhibitor of CYP1B1 showing IC_{50} value of 15 µM.

CYP2 and CYP3 family of enzymes are primarily involved in drug-drug interactions; therefore, the effect of these two compounds on CYP2D6 and CYP3A4 was also assessed. Results are shown in Table 3. Both compounds do not show any effect on these two enzymes, indicating their selectivity towards CYP1 family of enzymes and thus would be expected to be free from potential drug-drug interactions.

Table 2. *In-vitro* CYP1A1/CYP1B1 inhibition by isolated compounds **1-12** in Sacchrosomes™

Entry	% inhibition (± SD)[a] in Sacchrosomes at 10 µM	
	CYP1A1	**CYP1B1**
1	39 ± 1.5	10.5 ± 0.3
2	11.4 ± 0.4	-2.1 ± 0.2
3	29.6 ± 1.1	12.0 ± 0.3
4	31.5 ± 1.2	**67.2** ± 2.6
5	31.4 ± 1.1	7.5 ± 0.3
6	**67.2** ± 2.5	17.7 ± 0.6
7	36.8 ± 1.4	26.6 ± 0.8
8	10.8 ± 0.3	-1.3 ± 0.2
9	22.3 ± 0.8	3.1 ± 0.2
10	19.2 ± 0.5	38.2 ± 1.4
11	11.4 ± 0.3	-9.5 ± 0.3
12	27.5 ± 0.9	31.7 ± 1.1
ANF	97 ± 1.1	98 ± 1.0

[a]All % inhibition values represent the mean and standard deviations of three independent experiments.

The screening results led to the identification of *G. glabra* extracts, quercetin (**4**) and glabrol (**6**) as inhibitors of CYP1 family of enzymes. To demonstrate their potential utility in therapeutics, the effect of extracts and compounds was assessed in two assays: (a) reversal of cisplatin resistance in breast cancer cells; and (b) protection of human cells from benzopyrene B[a]P (a procarcinogen) medicated toxicity.

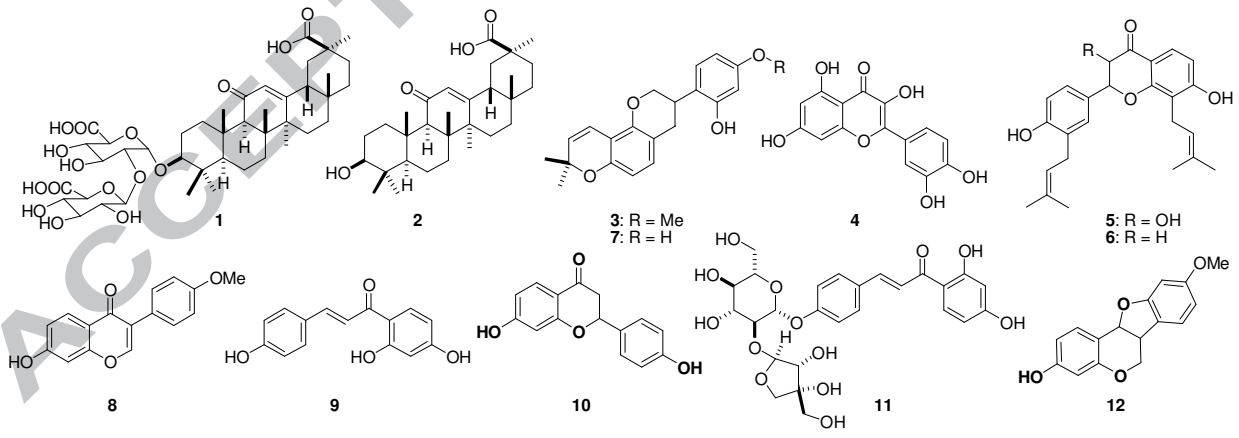

Figure 1. Chemical constituents isolated from *G. glabra* roots.

Table 3. IC_{50} values for in-vitro cytochrome P450 enzymes inhibition by selected compounds in Sacchrosomes™ and live cells

Entry	IC_{50} in µM (Sacchrosomes)™					IC_{50} in µM (HEK293 cells overexpressing respective CYP enzyme)				
	CYP1A1	CYP1B1	CYP1A2	CYP3A4	CYP2D6	CYP1A1	CYP 1B1	CYP1A2	CYP3A4	CYP2D6
4	>20	1.0 ± 0.3	>20	>20	>20	9.9 ± 0.7	2.2 ± 0.3	>20	>20	>20
6	17.6 ± 1.4	>20	>20	>20	>20	>20	15 ± 1.4	>20	>20	>20
ANF	0.01 ± 0.002	0.05 ± 0.01	0.03 ± 0.01	>10	>10	>10	>10	>10	>10	>10

Table 4. EC_{50} values of cisplatin after treatment of CYP1B1-overexpressing MDA-MB-468 cells with cisplatin and a CYP1B1 inhibitor[a]

Cell line	Extract/ compound	CYP1B1 IC_{50} of extract/ compound (μM)	CYP1B1 substrate[b]	cisplatin, EC_{50} (μM)
MDA-MB-468:: –	–	–	cisplatin	0.65 ± 0.28
MDA-MB-468::pcDNA3.1	–	–	cisplatin	0.76 ± 0.32
MDA-MB-468::pcDNA3.1/CYP1B1	–	–	cisplatin	6.70 ± 0.7
MDA-MB-468::pcDNA3.1/CYP1B1	GG-A003	18 μg/ml	cisplatin	1.6 ± 0.3
MDA-MB-468::pcDNA3.1/CYP1B1	GG-A002	16 μg/ml	cisplatin	1.4 ± 0.3
MDA-MB-468::pcDNA3.1/CYP1B1	**4**	2.2 μM	cisplatin	1.1 ± 0.2
MDA-MB-468::pcDNA3.1/CYP1B1	**6**	15 μM	cisplatin	5.2 ± 0.6
MDA-MB-468::pcDNA3.1/CYP1B1	ANF	>10 μM	cisplatin	5.1 ± 0.6

[a] The cells used were: (a) untransfected MDA-MB-468 cells (MDA-MB-468:: –), (b) MDA-MB-468 cells transfected with pcDNA3.1 (i.e. MDA-MB-468:: pcDNA3.1), the basic plasmid which does not contain a gene insert, and (c) MDA-MB-468 cells transfected with pcDNA3.1/hCYP1B1 (i.e. MDA-MB-468:: pcDNA3.1/hCYP1B1), a plasmid encoding the human *CYP1B1* gene. [b] range of concentrations of cisplatin (0.03 μM – 30 μM) were used, in the presence of 3 x IC_{50} values of GG-A003 and GG-A002 extracts and compound **4** (as determined in the human cell assay where cells were grown in suspension), whereas **6** and ANF were used at 20 μM concentrations. Thus, the test concentrations of GG-A003, GG-A002, compound **4**, **6**, and ANF were 54 μg/mL, 48 μg/mL, 6.6 μM, 20 μM and 20 μM, respectively. Adherent MDA-MB-468 cells, maintained in RPMI-1640 medium without phenol red with 10% fetal calf serum and 2 mM glutamine, were transfected with pcDNA3.1/hCYP1B1 (plasmid encoding the human *CYP1B1* gene). All values, presented in μM concentrations, represent the mean and standard deviations of three independent experiments.

Reversal of cisplatin-resistance in MDA-MB-468 cells. The overexpression of CYP1B1 enzyme causes resistance to anticancer drug cisplatin. MDA-MB-468 (ATCC, HTB-132) is a triple negative breast cancer cell line. Triple-negative breast cancer (TNBC) is an invasive carcinoma of the breast that lacks expression of (a) the estrogen receptor (ER), (b) progesterone receptor (PR) and (c) human epidermal growth factor receptor 2 (HER2). Drug resistance is a fundamental problem in TNBC management, and is responsible for most cases of treatment failure in patients with metastatic cancer. Cisplatin resistant ovarian cancer A2780 cells have been reported to overproduce CYP1B1.[6] In analogy to this observation, it is likely that cisplatin-resistant TNBC cells would also overexpress CYP1B1. Hence, a plasmid bearing the human CYP1B1 gene was transfected in MDA-MB-468 cells to mimic the situation that is likely to exist in cisplatin-resistant TNBC cells. Cisplatin manifests its toxicity in MDA-MB-468 cells that is transfected with an empty plasmid (that contains no *CYP1B1* gene), whereas the same cells transfected with a plasmid encoding *CYP1B1* gene is resistant (Table 4). The EC_{50} of cisplatin was increased from 0.65 to 6.70 μM (10-fold decrease in cisplatin's activity) in CYP1B1-transfected MDA-MB-468 cells. Then, we studied the ability of extracts and compounds possessing CYP1B1 inhibitory activity, to overcome cisplatin resistance in CYP1B1 overexpressing triple negative MDA-MB-468 cells. Both extracts GG-A002 and GG-A003 were found to restore the cisplatin-potency, as evidenced by the EC_{50} of cisplatin in extract treated cells. The EC_{50} was restored to 1.6 and 1.4 μg/mL from 6.7 μM, which is >90% reversal of resistance. Similar to the extracts, pure natural product quercetin (**4**) also restored the cisplatin activity (EC_{50} = 1.1 μM). However, glabrol (**6**) could only marginally reverse this resistance (Table 4).

Protection of human cells from B[a]P medicated toxicity. Aryl hydrocarbon hydroxylase (CYP1A1) is involved in the metabolic activation of aromatic hydrocarbons, e.g. CYP1A1 catalyses the oxidation of B[a]P to form B[a]P-7,8-epoxide which further oxidizes to B[a]P-7,8-dihydrodiol. With the help of epoxide hydrolase, B[a]P-7,8-dihydrodiol gets converted to B[a]P-7,8-dihydrodiol 9-10, epoxide which is a carcinogen. In this study, we took recombinant HEK293 cells that contain a plasmid that encodes the human *CYP1A1* gene and cells without the *CYP1A1* gene (i.e. untransfected HEK293 cells). The B[a]P EC_{50} values for cell viability were 1.2 ± 0.3 and 14 ± 1.2 μM, respectively, in these two types of cells (Table 5). Both extracts of *G. glabra* completely rescued human cells from B[a]P toxicity at 3 x IC_{50} concentration (Table 5). Compound **4** was also able to overcome CYP1A1-mediated B[a]P toxicity in CYP1A1 over-expressing HEK293 cells, as the EC_{50} of B[a]P was decreased from 1.2 to 8.8 μM (Table 5).

Molecular modeling of quercetin (4) and glabrol (6) with CYP1B1. Flavonoids **4** and **6** showed inhibition of CYP1B1 and this inhibition was selective over 3A4 and 2D6. To justify the observed trend in potency and selectivity, molecular modeling studies were

Table 5. EC_{50} values of B[a]P after treatment of CYP1A1-overexpressing adherent HEK293 cells with B[a]P and a CYP1A1 inhibitor [a]

Cell line	Extract/ compound	CYP1A1 IC_{50} of extract / compound (μM)	CYP1A1 substrate[b]	EC_{50} (μM) of B[a]P
HEK293:: –	–	–	B[a]P	14 ± 1.2
HEK293::pcDNA3.1	–	–	B[a]P	13.2 ± 0.9
HEK293::pcDNA3.1/CYP1A1	–	–	B[a]P	1.2 ± 0.3
HEK293::pcDNA3.1/CYP1A1	GG-A003	16 μg/ml	B[a]P	8.65 ± 0.8
HEK293::pcDNA3.1/CYP1A1	GG-A002	20 μg/ml	B[a]P	10.25 ± 1.0
HEK293::pcDNA3.1/CYP1A1	**4**	9.9 μM	B[a]P	8.8 ± 0.6
HEK293::pcDNA3.1/CYP1A1	**6**	>20 μM	B[a]P	2.3 ± 0.7
HEK293::pcDNA3.1/CYP1A1	ANF	>10 μM	B[a]P	3.2 ± 0.4

[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 (i.e. HEK293:: pcDNA3.1/hCYP1A1), a plasmid which encodes the human *CYP1A1* gene. [b]A range of concentrations of B[a]P (0.05 μM – 100 μM) were used, in the presence of 3 x IC_{50} values of GG-A003 and GG-A002 extracts (as determined in the human cell assay where cells had been grown in suspension), whereas compounds **4**, **6** and ANF (α-naphthoflavone) were used at 20 μM concentrations. Adherent HEK293 cells were used for all plasmid transfection. All values, presented in μM concentrations, represent the mean and standard deviations of three independent experiments.

performed. Quercetin (**4**) was found to display overlapping interactions exactly like alpha-napthoflavone. Quercetin showed π-π interactions with Phe 134 and Phe 231. Further, it displayed H-binding interactions with Asp 326 and Asn 265 residues of the CYP1B1 binding pocket (Figure 2A). The overlay image of quercetin with ANF in the binding cavity of CYP1B1 is shown in supporting information (section S3). In case of glabrol, its terminal hydroxyl group forms polar H-bond with the Asn 228 residue of F helix and both phenyl ring forms hydrophobic π-π interactions with C helix Phe134 and F helix Phe231 residue. The diprenylated rings showed favorable van der Waals interactions with receptor. However, despite of common interactions pattern, quercetin (**4**) and glabrol (**6**) displayed difference in inhibition potency towards CYP3A4 and CYP2D6 which is due to the difference in structural architecture of these enzymes (i.e. shape and amino acid residues that form edges of the cavity). In case of CYP2D6, they displayed only π-π hydrophobic interactions with the Phe483 residue. While in case of CYP3A4, glabrol interacts with Phe215 residue rather than corresponding Phe231 residue of CYP1A1 in F helix due to the open nature of the binding cavity.

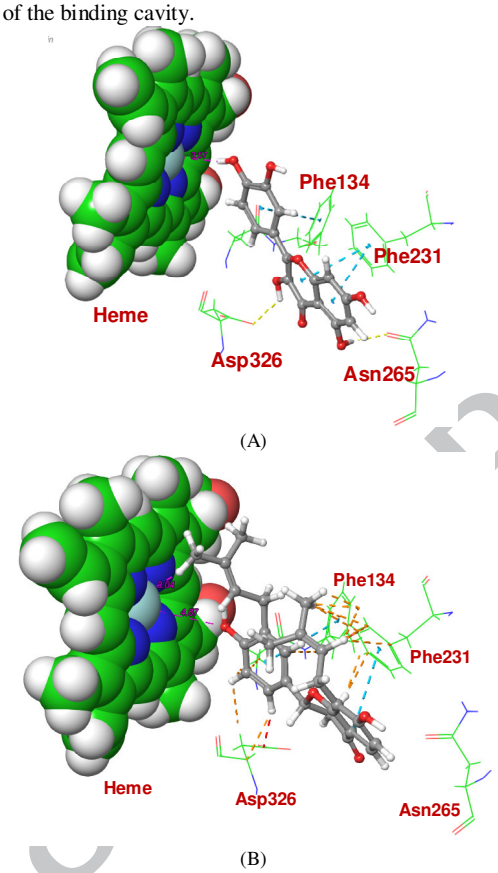

Figure 2. (A-B) Docked complex of compound **4** and **6** with CYP1B1, respectively. Yellow dotted lines indicate H-bonding, blue dotted lines indicates π-π interactions and orange dotted lines indicates van der Waals interactions between ligand and receptor.

In summary, we have identified that *G. glabra* possesses potent ability to inhibit CYP1B1 and CYP1A1 enzymes, and it protects cells from procarcinogens and overcomes cisplatin-resistance in CYP1B1 over-expressing triple negative breast cancer cells MDA-MB-468. Quercetin, a flavonoid isolated from this plant is also a potent inhibitor of CYP1B1 and it effectively reverses the cisplatin resistance in breast cancer cells. These results warrant further exploration of *G. glabra* and quercetin as resistance reversal agents in animal models.

Supporting information available. Experimental procedures and spectral data scans. This material is available free of charge via the internet at http://sciencedirect.com.

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