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Original article

Apoptosis inducing anthraquinone rhein and emodin differentially suppress human dehydroepiandrosterone sulfotransferase (hSULT2A1) and phenol sulfotransferases (hSULT1A1) in Hep-G2 and Caco-2 cells

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Abstract. The anti-cancer and apoptosis-inducing drugs rhein (4, 5-dihydroxyanthraquinone-2-carboxylic acid) and emodin (3-methyl-1, 6, 8-trihydro-xyanthrax-quinone) are clinically very important. They modulate cell cycle via tumor suppressor gene, immuno-receptors and ligand activated nuclear receptors. Our recent observation suggests for the first time that 10 days of treatment of either drug with various concentrations (0.01 to 100 μM) differentially suppressed the sulfotransferases (SULTs) activities and protein expressions in human hepatocellular carcinoma (Hep G2) and intestinal carcinoma (Caco-2) cell lines. SULTs are phase II drug metabolizing enzymes which catalyze the sulfuryl group transfer to hydroxyl containing endobiotics and xenobiotics. In the present investigation, dehydroepiandrosterone SULT (hSULT1A1) was markedly suppressed by these drugs in human cells. This is the first time report which demonstrates that rhein and emodin may regulate human SULTs. Our finding has important physiological and clinical implications. It will help in the understanding of the SULTs regulations by clinically important drugs and xenobiotics. In future, these drugs may be used in a better defined manner, taking into account its SULTs suppression effects with possible physiological consequences.

Keywords: Physiological role, rhein and emodin, sulfotransferase, suppression effect

1. Introduction

Sulfotransferases (SULTs) are members of phase II drug metabolizing enzymes which catalyze sulfuryl-group transfer to most of the endobiotics and maintain their physiological effective concentration. SULT expressions are shown to be regulated by several xenobiotics [1–4]. Clinically important drug rhein and emodin are evaluated here for

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their role in the expression of human SULT (e.g. phenol catalyzing hSULT1A1, dehydroxyepiandrosterone catalyzing hSULT2A1). SULTs regulations by rhein and emodin are not reported. Several reports reveal the regulations of the cytochrome group of enzymes (mainly CYPs) by these drugs [5, 6]. Rhein is a strong antibacterial compound which is also used in cancer chemotherapy. The inhibition of rat liver CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A enzymes by rhein is reported with the highest inhibition of CYP2E1 [7]. This inhibition may suggest that the interference on the pharmacokinetics of co-administered drugs are likely to occur [7]. The report reveals that rhein protects against acetaminophen-induced hepatic and renal toxicity by modulation of phase I enzymes activity [8]. This compound inhibits glucose uptake in tumor cells and modulates membrane-associated functions resulting in cell death [9]. The anti-mutagenic efficacy of both rhein and emodin are attributed by their ability to inhibit CYP1A1 [5]. Emodin was capable of inhibiting cellular proliferation, inducing apoptosis and preventing the tissue metastasis through signaling kinase (protein kinase C), transcription factor NF-κ-β, and mitogen-activated protein kinase (MAPK) signaling cascades [9]. Emodin has been shown to reverse carbon tetra chloride induced hepatic cytochrome P450 (CYP) enzymatic activities and tissue degenerations in rat [6].

Emodin, aloe-emodin and rhein inhibit migration and invasion of some human cancer cells through the inhibition of gene expression of matrix metalloproteinases (MMPs) [10]. Like rhein, emodin significantly attenuates hyperglycemia-induced nuclear translocation of NF-κ-B influencing some gene expression [11] and it also kills human breast carcinoma MCF-7 cell through apoptosis-related genes [12]. Emodin has been shown to inhibit UDP-glucuronosyltransferase (UGT) [13] thus modulating the cellular xenobiotic metabolism. But the SULTs regulations by either drug are not reported earlier.

The inhibition/ suppression of CYP expression by rhein and emodin is demonstrated and linked to their pathophysiological benefits. This is the first time; the present study elucidates the regulations of SULTs by these drugs. Our report may help in the understanding of xenobiotic regulations of SULTs and will also focus on the possible link between the regulation of SULT and the mechanistic efficacy of rhein and emodin. More defined use of these drugs may be possible in the future with the help of the knowledge of their possible role on SULTs regulations.

2. Material and methods

2.1. Chemicals

[^{14}C]β-naphthol (4.7 mCi/mmol), 3′-phosphoadenosine-5′-phosphosulfate (PAPS), and [1,2,6,7-^{3}H(N)], dehydroepiandrosterone ([^{3}H]DHEA, 60 Ci/mmol) [Sigma-Aldrich, St. Louis, MO]. SDS-polyacrylamide gel electrophoresis reagents were obtained from Bio-Rad (Hercules, CA). Western blot chemiluminescence reagent kits (Super Signal West Pico Stable Peroxide and Super Signal West Pico Luminol/Enhancer solutions) were purchased from Pierce Chemical (Rockford, IL). Nitrocellulose membrane (Immobilon-P; Millipore Corporation, Bedford, MA) used during the Western blot procedure (Fisher Scientific Co. Fair Lawn, NJ). Antibodies against human SULT2A1, human SULT1A1 and human β-actin were purchased from Panvera (Madison, WI). Protein assay reagent was purchased from Bio-Rad. All other reagents and chemicals were of the highest analytical grade available.

2.2. Cell culture and drug treatment

Both Hep G2 and Caco-2 cells were obtained from the American Type Culture Collection (Manassas, VA). Hep G2 Cells were grown and maintained in Dulbecco's Modified Eagles's Medium Nutrient Mixture F-12 Ham (Sigma) supplemented with L-glutamine and 15 mM HEPES, and 10% fetal bovine serum (FBS). Caco-2 cells were grown and maintained in Dulbecco's Modified Eagles's Medium (Sigma) supplemented with L-glutamine, 4500 mg/L glucose and 110 mg/L sodium pyruvate, and 20% fetal bovine serum (FBS). The cultures were incubated at 37°C in a humidified incubator containing 5% CO_2, 95% air [10]. After seeding at 0 days, on day 1, rhein or emodin dissolved in ethanol and added (0.01, 0.1, 1, 10 and 100 μM final) to the medium in properly marked plates. Control plates are added to the vehicle. The medium was refreshed every 3 days with the new addition of corresponding drug. On day ten the cells were harvested. Cytosols are prepared from Hep G2 and Caco-2 cells by suitable methods as explained earlier [14].

2.3. Cytosol preparation from Hep G2 and Caco-2 cells

Both Hep G2 and Caco-2 cells ($1–2 \times 10^7$) were harvested using 0.25% trypsin- EDTA solution (Sigma), washed with phosphate buffered saline, and then homogenized in 1 ml lysis buffer (3 mM 2-mercaptoethanol, 0.1 mM PMSF, 50 mM Tris, 250 mM sucrose; 0.1 mM EDTA, pH 7.5). The homogenate was then centrifuged at 12,000× g for 30 min and the supernatant was used in the studies.

2.4. Enzyme assays

2.4.1. PNPS assay

2-Naphthol sulfation activity (hSULT1A1) from Hep G2 cells was determined as previously described [14, 15]. Briefly, the activity was determined in a reaction mixture containing 50 mM Tris buffer, pH 6.2, 5 mM PNPS, 20 μM PAPS, and 0.1 mM β-naphthol. Hep G2 cell cytosols (50 μ g protein) were used as the enzyme source in a total reaction volume of 250 μl. After 30 min incubation at 37°C in a shaking water bath, the reaction was stopped by adding 250 μl of 0.25 M Tris, pH 8.7 (stop buffer). The reaction mixtures were read at 401 nm in a spectrophotometer. Specific activity (SA) was expressed as nanomoles per minute per milligram of protein. The data represent the average of three independent experiments.

2.4.2. Radioactive assay

hSULT1A1 activity from the Caco-2 cell cytosol (400 μ g protein) and hSULT2A1 activity from both Hep G2 and Caco-2 cell cytosol (50 μ g and 400 μ g protein respectively) were determined by this assay method. The respective amount of cytosol protein (as enzyme source) was added in a reaction mixture containing 50 mM Tris buffer, pH 6.2, 5 mM PNPS, 20 μM PAPS. For radioactive β-naphthol sulfation activity, (hSULT1A1) [^{14}C] β-naphthol (4.7 mCi/mmol; 0.1 mM final concentration) was used as substrate. To determine DHEA sulfation activity, (hSULT2A1) [^{3}H]DHEA (diluted to 0.4 Ci/mmol; 2 μM final concentration) was used as substrate. For all assays, 20 μM PAPS was used. All enzymatic reactions were performed in a total reaction volume of 250 μl. After 30 min 5 incubation at 37°C in a shaking water bath, the reaction was stopped by adding the stop buffer. Extraction was performed twice by the addition of 0.5 ml of water-saturated chloroform. After the final extraction, 100 μl of the aqueous phase was used for scintillation counting. Data from cell culture represents the average of two independent experiments. PAPS was eliminated from the controls of both assay methods. Assays were run in duplicate and the average of the results was used for enzyme activity calculations [14, 15].

2.5. Western blot analysis

Cytosol protein from Hep G2 cells (10 μg) and Caco-2 cells (15 μg) were used in a 12% polyacrylamide gel in an electrophoresis system (Novex, San Diego, CA). The protein bands were transferred onto a nitrocellulose membrane and blocked with 5% dry milk. The membranes with proteins from Hep G2 cells or Caco-2 cells were incubated separately with anti-hSULT1A1, anti-hSULT2A1 and anti-human β-actin antibody. After incubation and washing all membranes were incubated in secondary antibody (horseradish peroxidase-conjugated Immuno-Pure goat anti-rabbit IgG; H+L). The bands were developed with Super Signal West Pico Luminol Enhancer solution and Super Signal West Pico Stable Peroxidase solution and the bands on X-ray films were developed. Films were scanned and the densitometric analysis was performed in a Gel Documentation and Analysis System from Advanced American Biotechnology and with AAB software (Fullerton, CA) [14]. Densitometry analysis of protein bands is done using ImageJ Software and the values are plotted as relative densities.

2.6. Statistical analysis

Student's *t* test was performed to calculate the statistical significance with the difference between means of control and drug treated group. Data presented in the figures denotes the mean ± SE values generated from three independent experiments.

3. Results

3.1. hSULT1A1 and hSULT2A1 activity changes in Hep G2 and Caco-2 cells following rhein treatment

Figure 1 demonstrates the structure of rhein and emodin. The structural parity of these drugs with flavone and flavonol is shown.

The present results suggest that rhein at lower concentration (0.01–1 μM) does not influence the hSULT1A1 (2-naphthol sulfation) enzyme activity in Hep G2 cells (Fig. 2). But at higher concentration, e.g. 10 μM and 100 μM, it significantly inhibited this activity ($p < 0.01$ and $p < 0.001$ respectively). In Caco-2 cells, rhein does not significantly influence the hSULT1A1 activity.

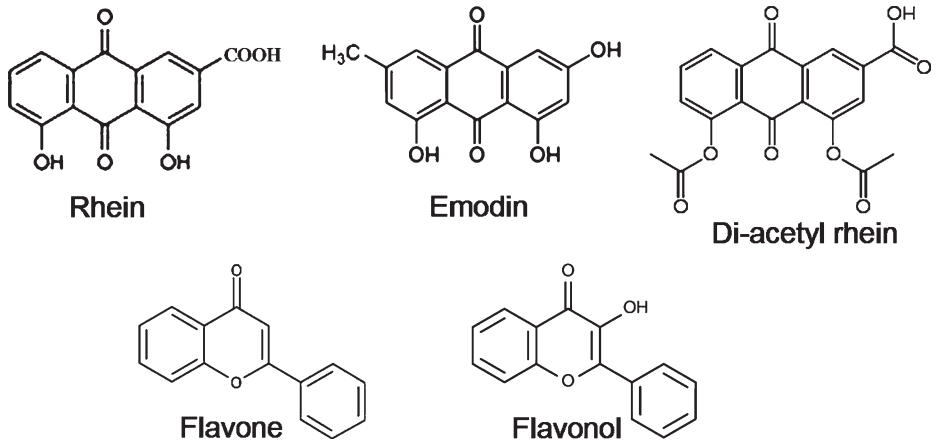

Fig. 1. Structure of rhein and emodin. Parity in the ring structures (with carboxylate group) of rhein, emodin with flavone and flavonol are shown.

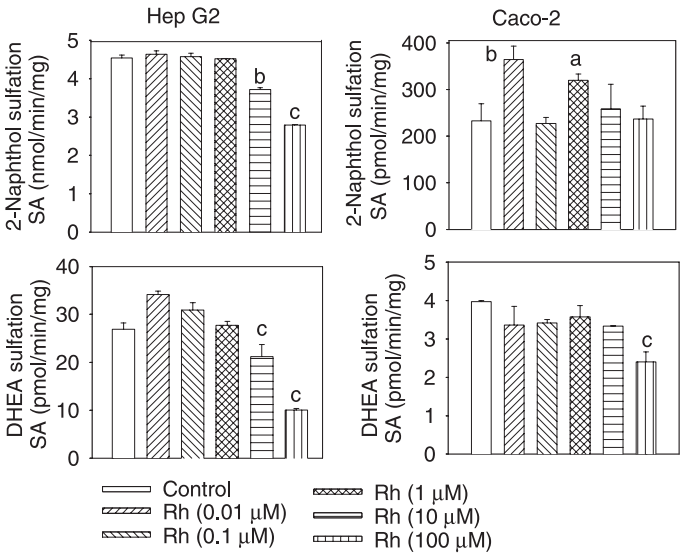

Fig. 2. hSULT1A1 (2-napthol sulfation) and hSULT2A1 (dehydrepiandrosterone sulfation) activities in Hep G2 and Caco-2 cells in response to different concentration of rhein exposure for 10 days. Each bar represents the mean ± SE values generated from three independent experiments. The level of significances (compared to control) is represented on the top of the bar. $^{a}p < 0.05$; $^{b}p < 0.01$; $^{c}p < 0.001$.

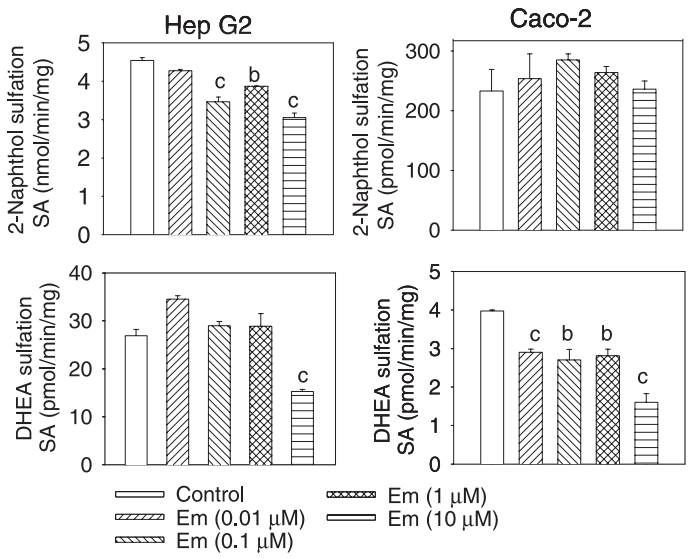

Fig. 3. hSULT1A1 (2-napthol sulfation) and hSULT2A1 (dehydrepiandrosterone sulfation) activities in Hep G2 and Caco-2 cells in response to different concentration of emodin exposure for 10 days. Each bar represents the mean ± SE values generated from three independent experiments. The level of significances (compared to control) is represented on the top of the bar. $^{a}p<0.05$; $^{b}p<0.01$; $^{c}p<0.001$.

hSULT2A1 activity is also evaluated. Result suggest that in Hep G2 cells, rhein inhibited DHEA sulfation activity in a concentration (1, 10 and 100 μM) dependant manner ($p<0.01$, $p<0.001$ and $p<0.001$ respectively). In Caco-2 cells, this activity was suppressed moderately at highest concentration of the drug (100 μM) (Fig. 2).

3.2. hSULT1A1 and hSULT2A1 activity in Hep G2 and Caco-2 cells following emodin treatment

In the present study, hSULT1A1 activity (2-napthol sulfation) decreased moderately in Hep G2 cells at higher concentration of emodin treatments (0.1–10 μM, $p<0.01$ and $p<0.001$), however, the hSULT1A1 activity is not influenced in the Caco-2 cells (Fig. 3). In case of DHEA sulfation, hSULT2A1 activity was significantly suppressed in both Hep G2 and Caco-2 cells. This suppression is found to be in higher extent in the cultured intestinal carcinoma cell lines. In Hep G2 cell, DHEA sulfation increased in the lowest concentration (0.01 μM) and then it decreased significantly in a dose dependant manner (Fig. 3).

3.3. Western blot analysis of hSULT1A1 and hSULT2A1 protein changes in Hep G2 and Caco-2 cells

Western blot results from normalized desitometric data (against housekeeping protein β-actin) suggest that hSULT1A1 protein expression is suppressed at highest (20 μM) rhein concentration (lane 3 of the upper panel of Fig. 4). However, protein amount did not change significantly in any emodin concentration. The hSULT2A1 protein expression significantly was suppressed in Hep G2 cells after rhein or emodin exposure for 10 days. This suppression is found to be dose-dependant at 2 μM and 20 μM for both drugs. The hSULT1A1 protein expression in Caco-2 cells did not alter in response to rhein or emodin with these concentrations. For hSULT2A1 expression, emodin dose dependently suppressed this protein and rhein suppressed this protein at 2 μM concentrations. The densitometry data suggest that hSULT2A1 expression significantly decreased in response to both rhein and emodin in the Caco-2 cells, but the suppression of hSULT1A1 is found to be significant only in HepG-2 cells after 10 days' rhein exposure.

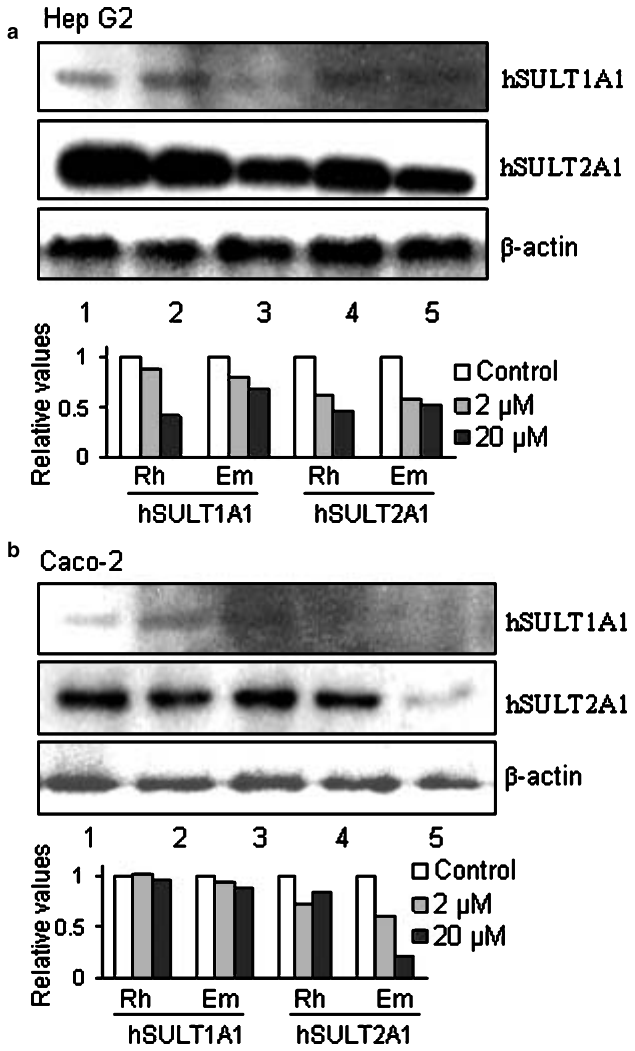

Fig. 4. Representative Western blot analysis showing human SULT proteins in Hep G2 and Caco-2 cells treated 10 days with different doses of ethanol. Lane 1 = control; 2 = 2 μM rhein; 3 = 20 μM rhein, 4 = 2 μM emodin; 5 = 20 μM emodin. Densitometry analysis of protein bands are done using ImageJ Software and then values are normalized against house keeping protein β-actin. The values are plotted as relative densities as bar diagram.

4. Discussion

The effect of rhein and emodin on SULTs has not been reported earlier. Our results suggest the differential suppression of hSULT1A1 and mainly hSULT2A1 by rhein and emodin in human cells. The suppression of hSULT2A1 is found to be higher than that of hSULT1A1 by these drugs. The patho-physiological consequences and clinical efficacy of these drugs might have some relations to this suppression effect. These regulations may also influence the pharmacokinetics of other drug metabolism [7]. Rhein is a weak anti-inflammatory, analgesic compound and it is a derivative of the di-acetyl rhein (for structure see Fig. 1). The report suggests that treatment with 100 μM rhein significantly increased mRNA expression of caspases and the Bax/Bcl-2 ratio resulting apoptotic cell death [16]. Rhein and aloe-emodin together showed a better *in vitro* anti-tumor effect than their individual application [17]. Rhein showed antiproliferative and apoptotic effects in human breast cancer MCF-7 cells via reactive oxygen species (ROS)-mediated activation of NF-κ-B and p53-signaling pathways [18]. Rhein plays its role by inducing cell

cycle arrest via downregulation of oncogene c-myc and apoptosis through the caspase-dependent pathway [19]. *In vitro*, the intrinsic clearance of the rhein via cytochrome P450 (CYP450), UDP-glucuronosyltransferase (UGT) and sulfotransferase (SULT) metabolism are demonstrated in rat [20].

Reports revealed that deacetylation of rhein occurs in the intestine, liver and other tissues [21]. *In vivo* metabolic studies detected the two major phase II metabolites of rhein in rat, one as a monosulfate and the other as a monoglucuronide [22, 23]. An enterohepatic recirculation of rhein is occurring in rat [23]. Rhein interferes with anion transporter-mediated renal elimination of drugs [24]. *In vitro* investigations with rat liver microsomes and V79 cells showed that rhein could be a substrate for the NADPH dependant enzymes as well as in the CYP1A1 [25]. The CYP 1A1/1A2 isoenzymes have been shown to be responsible for the oxidation of rhein. Our present study demonstrates that rhein significantly suppressed hSULT2A1 expression in Hep G2 cells in a dose dependent manner. Differential inhibitory effects of human phenol sulfotransferase and estrogen sulfotransferase by certain non-steroidal anti-inflammatory agents (NSAIAs) have been shown by meclofenamate (anthranilic acid) and aspirin. Some of those have a certain degree of structural parity (ring structure with carboxylate group) with the drugs studied in our present investigation [26]. The suppression of SULTs by these drugs may be mechanistically important for their clinical responses [26]. An anti-cancer drug, resveratrol, (a natural phenol and a phytoalexin) has been shown to suppress O-acetyltransferase and sulfotransferase activities in the breast cancer cell lines MCF-7 and ZR-75-1 [27]. Recently, anti-diabetic role of rhein and its nephro-protection efficacy have been demonstrated. Rhein minimizes ROS response and protect the mitochondria and pancreatic β-cells against diabetic cell-apoptosis [28]. It protects glucose induced epithelial-mesenchymal transition of renal cell by inhibiting integrin-linked kinase expression [29]. Rhein resists renal lesion in dyslipidemic mice with diabetic nephropathy by decreasing transforming growth factor-beta1 (TGF-beta1), fibronectin expression, plasma lipids and ApoE levels [30].

Nuclear receptor for vitamin D3 and 24-hydroxylase (CYP24) regulation is shown as an rhein target for intervention with diabetic/ nondiabetic nephropathy [31]. It is reported that liver xenobiotic receptors (LXRs) play an important role in regulating cholesterol homeostasis, and lipid and energy metabolism. Rhein is shown to bind directly to LXRs [32]. The expression levels of LXR target genes were suppressed by rhein in 3T3-L1 and HepG2 cells [32]. Chemo sensitization is an important drug response which has been observed by some anticancer phenolic phytochemicals e.g. genistein (phytoestrogen-isoflavones), epigallocatechin gallate (tea antioxidant), quercetin (flavonol-flavonoid), emodin (anthraquinone), and resveratrol [33]. Structural parity of some polyphenol phytochemicals with the rhein and emodin is shown in Fig. 1. Several of these drugs are shown to inhibit certain drug metabolizing enzymes [33].

In the present investigation rhein and emodin have been shown to suppress SULTs protein levels and enzyme activities. This suppression has been demonstrated at enzyme activity and corresponding protein amount level. The extent of activity inhibition may be attributed by the lowering of the enzyme protein amount resulted from drug associated impaired translation and/or post translational modifications. Suppression of target gene expression by LXRs-rhein complex [32] may suggest that SULT inhibition by rhein may also take place at the mRNA expression level. Other nuclear receptor-mediated gene regulatory processes also may be occurring. Further studies are required in this regard. Apart from this, the three ring structure with an oxygen group of rhein and emodin may be important for their SULT inhibition property (Fig. 1). The structure-activity relationship studies demonstrate that the interaction of flavones and flavonols with an enzyme required their three rings and an oxygen group in the side ring. These structural features favor their anti-mutagenic role via CYP inhibition [34]. The report reveals that aryl acetate- and aryl carboxylate-containing NSAIA drug meclofenamate inhibit human SULT1A1 and differentially inhibit SULT1E1 [26]. The inhibitory mechanism indicates that meclofenamate, and aspirin bind near enough to the substrate binding site of SULT to prevent catalysis [26]. The acetate and/or carboxilate group of the diacetyl rhein (known as Diacerein, used in osteoarthritis) is transformed into rhein in liver and intestine [21]. The chemical modification and activation of these drugs are helpful for their therapeutic mechanism. In relation to the apoptotic mechanism, several polyphenol anthraquinones present in some vegetable foods impart bioactivity by inducing oxidative stress via mitochondrial instability. Disintegration of the mitochondrial membrane and release of cytochrome C may initiate oxidative stress and apoptotic signaling [35]. Phytochemicals from several fruits (i.e. dark berries) induce apoptotic cell death by mitochondrial membrane depolarization, cell-cycle blockage and nitric oxide generation. Cell death was accompanied by G2/M and/or S phase arrest in the cell cycle [36]. Rhein and emodin have been shown to induce apoptosis by introducing DNA damage via the inhibition of DNA repair-associated gene expressions [37]. Sulfation associated modification of these drugs may be related to their therapeutic mechanism.

In conclusion, the present finding suggests the human SULTs suppression by clinically important drugs like rhein and emodin. These results indicate the possible role of SULTs regulation in the physiological and clinical mechanism played by these drugs or other anthraquinone drugs. We first time report here the rhein and emodin regulations of human SULTs. This study also will explore the xenobiotic regulation of SULT and their physiological consequences. Further studies are necessary to elucidate the suppression mechanism, drug-drug interaction and their clinical consequences.

Conflict of interest

None.

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