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Research paper

Inhibition of UGT1A1 by natural and synthetic flavonoids

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Abstract

Flavonoids are widely distributed phytochemicals in vegetables, fruits and medicinal plants. Recent studies demonstrate that some natural flavonoids are potent inhibitors of the human UDP-glucuronosyltransferase 1A1 (UGT1A1), a key enzyme in detoxification of endogenous harmful compounds such as bilirubin. In this study, the inhibitory effects of 56 natural and synthetic flavonoids on UGT1A1 were assayed, while the structure–inhibition relationships of flavonoids as UGT1A1 inhibitors were investigated. The results demonstrated that the C-3 and C-7 hydroxyl groups on the flavone skeleton would enhance UGT1A1 inhibition, while flavonoid glycosides displayed weaker inhibitory effects than their corresponding aglycones. Further investigation on inhibition kinetics of two strong flavonoid-type UGT1A1 inhibitors, acacetin and kaempferol, yielded interesting results. Both flavonoids were competitive inhibitors against UGT1A1-mediated NHPN-O-glucuronidation, but were mixed and competitive inhibitors toward UGT1A1-mediated NCHN-O-glucuronidation, respectively. Furthermore, docking simulations showed that the binding areas of NHPN, kaempferol and acacetin on UGT1A1 were highly overlapping, and convergence with the binding area of bilirubin within UGT1A1. In summary, detailed structure-inhibition relationships of flavonoids as UGT1A1 inhibitors were investigated carefully and the findings shed new light on the interactions between flavonoids and UGT1A1, and will contribute considerably to the development of flavonoid-type drugs without strong UGT1A1 inhibition.

Keywords: Flavonoids; UGT1A1; Structure-inhibition relationships

1. Introduction

Flavonoids are polyphenolic phytochemicals that are commonly present in the human diet, since they are abundant in vegetables, fruits, and a wide variety of other edible plants [1-3]. As an important class of bioactive compounds, flavonoids display many beneficial effects on human health, such as antioxidant, anti-inflammatory, anti-proliferative, anti-tumor, anti-microbial, and estrogenic-like activities, as well as regulatory effects on various enzymes [4-8]. Flavonoids-containing herbs, beverages (such as tea and coffee) or dietary supplements are often used for the prevention of various human diseases, such as diabetes, osteoporosis and cancer that are caused by free-radical damage, and as adjuvant treatment for liver injury, cardiovascular diseases and neurodegenerative disorders [9-12]. The positive effects on human health are combined with only very minor side effects that flavonoids have, prompting many researchers and biomedical companies to develop health-care foods or flavonoid-type drugs for clinical use. At present, some of the flavones from natural origin like silymarin (Milk Thistle), gingko flavone glycosides (Gingko Smart), naringenin (Natural Female Support), and synthetic flavonoid-type drugs (such as flavopiridol), are commercially available in the market. In China, several flavonoids-containing herbs, or flavone mixtures (such as baicalin and sliymarin) have been widely used for treatment of hepatitis [13-14]. Meanwhile, some natural flavones (such as oroxylin A, wogonin and vitexin) are undergoing phase I/II clinical trials in China, for auxiliary treatment of cancer or coronary heart diseases [15-17].

It is well-accepted that the drug-drug interaction potentials should be carefully investigated

during preclinical development process of drug candidates [18-19]. Over the past twenty-years, many studies have been conducted to explore the regulatory effects of natural and synthetic flavones on drug metabolizing enzymes or transporters [20-22]. The results demonstrated that several polyphenolic flavones (such as baicalein and diosmetin) can strongly inhibit the activities of some human cytochrome P450 enzymes (CYPs), while other polymethoxylated flavones (such as tangeretin and nobiletin) are potent inhibitors of the multidrug transporter P-glycoprotein [23-27]. These investigations suggested that flavones could reduce the elimination of many therapeutic drugs that are substrates for CYPs or P-glycoprotein, thereby causing clinically relevant herb/food-drug interactions (HDIs) [28]. The inhibitory effects of natural and synthetic flavones against phase II metabolizing enzymes, however, were rarely reported. This is somewhat surprising, considering that many flavones are substrates for phase II metabolizing enzymes such as the UDP-glucuronosyltransferases (UGTs) [29-30]. As UGTs substrates, flavones could lead to undesirable effects *via* competitive inhibition by occupying the ligand-binding pockets in the substrate domain of the UGT enzymes [31].

Mammalian UDP-glucuronosyltransferases are important endoplasmic reticulum-bound enzymes, which catalyze the conjugation of the glucuronic acid moiety from the donor UDP-glucuronic acid (UDPGA) to a variety of compounds, thus forming water-soluble glucuronides that are more easily eliminated [32-33]. In human, there are over twenty different UGTs and the majority of them are expressed in the liver and/or the small intestine [33-35]. Among all known human UGTs, UGT1A1 is of particular importance due to its crucial role in the metabolic elimination and detoxification of bilirubin, a major product of

hemoglobin metabolism, as well as several clinical drugs like cancer chemotherapy drug etoposide, the topoisomerase inhibitor SN-38 (the active metabolite of CPT-11), and the anti-AIDS agent raltegravir [36-39].

Impairment or considerable inhibition of UGT1A1 activity may lead to disorders in bilirubin metabolism, such as hyperbilirubinemia and jaundice, which may also be triggered by significant drug/herb-drug interactions (DDIs or HDIs) [40-42]. Previous studies have demonstrated that both therapeutic drugs and natural flavonoid-containing compounds in herbs or foods, including indinavir, erlotinib, regorafenib, licochalcone A, bavachin, corylifol A, and amentoflavone can strongly inhibit the catalytic activities of UGT1A1, thereby reduce bilirubin elimination and trigger clinically relevant drug–drug interactions [43-48]. Thus, it is important to systematically assay the inhibitory effects of flavonoids on the human UGT1A1 and to explore structure-inhibition relationships among them with respect to UGT1A1 inhibition. Such an investigation will be valuable for developing new flavonoid-type drugs with improved safety profiles, and for avoiding or minimizing the occurrence of adverse side effects *via* UGT1A1 inhibition by flavonoids-containing products.

In the present study, more than fifty flavonoids were collected and their inhibitory effects on the human UGT1A1 were investigated carefully. A florescence-based biochemical assay was constructed for high-throughput screening of UGT1A1 inhibitors, in which the newly developed fluorescent substrate for UGT1A1 was used as a probe [47, 49-50] and human liver microsomes (HLM) as the enzyme source. The potential structure-inhibition relationships of these structurally diverse flavonoids have been summarized and discussed. In

order to deepen our understanding of the interactions between flavonoids and UGT1A1, the inhibition kinetics of acacetin and kaempferol, the most potent UGT1A1 inhibitors among all tested flavonoids, were carefully examined, using two different probe substrates for UGT1A1. Our results strongly support the need to investigate the inhibitory effects of natural flavones and their derivatives toward human UGTs, especially those that are responsible for the elimination of endogenous toxins or clinical drugs with narrow therapeutic windows [32, 51-52].

2. Material and methods

2.1. Chemicals and reagents

Pooled human liver microsomes (HLMs, from 50 donors, lot no. X008067) were obtained from BioreclamationIVT (Baltimore, MD, USA). Tris-HCl, 7-hydroxycoumarin, uridine-5'-diphosphoglucuronic acid (UDPGA) trisodium salt and polyethylene glycol hexadecyl ether (Brij 58) were obtained from Sigma-Aldrich (St. Louis, MO, USA). N-3-carboxy propyl-4-hydroxy-1,8-naphthalimide (NCHN) and N-butyl-4-(4-hydroxyphenyl)-1,8-naphthalimide (NHPN) were chemically synthesized in our lab as previously reported [47, 53]. 5,6-dihydroxyflavone, 5,7-dihydroxyflavove, 6,7-dihydroxyflavove, and 3',4'-dihydroxyflavone were purchased from Alfa Aesar (Ward Hill, MA). 3-hydroxyflavone, 5-hydroxyflavone, 6-hydroxyflavone, 6-methoxyflavone and 7,8-dihydroxyflavone were purchased from TCI (Tokyo, Japan). 5-hydroxy-6-methoxyflavone, 7-methoxybaicalein, 6,7-dimethoxybaicalein,

5,6,7-trimethoxybaicalein and 8-nitrobaicalein were synthesized by the author (Dr. Ping Wang), the synthetic details and structural characterization of these compounds were described previously [54]. Norwogonin and 8-methoxykaempferol were purchased from ChemFaces (Wuhan, China). Isorhamnetin, eupatilin, icaritin, fisetin, and morin were purchased from Sichuan Weikeqi Biotechnology Company (Chengdu, Sichuan, China). Other flavonoids that were used in this study were purchased from Chengdu Pufei De Biotech Co., Ltd. (Chengdu, Sichuan, China). The flavonoids purity was above 98% by LC-UV analysis. All other reagents were of HPLC grade or of the highest grade commercially available.

2.2 Florescence-based enzyme inhibition assay

2.2.1 NCHN-O-glucuronidation based inhibition assay

The inhibitory effects of flavonoids on NCHN glucuronidation by the human UGT1A1, were investigated in HLM. A typical incubation mixture (total volume of 200 μL), consisted of HLM (0.2 mg/mL), 2 mM UDPGA, 5 mM $MgCl_2$, 50 mM Tris-HCl (pH = 7.4), Brij 58 (0.1 mg/mg protein) and the probe substrate NCHN. The NCHN concentration in the initial screening assays was 20 μM and the tested flavonoid concentrations were 1, 10 and 100 μM. In experiments for inhibition constant determination, the NCHN concentration was changed between 15 and 100 μM, whereas the flavonoids concentrations were between 1 and 80 μM. HLM were first pre-incubated with Brij 58 (0.1 mg/mg microsomal protein, make the structure of the enzyme flipping to remove the latency) on ice for 20 min and then at 37℃ for 3 min. The reaction was initiated by adding 10 μL of UDPGA and continued for 40 min (in the case of HLM) at 37℃, before termination by the addition of 200 μL acetonitrile. The

incubation mixtures were subsequently centrifuged at 20,000 ×g, 4℃ for 20 min and 200 μL aliquots of the supernatants were diverted into black 96-well plates for determining the fluorescence intensity of NCHN-O-glucuronide, in accordance with our previously published methods [43, 55-56]. A positive control (positive inhibitor, PPT) was also carried out under the same conditions. NCHN and each flavonoid compound were dissolved in DMSO to a final concentration of 1% (v/v).

2.2.2 NHPN-O-glucuronidation based inhibition assay

The inhibitory effects of flavonoids on UGT1A1 activity of selected flavonoids, acacetin and kaempferol, was also investigated using a second specific fluorescent probe substrate, NHPN. The incubation mixture in this case (total volume of 200 μL) contained 0.1 mg/mL HLM, 50 mM Tris-HCl (pH = 7.4), Brij 58 (0.1 mg/mg microsomal protein, make the structure of the enzyme flipping to remove the latency), 2 mM UDPGA, 5 mM $MgCl_2$, and NHPN at varying concentrations from 1 to 40 μM, for inhibition constant determination. The inhibitor concentrations (acacetin or kaempferol), in these inhibition constant determination analyses were ranged from 1 and 100 μM. All incubations, from preincubation to termination and subsequent centrifugation, were carried out as described above for NCHN O-glucuronidation, and the fluorescence determination of NHPN concentration was carried out as previously described [42, 46-47]. NHPN and each of the flavonoids were dissolved in DMSO, at a final concentration of 1% (v/v).

2.2.3 Inhibition kinetic analyses

The residual activity of UGT1A1 was calculated as follow: residual activity (%) = (the

fluorescence intensity of NCHNG or NHPNG formed in the incubation sample in the presence of inhibitor)/(the fluorescence intensity of NCHNG or NHPNG formed in the incubation samples in the absence of inhibitor) × 100%. The half maximal inhibition concentration (IC_{50}) was determined by using different concentrations of the flavonoids compounds with the same fluorescent substrate concentration. The inhibition kinetic modes and the corresponding inhibition constant (K_i) values were determined by performing a set of analyses in which the concentrations of both the fluorescent substrate and the flavonoid compound were changed [57-60]. The following equations for competitive inhibition Eq. (1), noncompetitive inhibition Eq. (2), or mixed inhibition Eq. (3) were used to calculate the K_i values.

$$V=(V_{max}S)/\ [K_m\ (+I/K_i)\ +S] \quad (1)$$

$$V=(V_{max}S)/\ [(K_m+S)\ (1+I/K_i)] \quad (2)$$

$$V=(V_{max}S)/\ [(K_m+S)\ (1+I/\alpha K_i)] \quad (3)$$

where V is the velocity of the reaction; V_{max} is the maximum velocity; S and I are the substrate and inhibitor concentrations, respectively; K_m is the Michaelis constant (substrate concentration at 0.5 V_{max}); K_i is the inhibition constant describing the affinity of the inhibitor towards the target enzyme (UGT1A1).

2.2.4 Molecular docking simulations

A knowledge-based comparative modelling method (SYBYL Advanced Protein Modeling Package) was used to construct a 3D structure of UGT1A1. Using the ORCHESTRAR

module in the APM software package, we established a full-atom model of UGT1A1 that is based on the amino acid sequence of the enzyme and several known structural homologues. The Homo sapiens sequence of UGT1A1 (accession number NP_000454.1) was obtained from NCBI. After searching for similarity among target sequences and homologues in the protein database using a fugue module, the crystal structure of UDP-glucosyltransferase GtfB (PDB code = 1IIR) was selected as the homology template. On the basis of this template, a backbone was constructed, as well as structurally-conserved regions (SCR) and models of variable regions of the structure. Finally, a three-dimensional model of UGT1A1 was obtained through following energy minimization optimization.

Using the Surflex-Dock software package, the ligand interaction mode and binding affinity of the compounds with the constructed UGT1A1 model was studied. Surflex-dock uses a patented search engine to link the ligands to the active site of the protein and uses an empirical scoring function to evaluate the binding. The established 3D model of UGT1A1 was prepared as an acceptor by adding missing residues and calculating the partial charge of AMBER7FF99. Four compounds, namely NCHN, NHPN, acacetin, and kaempferol, were optimized by energy minimization and conformational search using a Tripos field. The gas charge was calculated using the Gasteiger-Huckel charge method. The automated mode was used to generate the active site in combination with each substrate, and the float radius was set to 3. The protein-ligand complexes with the highest LibDock score were taken from the docking results and are depicted in full text.

2.2.5 Data analysis

The kinetic and inhibition experiments were performed in triplicate. All values obtained from experiments are expressed as mean ± SD. The IC_{50} and K_i values were obtained by nonlinear regression using Graph Pad Prism 6.0 software (Graph Pad Software, Inc., La Jolla, CA, USA). Goodness-of-fit parameters were employed to identify the most appropriate inhibition mode [61].

3. Results and discussion

3.1 Screening flavonoids for inhibition potentials against UGT1A1

In this study, to systematically explore the structure-inhibition relationships of flavones as UGT1A1 inhibitors, we have collected 56 flavones and assayed their inhibitory effects on the human UGT1A1, using a fluorescence-based biochemical assay [47]. Firstly, the inhibition potentials of all collected flavones against UGT1A1 catalyzing NCHN-O-glucuronidation were screened (**Figure 1**), taking advantage of the high fluorescence intensity and UGT1A1-specificity of NCHN as glucuronidation substrate in HLM [47]. The results revealed that most of the tested flavones inhibited UGT1A1-mediated NCHN-O-glucuronidation by 50% or more at the high concentration (**Figure 1**). However, there were some compounds, such as 2, 4, 16 and 42, that did not have a significant effect. A unique exception within the current flavonoids collection was the result with 6-methoxyflavone, compound no. **10**, that clearly activated UGT1A1 by about 2-fold (**Figure 1**).

After the first inhibition screen, the dose-inhibition curves of the flavonoids for UGT1A1 were depicted, using different inhibitor concentrations (**Figures S2 to S6**), and the derived IC_{50} values of all the tested flavonoids were listed in **Table 1**. In addition to the flavonoids in our collection, UGT1A1 inhibition by protopanaxatriol (PPT), a known UGT1A1 inhibitor, was used as a positive control [62-63]. Twenty-one flavones displayed strong inhibition of UGT1A1, with IC_{50} values lower than 10 μM (Table 1). Among them, 6,7-dimethoxybaicalein (**11**; IC_{50} value 5.4 μM), 7,4'-dihydroxyflavone (**15**; 3.6 μM), apigenin 7-O-methyl ether (**31**; 4.8 μM), hispidulin (**32**; 6.2 μM), acacetin (**37**; 3.6 μM), galangin (**45**; 5.0 μM), morin (**48**; 4.2 μM), kaempferol (**50**; 3.3 μM) and anhydroicaritin (**53**; 3.9 μM) were more potent inhibitors of UGT1A1 than the positive control PPT (IC_{50} = 6.5 μM). These findings demonstrate that many natural flavonoids can strongly inhibit the enzymatic activity of UGT1A1, suggesting that medicinal plants that are rich in flavonoids might trigger metabolic disorder of bilirubin metabolism *via* UGT1A1 inhibition.

3.2 Structure-inhibition relationships of flavonoids against UGT1A1

It appears from the results in **Table 1** that the inhibitory effects of flavonoids on UGT1A1 may depend on the number and position of phenolic hydroxyl groups in the flavonoid skeleton. The structure-inhibition relationships of flavonoids with respect to the human UGT1A1 catalyzing NCHN-O-glucuronidation, as derived from our experimental results, are summarized in **Figure 2**. It is suggested that a hydroxyl group at the C-3 site will enhance UGT1A1 inhibition, as indicated by the following pairs (or small group): 5,7-dihydroxyflavone **7** (9.40 μM) vs. galangin **45** (5.03 μM), apigenin **29** (18.57 μM) vs.

kaempferol **50** (3.34 μM), as well as luteolin **24** (7.78 μM) and quercetin **46** (7.47 μM). Another observation was that a hydroxyl group at the C-5 site in the A ring, forming either a catechol or a pyrogallol group, enhances the inhibitory effects on UGT1A1, as demonstrated by 6,7-dihydroxyflavone **12** (23.20 μM) vs. baicalein **18** (12.43 μM) and 6-hydroxyflavone **9** (17.29 μM) vs. 5,6-dihydroxyflavone **5** (16.29 μM). However, 5-OH that forms a resorcinol structure may decrease the inhibition activity, as shown by comparing 7,8-dihydroxyflavone **14** (30.89 μM) and norwogonin **35** (51.32 μM), as well as 7,4'-dihydroxyflavone **15** (3.65 μM) and apigenin **29** (18.57 μM). These findings suggested that, if not forming a resorcinol group, a catechol or pyrogallol group at the 5-OH position on the flavonoid skeleton could stimulate UGT1A1 inhibition.

Remarkably, the hydroxyl group at the C-8 site had a significantly negative impact on UGT1A1 inhibition, but inhibition was recovered when the 8-hydroxyl group was replaced by a methoxy group, such as in 5,7-dihydroxyflavone **7** (9.40 μM) and wogonin **22** (10.01 μM) vs. norwogonin **35** (51.32 μM), as well as kaempferol **50** (3.34 μM) and 8-methoxykaempferol **51** (6.56 μM) vs. herbacetin **49** (56.13 μM). These results suggested that introducing a hydrophilic group at the flavones' C-8 site would decrease UGT1A1 inhibition. To test this assumption, compound **41** and **42** were semi-synthesized by introducing a hydrophilic tertiary amine group at the C-8 site of baicalein and 7-hydroxyflavone, respectively. The results showed that the IC_{50} values of compound **41** and **42** were 69.20 μM and more than 100 μM, respectively. These findings strongly support introduction of a hydrophilic group at the C-8 of flavones as a way to reduce its inhibitory effect on UGT1A1. Meanwhile, such structural modification would also improve the

metabolic stability of these flavones, enhance their water solubility and could potentially reduce liver toxicity [64-65]. It should also be added that flavonoid glycosides displayed very weakly inhibition on UGT1A1-mediated NCHN-O-glucuronidation, in comparison to the corresponding aglycones. These experimental data may be regarded as a proof of concept that to improve the metabolic stability and the safety profiles of some investigational new drugs (especially for flavonoid-type drug candidates) without strong UGT1A1 inhibition.

3.3 Inhibition mechanism

Considering that the wide distribution of acacetin and kaempferol in herbs or vegetables, as well as their strong inhibition of the human UGT1A1, we further investigated the inhibition mechanism of these two flavonoids toward UGT1A1. In this part, two highly specific florescent probes for UGT1A1, NCHN and NHPN, were used to explore the interactions of these two UGT1A1 inhibitors with the ligand-binding sites of UGT1A1, as well as to investigate their inhibition kinetics. Notably, a recent study has demonstrated that NCHN and NHPN may bind at different ligand-binding sites within UGT1A1, only one of them, the NHPN site, also binds bilirubin [47]. Thus, NCHN and NHPN could be used as site-specific makers for UGT1A1 ligands, at least for the two important ligand-binding sites we are currently certain about [47].

3.3.1 UGT1A1 Inhibition by acacetin

In HLM, acacetin could inhibit UGT1A1-mediated both NCHN-O-glucuronidation and NHPN-O-glucuronidation activity at almost identical IC50 values of 3.57 μM, and 3.84 μM, respectively (**Figure 3A & 3B**). Further inhibition assays in HLM were carried out and the

results demonstrated that the inhibition of both NCHN- and NHPN-O-glucuronidation by acacetin was not time-dependent (**Figure S7**), suggesting that acacetin was a reversible inhibitor of this enzyme. Lineweaver-Burk plots demonstrated that acacetin functioned as a mixed inhibitor of NCHN-O-glucuronidation in HLM (**Figure 3C**), while competitive inhibitor of the NHPN-O-glucuronidation in HLM (**Figure 3D**). The Ki values of acacetin, in HLM, for NCHN-O-glucuronidation and NHPN-O-glucuronidation were 8.56±0.87 μM and 5.95±0.16 μM, respectively. However, careful analysis of the NCHN-O-glucuronidation inhibition kinetics by acacetin indicated that it is of a mixed type and the value of the second, only little higher αK_i value is 32.57±1.43 μM. This explains why the inhibition of HLM catalyzed NCHN glucuronidation by acacetin appeared first nearly as competitive.

3.3.2 *UGT1A1 Inhibition by kaempferol*

UGT1A1 inhibition by kaempferol was investigated in a similar way to the assays with acacetin, using both NCHN and NHPN as UGT1A1 probe substrates. Hence the inhibition kinetic mode was determined for each of the two reactions, and the corresponding K_i values of kaempferol for them were calculated. As shown in **Figure 4,** kaempferol efficiently inhibited both NCHN-O-glucuronidation and NHPN-O-glucuronidation in HLM, exhibiting IC_{50} values of 3.34 μM, and 2.33 μM, respectively (**Figure 4A & 4B**). Time-dependent inhibition assays demonstrated that in both cases the inhibition was not time-dependent (**Figure S7**), suggesting that kaempferol is also a reversible inhibitor of UGT1A1. Lineweaver-Burk plots revealed that kaempferol functions as a noncompetitive inhibitor toward NCHN-O-glucuronidation in HLM (**Figure 4C**). Unlike NCHN-O-glucuronidation,

the inhibition of NHPN-O-glucuronidation in HLM by kaempferol was clearly competitive (**Figure 4D**). The K_i values of kaempferol were 1.74±0.29 μM and 0.68±0.24 μM for the inhibition of NCHN- and NHPN- O-glucuronidation reactions, respectively. These findings indicate that kaempferol is a particularly strong inhibitor of the human UGT1A1. Moreover, this compound binds at the NHPN binding site, also named the bilirubin-binding site. Hence, our findings suggest that uptake of kaempferol into the liver, so that its concentration will approach 0.4-0.5 μM, could start interfering with bilirubin conjugation, perhaps even leading to hyperbilirubinemia in some individuals, particularly those that have low expression level of the enzyme, as in the rather common Gilbert's Syndrome. Acacetin is a strong inhibitor of UGT1A1 as well, even if not as strong as kaempferol. It might be added that when considering the possible risks of these and other flavonoids for bilirubin glucuronidation by UGT1A1, one also has to consider the rates of detoxification and excretion of the flavonoids themselves, let alone their generally poor bioavailability [66-68]. More investigation on these topics should be done in the future to fully clarify their risk to humans.

3.4 Docking simulations

In order to gain deeper insight into the inhibition of UGT1A1 by acacetin and kaempferol from the point of view of ligand-enzyme interactions, molecular docking simulations were performed, using a homology model of the human UGT1A1. A knowledge-based comparative modeling method (SYBYL Advanced Protein Modeling Package) was used to construct a 3D structure of UGT1A1. As shown in **Figure 5**, acacetin and kaempferol, as well as the two probe substrates of UGT1A1 (NCHN and NHPN), could be well-docked into the

catalytic domain of the enzyme, but the top scoring binding conformations of these 4 ligands differed considerably. The binding areas of NHPN, kaempferol and acacetin on UGT1A1 highly overlapped, while the binding area of NCHN was clearly separated and distinguished (**Figure 5**). Furthermore, docking simulations demonstrated that kaempferol could form strong interactions with UDPGA, Ser375 and His376 *via* hydrogen-bonding (**Figure S8),** while acacetin could form strong interactions with UDPGA, Ser375 and Glu289 (instead of His376) *via* hydrogen-bonding (**Figure S9)**. These findings are consistent with the experimental results, in which acacetin and kaempferol were both potent and non-competitive inhibitors toward UGT1A1-mediated NCHN-O-glucuronidation but functioned as competitive inhibitors toward UGT1A1-mediated NHPN-O-glucuronidation.

4. Conclusion

In summary, the inhibitory effects of flavonoids on UGT1A1, one of the most important human conjugative enzymes, have been systematically investigated. The results suggest that hydroxyl groups at the C-3 and C-7 positions of the flavones can enhance UGT1A1 inhibition, while flavonoids that carry glycoside groups displayed weak inhibition than corresponding aglycones. Detailed structure-inhibition relationships of flavonoids towards UGT1A1 have been summarized for the first time. Further investigation and inhibition kinetic analyses for acacetin and kaempferol, demonstrated that these two flavonoids are non-competitive UGT1A1 inhibitors toward UGT1A1-mediated NCHN-O-glucuronidation, but are competitive inhibitors toward UGT1A1-mediated NHPN-O-glucuronidation. The new

findings not only shed light on the structure of the UGT1A1 substrate binding site, but contribute valuable knowledge on the interactions between flavonoids and UGT1A1. All the information and knowledge that are presented here will be useful for avoiding potential food/herb-drug/endobiotic interactions in the clinic, as well as for the development of new flavonoid-type drugs without UGT1A1 inhibition.

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Figure legends

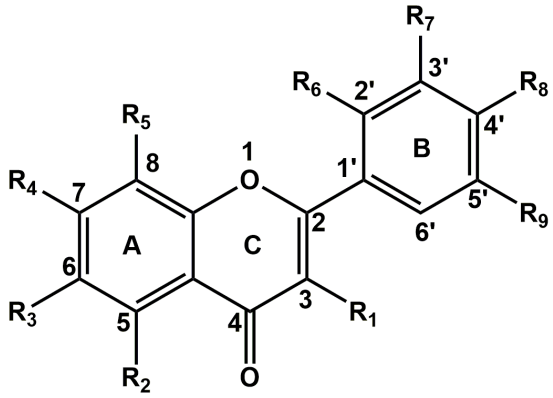
Figure 1 The inhibitory effects of all tested the flavonoids (final concentration, 100 μM) on UGT1A1-mediated NCHN-O-glucuronidation in HLM. The data are presented as the means of triplicate determinations.

Figure 2 The structure-inhibition relationship of flavonoids against UGT1A1-mediated NCHN-O-glucuronidation

Figure 3 Inhibition type analysis of acacetin against UGT1A1 in HLM. Acacetin exerted a dose-dependent inhibition towards UGT1A1-catalyzed NCHN glucuronidation and NHPN glucuronidation reactions (A and B, respectively). Lineweaver-Burk plots of acacetin against UGT1A1-mediated NCHN-O-glucuronidation and NHPN-O-glucuronidation, both in HLM (C and D, respectively). Each point represents the mean of triplicate determinations.

Figure 4 Inhibition type analysis of kaempferol against UGT1A1 in HLM. Kaempferol exerted a dose-dependent inhibition towards UGT1A1-catalyzed NCHN glucuronidation and NHPN glucuronidation reactions (A and B, respectively). Lineweaver-Burk plots of kaempferol against UGT1A1-mediated NCHN-O-glucuronidation and NHPN-O-glucuronidation in HLM (C and D, respectively). Each point represents the mean of triplicate determinations.

Figure 5 The binding poses of NPHN, NCHN, kaempferol and acacetin in the active pocket of UGT1A1. (A) An overview of UGT1A1 binding site with kaempferol. (B) A closer and more detailed view of kaempferol bound at the active pocket of UGT1A1. (C) An overview of UGT1A1 binding with acacetin. (D) A closer and more detailed view of acacetin bound at the active pocket of UGT1A1. The four molecules are depicted in white, cyan, yellow and magenta, respectively. NPHN, NCHN were used to represent ligand-binding site I and ligand binding site II. UDPGA is shown in balls and sticks type.



Flavonoids **1~56**

Table 1. The IC_{50} value of the flavones towards human UGT1A1 using NCHN as the fluorescent probe substrate

No.	Compound	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9	IC_{50} (μM)
1	3-Hydroxyflavone	OH	H	H	H	H	H	H	H	H	16.39±3.83
2	3-Methoxyflavone	OCH_3	H	H	H	H	H	H	H	H	>100
3	3,6-Dihydroxyflavone	OH	H	OH	H	H	H	H	H	H	23.83±2.44
4	5-Hydroxyflavone	H	OH	H	H	H	H	H	H	H	>100
5	5,6-Dihydroxyflavone	H	OH	OH	H	H	H	H	H	H	16.29±1.46
6	5-hydroxy-6-methoxyflavone	H	OH	OCH_3	H	H	H	H	H	H	11.53±1.84

7	5,7-Dihydroxyflavone	H	OH	H	OH	H	H	H	H	H	9.40±1.10
8	5,6,7-Trimethoxyflavone	H	OCH_3	OCH_3	OCH_3	H	H	H	H	H	9.86±1.01
9	6-Hydroxyflavone	H	H	OH	H	H	H	H	H	H	17.29±0.98
10	6-Methoxyflavone	H	H	OCH_3	H	H	H	H	H	H	**Activator**
11	6,7-Dimethoxybaicalein	H	OH	OCH_3	OCH_3	H	H	H	H	H	5.39±0.96
12	6,7-Dihydroxyflavone	H	H	OH	OH	H	H	H	H	H	23.20±1.99
13	7-Hydroxyflavone	H	H	H	OH	H	H	H	H	H	9.23±2.09
14	7,8-Dihydroxyflavone	H	H	H	OH	OH	H	H	H	H	30.89±2.97
15	7,4'-Dihydroxyflavone	H	H	H	OH	H	H	H	OH	H	3.65±0.30
16	7-hydroxy-4'-methoxyflavone	H	H	H	OH	H	H	H	OCH_3	H	>100
17	3',4'-Dihydroxyflavone	H	H	H	H	H	H	OH	OH	H	16.47±1.55
18	Baicalein	H	OH	OH	OH	H	H	H	H	H	12.43±0.69
19	Baicalin	H	OH	OH	**O-GluA**	H	H	H	H	H	39.29±3.67
20	Scutellarein	H	OH	OH	OH	H	H	H	OH	H	32.23±3.99
21	Scutellarin	H	OH	OH	**O-GluA**	H	H	H	OH	H	74.62±9.62
22	Wogonin	H	OH	H	OH	OCH_3	H	H	H	H	10.01±1.65
23	Wogonoside	H	OH	H	**O-GluA**	OCH_3	H	H	H	H	>100

24	Luteolin	H	OH	H	OH	H	H	OH	OH	H	7.78±0.57
25	Orientin	H	OH	H	OH	**C-Glu**	H	OH	OH	H	48.26±5.90
26	Cynaroside	H	OH	H	**O-Glu**	H	H	OH	OH	H	37.22±1.42
27	Oroxylin A	H	OH	OCH_3	OH	H	H	H	H	H	9.14±1.54
28	Oroxyloside	H	OH	OCH_3	**O-GluA**	H	H	H	H	H	>100
29	Apigenin	H	OH	H	OH	H	H	H	OH	H	18.57±2.94
30	Apigenin-7-O-glucronide	H	OH	H	**O-GluA**	H	H	H	OH	H	25.10±4.52
31	Apigenin 7-O-methyl ether	H	OH	H	OCH_3	H	H	H	OH	H	4.79±0.75
32	Hispidulin	H	OH	OCH_3	OH	H	H	H	OH	H	6.23±0.90
33	7-methoxybaicalein	H	OH	OH	OCH_3	H	H	H	H	H	11.08±1.97
34	8-Nitrobaicalein	H	OH	OH	OH	NO_2	H	H	H	H	7.50±0.71
35	Norwogonin	H	OH	H	OH	OH	H	H	H	H	51.32±9.99
36	Diosmetin	H	OH	H	OH	H	H	OH	OCH_3	H	17.70±2.81
37	Acacetin	H	OH	H	OH	H	H	H	OCH_3	H	3.57±0.37
38	Isovitexin	H	OH	**C-Glu**	OH	H	H	H	OH	H	60.90±7.42
39	Vitexin	H	OH	H	OH	**C-Glu**	H	H	OH	H	61.70±8.82
40	Eupatilin	H	OH	OCH_3	OH	H	H	OCH_3	OCH_3	H	12.61±2.01

41	8-((dimethylamino)-methyl)-baicalein	H	OH	OH	OH	**R'**	H	H	H	H	69.20±10.64
42	8-((dimethylamino)-methyl)-7-hydroxyflavonoid	H	H	H	OH	**R'**	H	H	H	H	>100
43	Flavopiridol	H	OH	H	OH	**R''**	Cl	H	H	H	>100
44	Lysionotin	H	OH	OCH_3	OH	OCH_3	H	H	OCH_3	H	9.18±1.25
45	Galangin	OH	OH	H	OH	H	H	H	H	H	5.03±0.29
46	Quercetin	OH	OH	H	OH	H	H	OH	OH	H	7.47±1.20
47	Quercitrin	**O-Glu**	OH	H	OH	H	H	OH	OH	H	12.39±1.25
48	Morin	OH	OH	H	OH	H	OH	H	OH	H	4.19±0.29
49	Herbacetin	OH	OH	H	OH	OH	H	H	OH	H	56.13±9.14
50	Kaempferol	OH	OH	H	OH	H	H	H	OH	H	3.34±0.40
51	8-Methoxykaempferol	OH	OH	H	OH	OCH_3	H	H	OH	H	6.56±0.56
52	Myricetin	OH	OH	H	OH	H	H	OH	OH	OH	10.46±1.55
53	Anhydroicaritin	OH	OH	H	OH	**prenyl**	H	H	OCH_3	H	3.93±0.49
54	Icariside I	OH	OH	H	**O-Glu**	prenyl	H	H	OCH_3	H	6.01±1.11
55	Isorhamnetin	OH	OH	H	OH	OH	H	OCH_3	OH	H	6.67±0.39
56	Fisetin	OH	H	H	OH	H	H	OCH_3	OH	H	11.49±1.92
57	Protopanaxatriol (PPT)	positive control									6.54±0.87

* GluA: Glucuronic acid; Glu: Glucose; rha: rhamnoside; R’ = $CH_2N(CH_3)_2$; R’’ = $CH(CH_2CH_2)(CHOHCH_2)NCH_3$

Table 2. Inhibition kinetic parameters of acacetin and kaempferol against UGT1A1-mediated **NCHN-** or **NHPN-O-**Glucuronidation in HLM

Inhibitor	Substrate	IC_{50} (μM)	K_i (μM)	The type of inhibition	αK_i (μM)	Goodness of fit (R^2)
Acacetin	NCHN	3.57±0.37	8.56±0.87	mixed	32.57±1.43	0.99
	NHPN	2.08±0.27	5.95±0.16	competitive	n/a	0.99
Kaempferol	NCHN	3.34±0.18	1.74±0.29	noncompetitive	n/a	0.97
	NHPN	2.33±0.33	0.68±0.24	competitive	n/a	0.97

* n/a: not applicable

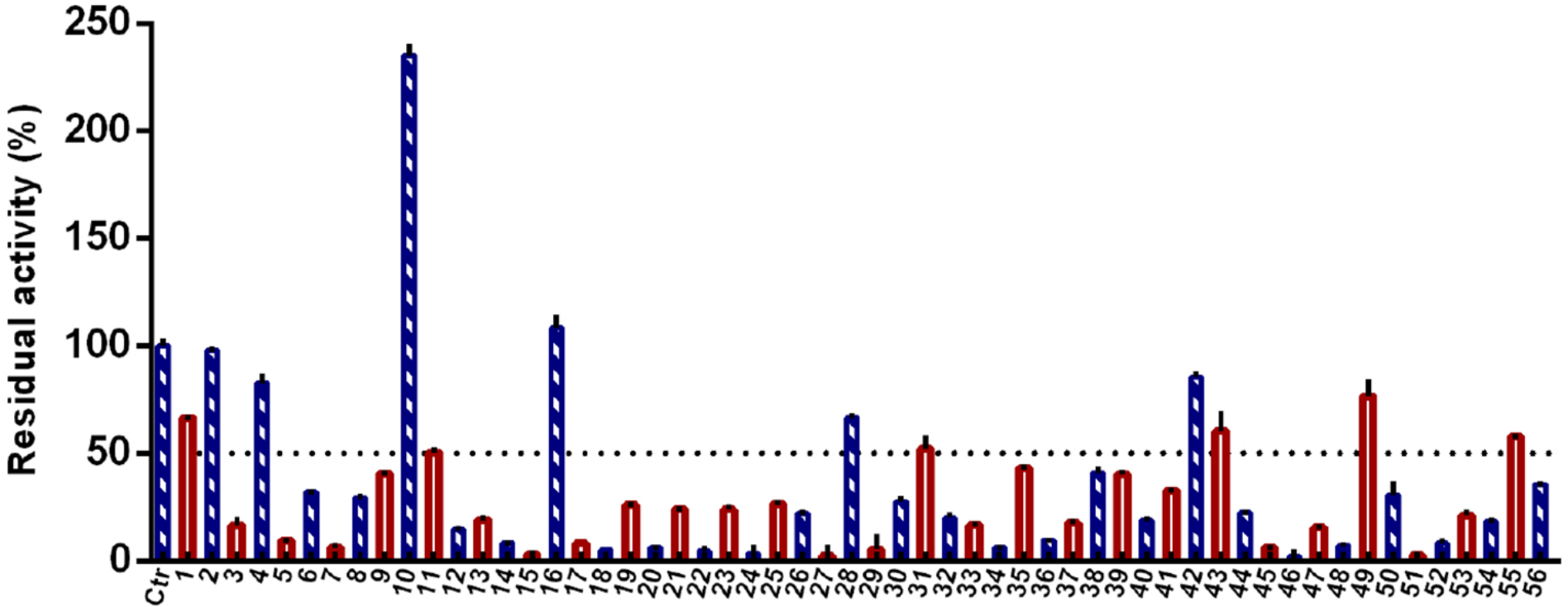
Highlights

1. 56 natural and synthetic flavonoids were collected and their inhibitory effects on UGT1A1 were assayed.

2. The structure-activity relationships of flavonoids as UGT1A1 inhibitors were summarized.

3. The inhibition mechanism of acacetin and kaempferol toward two different activities of UGT1A1 were carefully investigated.

4. Docking simulations were conducted to explore the interactions between UGT1A1 and acacetin/kaempferol.


Residual activity (%)
250
200
150
100
50
0
Ctr
1
2
3
4
5
6
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11
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Figure 1

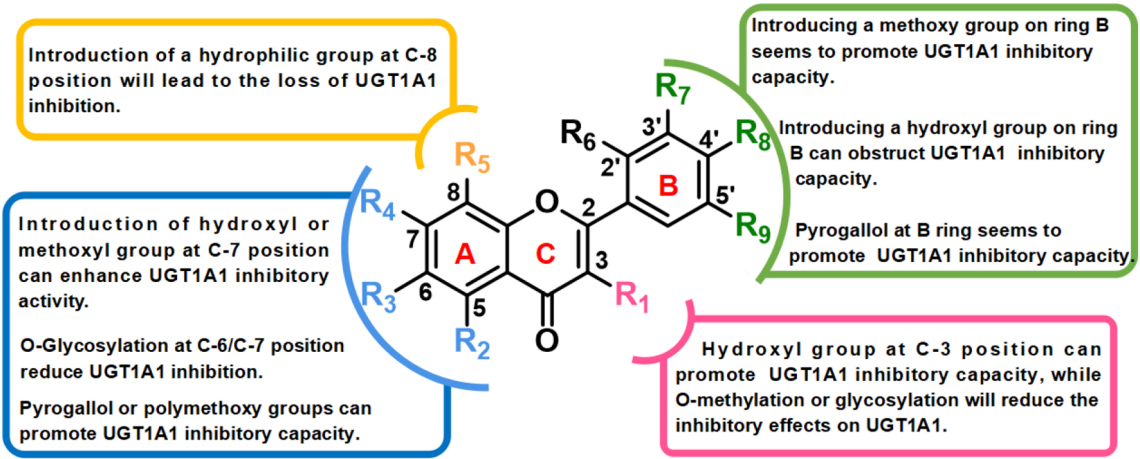
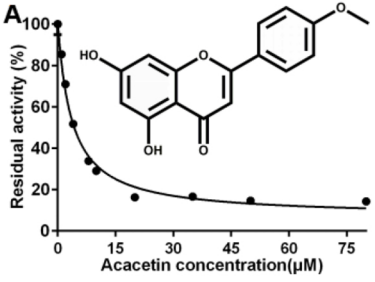
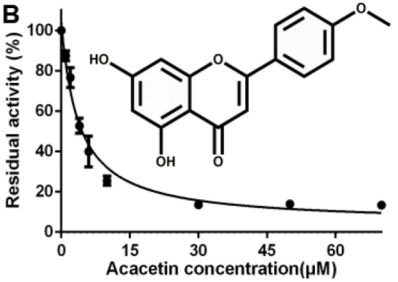
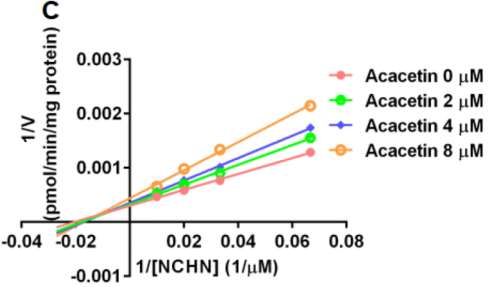

Introduction of a hydrophilic group at C-8 position will lead to the loss of UGT1A1 inhibition.
Introducing a methoxy group on ring B seems to promote UGT1A1 inhibitory capacity.
Introducing a hydroxyl group on ring B can obstruct UGT1A1 inhibitory capacity.
Pyrogallol at B ring seems to promote UGT1A1 inhibitory capacity.
Introduction of hydroxyl or methoxyl group at C-7 position can enhance UGT1A1 inhibitory activity.
O-Glycosylation at C-6/C-7 position reduce UGT1A1 inhibition.
Pyrogallol or polymethoxy groups can promote UGT1A1 inhibitory capacity.
Hydroxyl group at C-3 position can promote UGT1A1 inhibitory capacity, while O-methylation or glycosylation will reduce the inhibitory effects on UGT1A1.
R5
R4
R3
R2
R1
R6
R7
R8
R9
A
B
C
O
2
3
5
6
7
8
2'
3'
4'
5'

Figure 2


A
Residual activity (%)
Acacetin concentration(μM)
HO
O
OH
O


B
Residual activity (%)
Acacetin concentration(μM)
HO
O
OH
O


C
1/V
(pmol/min/mg protein)
1/[NCHN] (1/μM)
Acacetin 0 μM
Acacetin 2 μM
Acacetin 4 μM
Acacetin 8 μM

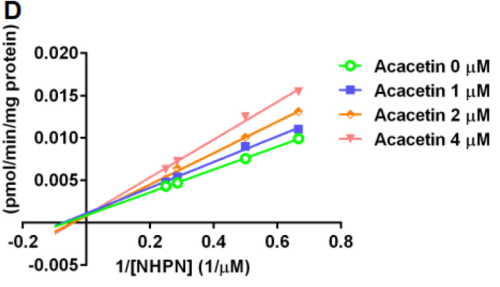

D
1/V
(pmol/min/mg protein)
1/[NHPN] (1/μM)
Acacetin 0 μM
Acacetin 1 μM
Acacetin 2 μM
Acacetin 4 μM

Figure 3

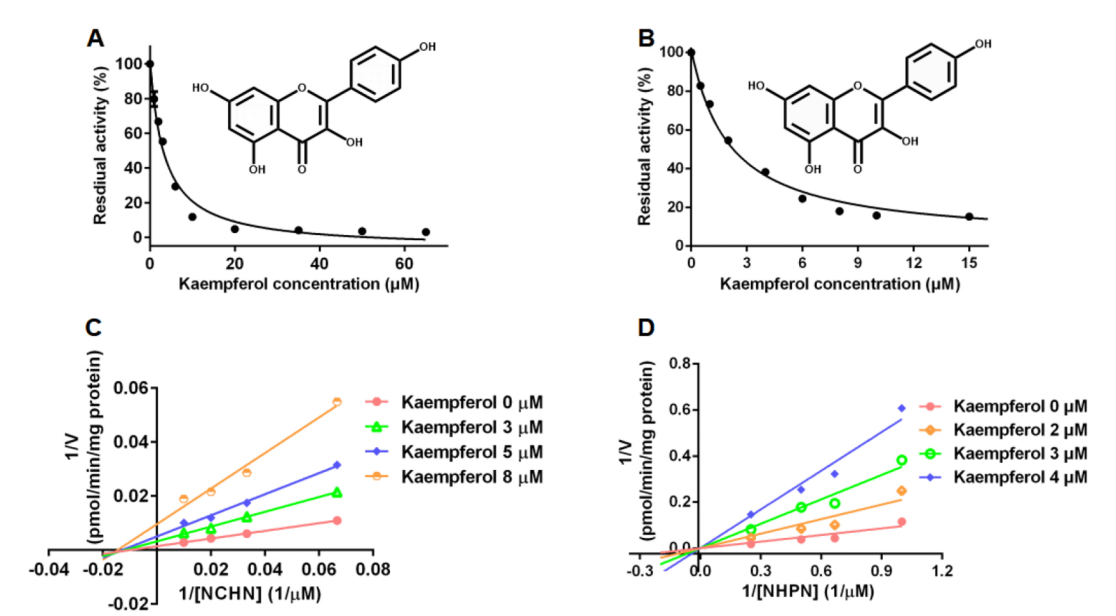

A
Residual activity (%)
Kaempferol concentration (μM)
B
Residual activity (%)
Kaempferol concentration (μM)
C
1/V (pmol/min/mg protein)
1/[NCHN] (1/μM)
Kaempferol 0 μM
Kaempferol 3 μM
Kaempferol 5 μM
Kaempferol 8 μM
D
1/V (pmol/min/mg protein)
1/[NHPN] (1/μM)
Kaempferol 0 μM
Kaempferol 2 μM
Kaempferol 3 μM
Kaempferol 4 μM

Figure 4

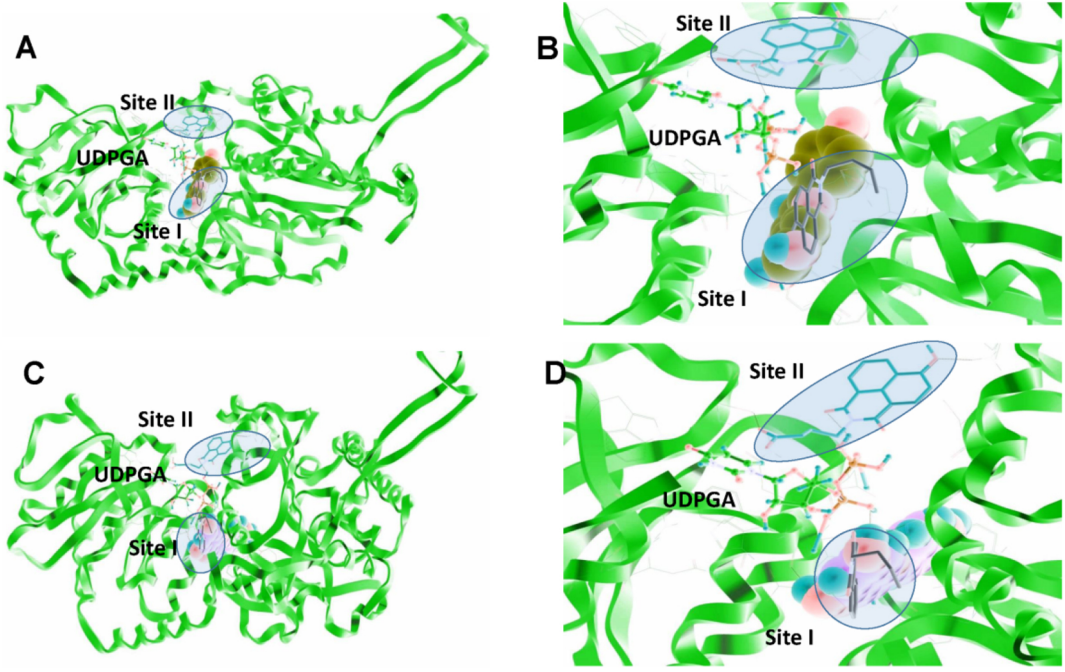

A
Site II
UDPGA
Site I
B
Site II
UDPGA
Site I
C
Site II
UDPGA
Site I
D
Site II
UDPGA
Site I

Figure 5