Effects of protein-protein interactions between human UGT1A isoforms on their catalytic properties

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Abstract

UDP-glucuronosyltransferases (UGTs, EC 2.4.1.17) are a family of membrane-bound enzymes that are involved in phase II of drug metabolism. Human UGTs are classified into two subfamilies, UGT1 and UGT2, based on similarities between their amino acid sequences and gene organization. To date, 19 human UGT isoforms have been identified. In this thesis, first, protein-protein interactions between UGTs and their effects on enzymatic activities were investigated. Thermal stability assay, immunoprecipitation, and native-PAGE analysis revealed the protein-protein interactions between UGT1A1, UGT1A4, UGT1A6, and UGT1A9. Kinetic analysis showed that kinetic parameters were changed by the coexpression of other isoforms. Second, the cause of the discrepancies of the inhibitory effects on glucuronidations between human liver microsomes and recombinant systems was elucidated. It was clarified that the substrates with high V_{max}/K_m values (> 200 μL/min/mg) showed prominent inhibitory effects toward the glucuronidation in human liver microsomes compared to those when using recombinant enzymes. Third, in silico techniques were used to investigate the cause of thermal stability of UGT1A9 protein. It was demonstrated that 13 amino acid residues, which are unique to UGT1A9, would collectively play an important role in the stabilization of the UGT1A9 conformation. These findings obtained in this study could provide information on UGT-UGT interactions and structural features of UGT protein.

Dissertation abstract

UDP-glucuronosyltransferases (UGTs, EC 2.4.1.17) are a family of membrane-bound enzymes that are involved in phase II of drug metabolism. They catalyze the formation of glucuronides by the transfer of glucuronic acid from a cofactor uridine 5'-diphosphoglucuronic acid (UDPGA) to hydroxyl, carboxyl, or amine groups of endogenous and exogenous

substrates. Human UGTs are classified into two subfamilies, UGT1 and UGT2, based on similarities between their amino acid sequences and gene organization. To date, 19 human UGT isoforms have been identified. UGTs have been reported to form homo- or hetero-oligomers, but the functional significance of the oligomerization is largely unknown. In this study, first, double expression systems were established and kinetic analyses were performed to investigate whether hetero-oligomerizations affect the enzymatic activity of UGTs. Second, the cause of the discrepancies between the inhibitory effects on glucuronidation in human liver microsomes and recombinant UGTs was determined. Third, the cause of the thermally stable property of UGT1A9 was investigated using *in silico* and *in vitro* techniques.

Effects of protein-protein interactions of human UGTs on the enzymatic activities

A number of studies suggested that mammalian UGTs form oligomers. However, whether or not human UGTs form homo- and hetero-oligomers remains to be determined. In addition, effects of the oligomerizations on UGT activities had not been cleared. In this study, the protein-protein interactions between UGTs and its effects on UGT activities were investigated. To confirm the formations of hetero-oligomers of UGTs, thermal stability assay, detergent resistance assay, immunoprecipitation, and native-PAGE were carried out using double expression systems in HEK293 cells. These experiments clearly demonstrated the hetero-oligomerizations of UGT1A1/UGT1A4 (native-PAGE), UGT1A1/UGT1A9 (thermal stability assay and detergent resistance assay), UGT1A4/UGT1A9 (thermal stability assay and detergent resistance assay), and UGT1A6/UGT1A9 (thermal stability assay, detergent resistance assay, and immunoprecipitation). Although hetero-oligomerizations of UGT1A1/UGT1A6 and UGT1A4/UGT1A6 were not detected, it is considered that those isoforms also interact with each other because UGT1A members share high amino acid sequence similarity. Kinetic parameters for UGT1A1, UGT1A4, UGT1A6, and UGT1A9-catalyzed glucuronidations were affected by other UGT isoforms. In conclusion, it was found that UGT1A1, UGT1A4, UGT1A6, and UGT1A9 interact each other, resulting in a change of the kinetics of the enzymatic activities. The results in this study suggest the difficulty of quantitative predictions of glucuronidation activities as well as the contribution of each UGT isoform to the activities in human liver microsomes [1, 2].

Product inhibition of UGT enzymes by UDP obfuscates the inhibitory effects of UGT substrates

Substantial progress has been made in identifying typical substrates for individual UGT isoforms. Although UGTs display broad and overlapping substrate specificities, some compounds are specific for certain UGT isoforms. Such substrates can be used as specific inhibitors to identify the UGT isoform(s) contributing to the glucuronidation of drugs. However,

previous reports have shown confusing results, namely that 4-nitrophenol and 1-naphthol, typical substrates for human UGT1A6, inhibited UGT1A1, UGT1A4, or UGT2B7-catalyzed glucuronidations in human liver microsomes, but did not inhibit the activities by recombinant UGTs. The present study found that 1-naphthol showed more potent inhibition of the UGT1A1- and UGT1A4-catalyzing activities in human liver microsomes than that by recombinant enzymes, whereas 1-naphthol more potently inhibited the UGT1A9-catalyzing activity by recombinant enzymes than that in human liver microsomes. As for the mechanism responsible for this discrepancy between human liver microsomes and recombinant enzymes, it was found that the prominent inhibition of UGT1A1 and UGT1A4 activities could be attributed to the inhibitory effects of UDP produced by UGT1A6-catalyzed 1-naphthol glucuronidation in human liver microsomes. In contrast, it was considered that the UGT1A6-catalyzed 1-naphthol glucuronidation in human liver microsomes attenuated the inhibitory effects of 1-naphthol on the UGT1A9 activity, since 1-naphthol has most potent inhibitory effects toward UGT1A9 within 1-naphthol, 1-naphthol *O*-glucuronide, and UDP. Thus, when human liver microsomes are used as an enzyme source for UGT inhibition studies, we should carefully interpret the results. Moreover, it was clarified that the substrates with high V_{max}/K_m values (> 200 μL/min/mg) showed prominent inhibitory effects toward the glucuronidation in human liver microsomes compared to those when using recombinant enzymes. In conclusion, it was demonstrated that UGT substrates with high turnover rates might confuse the identification of the UGT isoform responsible for the glucuronidation of drugs, when such compounds were used as inhibitors for the activities in human liver microsomes, owing to the production of UDP and/or glucuronide or reduction of the inhibitor per se. This finding is important for avoiding misinterpretations in the identification of UGT isoforms [3].

In silico and _in vitro_ approaches to elucidate the thermal stability of human UGT1A9

Human UGT1A9 is uniquely stable to heat treatment. To understand the unique property, the three-dimensional structure of UGT1A9 was constructed by homology modeling using a crystal structure of GtfA as a template. The structure of UGT1A8, which has the highest homology to UGT1A9 but is thermally instable, was also constructed as a reference. These structures contain two Rossmann fold-like domains in the N-terminus and C-terminus. Sequence alignment analysis revealed that 13 amino acid residues (42Arg, 91Lys, 92Ala, 106Tyr, 111Gly, 113Tyr, 115Asp, 152Asn, 173Leu, 219Leu, 221His, 222Arg, and 241Glu) are unique to UGT1A9 when compared with UGT1A7, UGT1A8, and UGT1A10. To examine the role of these residues in the conformational stability of UGT1A9, molecular dynamics simulation of the structures was carried out at 310 K and 360 K in aqueous solution for 3.0 nanoseconds. Root mean square deviation analyses revealed that 42Arg, 173Leu, 219Leu,

221His, and 222Arg were responsible for the thermal stability. Root mean square fluctuation analyses and dynamical cross correlation map revealed that 91Lys, 92Ala, 106Tyr, 111Gly, 113Tyr, 115Asp, 219Leu, 221His, 222Arg, and 241Glu were responsible for the thermal stability. *In vitro* study using mutants of these residues demonstrated that all of these amino acids might be collectively involved in the thermal stability of UGT1A9, although 152Asn would be most important. Thus, the results in this study could provide the molecular basis of the thermal stability of human UGT1A9.

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学位論文審査結果の要旨

UDP-glucuronosyltransferase (UGT) によって触媒されるグルクロン酸抱合反応は生体における外因性および内因性の化合物の主要な代謝経路である。UGT はスーパーファミリーを形成しており、薬物代謝の主要な臓器である肝臓には UGT1A1、UGT1A4、UGT1A6 および UGT1A9 が発現している。本研究では UGT の異分子種間における相互作用に着目し、熱安定性の測定、免疫沈降法および Native-PAGE によりタンパクレベルで相互作用していることを明らかにした。さらに速度論的解析を行うことで、UGT の異分子種間相互作用は酵素活性に影響を及ぼし、キネティックパラメータの変動を引き起こすことを初めて明らかにした。第二に、in vitro 実験において、ヒト肝ミクロソームと UGT 発現系を酵素源として用いた場合で阻害効果が大きく異なる原因について解析した。その結果、発現系では目的分子種のみ存在する一方で肝ミクロソームには多くの分子種が存在し、阻害剤の代謝反応によって生成した代謝産物 UDP が阻害作用を示すことが阻害効果の差の原因であることを明らかにした。第三に、UGT1A9 は他の分子種と比較し耐熱性であることを見出し、in silico におけるシミュレーション解析および in vitro における変異導入解析を行うことで、UGT1A9 に特異的に存在する 13 個のアミノ酸残基が協調的に耐熱性に重要な役割を果たしていることを明らかにした。本研究成果は UGT の in vitro 研究を行う上で重要な情報を与え、今後の UGT 研究の発展に大きく貢献するものであり、博士(薬学) に値すると判定した。