HCVコアタンパク質定量と細胞生存率解析

細胞ライセートまたは培養上清のHCVコアタンパク質は、GeneCopoeia (Rockville、 MD)を用いて定量した。また、細胞生存率は、CellTiter-Glo Luminescent Cell Viability Assay (Promega、 Madison、 WI)を用いて解析した。

トランスフェクション

細胞を24ウェルプレートに播種した後、siRNAをLipofectamine RNAiMAX (Invitrogen、San Diego、CA)により、プラスミドをTransIT-LT1 (Mirus、Madison、 WI)によりトランスフェクションした。

ルシフェラーゼレポーターアッセイ

miR-17-5pの標的配列を含むMAP3K8遺伝子の3'非翻訳領域をpGL3レポータープラスミドにサブクローニングした。標的配列に対する変異の導入はPCRによりおこなった。このルシフェラーゼレポータープラスミドとmiR-17-5p発現プラスミド（または空プラスミド）を、上記の方法により同時に細胞へ導入した。この48時間後にDual-Luciferase Reporter Assay System (Promega)によりルシフェラーゼ活性を解析した。

ウエスタンブロット

肝ホモジネートを調製し、これをSDS-polyacrylamide gel electrophoresisにかけて各タンパク質を分離した。タンパク質をニトロセルロース膜に転写した後、ブロッキングをおこなった。MAP3K8タンパク質は抗MAP3K8抗体 (ab70853、Abcam、 San Diego、 CA)により、内部標準タンパク質βアクチンは抗β-actin抗体(EP1123Y、 Abcam)により検出した。

IL28B遺伝子多型およびITPA遺伝子多型の解析

患者ゲノムDNAは全血からMagNA Pure LC and the DNA Isolation Kit (Roche Diagnostics)を用いて採取した。Interluekin 28B (IL28B)のrs8099917およびrs12979860、inosine triphosphatase (ITPA)のrs1127354はTaqMan SNP Genotyping Assaysにより解析した。

治療不応/部分応答因子に関わる統計解析

治療不応/部分応答因子同定にかかわる統計解析は、chi-square、Fisher's exact、Student's t、およびMann-Whitney two-tailed testsによりおこなった。また、SVRと関連する因子の同定には、多変量解析を用い、オッズ比（95%信頼区間）の計算もおこなった。統計計算結果はP値（両側）により示し、本値が<0.05を統計的優位とした。以上の統計計算にはSPSS(ver.17.0、 IBM-SPSS、Chicago、 IL)を用いた。

倫理面への配慮

本研究で解析した臨床検体は、倫理委員会の承認のもと、全てインフォームドコンセントを取得した後に採取した。検体の個人情報は外部に洩れることのないよう厳重に管理し、その試料等は個人情報管理者及び分担管理者を設け連結可能匿名化することにより、研究者に患者の特定ができないよう配慮した。また、本研究成果の発表にあたっては、患者の氏名などは一切公表しない。

C．研究結果

治療成績

全体として、62名（48%）の症例でSVRが認められ、36名（28%）の症例でRelapse、6名（5%）の症例で部分応答、26名（20%）の症例で治療不応答であった。以降、これら患者をSVR/relapse (S/R)と、部分応答/不応 (P/N) 群に分ける。P/N群にはγ-GTP高値、低アルブミン濃度の患者が多く、IL28Bの遺伝子多型マイナー型も多く認められた。

治療効果と関連するmRNA

mRNAマイクロアレイの結果、39種のmRNAの発現上昇と17種のmRNAの発現低下が治療不応と関連していた。発現上昇していたmRNAは、転写・翻訳、細胞周期、リン酸化、シグナル伝達、免疫応答、RNAスプライシング/mRNAプロセシングおよびウイルス産生の経路に関わる遺伝子由来であった。一方、発現低下していたmRNAは、異物代謝、脂質代謝、および酸化還元反応の経路に関わる遺伝子由来であった。これらの発現変動をより定量的に解析するため、定量的リアルタイムPCRをおこなった。その結果、MAP3K8 （$p=5.2461027$）、TMEM178 (transmembrane protein 178、 $p=7.31\times10^{-6}$)、PSME4 (proteasome activator subunit 4、 $p=2.43\times10^{-4}$)、およびEIF3B (eukaryotic translation initiation factor-3B、$p=3.16\times10^{-6}$)のmRNAが、P/N群においてS/R群よりも有意に高く発現していた。また、MAP3K8のタンパク質発現量も、P/N群においてS/R群よりも有意に高かった。

治療効果と関連するmiRNA

miRNAマイクロアレイの結果、76種のmiRNAの発現上昇と111種のmiRNAの発現低下が治療不応と関連していた。これらのうち、1)MAP3K8、TMEM178、PSME4、またはEIF3BのいずれかのmRNAを標的としうること、2)その発現変動が、MAP3K8、TMEM178、PSME4、またはEIF3BのいずれかのmRNAの発現変動と逆相関していること、を満たすmiRNAを探索した。その結果、MAP3K8に対してはhsa-let-7g*、hsa-miR-17-5p、-20b、-297、-374b、-494、-602、-668、および-1297が、TMEM178に対してはhsa-miR-106b*、-122-5pが、PSME4に対してはhsa-miR-492、-675-5pが見出された(EIF3Bに対するmiRNAは見いだされなかった)。

これらmiRNAの発現変動とmRNAの発現変動の逆相関をより定量的に解析するため、miRNAの定量的リアルタイムPCRをおこなった。その結果、miR-122-5p ($p=2.75\times10^{-8}$)、miR-675-5p ($p=1.00\times10^{-5}$)、およびmiR-17-5p ($p=1.73\times10^{-8}$)のmiRNAが、P/N群においてS/R群よりも有意に低く発現していた。特に、MAP3K8 mRNAはmiR-17-5pと高い逆相関が認められた ($r=-0.592$、$p=4.31\times10^{-3}$)。

治療応答関連解析

単変量解析で統計的差異が認められた因子について多変量解析をおこなったところ、P/N群に関連する因子として、rs8099917 [$p=3.67\times10^{-3}$、odds ratio (OR) = 7.51、95% confidence interval (CI) = 2.14-29.27]、miR-122-5p ($p=5.60\times10^{-4}$、OR= 0.11、95% CI = 0.03-0.38)、miR-17-5p ($p=2.02\times10^{-4}$、OR= 0.56、95% CI = 0.41-0.76)、およびMAP3K8 ($p=8.58\times10^{-3}$、OR= 2.86、95% CI = 1.31-6.25)が見出された。

HCV複製に対するMAP3K8の影響

MAP3K8は、細胞増殖、炎症、アポトーシスなど様々な細胞機能に関与する遺伝子として知られている。そこで、MAP3K8のHCV産生に対する役割を、siRNAを用いて検討した。MAP3K8 siRNAまたはコントロールsiRNAをHCV感染Huh7.5.1細胞に導入した結果、細胞上清中のHCVコアタンパク質量は有意に減少し、この時、細胞内のmiR-17-5p発現量は有意に上昇した。しかしながら、細胞内HCVコアタンパク質量に変化は認められなかった。したがって、MAP3K8はmiR-17-5pの発現抑制を介して、HCV粒子の産生・放出促進に関与すると考えられた。

miR-17-5pによるMAP3K8を介したHCV産生制御

次に、miR-17-5pの阻害または過剰発現におけるMAP3K8発現変動およびHCV産生変動を解析した。その結果、miR-17-5pを阻害するとMAP3K8の発現は上昇し、その過剰発現ではMAP3K8の発現は低下することが明らかとなった。また、miR-17-5pを阻害するとHCVコアタンパク質のの発現は上昇し、その過剰発現では低下することが明らかとなった。

したがって、miR-17-5pはMAP3K8を標的とすることでその発現を制御し、これよりHCV粒子産生に関与すると考えられた。

最後に、ルシフェラーゼアッセイにより、miR-17-5pによるMAP3K8 mRNAの3'非翻訳領域を介した発現制御を検証した。その結果、miR-17-5pの過剰発現によりMAP3K8の3'非翻訳領域を有するレポーターの活性は低下し、結合配列と考えられる領域に変異を導入すると、miR-17-5pによる発現抑制作用は認められなくなった。

D. 考察

本研究は、C型肝炎治療患者の治療応答に関連するmRNAとmiRNAの網羅的発現解析をおこなった初めての報告である。

これまでに治療応答不良患者においては、治療応答患者と比べ、治療前のインターフェロン応答遺伝子群(ISGs)の発現量が高いことが報告されている。したがって、このような患者では外からインターフェロンを投与しても十分なインターフェロン応答が得られず、そのため治療効果に乏しいと考えられる。このISGsの高発現常態はIL28B遺伝子多型と関連することが報告されており、さらに、IL28B遺伝子多型の解析と比べ、ISGs発現量を解析する方が治療応答予測により有効であるとする報告もある。しかしながら、今回の検討においては、ISGsの発現は確かにP/N群でS/R群よりも高い傾向にあったものの、これは統計的有意な差異には至らなかった。このように本研究と上記報告で異なる結果が得られた原因は定かではないが、人種、HCV遺伝子型、治療応答に対する定義、評価指標、治療方法などによる違いに起因する可能性があると考えられる。

MAP3K8は免疫応答や炎症反応に関わる機能を有すると考えられている。HCVはToll-like receptor 4 (TLR4)を活性化することが知られており、活性化TLR4はinhibition of kappa B kinase (IKK)を介してnuclear factor kappa B (NF-kB) p105を活性化することが知られている。通常、リン酸化されていないNF-kB p105はMAP3K8と不活性な複合体を形成しているが、NF-kBが活性化されることによ

り、MAP3K8は遊離する。この遊離したMAP3K8により、MAPK/ERK kinase (MEK)-extracellular signal-regulated kinase (ERK) 経路が活性化され、様々なサイトカインやケモカインが放出されると考えられている。

上記に加え、本研究では新たにMAP3K8がHCV治療抵抗性と関連することを見出した。本研究の結果から、MAP3K8はHCVの放出または粒子形成に関わると考えられるものの、HCVコアタンパク質発現量にも影響する可能性も現時点では否定できない。もしMAP3K8がHCVの放出や粒子形成に影響するのみであれば、miR-17-5pの過剰発現や阻害時に、MAP3K8の発現変動に伴い培養上清ばかりでなく細胞内のHCV発現量も変動したことは、他の因子の関与が無い限り説明できない。このような現象はHCVの複製・産生に関わる他の因子においても報告されている。したがって、MAP3K8はHCVライフサイクルの何等かの経路に作用するものと考えられるものの、現時点ではその詳細な機序は不明である。

これまでにmiR-17-5pは、細胞増殖または抑制遺伝子の発現制御をおこなうことにより、細胞依存的にがん抑制に働くともがん促進に働くとも考えられている。ウイルス複製に関しては、これまでにhsa-miR-17-5pが存在する遺伝子座はHIV複製に対して促進的に働くことが報告されているが、今回の我々の結果からは、miR-17-5pはHCV産生に対しては抑制的に働くことが明らかとなった。さらにこの分子機序として、miR-17-5pはMAP3K8の発現を転写および翻訳レベルで抑制すると考えられた。

一般に、miRNAは多くの標的mRNAを同時に制御しており、また、上述のとおりMAP3K8の活性は多岐に渡る細胞機能に影響を及ぼすと考えられる。したがって、これら因子はその下流に存在する多くの遺伝子が、HCVの複製や産生に関与する可能性が考えられる。例えばこれまでにmiR-17-5pはlow density lipoprotein receptor (LDLR)を標的としていることが知られており、LDLRの発現低下は肝細胞内脂質代謝を変動させると考えられる。細胞内脂質代謝はHCVの複製にとって重要な役割を担っていることが報告されていることから、miR-17-5pは肝細胞内の脂質代謝変動を介してHCV複製に関与する可能性も考えられる。

一方、MAP3K8は肝細胞リバビリン取り込みトランスポーターであるequilibrative nucleoside transporter 1 (ENT1) の発現・活性制御にも関わる可能性がある。これまでにPKC-zeta、 Raf-1、MEK、 and p38 MAPK経路によりENT1の発現が減少するこが報告されている。したがって、MAP3K8によりこれらシグナル経路が活性化されて、ENT1の発現や活性が減弱すると、リバビリンの取り込み量が低下し、それに伴い薬効発現が減弱する可能性も否定できない。

上述のとおり、MAP3K8/miR-17-5p経路がHCV複製にどの程度かかわっているか、また、MAP3K8/miR-17-5p経路の下流でどのような遺伝子が制御されているか、今後更なる*in vitro*・*in vivo*の検討が必要である。このような検討の中では、HCVの複製・産生に関わる様々な経路に着目した解析とともに、治療薬トランスポーターとの関連に着目した解析も進める必要があると考えられる。

E. 結論

患者由来試料を用いたmRNAおよびmiRNAの網羅的解析およびそれらの関連解析から、治療効果と関連する新たな分子 (MAP3K8およびmiR-17-5p) が明らかとなった。本結果は、HCV感染と治療薬薬効発現に関わる新たな経路を明らかとしたばかりでなく、今後二剤または三剤併用療法において患者治療効果を予測する因子を同定することにもつながる可能性もある。しかし、これら遺伝子がどのような分子経路を介してHCV複製・産生に作用するか明らかではなく、今後MAP3K8/miR-17-5pの標的遺伝子 (HCV複製に関わる遺伝子や治療薬取り込みトランスポーター遺伝子など) の同定やその治療薬標的としての可能性を明らかとしていく必要があると考えられる。

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G. 知的財産権の出願・登録状況

1. 特許取得

特になし

2. 実用新案登録

特になし

3. その他

特になし

III. 研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
	なし						

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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Ⅳ．研究成果の刊行物・別刷

（次項）

Differential inhibition features of direct acting anti-hepatitis C virus agents against human organic anion transporting polypeptide 2B1

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Abstract

Simeprevir (SMV), asunaprevir (ASV), daclatasvir (DCV) and sofosbuvir (SOF), which are direct-acting antiviral agents (DAAs), are expected to become essential pharmaceutical tools in the fight against the hepatitis C virus (HCV). However, because DAAs are taken orally, there is a potential risk of drug-drug interaction (DDI) at the absorption step of co-administered drugs in the small intestine. Since it is known that organic anion transporting polypeptide 2B1 (OATP2B1) is one of the key transporters that contribute to intestinal drug absorption, it is important to thoroughly understand the inhibition profiles of various DAAs in relation to the OATP2B1 function in order to avoid unexpected DDIs. Therefore, using a cell-based transport assay, this study aimed at clarifying such DAA inhibition characteristics towards the OATP2B1 function. Our results of co-incubation inhibition assays showed that SMV and ASV strongly inhibited estrone-sulfate (5 nM) uptake by OATP2B1 with 0.49 ± 0.12 and 0.16 ± 0.06 (μM) of half maximal inhibitory concentrations, respectively. Furthermore, it was found that SMV and ASV imposed long-lasting pre-incubation inhibitory effects on the OATP2B1 function that enhanced their co-incubation inhibition potencies. On the other hand, no (or much less significant) inhibitory effects were observed in SOF or DCV. To summarize, our results show that SMV and ASV are co-incubation, as well as long-lasting pre-incubation, inhibitors of the OATP2B1 function, and therefore those inhibitions may lead to clinically relevant DDIs when used with OATP2B1 substrates.

Key words: Direct-acting antiviral agents; organic anion transporting polypeptide 2B1; transporter; drug-drug interaction; hepatitis C virus; long-lasting inhibition

1. Introduction

Direct-acting antiviral agents (DAAs) are orally administered drugs that strongly inhibit hepatitis C virus (HCV) protein functions, and the introduction of telaprevir (TLV) and boceprevir (BOC) have resulted in significant improvements to therapy success rates [1]. In addition, the more recently-developed agents, including simeprevir (SMV), asunaprevir (ASV), daclatasvir (DCV), and sofosbuvir (SOF), are expected to further advance the success rates of the sustained virologic response to more than 80%, due to their higher efficacy and the lower risk of severe adverse effects [2]. Therefore, it is considered likely that these new agents will significantly contribute to reducing cirrhosis and liver cancer mortality rates.

However, it has also become evident that DAAs bring with them a potential risk of drug-drug interactions (DDIs) [3,4]. Furthermore, risk profiles are different among DAAs, and it has been reported that TLV and BOC interact significantly with various drugs, such as atorvastatin, cyclosporine, and tacrolimus [5,6]. It has also been shown that TLV and BOC are strong inhibitors of cytochrome P450 3A4 (CYP3A4) and organic anion transporting polypeptide (OATP) 1B1/1B3 [7-9]. The former is an important drug-metabolizing enzyme that is expressed in the liver and small intestine, while the latter are hepatocyte transporters that take up various drugs from the circulatory system. Thus, these inhibition properties are obviously the most probable mechanisms behind the DDIs observed with tacrolimus, cyclosporine, and atorvastatin.

In contrast, the potential DDI risks of recently-developed DAAs remain mostly uncertain. However, it has been shown that SMV and ASV are capable of inhibiting CYP3A4 and CYP2D6, respectively (the Sovriad and Sunvepra interview forms). More recently, it has been shown that SMV, ASV, and DCV, possess inhibitory effects on OATP1B1 and 1B3 functions [the Sovriad, Sunvepra, and Daklinza interview forms, and 10]. Therefore, even though there have been no reports showing detrimental DDIs between those newly-developed DAAs and co-administered drugs in their clinical trials, their DDI risk potential should be evaluated to the greatest extent in order to achieve better clinical management in HCV therapies, where the co-administration of multiple medications is often required.

In addition to the liver, it has been acknowledged that the small intestine is also a pivotal site of DDI occurrence, as exemplified by the finding that perpetrator drugs inhibit transporter-mediated absorption of the victim drug [11]. OATP2B1 is another member of the OATP family that is abundantly expressed in enterocytes at the apical side, where it plays an important role in absorption of a number of drugs such as

77 fexofenadine, aliskiren, montelukast, and celiprolol, by taking advantage of its substrate
78 polyspecificity [12,13]. It has previously been shown that grapefruit juice (GFJ), orange
79 juice, and apple juice can strongly inhibit OATP2B1-mediated uptake of fexofenadine,
80 aliskiren, and montelukast, thereby causing significant reduction in their area under the
81 plasma concentration-time curves (AUCs) *in vivo* [14-18]. Accordingly, OATP2B1 has
82 been recognized as a critical target of DDI studies.
83 When considering DDI at the OATP2B1 level, two recent findings should be
84 taken into account: one is that OATP2B1 is considered to have multiple transport
85 activity sites. For example, it has been shown that there are at least two sites that show
86 different affinity to estrone-sulfate (E_1S, a classical OATP2B1 substrate), which are
87 designated as the high or low E_1S affinity site (H-site or L-site), respectively [19], and it
88 appears that these sites are interchangeable in a substrate-dependent manner (e.g., the
89 H-site for E_1S uptake can be the L-site for another drug) [20]. Furthermore, OATP2B1
90 inhibitors have shown to exhibit interaction preferences related to those sites, as
91 exemplified by taurocholic acid (TCA) and testosterone (TST) as selective inhibitors of
92 the H- and L-sites for E_1S uptake, respectively [19].
93 Another finding is that apple and orange juices are first examples that not only
94 show co-incubation inhibitory effects, but also long-lasting pre-incubation inhibitory
95 effects on the OATP2B1 function [21]. Tentatively, co-incubation inhibition is defined
96 as the conventional transporter inhibitory effect that occurs in the presence of an
97 inhibitor, while long-lasting inhibition indicates an inhibitory effect that can last for
98 some time even after the removal of an inhibitor. It has been reported that the
99 long-lasting OATP2B1 inhibition property of apple juice appears to augment its
100 co-incubation inhibition property [21]. Therefore, it is possible that this newly-emerged
101 inhibition type may significantly affect the overall OATP2B1 inhibition profile of an
102 inhibitor.
103 With the above background in mind, it is self-evident that the OATP2B1
104 inhibition profiles of DAAs need to be clarified in order to thoroughly understand their
105 DDI risks with current, as well as future, orally administered drugs. Therefore, in the
106 present study, we aimed to characterize the interaction profiles of SMV, ASV, DCV, SOF,
107 and TLV, in relation to OATP2B1. Then, based on those results, we also sought to
108 clarify their potential DDI risks in relation to OATP2B1 substrates.
109

2. Material and methods

2.1. Development of human embryonic kidney 293 (HEK293) cells stably expressing OATP2B1

The OATP2B1 cDNA was subcloned into pcDNA3.1/Neo(-) vector (Life Technologies, Carlsbad, CA). The vector was transfected into HEK293 cells, and the cells expressing OATP2B1 at the highest level were isolated and named 2B1/HEK. Similarly, HEK293 cells carrying an empty pcDNA3.1/Neo(-) were prepared and named mock/HEK.

2.2. Total RNA isolation, cDNA synthesis, and reverse-transcription PCR (RT-PCR)

Total RNA isolation and cDNA synthesis of 2B1/HEK and mock/HEK were conducted using the methods described previously [10].

RT-PCR for detection of OATP2B1 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression was performed using each gene-specific primer set.

2.3. Western blotting

Western blotting for detection of OATP2B1 protein expression was performed essentially using the methods described previously [10].

2.4. Transport assay for validation of OATP2B1 expression in HEK293 cells

OATP2B1 activity was examined in 2B1/HEK and mock/HEK using transport assay, which was essentially the same as described in [10]. As OATP2B1 has two activity sites with different substrate affinity profiles, it has been shown that the function of each site can be independently evaluated using different substrate concentrations [19,20]. Based on this information, the E_1S (Sigma, St. Louis, MO) concentration was set to 5 nM for the H-site-mediated uptake or to 50 μM for the L-site-mediated uptake. [^{3}H]-labelled E_1S were obtained from PerkinElmer Life Science (Boston, MA). The Krebs-Henseleit buffer (KHB, pH 6.5) was used as an assay buffer. The uptake period was set to three min, based on the results of preliminary experiments showing the uptake level linearity.

Inhibition assays against OATP2B1 activity were performed using TCA (1 mM, an inhibitor of the H-site for E_1S uptake), TST (1 mM, an inhibitor of the L-site for E_1S uptake), and bromosulfophthalein (BSP, 100 μM, an inhibitor of both sites). These inhibitors were purchased from Sigma, and solubilized in dimethyl sulfoxide (DMSO).

2.5. Transporter inhibition assays

TLV, SMV, ASV, DCV, and SOF were purchased from Shanghai Biochempartner (Shanghai, China), ChemScene LLC (Monmouth Junction, NJ), AdooQ BioScience LLC (Irvine, CA), ChemScene LLC, and Medchemexpress LLC (Princeton, NJ), respectively, and dissolved in DMSO. Transporter inhibition assays (co-incubation method, pre-incubation method, long-lasting pre-incubation method, as well as pre- and co-incubation combination method) were conducted essentially based on the above-described method (the Section 2.4) and the method described previously [10]. The OATP2B1 activity level was calculated by subtracting the value obtained from mock/HEK from the value obtained from 2B1/HEK. Inhibitor concentrations are indicated in the figure legends. A concentration that inhibited OATP2B1 activity level by 50% in co-incubation assay ($IC_{50(co)}$) or in pre- and co-incubation combination assay ($IC_{50(co\&pre)}$) was calculated using the formula: Control (%) = $100/(1 + I/IC_{50})$, where control (%) represents the transporter-mediated uptake in the presence of various inhibitor concentrations (I) relative to that in the absence of inhibitor.

2.6. Evaluation of DDI risk potential of DAA through inhibition of OATP2B1 function

The DDI risk potential of a DAA was evaluated according to a method using $[I]_2/IC_{50}$, where $[I]_2$ is the estimated maximum intestinal drug concentration defined by $[I]_2$ = Dose/250 mL [22].

2.7. Statistical analysis

Student's t-test was performed using a statistical software package (Statcell, OMS, Saitama, Japan) in order to determine whether the differences between two values were significant.

2.8. Others

Method details, including specific materials utilized, are provided in the supplemental material.

3. Results

3.1. Development of HEK293 cells stably expressing OATP2B1

To begin with, validation experiments of OATP2B1 expression in 2B1/HEK were performed. As shown in Figs. 1A and 1B, OATP2B1 mRNA and its protein were exclusively expressed in 2B1/HEK, while GAPDH mRNA or Na^+/K^+ ATPase protein was detected in both 2B1/HEK and mock/HEK cells. Then, the OATP2B1 functionality in 2B1/HEK was examined using low E_1S concentration (5 nM for the H-site) and high E_1S concentration (50 μM for the L-site) (Fig. 1C). The results showed that OATP2B1-mediated E_1S uptake via the H- and L-sites were observed in 2B1/HEK. The uptake activity mediated by the H-site was completely inhibited by TCA and BSP, but was enhanced, rather than repressed, by TST. This enhancement effect of TST is consistent with the previous result [19], although the reason behind it is currently unknown. On the other hand, the uptake activity mediated by the L-site was strongly inhibited by both TST and BSP, while the effect of TCA was modest.

Taken together, these results confirmed that OATP2B1 was functionally expressed in 2B1/HEK and that it appeared to possess the two previously identified substrate affinity sites.

3.2. Characterization of inhibition profile of DAAs on OATP2B1 function

The inhibition profiles of DAAs on E_1S uptake activity by OATP2B1 through the H- and L-sites were separately examined by a co-incubation method (Fig. 2). The results showed that, among the DAAs examined, SMV and ASV significantly inhibited the OATP2B1 function at both sites. The $IC_{50(co)}$ values (μM) of SMV for the H- and L-sites were 0.49 ± 0.12 and 10.15 ± 2.80, while those of ASV were 0.16 ± 0.06 and 0.92 ± 0.08, respectively. In addition, it was found that TLV reduced the OATP2B1 function level in an L-site specific manner ($IC_{50(co)}$ = 16.22 ± 2.73 μM). In contrast, DCV appeared to have weak inhibitory effects on OATP2B1 activity, and SOF did not significantly suppress the activity. Therefore, these results indicated that SMV and ASV were potent OATP2B1 inhibitors at both sites, and that TLV was an L-site specific inhibitor. $IC_{50(co)}$ values are summarized in Table 1.

3.3. Characterization of pre-incubation inhibitory effects of DAAs on OATP2B1 function

In addition to co-incubation inhibition, the pre-incubation inhibition effects of DAAs on the OATP2B1 function were examined. Consistent with the co-incubation assay results, the pre-incubation results showed that SMV (1 and 10 μM) significantly inhibited the OATP2B1 activity level to 40.3 ± 5.9 and 29.2 ± 5.5 (%) of the control level for the H-site (see black circles of the figure for SMV in Fig. 3), and to 39.9 ± 0.8 and 13.6 ± 6.8 (%) of the control level for the L-site (see white circles of the same figure), respectively. These values showed that the inhibition profiles of SMV on the H- and L-sites were similar to each other, despite their different co-incubation inhibition potencies. Similarly, ASV (0.1 and 1 μM) showed substantial repressive effects on the OATP2B1 function, the residual activity levels of which were 48.1 ± 3.1 and 14.8 ± 5.6 (%) of the control level for the H-site (see black circles of the figure for ASV in Fig. 3), and 74.4 ± 18.5 and 31.1 ± 5.6 (%) of the control level for the L-site (see white circles of the same figure), respectively. In contrast, no pre-incubation effect of TLV, DCV and SOF was observed on the OATP2B1 function, at least at concentrations up to 10 μM.

Given that SMV and ASV showed notable pre-incubation inhibitory effects on the OATP2B1 function, their continuous effects were further characterized by a long-lasting pre-incubation method (Fig. 4). The results showed the differential long-lasting profiles of their pre-incubation inhibitory effects. The inhibitory effects of SMV and ASV on the H-site functions were maintained more than three hours, and for at least one hour, respectively. In contrast, the effects on the L-site dissipated after approximately one hour.

Taken together, these results showed that, among the DAAs, SMV and ASV imposed long-lasting repressive effects on the OATP2B1 function in a site-specific manner.

3.4. Determination of cooperative pre- and co-incubation inhibition of SMV and ASV on the OATP2B1 function

To investigate the possibility that the pre-incubation effects of SMV and ASV influenced their co-incubation effects, we assessed their cooperative inhibition properties on the OATP2B1 function by determining their $IC_{50(co\&pre)}$ values using pre- and co-incubation combination methods (Fig. 5 and Table 2). The results showed that $IC_{50(co\&pre)}$ values of SMV against the H- and L-sites were, respectively, 2.6-fold and 20.3-fold lower than those of $IC_{50(co)}$. Likewise, the $IC_{50(co\&pre)}$ values of ASV against the H- and L-sites were, respectively, 2.0-fold and 2.7-fold lower than those of $IC_{50(co)}$.

Therefore, pre-incubation with SMV or ASV was found to remarkably enhance their co-incubation inhibitory effects on the OATP2B1 function in a site-dependent manner.

3.5. Evaluation of potential DDI risk of DAAs at OATP2B1 level

Taking into consideration the above-identified inhibition characteristics of DAAs, it was deemed worthwhile to evaluate their OATP2B1-mediated DDI risks. Although a standard evaluation method for OATP2B1-mediated DDI risk has not yet been established, the International Transporter Consortium has proposed using $[I]_2/IC_{50}$ values as criteria for ascertaining whether a new molecular entity has P-glycoprotein-mediated DDI risk potential at the small intestine in drug development [22]. (where $[I]_2$ is the estimated maximum intestinal drug concentration defined by $[I]_2$ = Dose/250 mL)

Utilizing this method, the OATP2B1-mediated DDI risks of SMV and ASV were tentatively estimated (Table 3). The results showed that the $[I]_2/IC_{50(co)}$ of SMV values for the H- and L-sites were 1,632 and 78.8, respectively, and that those of the ASV values were 3,344 and 582, respectively. All these values were higher than 10, which was suggested as a cut-off value [22]. Furthermore, it was clear that these values would be significantly higher if, by using $IC_{50(co\&pre)}$, instead of $IC_{50(co)}$, pre-incubation effects were taken into consideration.

On the other hand, the results of similar calculations showed that the $[I]_2/IC_{50(co)}$ value of TLV for the L-site was 272, and those of DCV for the H- and L-sites were 9.2 and 6.5, respectively.

4. Discussion

Our results have found that, among the DAAs examined, SMV and ASV show greatest impact on OATP2B1 function. While SMV and ASV are co-incubation inhibitors of OATP2B1 at both the H- and L-sites, and that their H-site inhibition potencies are comparable with each other, it was found that ASV has higher inhibition potency for the L-site function. Because, based on results of PubChem Compound database analysis (Fig. S1), their physicochemical properties appear to be remarkably similar, the dissimilarity may result from structural differences. For example, SMV has a macrocyclic moiety, while ASV does not (Fig. S1).

The co-incubation inhibition potencies of SMV and ASV (especially for the H-site) can be classified among the highest levels identified so far [23], and our DDI risk evaluation results consistently indicate that a substantial risk of interactions exists with OATP2B1 substrate drugs when they are co-administered. The evaluation method used in this study was based on the estimated maximum intestinal drug concentration ($[I]_2$), where dose is divided by administration volume (250 mL). It has been reported that, in the fasted state, the fluid volumes of the human stomach and small intestine are 45 and 105 mL (mean of 12 individuals), respectively [24]. Thus, the *in vivo* SMV or ASV concentration in the small intestine might not be drastically different from the $[I]_2$ value if the drug was taken in the fasted state. However, these drugs are usually taken after meals, and it has been shown that fluid distribution in the intestine is actually scattered as small pockets [24]. These facts indicate that it is remarkably difficult to precisely predict intestinal drug concentrations at the drug absorption site. Nevertheless, it is clear that the $[I]_2/IC_{50(co)}$ values of SMV and ASV are markedly higher than the proposed cut-off value, and would become even higher if, by using $[I]_2/IC_{50(co\&pre)}$, their pre-incubation effects were taken into consideration. Additionally, although it will be necessary to await further extensive studies in order to ascertain whether the long-lasting pre-incubation effects observed in *in vitro* actually have clinical significance, it has been reported that long-lasting intestinal Oatps inhibition by cyclosporine A contributes to a reduction in the fexofenadine absorption level in rats [25]. Therefore, the possibility that long-lasting SMV or ASV pre-incubation may significantly inhibit the OATP2B1 function in clinical settings cannot be ruled out. For example, it can be speculated that the DDI assessment results observed at times when an OATP2B1 substrate is administered simultaneously with SMV or ASV may be different from those times when it is administered after multiple doses of SMV or ASV. This has the potential of drawing misleading conclusions in a single dose study.