약학석사 학위논문

Impact of histone deacetylase inhibitor Romidepsin on the expression and function of organic anion transporter polypeptide 2B1 (OATP2B1) in liver cancer cell lines

히스톤 탈아세틸화효소 억제제 romidepsin이 간암 세포주에서 유기음이온수송체 2B1 (OATP2B1)의 발현과 기능에 미치는 영향

2020 년 2 월

서울대학교 대학원

약학과 약제과학 전공

최 지 윤

Impact of histone deacetylase inhibitor romidepsin on the expression and function of organic anion transporter polypeptide 2B1 (OATP2B1) in liver cancer cell lines

지도교수 이 우 인

이 논문을 약학석사 학위논문으로 제출함

2019 년 12 월

서울대학교 대학원

약학과 약제과학 전공

최 지 윤

최지윤의 석사 학위논문을 인준함

2019 년 01 월

위 원 장 정 석 재 (인)

부 위 원 장 김 대 덕 (인)

위 원 이 우 인 (인)

Abstract

Impact of histone deacetylase inhibitor romidepsin on the expression and function of organic anion transporter polypeptide 2B1 (OATP2B1) in liver cancer cell lines

Jiyoon Choi
Department of Pharmaceutical Sciences
The Graduate School
Seoul National University

Romidepsin (RMD) is an epigenetic modulator that inhibits histone deacetylase and it is currently approved for the treatment of T-cell lymphoma (cutaneous or peripheral). Efficacy of RMD requires its metabolic conversion into an active form in hepatocytes, but little is known about the mechanism by which RMD enters and accumulates in hepatocytes. As one of uptake transporters in hepatocytes, organic anion transporter polypeptide 2B1 (OATP2B1) may interact with RMD, but such possibility was not carefully examined. Moreover, it remains unknown whether RMD can impact the expression and function of OATP2B1. In the current study, we

investigated whether RMD can interact with OATP2B1 using a fluorescent probe substrate (dibromofluorescein, DBF) and HEK293 cells stably expressing OATP2B1. In addition, we examined whether RMD can alter the expression of OATP2B1 in established hepatic cancer cell line models. Our results indicated that RMD does not inhibit the transport activity of OATP2B1, suggesting that it is unlikely to be a substrate of OATP2B1. When three hepatic cancer cell lines were treated with RMD (1~10 nM; 24 hours), the level of OATP2B1 mRNA decreased in a concentration-dependent manner, followed by recovery upon drug removal. Similar results were obtained using with another histone deacetylase inhibitor (suberoylanilide hydroxamic acid, 0.1~5 μM). Our immunoblotting results indicated that the treatment with RMD led to a decreased OATP2B1 protein levels. Taken together, RMD is not likely an OATP2B1 substrate, but may decrease OATP2B1 levels. Further investigations are warranted to examine the potential impact of RMD on the expression and activity of OATP2B1, thereby the hepatic accumulation of other drugs.

Keywords: Romidepsin, Epigenetics, Drug transporters, Organic anion transporting polypeptide 2B1(OATP2B1)

Student Number: 2018-26399

Contents

List of Figures

Introduction

Romidepsin (RMD) is a natural product isolated from Chromobacterium violaceum and currently approved for the treatment of cutaneous T-cell lymphoma (CTCL) and peripheral T-cell lymphoma (PTCL).[1-3] RMD belongs to a class of histone deacetylase inhibitors (HDACi) and mainly inhibits HDAC1, keeping chromatin at the transcriptionally active state.[4,5] RMD is a prodrug and requires the reduction of disulfide bonds by glutathione and its conversion into an active monocyclic dithiol form inside the cell.[6]

In preclinical animals, the pharmacokinetic profiles of RMD suggested its potential interactions with transporters. When mass balance studies were carried out using a radiolabeled drug in bile duct cannulated rats, the results indicated that RMD is primarily excreted into bile (66% of the administered dose at 48 h) after the intravenous (IV) dosing of 0.3 mg/kg.[7] RMD undergoes metabolism to more than 20 metabolites in the liver S9 and microsomal fractions, but in rats receiving the radiolabeled RMD no single metabolite accounted for more than 5% of total radioactivity.[8] The metabolism was mediated mainly by cytochrome P450 3A4 (CYP3A4) and to a minor extent by CYP3A5, CYP1A1, CYP2B6, and CYP2C19.[9] RMD is a substrate of the efflux transporter P-

glycoprotein (P-gp).[10] Currently, the mechanism and involved transporters by which RMD accumulates in the liver is unknown. In vitro studies ruled out the involvement of organic anion transporting polypeptide (OATP) 1B1/1B3, organic anion transporter (OAT) 1/3, organic cation transporter (OCT) 2 in the hepatic uptake of RMD.[11]

In addition to OATP1B1 and OATP1B3, OATP2B1 is also expressed on the basolateral membrane of hepatocytes and it is increasingly recognized to play a role in the intestinal drug absorption, drug-drug interaction (DDI) and food-drug interaction (FDI).[12] So far, there is no experimental evidence whether RMD is a substrate of OATP2B1. Epigenetic modulation may be involved in the regulation of OATP2B1 expression in various tissues and disease settings. For instance, analyses of samples from patients with non-alcoholic fatty liver disease (NAFLD) suggested that changes in DNA methylation may impact the expression of OATP2B1.[13] Further mechanistic investigations are not available yet and it is possible that RMD can affect OATP2B1 expression via epigenetic modulation.

In this study, we investigated whether RMD is an inhibitor or substrate of OATP2B1. Using in vitro cell line model stably expressing OATP2B1, we assessed whether RMD can inhibit the transport activity of OATP2B1 upon co-incubation with the

fluorescent substrate 4',5'-dibromofluorescein (DBF). In separate experiments, we assessed the impact of RMD treatment on the expression level and subsequent recovery of OATP2B1 mRNA and protein.

1. Materials and methods

2.1. Materials and Cell Lines

4',5'-dibromofluorescein (DBF), sodium butyrate (NaB), suberoylanilide hydroxamic acid (SAHA) and poly-L-lysine were purchased from Sigma-Aldrich (St. Louis, MO). Sulfasalazine (SSZ) was from Cayman Chemical Company (Ann Arbor, MI). Romidepsin (RMD) was purchased from MedChemExpress (Princeton, NJ). HEK293 cell and human hepatic cancer cell lines (HepG2, Huh7 and PLC/PRF/5) were purchased from the Korean Cell Line Bank (KCLB, Korea). HEK293 and HepG2 cells were maintained in Minimum Essential Eagle's Medium (MEM, Sigma-Aldrich (St. Louis, MO)) supplemented with 10% fetal bovine serum (FBS) (Welgene, Gyeongsan, Korea), 1% non-essential amino acid solution (Sigma-Aldrich) and 100 U/mL of penicillin/streptomycin (ThermoFisher scientific, Waltham, MA). Huh7 and PLC/PRF/5 were maintained in RPMI1640 purchased from Welgene (Gyeongsan, Korea) with the same supplements used for MEM. The polyclonal antibody against the N-terminal region of OATP2B1 was purchased from ThermoFisher scientific (PA5-42453, Waltham, MA). An antibody against Myc tag (2276), histone H3 (4499), acetylated histone H3 (9649) and GAPDH (2118) was from Cell Signaling

Technology (Danvers, MA). The plasmid to express OATP2B1 fused with C-terminal Myc and DDK epitopes (SLCO2B1 (Myc-DDK-tagged), transcript variant 1; RC207759) was purchased from Origene (Rockville, MD).

2.2. Generation and culture of HEK293 cells stably expressing OATP2B1

HEK293 cells were grown in 100 mm cell culture dish in the recommended complete media at 37℃ in a humidified 5% CO2 incubator. HEK293 cells were transfected with the expression plasmid (pcDNA3.1/Zeo, Invitrogen, Carlsbad, CA) containing the full open reading frame sequence for human OATP2B1 using Lipofectamine 2000 (Invitrogen). Stably transfected clones were selected by adding zeocin (150μg/mL, InvivoGen, San Diego, CA) to the media, and individual clones were subsequently screened for their functional activities using DBF. Selected clones were maintained in the culture media containing zeocin (50μg/mL) and used for further experiments.

2.3. Transient transfection of HEK293 cells expressing OATP2B1

HEK293 cells were transfected with the expression plasmid for OATP2B1 fused with the N-terminal Myc and DDK epitopes

(SLCO2B1 (Myc-DDK-tagged)/pCMV6-entry, Origene, Rockville, MD) using FuGENE® HD Transfection Reagent (Promega). At 24 h post-transfection, 5 mM NaB was added to boost the expression of OATP2B1 in HEK293 cells. At 48 h Post transfection, cells were harvested for immunoblotting.

2.4. In vitro treatment with epigenetic modulators

HepG2, Huh7 and PLC/PRF/5 cells were treated with two clinically used histone deacetylase inhibitors (HDACi): SAHA (0.1~5 μM) and RMD (1~10 nM). Different concentrations were used for SAHA and RMD, considering the differences in their potency and clinically relevant concentrations. Cells were plated onto 6-well plates at the density of 2.0×105 cells/well cell (n=3). At 24 hours post-plating, the drug treatment was initiated by adding media containing varying concentrations of SAHA and RMD. After 24 hours, cells were harvested and subjected to mRNA and protein analyses. To examine whether and when drug-induced changes recover following the drug removal, cells were treated with the respective drugs for 24 hours, washed with warm Dulbecco's Phosphate-Buffered Saline (DPBS) and placed in drug-free media. At 24 hours post-washing, cells were harvested for RNA and protein analysis.

2.5. RNA extraction and Quantitative RT-PCR analysis

RNA was extracted from hepatic cancer cell lines (Huh7, PLC/PRF/5, HepG2) using PureLinkTM RNA Mini Kit (Thermo Fisher Scientific, Fremont, CA, USA) according to the manufacturer's instructions. The isolated RNA (500 ng) was converted to cDNA using the PrimeScript RT Reagent Kit (Takara, Shiga, Japan). Quantitative RT-PCR was performed with the resulting cDNA carried out with TOPrealTM qPCR 2x PreMIX containing SYBR Green (Enzynomics, Daejeon, Korea) to determine the mRNA level of OATP2B1 using the StepOnePlusTM Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The following primers were used to quantify OATP2B1 according to the previous report[14] (forward primer '5-GGACAACAGCCAGGTTTTCTACAC-3', reverse primer '5-AGGAAGGGCACCACCAGATG-3'). The PCR conditions were as follows: Initial denaturation (95 ℃, 10 min) followed by 40 cycles of denaturation (95 ℃, 10 min), annealing (60℃, 15sec), and extension (72 ℃, 30 sec). The fold changes in the OATP2B1 expression (compared to the vehicle control) were calculated using $2^{-\triangle\triangle Ct}$ method and normalized to that of β-actin (housekeeping control). Each RT-PCR run included negative controls containing no template.

2.6. Cellular Uptake Study using HEK293 cells stably expressing OATP2B1.

For uptake studies, cells were seeded in 24-well dishes coated with poly-L-lysine and cultured to >80% confluence. Cells were washed twice with pre-warmed DPBS after the medium was removed and equilibrated in pre-warmed Optimem (ThermoFisher Scientific) at 37 ℃ for 10 min. The uptake was started by replacing Optimem containing DBF with or without an inhibitor. Uptake transport was terminated by aspirating the media and placing the plate on ice at specified time points. Cells were washed three times using ice cold-DPBS and lysed with by probe sonication using lysis buffer (0.5% Triton X-100 (Georgiachem, Norcross, GA) containing DPBS). Fluorescence signals in cell lysates were measured at excitation/emission wavelengths of 460/515 using a multireader (SpectraMAX M5, Molecular Devices). Protein amount was quantified by BCATM Protein Assay (pierce, ThermoFisher Scientific) and used for data normalization.

DBF and SSZ were used as a probe substrate and an inhibitor for OATP2B1, respectively, based on the previous reports.[15,16] From preliminary experiments, the incubation time of 3 min was identified to be the time period where the cellular uptake of DBF is proportional to time. To characterize the transport kinetics

for DBF in HEK293 cells expressing OATP2B1, cellular retention of DBF was determined following the addition of varying concentrations of DBF (0.1 – 20 μM). In order to assess the extent of non-OATP2B1-mediated uptake (likely via passive diffusion), the reported substrate of OATP2B1, SSZ was added. The effect of RMD on the uptake of DBF was assessed by co-incubation with DBF. Cellular uptake rates were calculated by normalizing per incubation time and total protein amount. Transport kinetic parameters (K_m, V_{max} and PS_{diff}) were determined by nonlinear regression analysis using GraphPad Prism v.5.01 (GraphPad Software, Inc. La Jolla, CA). The experimental data were fitted to the following equation; $V = [S]* V_{max} /([S]+ K_m) + PS_{diff} *[S]$, where V is the overall permeability rate, [S] is the concentration of DBF, V_{max} is the maximum velocity, K_m is Michaelis constant and PS_{diff} is the permeability clearance via passive diffusion.

2.7. Immunoblotting Analysis

Cell lysates were prepared in modified RIPA buffer (10 mM Tris, 150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, pH 7.4) containing protease inhibitors (Complete®; Roche, Basel, Switzerland). After probe sonication, samples were incubated on ice for 30 min and then centrifuged at 16000 rpm for 10 min to

remove cell debris. After protein content determination by BCA method, equivalent amounts of protein mixed with 4X Laemmli buffer (Bio-Rad, Richmond, CA) were incubated at 37° C for 20 min (to minimize protein aggregation). Samples were resolved on 7.5% or 14% SDS-polyacrylamide gel electrophoresis. Semi-dry transfer system (Bio-Rad) was used to transfer protein from gels to the polyvinylidene fluoride membrane. Membranes were incubated for 1 hour at room temperature with the blocking buffer (5% skim milk in 0.05% Tween-20 in tris-buffered saline (TBST)) and probed with antibodies against OATP2B1 (1:500), acetylated histone H3 (1:1000), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:1000), diluted in blocking buffer at 4° C overnight. Membranes were incubated with respective secondary antibodies conjugated with horseradish peroxidase. The immune-reactive bands were visualized using an enhanced chemiluminescence substrate (SuperSignal; Thermo Fisher Scientific, Waltham, MA).

For re-probing, membranes were washed with TBST for 5 min and incubated in stripping buffer (1 M tris-HCl pH6.8, 20% SDS, 0.7% ß-mercaptoethanol in double distilled water (DDW)) for 30 min at 50° C. The stripped membranes were rinsed with TBST 6 times for 5 min prior to subsequent analysis.

3. Results

3.1. RMD have no substantial effect on cellular uptake of DBF in HEK293 cells stably expressing OATP2B1

We calculated kinetic parameters (K_m, V_{max}) with the established HEK293-OATP2B1 model (Fig 1A). The Kinetic parameter of OATP2B1 mediated DBF transport was calculated by fitting data to equation containing active and passive component (K_m = 0.491 ± 0.226 μM, V_{max} = 19.57 ± 2.8 pmol/min/mg). Unlike concentration dependent inhibitory effect of SSZ(Fig. 1B), co-incubation of RMD did not inhibit uptake of DBF in HEK293-OATP2B1 when RMD in nM range for in vitro HDAC inhibition (Fig.1C) and μM range to consider protein binding condition in vivo (Fig.1D) was used.

3.2. RMD led to downregulate OATP2B1 mRNA in hepatic cell lines

RMD downregulated the transcription level of OATP2B1 in three different hepatic cancer cell lines (Fig. 2A). To determine whether the effect was a class effect of HDACi rather than RMD alone, SAHA was additionally treated and the relative mRNA expression was analyzed (Fig. 2B). Consistent with RMD, relative

expression of OATP2B1 mRNA was decreased by SAHA.

3.3. Identification of histone acetylation followed by HDACi treatment in Huh7 cell line

We conducted western blot to determine whether the decrease of OATP2B1 mRNA due to HDACi 24hours treatment in the hepatic cancer cell lines was associated with the effect of histone acetylation (Fig. 3). When the acetylation of histone H3 (H3) was confirmed by western blot using RMD in huh7 (Fig. 3A), HDACi (SAHA 0.1~0.5 μM, RMD 1~10 nM at 24 hours) treatment increased acetylated histone h3 (AcH3) concentration-dependently. In order to determine whether the protein increase trend of AcH3 was the only effect of RMD or class effect of HDACi drug, SAHA treatment was performed under the same conditions and the amount of protein was confirmed by western blot(Fig. 3B). The expression level of AcH3 increased depending on the concentration of SAHA.

3.4. Downregulated OATP2B1 mRNA due to RMD treatment recovered in 24 hours followed by the use of drug free media

To identify recovery profile of mRNA after 24 RMD (10 nM) treatment, we analyzed mRNA level after incubation in drug free

media for 24h. In contrast to substantial decrease 2B1 mRNA level after 24h RMD treatment, 24h incubation in drug free media showed no difference in Huh7, HepG2 and PLC/PRF/5 cells (Fig. 4B).

3.5. HDACis downregulate OATP2B1 protein in hepatic cell lines

To determine whether the mRNA reduction of OATP2B1 by RMD treatment also affects the protein expression level of OATP2B1, protein level was confirmed after 24 hours of RMD treatment by concentration (0~10 nM) in three hepatic cancer cell lines (Huh7, HepG2, PLC/PRF/5). We found that the amount of OATP2B1 protein tended to slightly decrease in concentration dependent manner (Fig. 5).

3.6. Downregulated OATP2B1 protein due to RMD treatment continued to decrease followed by the use of drug free media

To identify recovery profile of protein after 24 RMD (10 nM) treatment, we analyzed protein level after incubation in drug free media for 24h. We found that protein expression level of OATP2B1 tended to decrease over time even after the drug was removed (Fig. 6).

4. Discussion

Investigations on potential interactions of RMD with OATP2B1 may provide some information on hepatic disposition pathways and predicting potential transporter-mediated DDI. In the present study, we describe RMD is not likely to be a substrate of OATP2B1 and RMD can cause a decrease in OATP2B1 expression as epigenetic modulator.

In our study, the transporter kinetic parameter we obtained was different from the previous report (Fig. 1A). K_m value and V_{max} value of our HEK293-OATP2B1 on DBF was smaller than the previous report (current study: K_m = 0.491 ± 0.226 μM, V_{max} = 19.57 ± 2.8 pmol/min/mg vs Izumi et al, 2016: K_m = 10 ± 5.1 μM, V_{max} = 236 ± 41 pmol/min/mg).[15] V_{max} value may vary between models depending on the amount of transporter protein expression. However, the difference in K_m value which is independent of transporter expression and is indicative intrinsic affinity is hard to understand. Possible reason of the difference is that may occur because of the experimental conditions (buffer, method) of each group used. For example, another substrate of OATP2B1 E_1S K_m value vary more than 13-fold between previous reports.[17]

In our study, there is a limitation that only one probe

substance (DBF) is used to identify inhibitory effect of RMD on OATP2B1 function (Fig. 1). We cannot rule out the possibility that the inhibitory potency depends on substrate. The same superfamily transporter OATP1B3, for example, has shown that inhibitory level of oleanolic acid on OATP1B3 function decreased when substrates changed from estron-3-sulfate to estradiol-17β-glucuronide.[18] OATP superfamily transporters, including OATP2B1, are also known to have multiple substrate binding sites.[19,20] Therefore, further study will be necessary to obtain clear evidence that RMD is not a substrate or inhibitor of OATP2B1.

In this study, we observed that transcription level of OATP2B1 is downregulated by HDACi(RMD, SAHA) in 3 hepatic cancer cell lines, but the underlying detail mechanism is yet unknown(Fig. 2, Fig. 4). HDACi is generally known to modulate chromatin condensation by acetylation of the histones affecting transcription actively.[21] However, our result showed that the transcriptional expression of OATP2B1 was reduced by HDACis. As such, there are studies confirming the inhibition of gene transcription, contrary to the expected results considering the mechanism of HDACi.[22,23] Possible mechanism is the involvement of transcriptional activation of unknown OATP2B1 gene suppressor(s) or nuclear receptor. Several nuclear receptors have been reported

to be involved in the regulation of OATP2B1. Liver specific transcription factor hepatic nuclear factor 4α (HNF4α) reported to regulate expression of major OATP2B1 protein form(short OATP2B1 1e variant).[24] Also, nuclear receptor shown to involved in OATP2B1 mRNA expression in human hepatocytes, demonstrated by using chemical activators phenobarbital (CAR activator) & 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (AhR activator).[25] Further studies are needed to understand more precise underlying mechanisms.

The continued decrease in the expression level of OATP2B1 even after the drug was removed (Fig. 6) suggests the possibility of causing pharmacokinetic changes in patients receiving a combination of RMD and OATP2B1 substrate drugs. Erlotinib is a clinically used selective OATP2B1 substrate.[26] Also, the possibility of using these two drugs for increasing the anti-tumor effect have been reported.[27-29] Therefore, it's worthwhile for clinical relevance to identify the possibility of DDI between RMD and Erlotinib with involvement of OATP2B1 function.

In summary, the present study report novel findings that RMD is neither an inhibitor nor substrate of OATP2B1. Accumulation mechanism of RMD into liver is conducted by other mechanism. Also, we have demonstrated an inhibitory effect of

HDACis on OATP2B1 expression. Thus, patients who are treated with HDACis, may have potential alteration of OATP2B1-mediated uptake and change the disposition of drugs transported by OATP2B1 (e.g., erlotinib, statins). Further investigations needed to assess the potential for drug-drug interactions between RMD and OATP2B1 substrate drugs for the clinical implications of our study.

5. Future directions

본 연구에서는 OATP2B1을 발현하는 HEK293 세포 모델을 이용해 RMD이 OATP2B1의 기질과 함께 co-incubation 되었을 때 transport 기능에 영향을 미치지 못한다는 것을 실험을 통해 확인하고, RMD의 간세포 축적 메커니즘에 OATP2B1이 관여하지 않음을 밝혔다. 하지만 RMD의 OATP2B1 기능 억제 확인 기질로서 한가지 물질(DBF)만을 다루었다는 한계가 있다. 저해 potency가 기질에 따라 다를 수 있는 가능성을 배제할 수 없다. 실제로 같은 상과(superfamily) 수송체인 OATP1B3의 경우, 물질의 저해 수준이 1B3의 어떤 기질을 쓰냐에 따라서 저해 정도가 달랐다는 연구결과가 있다.[18] 또한 OATP2B1을 포함한 OATP 상과 수송체들은 기질과의 다중 결합 부위가 가능성이 알려져 있다.[19,20] 따라서 이에 대한 추가 연구가 필요하다. 이를 위한 방법으로는 OATP2B1의 probe 기질로써 서로 다른 결합 부위를 가질 수 있다고 알려진 estrone-3-sulfate, pravastatin, fexofenadine 등이 RMD와 함께 co-incubation 되었을 때 경쟁적 저해 여부로 확인할 수 있다. OATP2B1이 RMD의 기질이 아님을 더욱 잘 설명할 것이며, RMD의 활성 대사체로 변화를 위한 간세포 축적 메커니즘에 대한 정보를 제공할 수 있을 것이다.

또한 본 연구에서는 간암 세포 모델에서 RMD가 OATP2B1의 유전자와 단백질의 발현량을 감소시킬 수 있는 가능성을 확인하고 조절 기전으로 후생유전학적 변화가 관여되었음을 밝혔다. 하지만 이것이 실

제 임상적으로 의미가 있는 결과인지 추가 연구가 필요하다. 간 및 간암 세포 모델에는 OATP2B1뿐만 아니라 다양한 수송체들이 존재한다.[30,31] 따라서 다른 수송체에 의한 약물의 transport를 배제할 수 있도록 OATP2B1에 특이적인 기질이며 임상적으로 사용되는 약물인 erlotinib을 사용할 수 있다. RMD로 인한 OATP2B1 의 발현량이 변화한 상태에서 erlotinib의 transporter kinetic parameter를 구하여 RMD와 erlotinib이 임상적으로 함께 사용될 때 잠재적인 상호작용을 예측하는 것에 중요한 통찰력을 제공할 수 있을 것이다.

6. References

1. Ueda H, Nakajima H, Hori Y, Goto T, Okuhara M 1994. Action of FR901228, a novel antitumor bicyclic depsipeptide produced by Chromobacterium violaceum no. 968, on Ha-ras transformed NIH3T3 cells. Bioscience, biotechnology, and biochemistry 58(9):1579-1583.
2. Kim M, Thompson LA, Wenger SD, O'Bryant CL 2012. Romidepsin: a histone deacetylase inhibitor for refractory cutaneous T-cell lymphoma. Annals of Pharmacotherapy 46(10):1340-1348.
3. Khot A, Dickinson M, Prince HM 2013. Romidepsin for peripheral T-cell lymphoma. Expert review of hematology 6(4):351-359.
4. Kuo MH, Allis CD 1998. Roles of histone acetyltransferases and deacetylases in gene regulation. Bioessays 20(8):615-626.
5. Furumai R, Matsuyama A, Kobashi N, Lee K-H, Nishiyama M, Nakajima H, Tanaka A, Komatsu Y, Nishino N, Yoshida M 2002. FK228 (depsipeptide) as a natural prodrug that inhibits class I histone deacetylases. Cancer research 62(17):4916-4921.
6. Xiao JJ, Byrd J, Marcucci G, Grever M, Chan KK 2003. Identification of thiols and glutathione conjugates of depsipeptide FK228 (FR901228), a novel histone protein deacetylase inhibitor, in the blood. Rapid communications in mass spectrometry 17(8):757-766.
7. NDA022393_romidepsin pharmacology review p66.
8. NDA022393_romidepsin pharmacology review p57.
9. Shiraga T, Tozuka Z, Ishimura R, Kawamura A, Kagayama A 2005.

Identification of cytochrome P450 enzymes involved in the metabolism of FK228, a potent histone deacetylase inhibitor, in human liver microsomes. Biological and Pharmaceutical Bulletin 28(1):124-129.

10. Xiao JJ, Foraker AB, Swaan PW, Liu S, Huang Y, Dai Z, Chen J, Sadée W, Byrd J, Marcucci G 2005. Efflux of depsipeptide FK228 (FR901228, NSC-630176) is mediated by P-glycoprotein and multidrug resistance-associated protein 1. Journal of Pharmacology and Experimental Therapeutics 313(1):268-276.
11. ISTODAX® (romidepsin) label 2018.
12. McFeely SJ, Wu L, Ritchie TK, Unadkat J 2018. Organic anion transporting polypeptide 2B1–More than a glass-full of drug interactions. Pharmacology & therapeutics.
13. Schiöth HB, Boström A, Murphy SK, Erhart W, Hampe J, Moylan C, Mwinyi J 2016. A targeted analysis reveals relevant shifts in the methylation and transcription of genes responsible for bile acid homeostasis and drug metabolism in non-alcoholic fatty liver disease. BMC genomics 17(1):462.
14. Libra A, Fernetti C, Lorusso V, Visigalli M, Anelli PL, Staud F, Tiribelli C, Pascolo L 2006. Molecular determinants in the transport of a bile acid-derived diagnostic agent in tumoral and nontumoral cell lines of human liver. Journal of Pharmacology and Experimental Therapeutics 319(2):809-817.
15. Izumi S, Nozaki Y, Komori T, Takenaka O, Maeda K, Kusuhara H, Sugiyama Y 2016. Investigation of Fluorescein Derivatives as

Substrates of Organic Anion Transporting Polypeptide (OATP) 1B1 To Develop Sensitive Fluorescence-Based OATP1B1 Inhibition Assays. Mol Pharm 13(2):438-448.

16. Karlgren M, Vildhede A, Norinder U, Wisniewski JR, Kimoto E, Lai Y, Haglund U, Artursson P 2012. Classification of inhibitors of hepatic organic anion transporting polypeptides (OATPs): influence of protein expression on drug-drug interactions. Journal of medicinal chemistry 55(10):4740-4763.
17. Sugiyama Y, Steffansen B. 2013. Transporters in drug development: discovery, optimization, clinical study and regulation. ed.: Springer Science & Business Media.
18. Roth M, Araya JJ, Timmermann BN, Hagenbuch B 2011. Isolation of modulators of the liver-specific organic anion-transporting polypeptides (OATPs) 1B1 and 1B3 from Rollinia emarginata Schlecht (Annonaceae). J Pharmacol Exp Ther 339(2):624-632.
19. Hoshino Y, Fujita D, Nakanishi T, Tamai I 2016. Molecular localization and characterization of multiple binding sites of organic anion transporting polypeptide 2B1 (OATP2B1) as the mechanism for substrate and modulator dependent drug-drug interaction. MedChemComm 7(9):1775-1782.
20. Noe J, Portmann R, Brun ME, Funk C 2007. Substrate-dependent drug-drug interactions between gemfibrozil, fluvastatin and other organic anion-transporting peptide (OATP) substrates on OATP1B1, OATP2B1, and OATP1B3. Drug Metab Dispos 35(8):1308-1314.

21. Glaser KB 2007. HDAC inhibitors: clinical update and mechanism-based potential. Biochemical pharmacology 74(5):659-671.
22. Bode KA, Schroder K, Hume DA, Ravasi T, Heeg K, Sweet MJ, Dalpke AH 2007. Histone deacetylase inhibitors decrease Toll-like receptor-mediated activation of proinflammatory gene expression by impairing transcription factor recruitment. Immunology 122(4):596-606.
23. Hu Y, Zhang L, Zhao L, Li J, He S, Zhou K, Yang F, Huang M, Jiang L, Li L 2011. Trichostatin A selectively suppresses the cold-induced transcription of the ZmDREB1 gene in maize. PLoS One 6(7):e22132.
24. Knauer MJ, Girdwood AJ, Kim RB, Tirona RG 2013. Transport function and transcriptional regulation of a liver-enriched human organic anion transporting polypeptide 2B1 transcriptional start site variant. Molecular pharmacology 83(6):1218-1228.
25. Jigorel E, Le Vee M, Boursier-Neyret C, Parmentier Y, Fardel O 2006. Differential regulation of sinusoidal and canalicular hepatic drug transporter expression by xenobiotics activating drug-sensing receptors in primary human hepatocytes. Drug Metabolism and Disposition 34(10):1756-1763.
26. Bauer M, Matsuda A, Wulkersdorfer B, Philippe C, Traxl A, Özvegy-Laczka C, Stanek J, Nics L, Klebermass EM, Poschner S 2018. Influence of OATPs on hepatic disposition of erlotinib measured with positron emission tomography. Clinical Pharmacology & Therapeutics 104(1):139-147.

27. Zhang W, Peyton M, Xie Y, Soh J, Minna JD, Gazdar AF, Frenkel EP 2009. Histone deacetylase inhibitor romidepsin enhances anti-tumor effect of erlotinib in non-small cell lung cancer (NSCLC) cell lines. Journal of Thoracic Oncology 4(2):161-166.

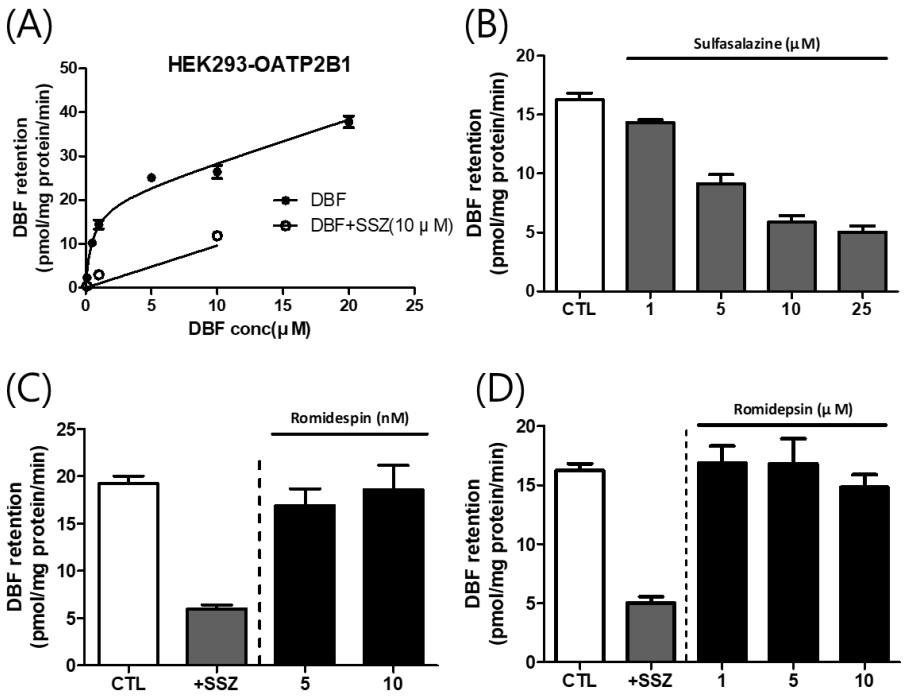
28. Gerber DE, Boothman DA, Fattah FJ, Dong Y, Zhu H, Skelton RA, Priddy LL, Vo P, Dowell JE, Sarode V 2015. Phase 1 study of romidepsin plus erlotinib in advanced non-small cell lung cancer. Lung cancer 90(3):534-541.

29. Karpel-Massler G, R Wirtz C, Halatsch M-E 2011. Drug combinations enhancing the antineoplastic effects of erlotinib in high-grade glioma. Recent patents on anti-cancer drug discovery 6(3):384-394.

30. Jetter A, Kullak-Ublick GA 2019. Drugs and hepatic transporters: A review. Pharmacol Res:104234.

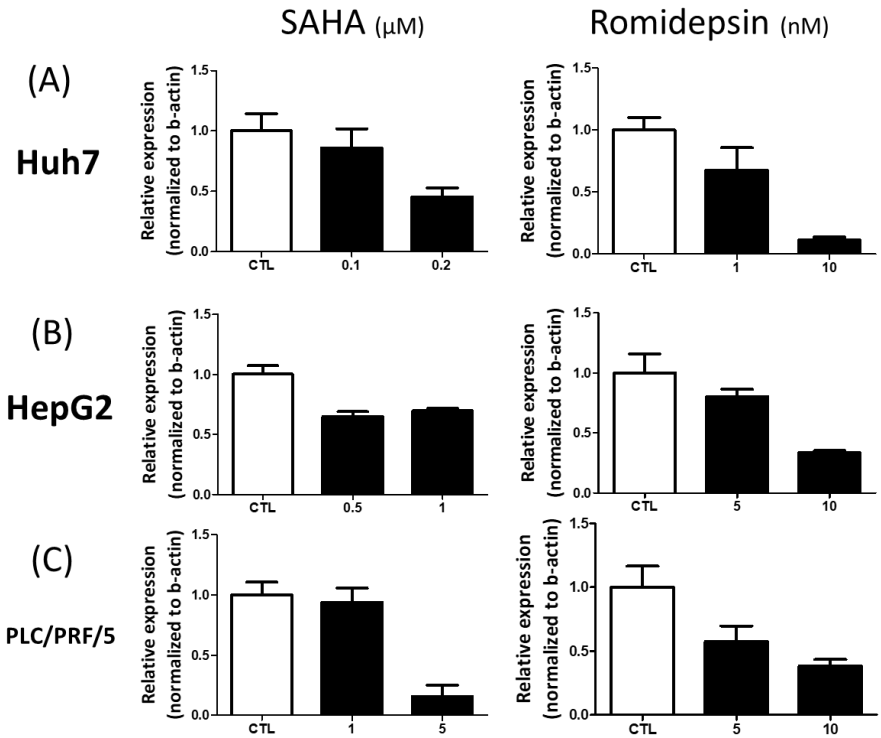
31. Jouan E, Le Vée M, Denizot C, Parmentier Y, Fardel O 2017. Drug transporter expression and activity in human hepatoma HuH-7 cells. Pharmaceutics 9(1):3.

Figure 1. No inhibitory effect of romidepsin (RMD) on the transport activity of OATP2B1 assessed by the uptake of the fluorescent probe dibromofluorescein (DBF).



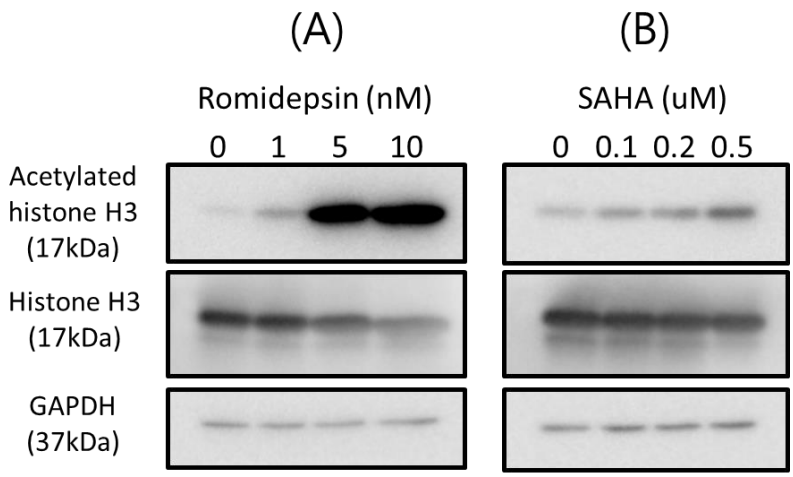
(A) Kinetic profiles of DBF uptake in HEK293 cells stably overexpressing OATP2B1 (HEK293-OATP2B1). Cellular retention of DBF was measured after incubating the cells with varying concentrations of DBF for 3 min. Co-incubation with SSZ (10 μM) was used to inhibit OATP2B1-mediated uptake, thereby assess the permeability clearance via passive diffusion. The kinetic parameters were obtained by non-linear regression analysis to an equation including both saturable and non-saturable components. (B) Results showing the concentration-dependent inhibition of cellular retention of DBF by SSZ. (C, D) Results showing no inhibition of cellular retention of DBF by RMD in nM and μM ranges. SSZ (25 μM) was included as a positive control.

Figure 2. Changes in the OATP2B1 mRNA levels following the treatment of two different histone deacetylase inhibitors (suberoylanilide hydroxamic acid, SAHA; romidepsin, RMD) in three hepatic cancer cell lines (Huh7 , HepG2 and PLC/PRF/5) (n=3).



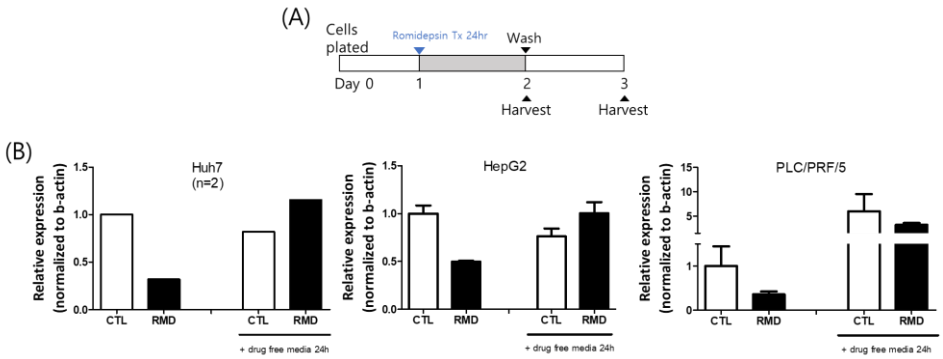
Cells were plated onto 6-well plates with at the plating density of 2.0×105 cells/well. At 24 hours post-plating, the drug treatment was initiated by adding media containing varying concentrations of RMD (1~10 nM) (A) and SAHA (0.1~5 μM) (B). After 24 hours, cells were harvested and subjected to mRNA analyses. The isolated RNA was converted to cDNA and analyzed by quantitative RT-PCR. The fold changes in the OATP2B1 expression (compared to the vehicle control) were calculated using 2-△△Ct method and normalized to that of β-actin (housekeeping control). Each RT-PCR run included negative controls containing no template. (CTL= control group)

Figure 3. Accumulation of acetylated histone H3 following the treatment of romidepsin (RMD) or suberoylanilide hydroxamic acid (SAHA) in Huh7 cell line.



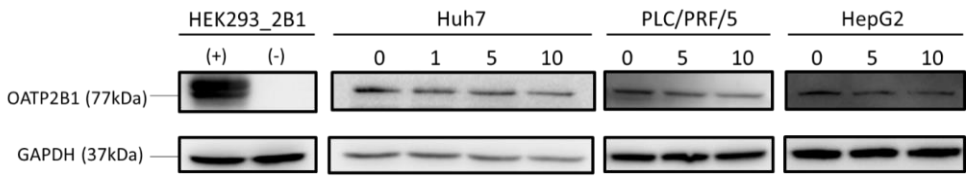
Huh7 cells were plated onto 6-well plates at the density of 2.0×105 cells/well cell (n=3). At 24 hours post-plating, the drug treatment was initiated by adding media containing varying concentrations of RMD (1~10 nM) (A) and SAHA (0.1~0.5 μM) (B). After 24 hours, cells were harvested and subjected to protein analyses. Equivalent amount of protein in cell lysate (10 μg) samples were loaded for electrophoresis. Acetylated histone H3 (AcH3) was primarily probed with antibody. After visualizing immune-reactive bands using an enhanced chemiluminescence, remaining antibodies were removed by membrane stripping to probe histone H3. Histone H3 were shown as the target amount control and GAPDH were shown as gel loading control.

Figure 4. OATP2B1 mRNA levels following the treatment of romidepsin (RMD) for 24 hours and subsequent incubation in drug-free media for 24 hours in three hepatic cancer cell lines.



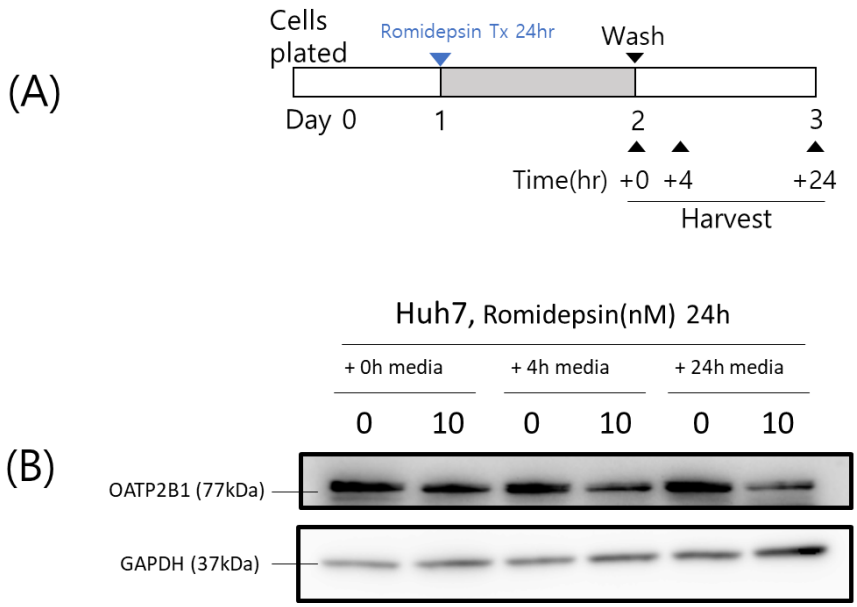
(A) Method scheme: Cells were plated onto 6-well plates with at the plating density of 2.0×105 cells/well. At 24 hours post-plating, the drug treatment was initiated by adding media containing RMD (10 nM). After 24 hours RMD treatment, cells were washed with DPBS and placed in drug-free media. At 24 hours post-washing, cells were harvested for RNA analyses. (B) Harvested cells (Huh7, HepG2 and PLC/PRF/5) were extracted and synthesized to cDNA and analyzed by quantitative RT-PCR. The fold changes in the OATP2B1 expression (compared to the vehicle control) were calculated using $2^{-\triangle\triangle Ct}$ method and normalized to that of β-actin (housekeeping control). Unless written, every experiments were conducted n = 3. (CTL= control group)

Figure 5. Changes in the OATP2B1 protein levels following the treatment of romidepsin (RMD) with Huh7, HepG2 and PLC/PRF/5 cells.



Cells were plated onto 6-well plates at the density of 2.0×105 cells/well cell (n=3). At 24 hours post-plating, the drug treatment was initiated by adding media containing varying concentrations of RMD (1~10 nM). After 24 hours, cells were harvested and subjected to protein analyses. Equivalent amount of protein in cell lysate (18 μg) samples were loaded for electrophoresis. GAPDH were shown as gel loading control. (+) Positive control: transient transfected HEK293 expressing OATP2B1 (Myc-DDK tagged) transcript variant1 in pCMV6_Entry plasmid, (-) Negative control: non transfected HEK293

Figure 6. Changes in the OATP2B1 protein levels following the treatment of romidepsin (RMD) and additional drug free media culture in Huh7 cells.



(A) Method scheme: Cells were plated onto 6-well plates with at the plating density of 2.0×105 cells/well. At 24 hours post-plating, the drug treatment was initiated by adding media containing RMD (10 nM). After 24 hours RMD treatment, cells were washed with DPBS and placed in drug-free media. At 0, 4, 24 hours post-washing, cells were harvested for protein analyses. (B) Equivalent amount of protein in cell lysate (18 μg) samples were loaded for electrophoresis. GAPDH were shown as gel loading control.

7. 국문초록

Romidepsin은 피부T세포림프종(CTCL)과 말초T세포림프종(PTCL)의 치료에 승인된 약물로서 히스톤 탈아세틸화효소를 억제하는 후생유전학적 메커니즘으로 약효를 나타낸다. Romidepsin은 활성 대사체로 간세포에서 변화하는 것이 약효에 필수적이지만, 약물의 간세포 축적 메커니즘은 알려져 있지 않다. 다양한 외인성 및 내인성 화합물의 간세포내 거동에 영향을 줄 수 있다고 알려져 있는 OATP2B1는 후생유전학적 조절기전에 의하여 유전자 전사량 변화가 보고 된 바 있다. 기존 문헌에서는 romidepsin이 OATP2B1의 발현과 기능에 미치는 영향에 대해 연구된 바가 없었으며, 따라서 본 논문에서는 romidepsin이 OATP2B1의 기질로서 작용하는지 알아보고 romidepsin이 후생유전학적 측면에서 OATP2B1의 발현에 영향을 주는지 알아보고자 하였다. Romidepsin의 기질성을 확인하기 위해 OATP2B1를 발현하는 HEK293 stable cell line을 구축하였다. OATP2B1에 대하여 선택적인 형광 기질인 dibromofluorescein을 사용하여 romidepsin은 경쟁적 저해를 유발하지 않는 것을 확인하였다. OATP2B1의 발현 조절에 미치는 영향을 연구하기 위하여 3가지 간암 세포주에 24시간 동안 romidepsin(1~10 nM) 처리하였을 때 OATP2B1의 mRNA가 농도의존적으로 감소하며 약물의 제거 후 24시간 이내에 발현 정도가 회복되는 것을 확인하였다. 히스톤 탈아세틸화효소 억제제 물질인 SAHA(0.1~5 uM)와 Na butyrate(0.5~2 mM) 처리했을 때도 유사한 결과로

OATP2B1의 mRNA가 감소하였다. Romidepsin의 농도가 증가할수록 OATP2B1의 단백질 양 또한 감소하는 경향을 보였다. 본 연구 결과는 romidepsin이 OATP2B1 기질이 아닐 가능성이 높으며 romidepsin에 지속적으로 간세포가 노출될 경우 OATP2B1의 mRNA와 단백질 발현량을 감소시켜 간세포 약물축적에 영향을 미칠 가능성을 제시하였다. 이러한 가능성은 추후 간암세포주를 사용하여 검증할 예정이다.

주요어: Romidepsin, 후생유전학, 약물수송체, 유기음이온수송체 2B1(OATP2B1)

학번: 2018-26399