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E297G mutated bile salt export pump (BSEP) function enhancers derived from GW4064: Structural development study and separation from farnesoid X receptor-agonistic activity

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ABSTRACT

Bile salt export pump (BSEP) is a member of the ATP-binding cassette transmembrane transporter family and mediates biliary excretion of bile acids from hepatocytes. Several BSEP mutants, including Glu297Gly (E297G) and Asp482Gly (D482G), cause progressive familial intrahepatic cholestasis type 2. We previously found that compounds based on GW4064, a representative farnesoid X receptor (FXR) agonist, enhanced E297G BSEP transport activity. Here, we conducted a structure–activity relationship analysis of GW4064 derivatives aimed at separating E297G BSEP-function-promoting activity and FXR-agonistic activity. Among newly synthesized reversed-amide derivatives of previously reported GW4064 analogs **2a**–**2f**, we identified **7c** as a selective BSEP function enhancer.

Bile salt export pump (BSEP) is a member of the ATP-binding cassette transmembrane transporter family and mediates the biliary excretion of monovalent bile acids (such as taurocholate) from hepatocytes.[1–3] It is well known that several BSEP mutants cause progressive familial intrahepatic cholestasis type 2 (PFIC2) which is a recessive hereditary liver disease characterized by cholestasis and jaundice in the first year of life, leading to cirrhosis and death before adulthood unless liver transplantation is carried out.[4] Among the PFIC2-causing mutations, two missense mutations, that is, E297G (Glu297Gly) and D482G (Asp482Gly), are frequently observed in PFIC2 patients.[5,6] The E297G mutant is considered to be folding-defective (misfolded), and as a result, this mutant BSEP is retained within the cell on endoplasmic reticulum (ER), and does not acquire the usual golgi-related glycosylation. Hayashi et al. reported that the E297G and D482G mutations cause a reduction of BSEP on plasma membrane, but importantly, they found that the transport function of both mutated BSEPs is almost normal if they are correctly localized.[7] Furthermore, Hayashi et al. reported that sodium 4-phenylbutyric acid (4-PBA) enhanced the cell-surface expression and transport capacity of E297G BSEP by prolonging the half-life of cell-surface-resident BSEP, through modulating its ubiquitination status and AP2-mediated internalization.[6–8]

In our previous work, we found that bile acids showed moderate E297G BSEP-function-promoting activity, and chenodeoxycholic acid (CDCA) was the most potent function-promoter among the tested bile acids.[9] Furthermore, compounds based on GW4064 [a representative farnesoid X receptor (FXR) agonist[10]] (Fig. 1) enhanced the BSEP transport activity. Among the tested compounds, compound **2d** possessed the most potent E297G BSEP-function-promoting activity at 10 μM (Table 2). We speculated that these compounds enhanced the transport capacity by increasing the cell-surface expression of the mutated BSEP.

On the other hand, it was reported that GW4064 derivatives (**2a**, **2b**, **2d**, **2e**) possess moderate to potent FXR-agonistic activity. FXR is a well-characterized member of the so-called metabolic subfamily of nuclear receptors, and is a transcriptional sensor for bile acids.[12,13] It is well known that FXR-agonists such as CDCA and GW4064 increase endogenous BSEP mRNA level and lower the bile acid pool.[14] We considered that the contribution of FXR-agonistic activity to the improvement of BSEP function in our assay system was negligible, because we had calculated the ratio of the amount of [^{3}H]taurocholate ([^{3}H]TC) accumulated in MDCK II cells expressing WT or E297G BSEP versus that in the MDCK II cells expressing GFP to confirm the effect of GW4064 derivatives on the function of BSEP.

A reduction in the bile acids pool, which may be caused by FXR-agonists (vide supra), is likely to cause inactivation of TGR5, which is a member of the G protein-coupled receptors (GPCR), and hence facilitates the development of obesity and diabetes.[15]

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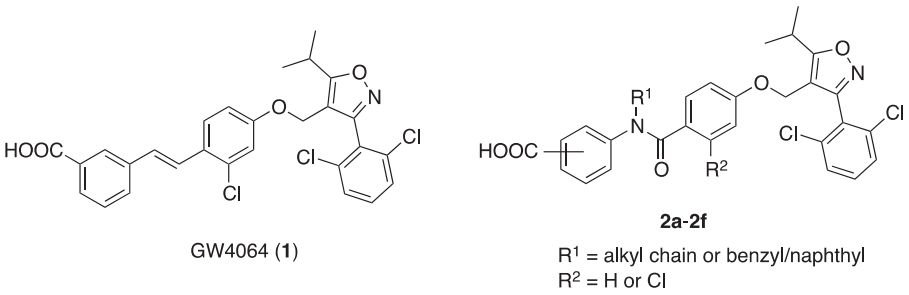
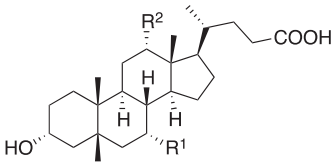


Figure 1. Structure of GW4064 and E297G BSEP function enhancers.

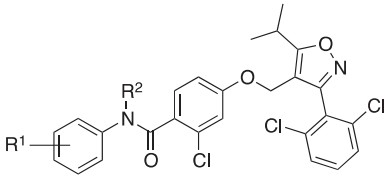
Table 1
E297G BSEP-function-promoting activity and FXR-agonistic activity of bile acids



Compd	R^1	R^2	Accumulation of [^{3}H]TC[a] (%) at 100 μM	FXR-agonistic activity EC_{50}[c] (μM)
CA (**3**)	OH	OH	40[b]	NA[d]
DCA (**4**)	H	OH	65[b]	50
CDCA (**5**)	β-OH	H	46[b]	27
UDCA (**6**)	α-OH	H	43[b]	NA[d]

[a] The amount of [^{3}H]TC accumulated in MDCK II cells expressing E297G BSEP was defined as 100%.
[b] Date taken from our previous report.[9]
[c] The EC_{50} value is the molar concentration of the test compound that affords 50% of the maximal reporter activity.
[d] NA = No activity at 10 μM.

Table 2
FXR-agonistic activity of E297G BSEP function enhancers



Compd	R^1	R^2	Accumulation of [^{3}H]TC[a] (%) at 10 μM	FXR-agonistic activity EC_{50}[c] (μM) and efficacy[d] (%)
CDCA (5)			50[b]	27 (41)
GW4064 (1)			33[b]	0.07 (100)
2a	3-COOH	H	111[b]	3.0 (61)
2b	3-COOH	Me	66[b]	3.1 (45)
2c	3-COOH	*n*-Bu	49[b]	1.1 (43)
2d	3-COOH	Bn	31[b]	2.5 (31)
2e	3-COOH	Phenethyl	53[b]	0.84 (30)
2f	3-COOH	1-Naphthyl	59[b]	3.0 (28)
2g	2-COOH	Bn	80[b]	NA[e]
2h	4-COOH	Bn	35[b]	2.6 (41)
2i	3-COOMe	Bn	93[b]	NA[e]

[a] The amount of [^{3}H]TC accumulated in MDCK II cells expressing E297G BSEP was defined as 100%.
[b] Data taken from our previous report.[9]
[c] The EC_{50} value is the molar concentration of the test compound that affords 50% of the maximal reporter activity.
[d] The effect of GW4064 on FXR activation efficacy was defined as 100%.
[e] NA = No activity at 10 μM.

Therefore, in order to understand the biology and/or pharmacology of BSEP in more detail, and to examine whether or not FXR-agonistic activity would be problematic for PFIC2 treatment, we require selective BSEP function enhancers lacking FXR-agonistic activity as investigative tools. Here, we investigated the effect of the previously reported compounds on FXR activation and analyzed

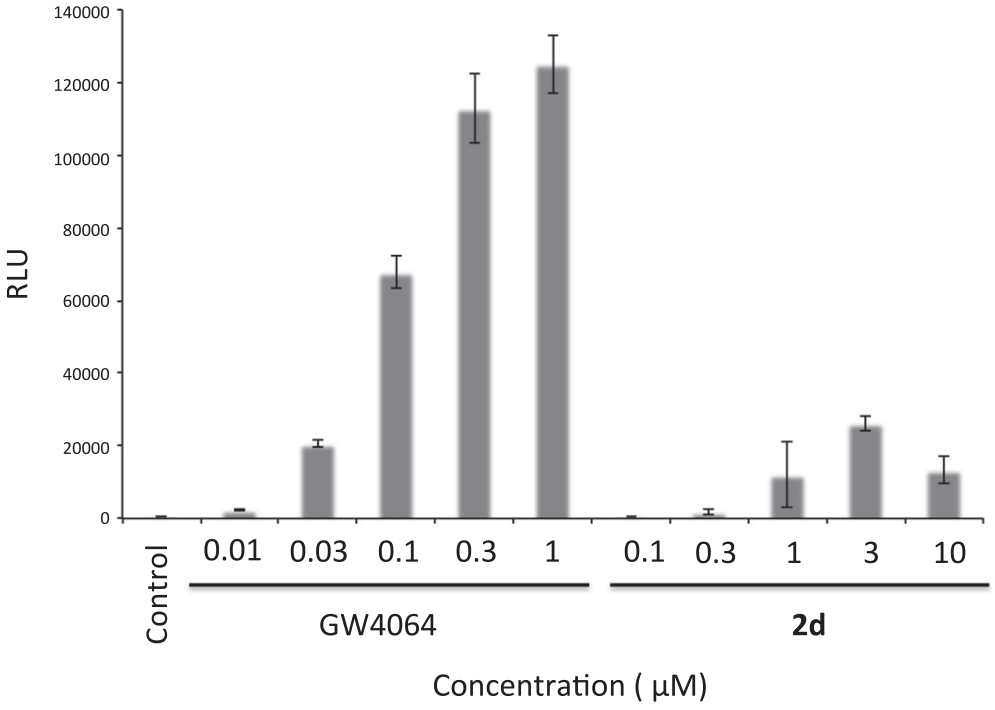
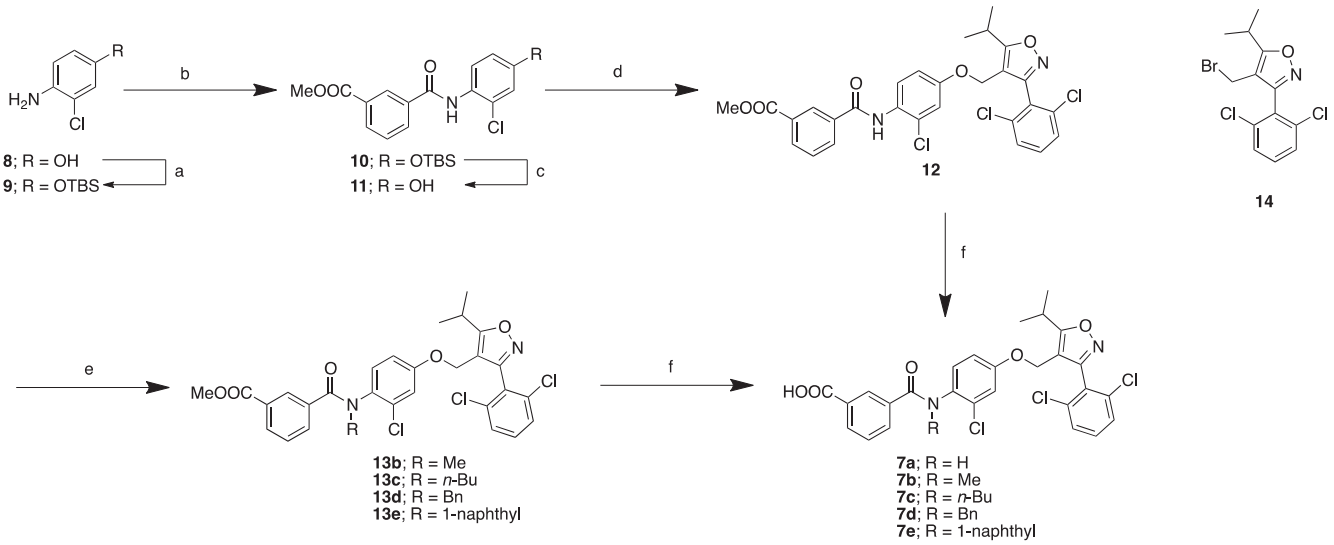


Figure 2. Maximal FXR transcriptional activation activity (efficacy) of GW4064 and **2d**.



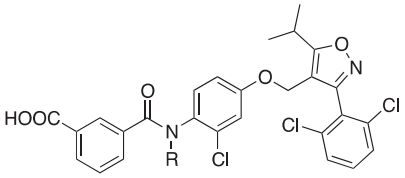
Scheme 1. Synthetic route to the present series of reverse amide compounds. Reagents and conditions: (a) TBSCl, imidazole, DMF, rt, quant; (b) monomethylisophthalate, MsCl, TEA, THF, 0 °C–rt, 78%; (c) TBAF, THF, 0 °C–rt, quant; (d) isoxazole derivative (**14**), K_2CO_3, DMF, rt, 87%; (e) R-Br or R-I, NaH, DMF, 0 °C–rt, 28–95%; (f) LiOH, THF, H_2O, rt, 29–81%.

their structure–activity relationship (SAR) for FXR-agonistic activity. We also describe the application of these results to the design and synthesis of selective BSEP function enhancer candidates.

To evaluate the activities of the compounds, we selected two assay systems. One was an accumulation assay to evaluate the E297G BSEP-function-promoting activity, using Madin-Darby canine kidney II (MDCKII) cells expressing Na^+-taurocholate cotransporting polypeptide (NTCP) and E297G BSEP, as described previously.[9] With this method, we evaluated the amount of [^{3}H]TC accumulated in MDCK II cells expressing E297G BSEP; this accumulation is determined by several parameters, including the cellular efflux of [^{3}H]TC via BSEP. The other was a reporter gene assay to evaluate the FXR-agonistic activity, including the EC_{50} value, and efficacy compared with those of the full agonist GW4064, using CMX-GAL4N-hFXR as the recombinant receptor gene, TK-MH100x4-LUC as the reporter gene, and the CMX β-galactosidase gene for normalization, as previously reported.[11]

We first tested the FXR-agonistic activity of various bile acids, that is, cholic acid (CA; **3**), deoxycholic acid (DCA; **4**), chenodeoxycholic acid (CDCA; **5**), and ursodeoxycholic acid (UDCA; **6**). We previously reported that these bile acids possess moderate E297G BSEP-function-promoting activity.[9] Compounds **4** and **5** showed weak FXR-agonistic activity, whereas compounds **3** and **6** showed no FXR-agonistic activity (Table 1). These results suggest that E297G BSEP-function-promoting activity can be separated from FXR-agonistic activity.

Table 3
E297G BSEP-function-promoting activity and FXR-agonistic activity of **7a**–**7e**



Compd	R	Accumulation of [^{3}H]TC[a] (%) at 10 μM	FXR-agonistic activity EC_{50} (μM) and efficacy[b] (%)
CDCA		50	27 (41)
GW4064		33	0.07 (100)
2d		31	2.5 (31)
7a	H	75	NA[c]
7b	Me	60	NA[c]
7c	*n*-Bu	42	NA[c]
7d	Bn	50	>10
7e	1-Naphthyl	59	0.41 (35)

[a] The amount of [^{3}H]TC accumulated in MDCK II cells expressing E297G BSEP was defined as 100%.
[b] The effect of GW4064 on FXR activation efficacy was defined as 100%.
[c] NA = no activity at 10 μM.

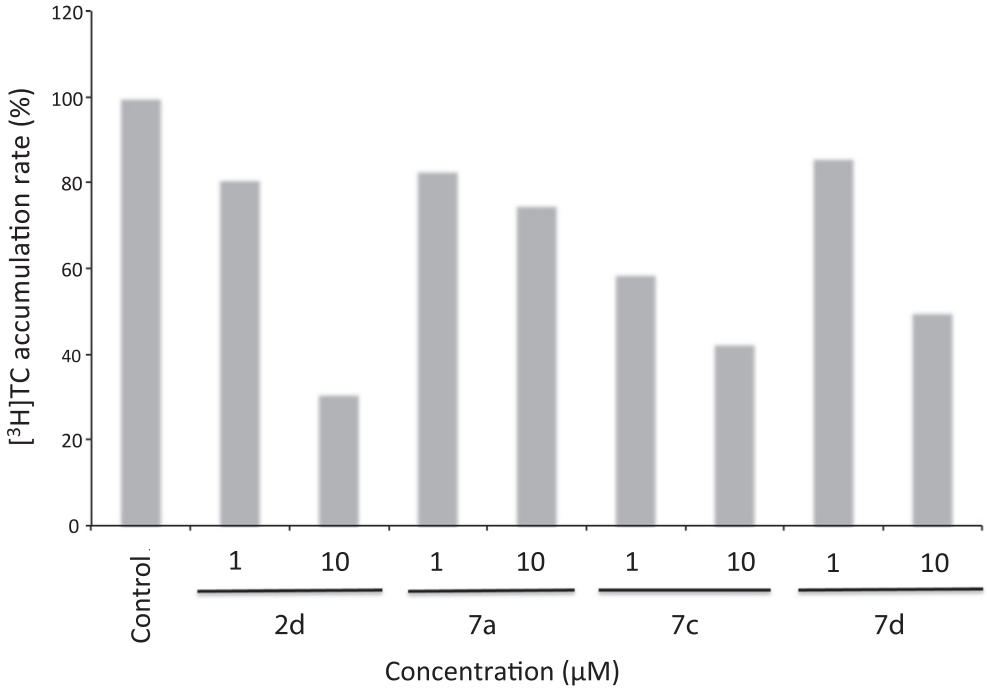


Figure 3. Concentration dependency of the effect of **2d**, **7a**, and **7c**–**7d** on [^{3}H]TC accumulation in MDCK II cells expressing E297G BSEP. The amount of [^{3}H]TC accumulated in MDCK II cells expressing E297G BSEP was defined as 100%.

Next, we investigated the FXR-agonistic activity of our previously reported E297G BSEP function enhancers derived from GW4064 (**2a**–**2i**, Table 2). E297G BSEP function enhancers **2a**–**f** and **2h** showed weak FXR-agonistic activity, while **2g** and **2i** were inactive. Broadly speaking, the EC_{50} values for FXR-agonistic activity of **2a**–**2f** and **2h** were about one order of magnitude higher (0.84–3.1 μM) than that of GW4064 (**1**, 0.07 μM). We previously reported the SAR of GW4064 derivatives for BSEP function enhancing activity, focusing on the stilbene moiety of GW4064, particularly the two-atom moiety connecting the two aromatic rings of the stilbene skeleton, which corresponds to the secondary amide structure of **2a** and tertiary amide structures of **2b**–**2f** [–N(R^1)–C(O)– of **2a–f** in Figure 1; designated as the central portion of the molecule in this paper]. Among them, *N*-benzyl derivative **2d** showed the most potent BSEP function enhancing activity, and decreased [^{3}H]TC accumulation with the potency of 31% at 10 μM (Table 2). The FXR-agonistic activities of **2a**–**2f** are summarized in Table 2. The secondary amide derivatives **2a** and *N*-alkyl/benzyl/phenethyl/naphthyl amide derivatives **2b**–**2f** activated FXR in the order of **2e** > **2c** > **2d** > **2a** = **2b** = **2f**. These results indicated that the size of the binding pocket of FXR hosting the central portion of GW4064 derivatives might be sufficient to accommodate a phenethyl or smaller group, being similar in this respect to the corresponding site of BSEP.

Then, we turned our attention to the SAR of the carboxyl group of the benzoic acid moiety. In the case of GW4064, a brief SAR report from GSK researchers disclosed that a carboxyl group is essential and is preferably located at the 3-position (*meta*-position) for

FXR-agonistic activity.[10] We evaluated the FXR-agonistic activity of regio-isomers, that is, the carboxyl group was shifted to the *ortho*-(**2g**) or *para*-position (**2h**) from the *meta*-position, as well as the effect of esterification of the *meta*-carboxyl group (**2i**) (Table 2). *ortho*-Isomer **2g** and ester derivative **2i** showed neither E297G BSEP-function-promoting nor FXR-agonistic activity, whereas the *para*-isomer (**2h**) showed these activities with similar potency to the *meta*-isomer **2d**. These results might indicate that the carboxyl group is similarly recognized by the binding pockets of BSEP and FXR.

As expected, E297G BSEP function enhancers (**2a**–**2f**, **2h**) also showed FXR-agonistic activity (Table 2). As shown in Table 2, substitution of the double bond at the central portion of GW4064 (**1**) to an amide bond, that is, compound **2d**, had no effect on E297G BSEP-function-promoting activity, but decreased the potency of FXR-agonistic activity, that is, the EC_{50} values for GW4064 (**1**) and **2d** are 0.07 μM and 2.5 μM, respectively. The above-mentioned SAR analysis of FXR-agonistic activity was based on the measured EC_{50} values. However, the efficacy of FXR activation should also be considered. It is well known that full agonists such as GW4064 fully activate FXR (100% efficacy), whereas partial agonists show lower levels of maximal transcriptional activation than the full agonist. As mentioned above, the EC_{50} value of compound **2d** is higher than that of GW4064 (**1**), and in addition, the efficacy of FXR transcriptional activation activity of **2d** is only 21% compared to that of the full agonist GW4064 (Fig. 2). So, we speculated that the N-substituted amide-type central portion of the molecule might be the key to separating E297G BSEP-function-promoting activity from FXR-agonistic activity. To further investigate the SAR of the N-substituted amide-type central portion, we designed and synthesized reversed-amide derivatives, **7a–e**. The synthetic route for the preparation of the reversed-amide derivatives is outlined in Scheme 1. Substituted isoxazole **14** was prepared by the method reported previously. 4-Amino-2-chlorophenol (**8**) was protected with a TBS group and condensed with monomethylisophthalate to afford amide **10**. After deprotection of the hydroxyl group, the resulting compound **11** was reacted with isoxazole derivative **14** to afford reversed-amide derivative **12**, whose saponification gave **7a**. Finally, N-alkylation of **12** by saponification of the esters **13b**–**e** yielded carboxylic acid derivatives **7b**–**7e**.

We evaluated the E297G BSEP-function-promoting activity and FXR-agonistic activity of the prepared reversed-amide derivatives **7a–7e** (Table 3 and Fig. 3). These compounds possessed moderate to potent E297G BSEP-function-promoting activity. Among the reversed-amide derivatives, *N*-butyl derivative **7c** possessed the most potent activity (accumulation of [^{3}H]TC was reduced to 42% and 59% at 10 μM and 1 μM, respectively, Table 3 and Fig. 3). Concerning FXR-agonistic activity, secondary reversed-amide derivative **7a** and *N*-alkyl reversed-amide derivatives **7b**–**7c** showed no activity over the concentration range examined (up to 10 μM), whereas *N*-benzyl/naphthyl derivatives (**7d**, **7e**) showed clear FXR-agonistic activity (EC_{50} values for FXR transcriptional activation activity of **7d** and **7e** are >10 and 0.41 μM, respectively, Table 3).

In summary, we confirmed that bile acids and **2a**–**2f** exhibit both FXR-agonistic activity and E297G BSEP-function-promoting activity. Based on SAR considerations, we designed and synthesized reversed-amide derivatives of previously reported GW4064 analogs **2a**–**2f** and identified one of the compounds, **7c**, as a selective BSEP function enhancer. Further SAR study and pharmacological evaluation of representative compounds are in progress. Comparison of the pharmacological effects elicited by a BSEP selective enhancer such as **7c** and BSEP/FXR dual activators such as **2d** should lead to a better understanding of the effect of FXR activation on PFIC2 treatment.

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