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Structure–activity relationships of oxysterol-derived pharmacological chaperones for Niemann–Pick type C1 protein

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ABSTRACT

Niemann–Pick disease type C is a fatal neurodegenerative disease, and its major cause is mutations in *NPC1* gene. This gene encodes NPC1 protein, a late endosomal polytopic membrane protein required for intracellular cholesterol trafficking. One prevalent mutation (I1061T) has been shown to cause a folding defect, which results in failure of endosomal localization of the protein, leading to loss-of-function phenotype. We have previously demonstrated that several oxysterols and their derivatives act as pharmacological chaperones; binding of these compounds to NPC1^{I1061T} mutant protein corrects the localization/maturation defect of the mutant protein. Here, we disclose detailed structure–activity relationships of oxysterol derivatives as pharmacological chaperones for NPC1^{I1061T} mutant.

Niemann–Pick disease type C is a fatal, heritable neurological disorder characterized by massive accumulation of cholesterol and other lipids in the late endosomal compartment.[1] Mutation in the *NPC1* gene,[2] which encodes late endosomal membrane protein NPC1, is carried by about 95% of patients.[3–5] This protein binds cholesterol[6–9] and is essential for intracellular cholesterol trafficking; thus, loss of function of the protein leads to late endosomal accumulation of cholesterol derived from endocytosed low-density lipoprotein (LDL). A major and well-characterized mutation in NPC1, I1061T (Ile1061 to Thr),[10] causes loss of function due to folding defect and instability, though the intrinsic function of the protein is retained.[11,12] In general, folding-defective membrane proteins are picked up by the cellular quality control system at the endoplasmic reticulum (ER), and are retained in the ER and degraded by proteasome through a process called ER-associated degradation (ERAD). I1061T mutant proteins are indeed retained and rapidly degraded in the ER, resulting in loss of functional NPC1 in late endosomes.[11]

Since the late 1990s, accumulating evidence has shown that loss-of-function phenotypes of folding-defective mutants can be corrected by using selective, small-molecular ligands.[13–23] These molecules, called pharmacological chaperones, are considered to rescue folding-defective mutant proteins via direct binding to the folding intermediates in the ER.[13] Binding of the pharmacological chaperone stabilizes mutant proteins, thus preventing their degradation via ERAD, and assists their exit from the ER. As rescued mutant proteins are, in most cases, at least partially functional at the correct location, overall cellular function can be restored by correction of mislocalization, mediated by pharmacological chaperones.

We previously reported that 25-hydroxycholesterol (**1**, 25HC, Fig. 1), mo56HC (**2**), and some other oxysterol derivatives including mo56CFA (**3**) act as pharmacological chaperones for NPC1 protein. These compounds corrected the folding-defective phenotypes of NPC1^{I1061T} mutant proteins, including localization/maturation defect, instability, and endosomal cholesterol accumulation in patient-derived fibroblasts, via direct binding to a putative second sterol-binding site[24,25] distinct from the N-terminal domain,[7,8,26,27] which is a well-characterized sterol-binding site on NPC1. Here, we disclose detailed structure–activity relationships of oxysterol derivatives as pharmacological chaperones for NPC1^{I1061T} mutant protein.

In our previous report,[24] we evaluated the relative efficacy of the sterol derivatives by quantitatively analyzing the altered localization of NPC1 proteins (Fig. 2B), by means of colocalization analysis with late-endosomal marker LAMP1. However, the colocalization analysis requires time-consuming immunocytochemical staining of LAMP1, and is not suitable for structure–activity relationship studies, where large numbers of derivatives need to be assayed. In order to increase throughput in the present work, we used a phenotypic analysis instead of colocalization analysis.[23] As shown in Figure 2A, the localization pattern of NPC1^{I1061T} mutant protein was classified on a cell-by-cell basis into three

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0960-894X/

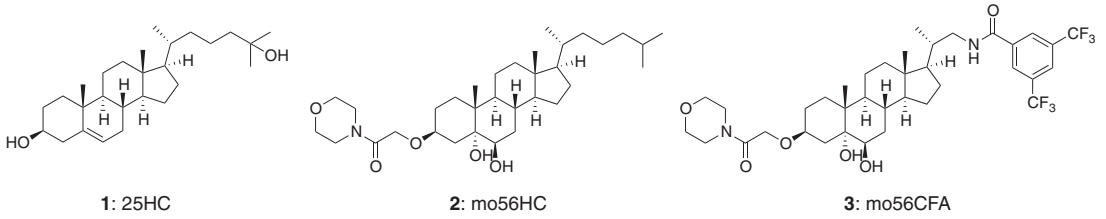


Figure 1. Structures of pharmacological chaperones for NPC1 mutant protein.

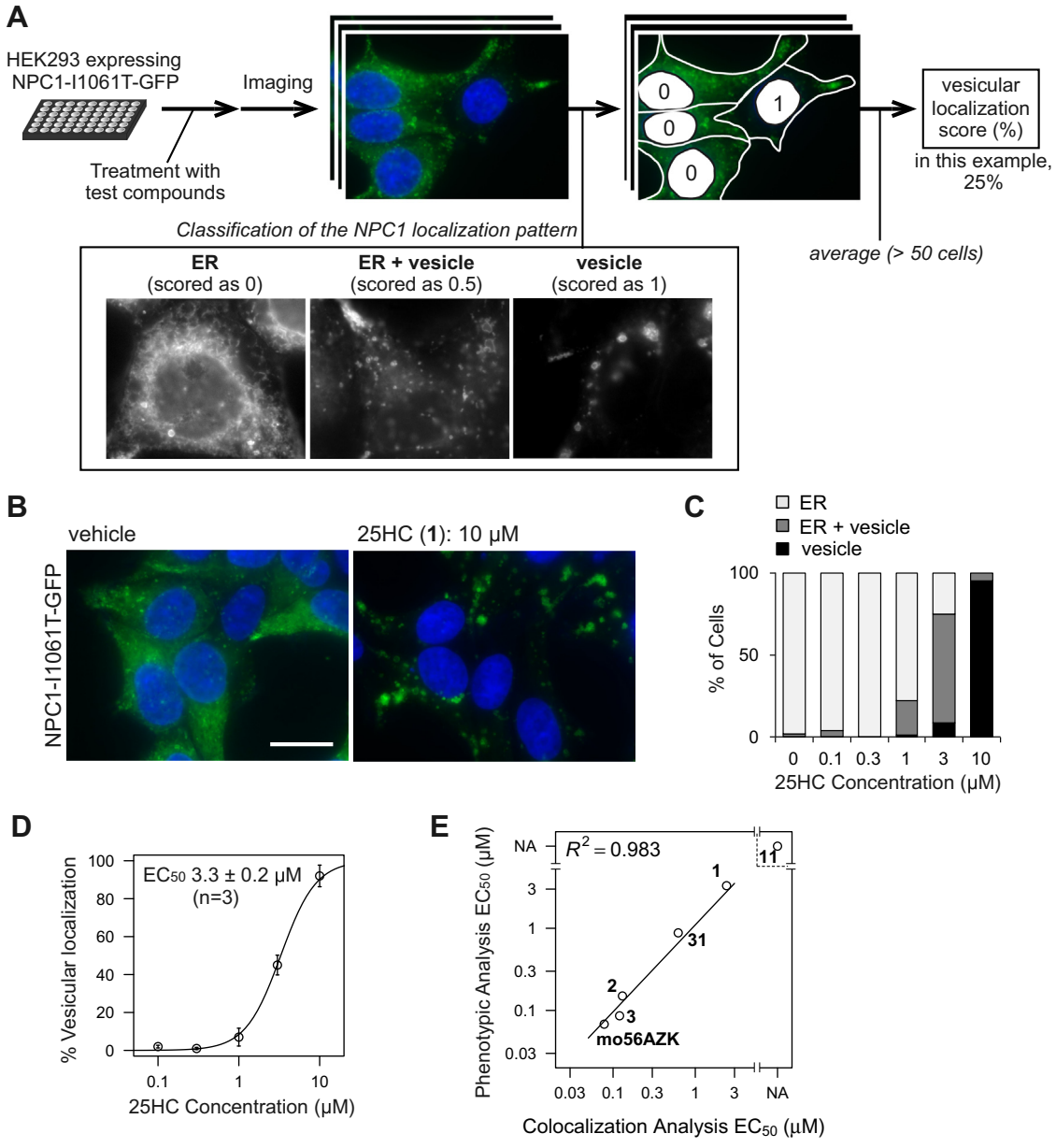


Figure 2. Phenotypic analysis of pharmacological chaperone-mediated correction of NPC1^{I1061T} mislocalization. (A) A schematic diagram illustrating phenotypic assay procedure. Cells stably expressing NPC1^{I1061T}-tGFP were treated with test compounds for 24 h, and after fixation and staining with Hoechst 33342 (a nuclear stain), images were acquired. Each cell in the images was classified into three categories, ER, ER+vesicle, and vesicle, and score (0, 0.5, and 1, respectively) was assigned to each cell. By averaging the scores over more than 50 cells per condition, we obtained 'vesicular localization score (%)', which should largely correspond to the percentage of correctly localized NPC1^{I1061T}-tGFP protein. Representative images of cells with ER, ER+vesicle, and vesicular localization of NPC1-tGFP are also shown in the lower panel. (B) Representative images showing localization of NPC1^{I1061T}-tGFP with or without 10 μM 25HC (**1**) treatment. Scale bar, 20 μm. (C) Representative result of a single phenotypic assay showing the dose-dependent correction of NPC1 mislocalization by 25HC. (D) Determination of EC_{50} value for 25HC (**1**). The EC_{50} value represents mean ± standard deviation (three independent experiments). (E) Correlation between EC_{50} values obtained from colocalization assay[24] and those from phenotypic assay.

categories, ER (scored as 0), mixed localization (scored as 0.5), and vesicular localization (scored as 1), and the percentage of cells with vesicular localization was obtained by averaging the score. By means of this procedure, EC_{50} of 25HC was estimated as 3.3 μM

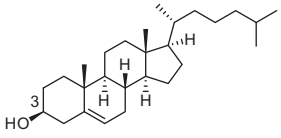
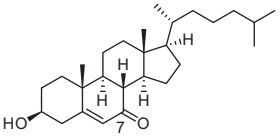
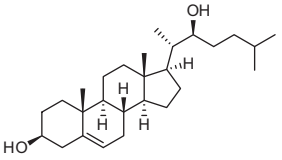
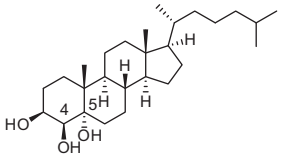
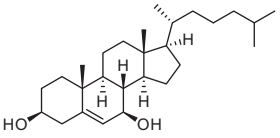
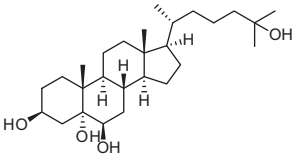
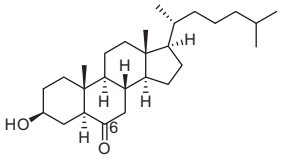
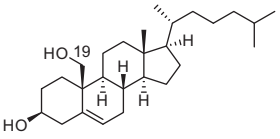
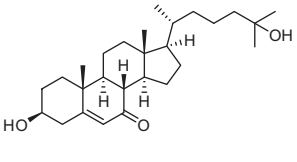
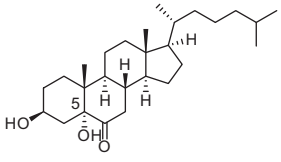
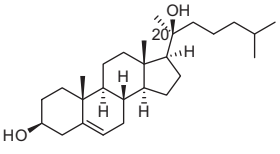
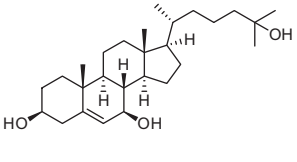
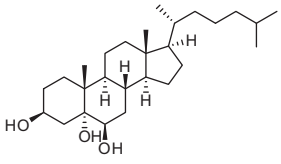
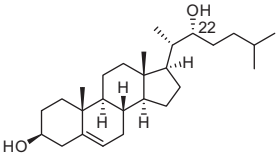
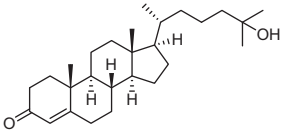
(Fig. 2B–D), which is in good agreement with the EC_{50} value obtained from colocalization analysis (2.4 μM). The localization change of NPC1 mutant was very clear, and this scoring procedure gave similar results even when conducted in a double-blinded manner. We also obtained EC_{50} values of representative oxysterol derivatives described in our previous report, and confirmed that the EC_{50} values obtained from our previous colocalization assay[24] and the present phenotypic assay were well correlated (Fig. 2E). The phenotypic assay could detect a 2-fold difference in EC_{50} values with confidence, and the throughput and assay quality were sufficient for structure–activity relationship study.

After the discovery of 25HC as a pharmacological chaperone for NPC1 mutant,[24] we first examined whether other hydroxylated or oxidized sterols could also act as pharmacological chaperones. Cholesterol (**4**) was found to be inactive under our conditions (up to 10 μM, ethanol or DMSO as co-solvent), probably because of its extremely low solubility in water.[28–30] Although hydroxylation of the A- or B-ring of the steroidal core was well tolerated (**5**–**10**), 19-hydroxycholesterol (**11**), bearing a hydroxyl group on the methyl group between the A- and B-rings, was totally inactive. As for hydroxylation of the isooctyl side chain, 20(*S*)-hydroxylated sterol (**12**) had no activity. Interestingly, 22(*S*)-hydroxycholesterol (**14**) was more potent than the 22(*R*)-hydroxylated counterpart (**13**), implying the presence of unfavorable interaction between the 22(*R*)-hydroxyl group and NPC1 protein. The combination of 25-hydroxylation and B-ring hydroxylation/oxidation resulted in slightly more potent derivatives (**15**–**17**) than 25HC, but their activity did not exceed that of 5α,6β-hydroxylated sterol (**8**). 3-Keto derivative of 25HC retained comparable activity to 25HC, indicating that the presence of a hydrogen bond donor at this position is not required. The structure–activity relationships were different from those observed for other oxysterol-binding proteins, including liver-X receptor,[31,32] Smoothened,[33] EBI2,[34,35] and Insig;[36] for example, while 20(*S*)-hydroxycholesterol activates the Hedgehog pathway by acting as a potent agonist for Smoothened protein, this sterol is not active toward NPC1.

As the position and stereochemistry of a hydroxyl group on the side chain moiety greatly affected pharmacological chaperone potency, we further examined structure–activity relationships of the isooctyl side chain moiety (Table 2). Sterols without the side chain (**19** and **20**) showed significantly diminished activity, indicating the importance of the side chain moiety for interaction with NPC1 protein. Comparison of **19** and **20** indicated that a hydroxyl group at C-17 is disfavored. This may imply that the binding pocket around C-17 has a hydrophobic character, and this is consistent with the fact that hydroxylation at C-20 (adjacent to C-17) (**12**) was not tolerated (Table 1). While sterols with a one-carbon shorter side chain than 25HC (**21**–**23**) were inactive, the *N*,*N*-dimethylamide derivative (**24**), which bears a dimethyl group at the position corresponding to the dimethyl group of 25HC, was as potent as 25HC. *N*,*N*-Diethylamide (**25**) was slightly more potent, but larger *N*,*N*-dibutylamide (**26**) lacked activity, implying that the binding pocket on NPC1 surrounding this part of the sterol is relatively hydrophobic but of limited size.

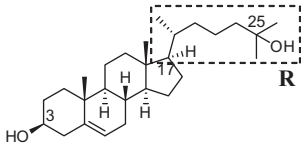
Next, we examined the effect of a 3-OH substituent on pharmacological chaperone activity. Although methylation of the

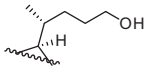
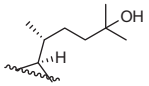
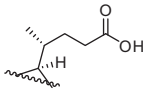
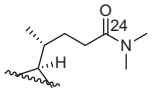
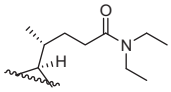
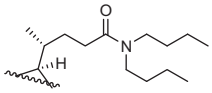
Table 1
Structure–activity relationships of oxysterols as pharmacological chaperones for $NPC1^{I1061T}$ mutant

No.	Compound	EC_{50}[a] (μM)	No.	Compound	EC_{50}[a] (μM)	No.	Compound	EC_{50}[a] (μM)
4		NA	**9**		8.7	**14**		2.9
5		4.3	**10**		3.4	**15**		1.7
6		3.4	**11**		NA	**16**		1.5
7		1.7	**12**		NA	**17**		1.9
8		1.7	**13**		NA	**18**		4.0

[a] HEK293 cells stably expressing $NPC1^{I1061T}$-tGFP were treated with the indicated compound. After 24 h, localization of $NPC1^{I1061T}$-tGFP was examined and localization change was quantified by phenotypic analysis (see Supplemental information for experimental procedures). Each EC_{50} value represents a single experiment, or an average of two or three independent assays. NA, no activity at 10 μM.

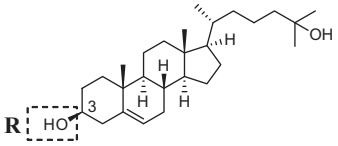
Table 2
Effect of side-chain structure on pharmacological chaperone efficacy for NPC1^{I1061T}

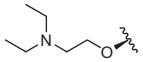
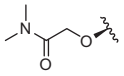
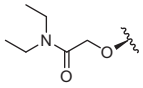
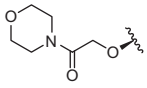


No.	R	EC_{50}[a] (μM)
19		>10
20		NA
21		NA
22		NA
23		NA
24		3.1
25		1.7
26		NA

[a] See Supplemental information for experimental detail of the phenotypic assay. Each EC_{50} value represents a single experiment, or an average of two or three independent assays. NA, no activity at 10 μM. >10, slightly active at 10 μM but EC_{50} could not be determined due to low solubility at higher concentration.

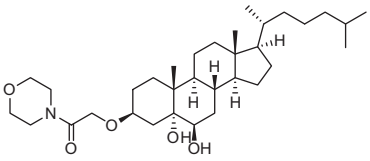
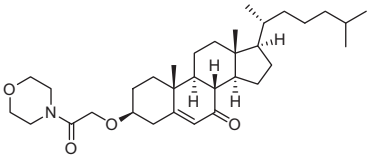
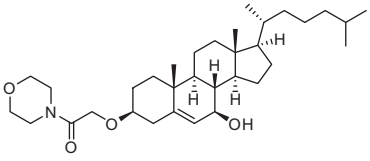
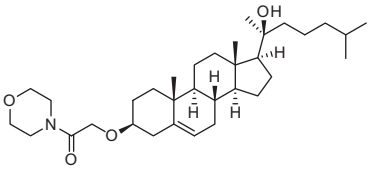
Table 3
Effect of 3-hydroxyl substituent on pharmacological chaperone activity for NPC1^{I1061T} mutant



No.	R	EC_{50}[a] (μM)
27		NA
28		9.9
29		1.4
30		4.8
31		0.88

[a] See Supplemental information for experimental detail of the phenotypic assay. Each EC_{50} value represents a single experiment, or an average of two or three independent assays. NA, no activity at 10 μM.

Table 4
Pharmacological chaperone activity of oxysterol derivatives with morpholine amide at the 3-position

No.	Compound	EC_{50}[a] (μM)
2		0.15
32		0.29
33		0.36
34		NA

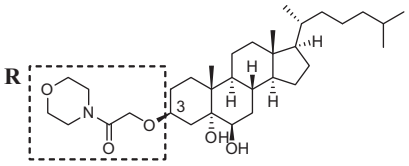
[a] See Supplemental information for experimental detail of the phenotypic assay. Each EC_{50} value represents a single experiment, or an average of two or three independent assays. NA, no activity at 10 μM.

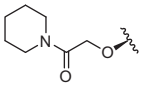
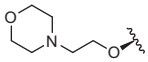
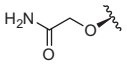
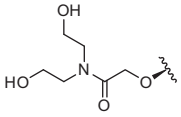
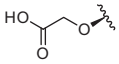
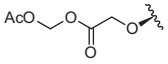
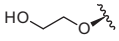
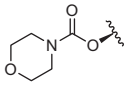
3-OH (**27**) resulted in a significant decrease of activity, derivatives with more polar substituents retained the activity (Table 3). In the amide-containing series, clear preference for a polar group was observed; the EC_{50} values were in the order of diethylamide (**30**) > dimethylamide (**29**) > morpholine amide (**31**), and **31** showed a 4-fold increase in activity compared with the parental oxysterol, 25HC. Removal of amide carbonyl resulted in a two-fold decrease in activity (compare **28** and **30**), implying the importance of the carbonyl oxygen for interaction with the NPC1 protein.

As the morpholine amide group was found to be optimal as a 3-OH substituent, we introduced this substituent into several B-ring oxidized sterols and 20(*S*)-hydroxycholesterol (**12**). For all of the B-ring oxidized derivatives tested (Table 4), introduction of the morpholine amide substituent on 3-OH enhanced the activity. Furthermore, consistent with the results obtained for sterols with an unsubstituted 3-OH group (Table 1), the 5α,6β-hydroxylated derivative (**2**) was more potent than the 25-hydroxylated derivative (**31** in Table 3), 7-keto derivative (**32**) or 7-hydroxylated derivative (**33**). 20(*S*)-Hydroxylated derivative (**34**) was totally inactive, which is consistent with the detrimental effect of 20(*S*)-hydroxylation on the activity (see **12** in Table 1).

Selecting the most potent derivative **2** (Table 4) as a starting point for the next round of optimization, we examined the structure–activity relationships of 3-OH substituents in more detail (Table 5). Substitution of the oxygen with CH_2 on the morpholine ring (**35**) resulted in one order of magnitude lower potency, implying the importance of the morpholine oxygen for interaction with NPC1 protein. As with **28** and **30** in Table 3, the importance of the carbonyl oxygen was recapitulated (compare **31** in Table 3 and **36** in Table 5). Relatively polar groups (**37**, **38**, and **41**; Table 5), including terminal amide (**37**), were tolerated, but free carboxylic acid or its protected form (**37** and **38**) were not tolerated at all. The morpholine carbamate (**42**), which is less polar than morpholine amide **31**, was only weakly active, suggesting the importance

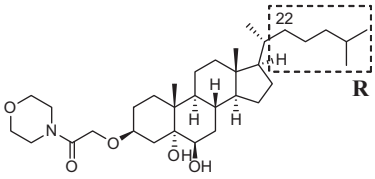
Table 5
Detailed structure–activity relationships of the 3-OH moiety

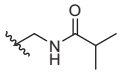
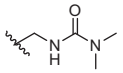
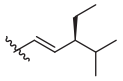
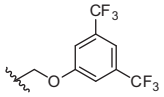
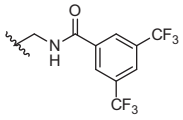


No.	R	EC_{50}[a] (μM)
35		1.8
36		1.0
37		0.84
38		0.56
39		NA
40		NA
41		3.4
42		6.1

[a] See Supplemental information for experimental detail of the phenotypic assay. Each EC_{50} value represents a single experiment, or an average of two or three independent assays. NA, no activity at 10 μM.

Table 6
Structure–activity relationship of the side chain moiety



No.	R	EC_{50}[a] (μM)
43		NA
44		0.37
45		6.8
46		0.36
47		>10
3		0.086

[a] See Supplemental information for experimental detail of the phenotypic assay. Each EC_{50} value represents a single experiment, or an average of two or three independent assays. NA, no activity at 10 μM. >10, slightly active at 10 μM.

of appropriate positioning of the morpholine ring and carbonyl oxygen. Overall, morpholine amide was confirmed to be the best substituent.

With the morpholine amide substitution and 5α,6β-hydroxylation fixed, we conducted a second round of optimization of the side chain moiety. The previous examination (Table 2) suggested the importance of a dimethyl group, which corresponds to the C-26 and C-27 methyl groups of 25HC, and preference for a hydrophobic group at this position. Therefore, in this round of optimization, we tested derivatives with a dimethyl group at the corresponding position (**44**–**46**) and more hydrophobic derivatives (**46**, **47**, and **3**) (Table 6). As in the case of 3-OH unsubstituted derivatives (Table 2), a derivative with a truncated side chain (**43**) was inactive, supporting the importance of this side chain moiety. Although a derivative with an amide-containing side chain (**44**) retained activity, incorporation of urea structure (**45**) resulted in marked loss of activity. 24-Ethyl-substituted derivative (**46**) also retained activity, implying that the binding pocket can accommodate a slightly larger substituent. So, we next tested derivatives with a larger and more hydrophobic side chain (**47** and **3**). While the ether-linked derivative (**47**) was almost inactive, the amide-linked derivative was found to be slightly more active than the parental derivative with an isooctyl side chain (**2** in Table 4).

In summary, we have examined structure–activity relationships of oxysterol derivatives as pharmacological chaperones for $NPC1^{I1061T}$ mutant. As a result, we obtained a nearly 40-fold more potent derivative **3**, starting from 25HC (**1**). Although it might be possible to obtain even more potent compounds through more extensive optimization, it seems preferable to search for non-steroidal pharmacological chaperones, as sterol-based structures might not possess sufficient metabolic stability for potential clinical application. Screening and optimization studies of a series of non-steroidal pharmacological chaperones are in progress, and the results will be reported in due course. In addition to this de novo drug discovery approach, a drug-repurposing approach (i.e., finding pharmacological chaperones among already-approved drugs) would be of great interest, as such compounds have the potential to be rapidly translated into the clinical setting.[37]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.05.064.

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