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Human ACAT inhibitory effects of shikonin derivatives from *Lithospermum erythrorhizon*

Sojin An,[a,c] Yong-Dae Park,[a,b] Young-Ki Paik,[c] Tae-Sook Jeong[a,*] and Woo Song Lee[a,b,*]

[a]*National Research Laboratory of Lipid Metabolism and Atherosclerosis, KRIBB, Daejeon 305-806, Republic of Korea*
[b]*Molecular Science, University of Science and Technology, Daejeon 305-806, Republic of Korea*
[c]*Department of Biochemistry, Yonsei University, Seoul 131-749, Republic of Korea*

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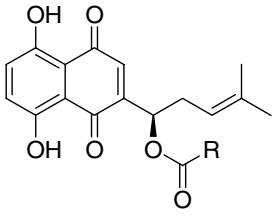
Abstract—Three naphthoquinones were isolated by bioassay-guided fractionation from the $CHCl_3$ extracts of roots of *Lithospermum erythrorhizon*. They were identified as acetylshikonin (**1**), isobutyrylshikonin (**2**), and β-hydroxyisovalerylshikonin (**3**) on the basis of their spectroscopic analyses. The compounds **1**–**3** were tested for their inhibitory activities against human ACAT-1 (hACAT-1) or human ACAT-2 (hACAT-2). Compound **2** preferentially inhibited hACAT-2 (IC_{50} = 57.5 μM) than hACAT-1 (32% at 120 μM), whereas compounds **1** and **3** showed weak inhibitory activities in both hACAT-1 and -2. To develop more potent hACAT inhibitor, shikonin derivatives (**5**–**11**) were synthesized by semi-synthesis of shikonin (**4**), which was prepared by hydrolysis of **1**–**3**. Among them, compounds **5** and **7** exhibited the strong inhibitory activities against hACAT-1 and -2. Furthermore, we demonstrated that compound **7** behaved as a potent ACAT inhibitor in not only in vitro assay system but also cell-based assay system.

Acyl-CoA: Cholesterol acyltransferase (ACAT, E.C.2.3.1.26) is the primary enzyme responsible for the intracellular esterification of free cholesterol with fatty acyl-CoA to produce cholesterol esters in various tissues.[1] Accumulation of cholesteryl ester leads to the formation of foam cells and a hallmark of atherosclerosis lesions. Two isoenzymes, ACAT-1 and -2, have been identified.[2] Under normal physiological conditions, ACAT-1 is present in many tissues, including macrophages, and ACAT-2 is present in intestinal epithelial cells and hepatocytes.[3] Inhibition of ACAT was expected to reduce plasma lipid levels by inhibiting intestinal cholesterol absorption and to prevent progression of atherosclerotic lesions by inhibiting the accumulation of cholesteryl esters in macrophage.[4] However, the known ACAT inhibitors[5] have no beneficial effects on the regression of coronary atherosclerosis.[6] Nissen et al. have drawn a bold conclusion that nonselective ACAT inhibition is not effective for treatment of atherosclerosis and is probably harmful.[6] Thus, ACAT inhibitors with different preferences on ACAT-1 and -2 may exert more favorable effects on atherosclerosis.

During search for new ACAT inhibitors from natural sources,[7] we found that the $CHCl_3$ extracts of *Lithospermum erythrorhizon* roots at 100 μg/mL inhibited hACAT-1 and -2 activities in 87% and 62%, respectively. Subsequent bioactivity-guided fractionation of the $CHCl_3$ extracts led to the isolation of three shikonin derivatives[8] that were identified as acetylshikonin (**1**), isobutyrylshikonin (**2**), and β-hydroxyisovalerylshikonin (**3**) on the basis of their spectroscopic analyses (Fig. 1).[9] Although shikonin derivatives isolated from the extracts of *L. erythrorhizon* roots have been reported to confer many medicinal properties such as antibacterial, wound



1 R = CH_3
2 R = $CH(CH_3)_2$
3 R = $CH_2C(CH_3)_2OH$

Figure 1. Structures of isolated shikonin derivatives (**1**–**3**) from the roots of *L. erythrorhizon*.

Keywords: ACAT inhibitor; Shikonin derivatives.
* Corresponding authors. Tel.: +82 42 860 4558; fax: +82 42 861 2675 (T.S.J.); tel.: +82 42 860 4278; fax: +82 42 861 2675 (W.S.L.); e-mail addresses: tsjeong@kribb.re.kr; wslee@kribb.re.kr

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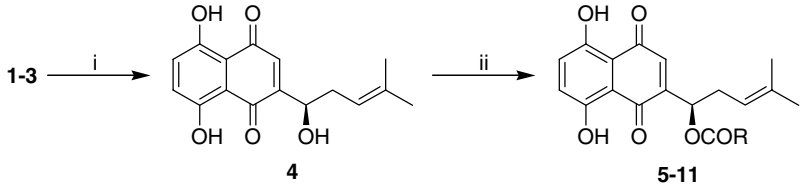
healing, antiinflammatory, antithrombotic, and antitumor effects,[10] their ACAT inhibitory activities have not been reported. In this study, we wish to describe the hACAT-1 and -2 inhibitory activities of the isolated and semi-synthetic shikonin derivatives (**1**–**11**).

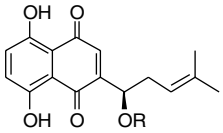
To investigate the best pharmacophore at 1′-OH of shikonin (**4**) against hACAT inhibitory activity, shikonin (**4**) was prepared by hydrolysis of compounds **1**–**3** with 1 N NaOH. Then, acylshikonins (**5**–**11**) were synthesized by a selective acylation at 1′-OH of shikonin (**4**) in the presence of *N*-(3-dimethylaminopropyl)-*N*′-ethyl carbodiimide (EDC) and 4-(dimethylamino) pyridine (DMAP) as shown in Scheme 1.

Three active compounds were isolated from $CHCl_3$ extracts and identified to be acetylshikonin (**1**), isobutyrylshikonin (**2**), and β-hydroxyisovalerylshikonin (**3**). The biological activities of shikonin derivatives **1**–**11** were assessed against expressed hACAT-1 or -2 from Hi5 cells[11] and confirmed by the positive control with oleic acid anilide which inhibited hACAT-1 and -2 with IC_{50} values of 0.14 and 0.17 μM,[11] respectively. The biological data for shikonin derivatives (**1**–**11**) have been shown in Table 1. Acetylshikonin (**1**) inhibited hACAT-1 and -2 with IC_{50} values of 128.9 and 112.2 μM, respectively. Isobutyrylshikonin (**2**) dominantly inhibited hACAT-2 not hACAT-1; the IC_{50} value for hACAT-2 was 57.5 μM, while hACAT-1 was inhibited only 32% at 120 μM. β-Hydroxyisovalerylshikonin (**3**) having 3-hydroxy-3-methylbutanoyl was less potent in both of enzymes. Shikonin (**4**) inhibited hACAT-1 with 2-fold greater potency (IC_{50} = 70.4 μM) compared to hACAT-2 (IC_{50} = 138.2 μM). Interestingly, compound **5** involving *n*-propanoyl exhibited higher activity against both hACAT-1 and -2 compared with the corresponding analogue **1** having acetyl moiety, whereas *n*-butanoyl-substituted analogue **6** showed an attenuated potency. The compound **7** substituted with 3-methylbutanoyl showed potent inhibitory activity with IC_{50} values of 13.8 and 25.1 μM, respectively. These results clearly demonstrated that simple extension of carbon chain without any polarity change is enough to significantly enhance activity. Substitution of *n*-pentanoyl, **8**, at this position, however, led to decrease in potency. Keeping the three or four acyl chain lengths indicated that perhaps specific lipophilic interaction between enzymes and acyl chains at 1′-OH of shikonin (**4**) was possible. To investigate the lipophilicity, compound **9** was examined for inhibitory potency. It was equipotent to **8** in hACAT-2, whereas compound **9** having more lipophilic group led to 3-fold increase in potency in hACAT-1. Compound **10** having linoleoyl as the long acyl residue resulted in a significant loss in potency. In addition, **11** introduced with benzoyl group showed a slight increase in potency in hACAT-1, but the potency was increased



Scheme 1. Reagents: (i) 1 N NaOH (1.2 equiv), MeOH; (ii) EDC (1.1 equiv), DMAP (0.2 equiv), RCOOH (1.1 equiv), CH_2Cl_2.

Table 1. ACAT inhibitory activities of shikonin derivatives (**1**–**11**) in in vitro assay system



Compound	R	Yield (%)	IC_{50}[a] (μM)	
			hACAT-1	hACAT-2
1	Acetyl	—	128.9	112.2
2	Isobutanoyl	—	32%[b]	57.5
3	3-Hydroxy-3-methylbutanoyl	—	186.9	169.8
4	H	71	70.4	138.2
5	*n*-Propanoyl	67	22.4	28.7
6	*n*-Butanoyl	59	77.6	49.6
7	3-Methylbutanoyl	57	13.8	25.1
8	*n*-Pentanoyl	62	94.8	84.5
9	4-Pentenoyl	53	30.7	71.4
10	Linoleoyl	70	5%[b]	4%[b]
11	Benzoyl	28	53.7	56.2

[a] In vitro ACAT inhibitory activity was measured using expressed human ACAT-1 and -2. Data were shown as mean values of two independent experiments performed in duplicate.

[b] Percentage at 120 μM.

approximately 2-fold in hACAT-2 with IC_{50} of 56.2 μM as compared to shikonin (**4**). To investigate that these compounds function as ACAT inhibitor in physiological condition, we established stable cell line expressing hACAT-1 or -2 from AC-29 cells, ACAT-deleted CHO cells, using Flp-In™ system.[12] The expression of hACAT-1 and -2 was confirmed by immunoblot analysis (Fig. 2) and fluorescence microscopy using NBD cholesterol (Fig. 3). Among compounds **1**–**11**, the most potent ACAT inhibitor, compound **7**, was tested in cell–base-fluorescent ACAT assay system.[13,14] As shown in Figure 4, compound **7** inhibited significantly the enzymatic activities of both hACAT-1 and -2 in cell-based assay system with even lower IC_{50} values (IC_{50} = 0.6 μM for hACAT-1 and IC_{50} = 0.8 μM for hACAT-2) than that of in vitro assay system. However, compound **7** exhibited no selectivity against hACAT isoforms in both assay systems, showing similar fold difference of IC_{50} value between hACAT-1 and -2 (1.8-fold for in vitro assay and 1.3-fold for cell-based assay).

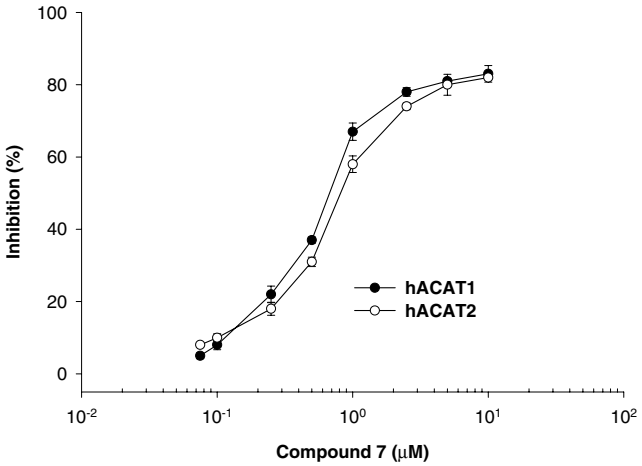


Figure 4. hACAT inhibitory activity of compound **7** in cell-based fluorescent assay system.

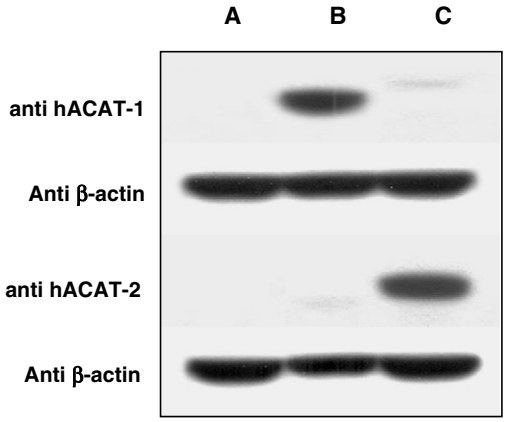


Figure 2. Human ACAT-1 or -2 expression in Flp-In AC29 cells. The expression of hACAT-1 or 2 was identified in cell lysates isolated from Flp-In AC29 cells (A) and Flp-In AC29 cells stably expressing hACAT-1 (B) or hACAT-2 (C) using immunoblot analysis. The intensity of anti-β-actin band was used as an internal control to compensate loading error.

According to these results, it was found that the inhibitory potency was strongly dependent on the size and length of acyl group. The chain length of acyl group for the maximal inhibition of hACAT was found to be three or four carbon atoms. These data suggest that there may be a size limitation of acyl moiety for the effective interaction of acylshikonins with hACAT and human microsomal ACAT-1 and -2 enzymes have proven to be very sensitive to the property of compounds. Previous studies have demonstrated that the selective inhibition of ACAT-1 and -2 provides evidence for uniqueness in structure and function of these two enzymes.[11,15] Chang et al. proposed that the putative ACAT catalytic domains, which includes His-460 for hACAT-1[16a] and His-434 for ACAT-2,[16b] are located within a hydrophobic membrane, enabling the enzyme to produce cholesteryl ester in the same membrane. In

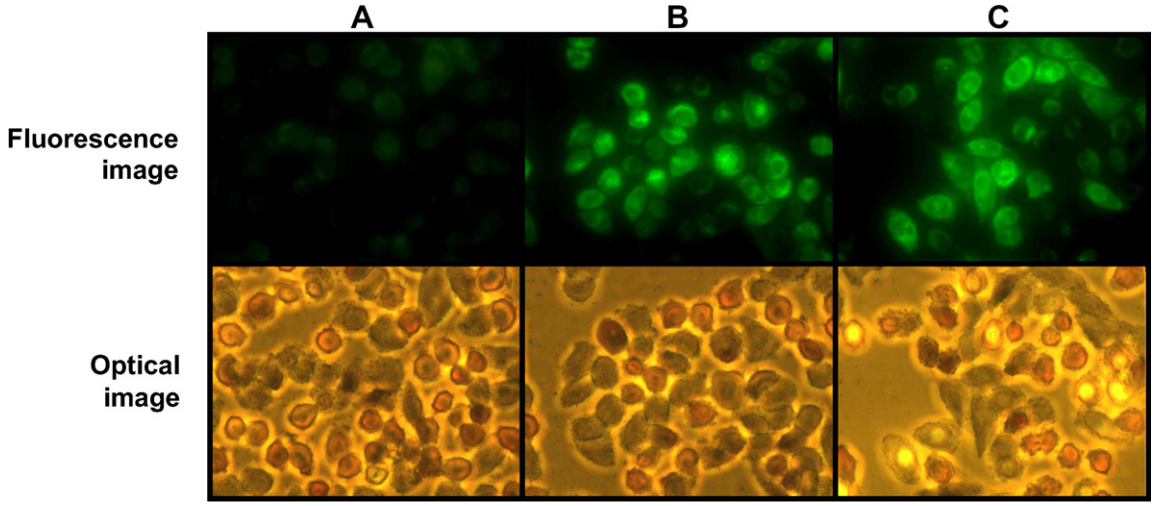


Figure 3. Fluorescence microscopy after incubation of cells with NBD-cholesterol. Flp-In AC29 cells (A) and Flp-In AC29 cells stably transfected with hACAT-1 (B) or hACAT-2 (C) were incubated with 5 μg/mL NBD-cholesterol for 3 h and fixed with 2% paraformaldehyde. The fluorescence images were viewed with green channel filters (488 nm excitation, 540 nm emission), and their corresponding optical images were shown below each fluorescence image. The results shown are representative of two separate experiments. Magnification 400×.

this context, different inhibitory potency of shikonin derivatives against hACAT-1 and -2 may have resulted from the degree of lipophilicity provided by acyl chain and, providing optimal lipophilicity, three to four carbon length of acyl chain could increase a chance to interact with catalytic site of hACAT-1 and -2 located in the hydrophobic membrane. Recently, An et al.[17] revealed that His-386, another histidine residue imbedded in a hydrophobic membrane, has the critical role for catalysis of hACAT-1, whereas His-360 and His-399, cytoplasmic histidine residues, are essential for catalysis of hACAT-2 suggesting catalysis in the plane of membrane is limited in ACAT-1 and that catalysis of ACAT-2 takes place in the cytoplasmic side as well as hydrophobic membrane. We discovered that compound **2** containing isopropyl moiety preferentially inhibited hACAT-2 than hACAT-1, whereas compound **5**, equivalent compound with compound **2** except containing *n*-propyl moiety instead of isopropyl moiety, considerably inhibited both hACAT-1 and -2. Thus, we could speculate that this isopropyl moiety of shikonin derivatives might specifically interact with catalytic site of hACAT-2 located in the cytoplasmic side.

In summary, we have discovered a new class of hACAT inhibitors. Shikonin derivatives (**1**–**3**) were isolated by bioassay-guided fractionation from the $CHCl_3$ extracts of *L. erythrorhizon* roots. The compounds **5**–**11** were modified using semi-synthesis of shikonin (**4**). Among them, compound **7** exhibited the most potent hACAT-1 and -2 inhibitory activities with IC_{50} values of 13.8 and 25.1 μM, respectively. Notably, we confirmed the ACAT inhibition capacity of compound **7** in cell-based assay system, newly established in our laboratory via construction of stable cell line expressing hACAT-1 or -2. Further structure–activity relationship (SAR) studies are being pursued to find more potent and selective inhibitors. Although the negative effects of nonselective ACAT inhibitors have been reported, hACAT-1 or -2 selective inhibitor may prove to have clinical benefit to reduce atherosclerosis via directly reducing the size of the lipid-rich core in the atherosclerotic plaques[18] or the absorption of cholesterol in intestine,[19] respectively. Thus, it is important to continue the search for ACAT isotype-selective inhibitors.

Acknowledgment

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8. The dried roots of *L. erythrorhizon* (500 g) were cut into small pieces and extracted with $CHCl_3$ (2 L) at room temperature for 3 days. After filtration, the solution was evaporated to remove $CHCl_3$. The extract (11 g) showed the inhibitory activity against hACAT-1 and -2 (87% and 62% inhibition at 100 μg/mL), and was chromatographed on a silica gel (230–400 mesh, Merck, Ø50 × 150 mm), eluting with *n*-hexane/EtOAc (10:1, 5:1, 1:1, and EtOAc), to yield eight fractions. Each fraction was monitored by in vitro ACAT activity assay. Purification of fraction 2 was carried out on Sephadex LH-20 column eluting with MeOH to yield five subfractions. Subfraction 1 was further purified by silica gel (230–400 mesh, Merck, Ø50 × 150 mm) chromatography, eluted with *n*-hexane/EtOAc (10:1) to yield **1** (50 mg). Active fraction 1 was separated by RP C_{18} (40–63 mesh, Merck, Ø30 × 100 mm) column chromatography, eluted with MeOH/H_2O (9:1) to yield **2** (30 mg). Fraction 4 was purified by chromatography on silica gel (230–400 mesh, Merck, Ø50 × 150 mm), eluted with *n*-hexane/EtOAc (5:1) to yield **3** (70 mg).
9. Physical and spectroscopic data: compound **1**: $C_{18}H_{18}O_6$; HREI-MS *m/z*: 330.1103 (calcd for $C_{18}H_{18}O_6$ $[M]^+$: 330.1104); ^1H NMR ($CDCl_3$, 300 MHz): δ 12.57 (1H, s), 12.41 (1H, s), 7.18 (2H, s), 6.98 (1H, s), 6.02 (1H, dd, J = 6.9, 4.2 Hz), 5.12 (1H, t, J = 7.2 Hz), 2.62 (1H, m), 2.46 (1H, m), 2.14 (3H, s), 1.69 (3H, s), 1.57 (3H, s); ^{13}C NMR ($CDCl_3$, 300 MHz): δ 178.2, 176.7, 169.7, 167.5, 166.9, 148.2, 136.1, 132.8, 132.7, 131.4, 117.6, 111.8, 111.5, 69.5, 32.8, 25.7, 20.9, 17.9. Compound **2**: $C_{20}H_{22}O_6$; HREI-MS *m/z*: 358.1416 (calcd for $C_{20}H_{22}O_6$ $[M]^+$: 358.1418), ^1H NMR ($CDCl_3$, 300 MHz): δ 12.58 (1H, s), 12.42 (1H, s), 7.18 (2H, s), 6.97 (1H, s), 6.02 (1H, dd, J = 7.5, 4.2 Hz), 5.12 (1H, t, J = 7.2 Hz), 2.63 (2H, m), 2.48 (1H, m), 1.69 (3H, s), 1.58 (3H, s), 1.21 (6H, dd, J = 6.9, 3.0 Hz); ^{13}C NMR ($CDCl_3$, 300 MHz): δ 178.3, 176.8, 175.8, 167.4, 166.8, 148.6, 135.9, 132.8, 132.7, 131.3, 117.8, 111.8, 111.6, 69.0, 34.0, 32.9, 25.7, 18.9, 18.8, 17.9. Compound **3**: $C_{21}H_{24}O_7$; HREI-MS *m/z*: 388.1522 (calcd for $C_{21}H_{24}O_7$ $[M]^+$: 388.1521); ^1H NMR ($CDCl_3$, 300 MHz): δ 12.56 (1H, s), 12.37 (1H, s), 7.15 (2H, s), 7.01 (1H, s), 6.07 (1H, dd, J = 7.5, 4.2 Hz), 5.10 (1H, t, J = 7.2 Hz), 3.14 (1H, s), 2.62 (1H, m), 2.57 (2H, s), 2.48 (1H, m), 1.67 (3H, s), 1.57 (3H, s), 1.29 (6H, d, J = 4.2 Hz); ^{13}C NMR ($CDCl_3$, 300 MHz): δ 176.9, 175.4, 171.6, 168.5, 167.9, 147.4, 136.3, 133.2, 133.0, 131.3, 117.6, 111.7, 111.5, 69.7, 69.0, 46.4, 32.8, 29.2, 29.0, 25.7, 17.9.
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12. Generation of cell lines expressing hACAT-1 or hACAT-2: we generated stable cell line expressing human ACAT-1 or -2 (Accession No. BC028940 and AF059203 for ACAT-1 and ACAT-2, respectively) from AC-29 cells, ACAT-deleted CHO cells, using Flp-In™ system (Invitrogen) according to the manufacturer's suggestion. AC-29 cells were kindly gifted by Dr. Rudel, L. L.[15]. Briefly, Flp-In AC-29 cells containing five copies of integrated FRT sequence were generated by transfection of pFRT/lacZeo into AC-29 cells. FRT site-integrated clones were selected by 100 μg/mL zeocin (Invitrogen) for 1 week. Several colonies were picked and maintained in F-12 medium supplemented with 10% fetal bovine serum (FBS) and 100 μg/mL zeocin. The presence and number of FRT-site were verified by β-galactosidase assay and Southern blot analysis, respectively. To construct stable cell lines expressing hACAT-1 or hACAT-2, Flp-In AC-29 cells were plated on 60-mm dishes and transfected with 1.8 μg of the Flp-In recombinase-encoding pOG44 vector and either 0.2 μg of pcDNA5/FRT-hACAT-1 or pcDNA5/FRT-hACAT-2. After 2 days, we proceeded to select the single colony with 500 μg/mL hygromycin (Invitrogen) for 2 weeks. Colonies were picked up, expanded, and assayed for expression of hACAT-1 or -2. Established cell lines were maintained in F-12/10% FBS containing 100 μg/mL hygromycin.
13. Cell-based fluorescent ACAT assay:[15] a total of 30,000 cells per well were plated on 96-well culture plates and allowed to recover overnight. Assays were done with cells at least 80% confluent. Cells were incubated in Ham's F-12 medium supplemented with 1% Eagle's vitamins and 10% heat-inactivated FBS containing 5 μg/mL NBD-cholesterol as methyl-β-cyclodextrin complex with or without ACAT inhibitor for 9 h. NBD-cholesterol was added from a 5-mg/mL stock solution in methanol, and methanol concentrations in the medium did not exceed 0.1%. Flp-In AC-29 cells incubated with NBD-cholesterol were used to determine background fluorescence attributable to free NBD-cholesterol. After incubation, the medium was removed, and the cells were washed two times with cold balanced salt solution (BSS, Invitrogen). The fluorescent intensities of 96-well culture plates were read from the top using VICTOR3 fluorescent plate reader (Perkin-Elmer) equipped with 485 nm excitation and 535 nm emission filters. After measurement, cellular protein was digested through incubation with 25 μL of 0.4 N NaOH for 2 h. The fluorescent intensity was normalized by cellular protein content and calculated by subtracting the background fluorescent intensity from the total fluorescent intensity.
14. Methyl-β-cyclodextrin (MβCD)–NBD-cholesterol complex preparation:[20] 100 μM of MβCD was dissolved in 10 mL F-12 medium, and 6 μM NBD-cholesterol, and 10 μg/mL BSA were added to this solution with vigorous vortex. The dispersion was sonicated at room temperature for 5 min using a Fisher model 60 sonic dismembrator at setting 5. The solution was filtered with 0.45 μm membrane filter and kept in a glass tube under argon at 4 °C for up to a week.
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