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Supplementary Information

For

Design and Synthesis of Water-soluble Grifolin Prodrugs for DNA Methyltransferase 1 (DNMT1) Down-regulation

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Supplementary Figures

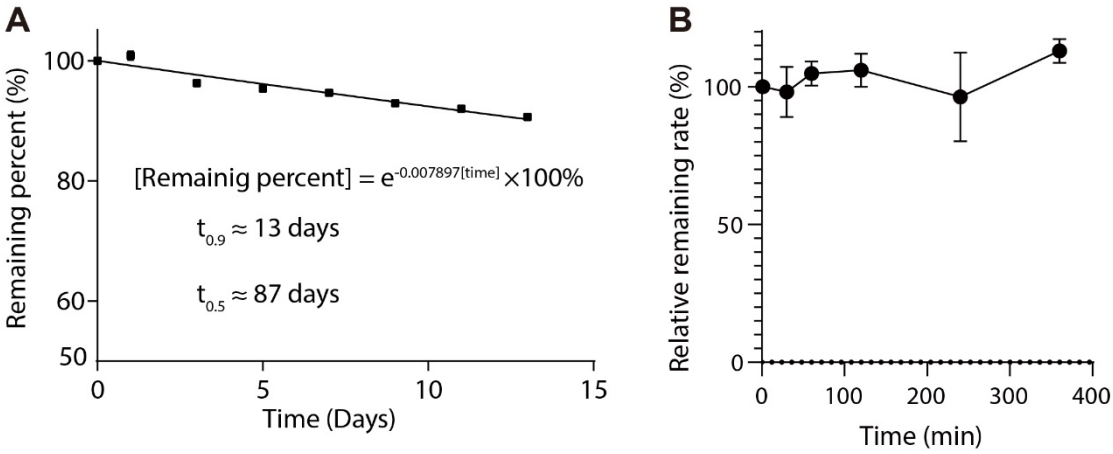


Fig. S1 Degradation rate of PEG5-Grifolin. (A) Degradation rate of PEG5-Grifolin in PBS at room temperature. PEG5-Grifolin was dissolved in PBS to make the 40 mM solution. The remaining percent was calculated as the ratio of remaining concentration of PEG5-Grifolin at 25℃ to the sample stored at -78℃ after the indicated time. Exponential curve prediction model was used. (B) Degradation rate of PEG5-Grifolin in mice plasma at room temperature. Sulfanilamide was used as internal standard substrate. Internal standard substrate and PEG5-Grifolin was dissolved in plasma. After incubating in plasma for indicated time, the plasma was injected into HPLC. (A-B)The data was measured by HPLC with three replicas.

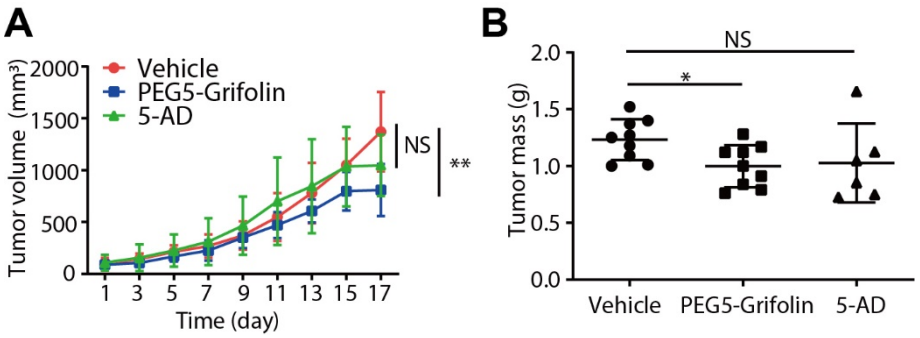


Fig. S2 PEG5-Grifolin hampers tumor growth *in vivo*. (A) Effect of PEG5-Grifolin on C666-1 NPC cell xenograft in athymic BALB/c nude mice. Nude mice bearing C666-1 cells were randomly separated into 3 groups (n = 8) and treated with PBS (vehicle), PEG5-Grifolin (90 mg/kg) or 5-Azacytidine (1 mg/kg) every other day for 17 days. Tumor volume was examined every other day and shown in the graph. (B) At the end of the experiment, the mice were sacrificed and the tumors were separated. Tumor mass of each group was weighed and shown in the graph. Data are shown

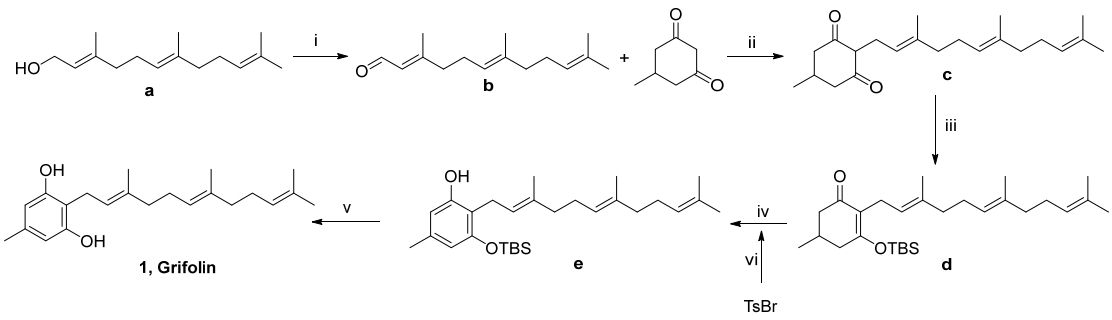
as mean values ± S.D. of independent, triplicate experiments. The asterisks (*,**) indicate significant differences ($p < 0.05$, $p < 0.01$, respectively). NS, no significance.

Table S1 The IC_{50} of PEG5-Grifolin, grifolin and 5-AD on viability of tumor cells and normal cells.

Cell line	PEG5-Grifolin (μM)	5-AD (μM)
Beas2b	34.6	7.9
293T	45.4	14.4
C666-1	17.3	-

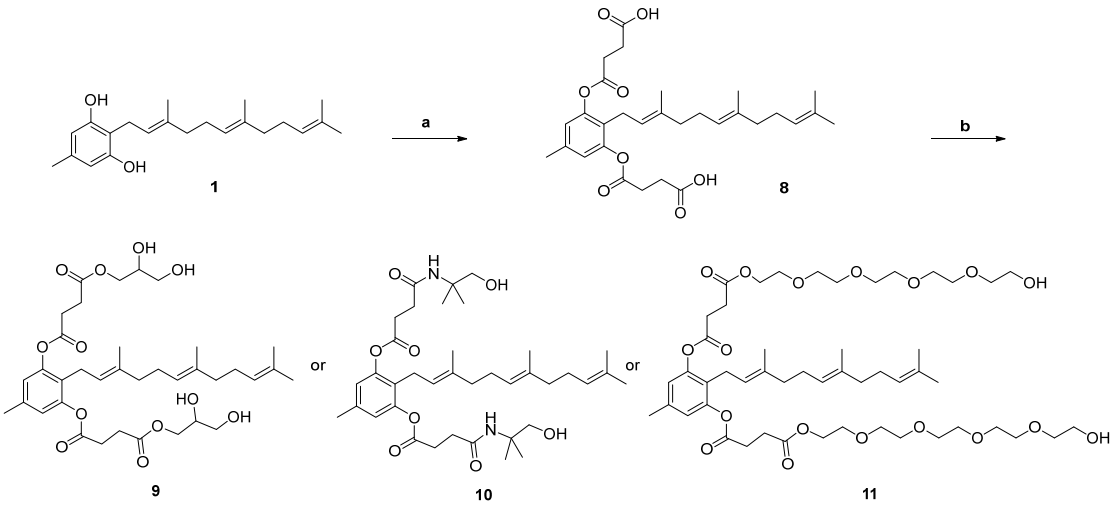
Materials and Methods

All commercial materials (Adamas-beta, Bidepharmatech, Energy Chemical, etc) were used without further purification. All solvents were analytical grade. The grifolin was prepared by the procedure reported by Justin T. Mohr in 2016[1]. The ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker AVANCE^{III}400 MHz spectrometer in $CDCl_3$ using solvent peak as a standard. All ^{13}C-NMR spectra were recorded with complete proton decoupling. Normal-resolution mass spectral analyses were performed with Waters AQUITY UPLC-MS/MS. High-resolution mass spectral analyses were performed with Waters Xevo G2 QTof. Analytical TLC was performed on Yantai Chemical Industry Research Institute silica gel 60 F254 plates and flash column chromatography was performed on Qingdao Haiyang Chemical Co. Ltd silica gel 60 (200-300mesh). 5-AD (Decitabine) was purchased from MedChemExpress. The reagents for genomic DNA extraction were purchased from QIAGEN.



Scheme S1 Synthetic route towards grifolin[1]

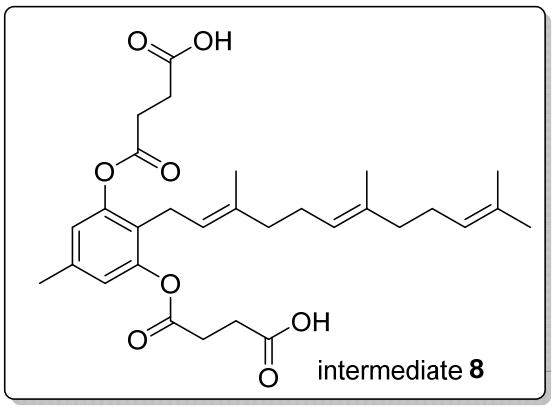
Synthesis and Data of Grifolin Prodrug



Scheme S2 Synthetic route of prodrugs. **a**) succinic anhydride, pyridine, DMAP, DCM, 12 h 51%; **b**) glycerol, pentaglycol or 2-amino-2-methylpropan-1-ol, EDCI, DMAP, THF, r.t., corresponding solvent, 43%~69%.

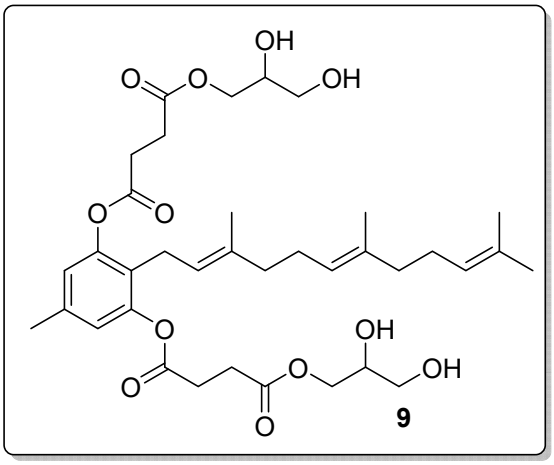
General Procedure

264 mg intermediate **8** (0.5 mmol, 1 equiv.) was dissolved in 20 ml solvent and reacted with 287 mg EDCI (1.5 mmol, 3 equiv.) under ice-bath. After stirred 10 minutes, the solution above was dropwise added into 10 ml solvent containing 6.0 equiv. corresponding substrate. Then the reaction was kept at room temperature for 12 h. After the TLC indicated the reaction finished, the solution was removed, then washed with saturated NaCl solution and extracted by ethyl acetate. After remove the solvent, the mixture was purified by silica-gel column to give the desired compound.



4,4'-((5-methyl-2-((2E,6E)-3,7,11-trimethyldodeca-2,6,10-trien-1-yl)-1,3-phenylene)bis(oxy))bis(4-oxobutanoic acid) (8)

To a 50 ml bottle, 328 mg grifolin was dissolved in 15 ml DCM. The bottle then was charged with nitrogen as the protective atmosphere. Next, 790 mg anhydrous pyridine was added. 1 g succinic anhydride was pre-dissolved in 10 ml DCM. Then succinic anhydride solution was added into grifolin solution by syringe in 2 h. The reaction was kept and stirred in room temperature for another 24 h. After the TLC indicated the reaction finished, the solution was removed, then washed with saturated NH_4Cl and extracted by ethyl acetate. Next, solvent was removed and puri-fied by silica-gel column (DCM: MeOH 100:1 to 20:1). Finally, 269 mg desired intermediate **8** was obtained, the isolate yield was 51%. Intermediate **8** was unstable and should be used for the next steps as soon as possible.



9

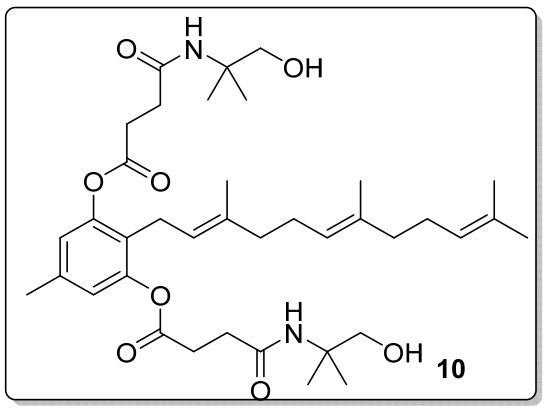
Bis(2,3-dihydroxypropyl) O,O'-(5-methyl-2-((2E,6E)-3,7,11-trimethyldodeca-2,6,10-trien-1-yl)-1,3-phenylene) disuccinate. (**9**)

SMILES code:

CC1=CC(OC(CCC(OCC(O)CO)=O)=O)=C(C/C=C(C)/CC/C=C(C)/CC/C=C(C)/C)C(OC(CCC(OCC(O)CO)=O)=O)=C1

Followed the general procedure, THF/Dioxane (1:1) was used as the solvent. Glycerol (3.0 mmol, 6 equiv.) was used as the corresponding substrate. Finally, 213 mg compound **9** was obtained with 63% isolated yield. ^{1}H-NMR (400MHz, $CDCl_3$, ppm): 6.76(s, 2H), 5.07(m, 3H) , 4.23-4.14(m, 4H), 3.89(t, J = 4.24, 2H), 3.64(dd, J = 2.92, J = 11.36, 2H), 3.55(dd, J = 5.76, J = 11.16, 2H), 3.11(d, J = 6.32, 2H), 2.90(t, J = 5.76, 4H), 2.74(m, 8H), 2.30(s, 3H), 2.05-1.93(m, 8H), 1.72(s, 3H), 1.67(s, 3H), 1.58(s, 3H), 1.57(s, 3H). ^{13}C-NMR (100MHz, $CDCl_3$, ppm): 172.50, 171.24, 149.36, 137.49, 136.09, 135.31, 131.45, 124.47, 124.05, 123.68, 121.20, 121.01, 70.13, 65.75, 63.32, 39.81, 39.72, 29.28, 29.10, 26.85, 26.68, 25.84, 23.70, 21.09, 17.82, 16.42, 16.15. LC-MS: calculated for

$C_{36}H_{53}O_{12}$ $[M+H]^+$: 677.35, found 677.45. HRMS: calculated for $C_{36}H_{51}O_{12}$ $[M-H]^-$: 675.3386, found 675.3389

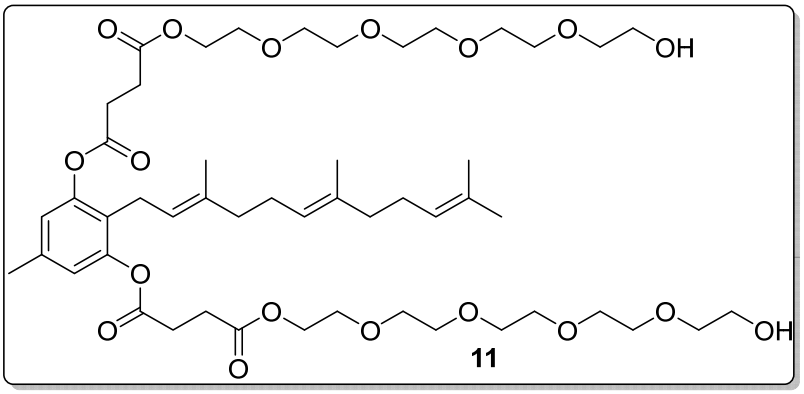


10

5-methyl-2-((2E,6E)-3,7,11-trimethyldodeca-2,6,10-trien-1-yl)-1,3-phenylene bis(4-((1-hydroxy-2-methylpropan-2-yl)amino)-4-oxobutanoate). (**10**)

SMILES code: CC1=CC(OC(CCC(NC(C)(C)CO)=O)=O)=C(C/C=C(C)/CC/C=C(C)/CC/C=C(C)/C)C(OC(CCC(NC(C)(C)CO)=O)=O)=C1

Followed the general procedure, DCM was used as the solvent. 2-amino-2-methylpropan-1-ol was used as the corresponding substrate. Finally, 230 mg compound **10** was obtained with 69% isolated yield. ^{1}H-NMR (400MHz, $CDCl_3$, ppm): 6.75(s, 2H), 5.75(s, 2H) , 5.07(m, 3H), 4.56(s, 2H),3.56(s, 4H), 3.11(d, J = 5.68, 2H), 2.90(t, J = 6.12, 4H), 2.51(m, 4H), 2.29(s, 3H), 2.01-1.93(m, 8H), 1.70(s, 3H), 1.66(s, 3H), 1.58(s, 3H), 1.57(s, 3H), 1.26(s, 12H). ^{13}C-NMR (100MHz, $CDCl_3$, ppm): 172.00, 171.60, 149.45, 137.36, 135.95, 135.24, 131.43, 124.46, 124.09, 123.58, 121.26, 120.95, 70.35, 56.38, 39.81, 39.75, 31.53, 29.82, 29.62, 26.85, 26.73, 25.84, 24.73, 23.69, 21.11, 17.82, 16.44, 16.16. LC-MS: calculated for $C_{38}H_{59}N_2O_8$ $[M+H]^+$: 671.43, found 671.33. HRMS: calculated for $C_{38}H_{57}N_2O_8$ $[M-H]^-$:669.4120, found 669.4124



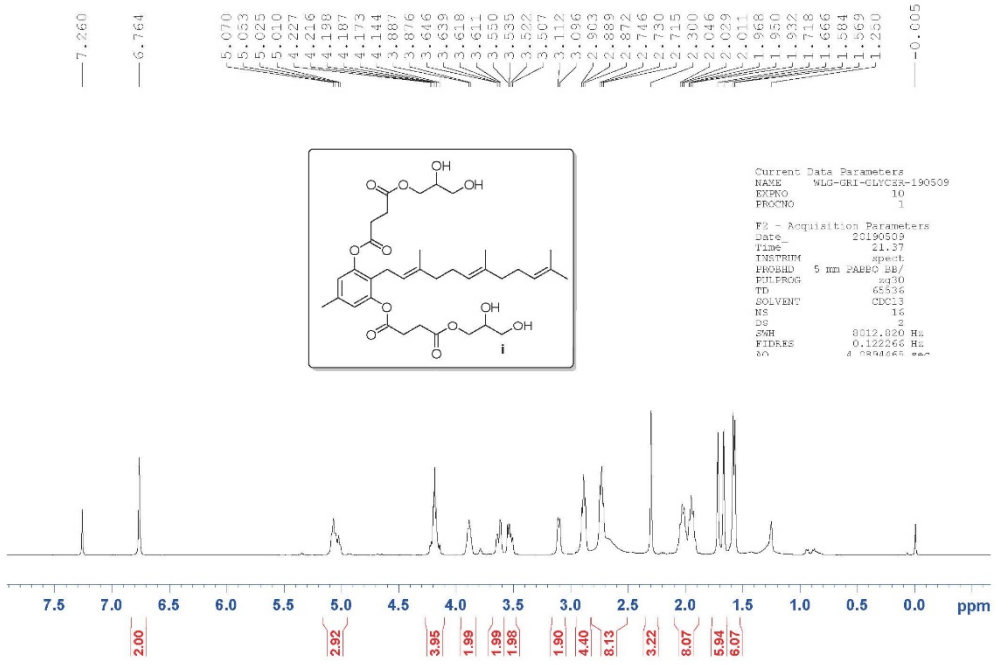
Bis(14-hydroxy-3,6,9,12-tetraoxatetradecyl) O,O'-(5-methyl-2-((2E,6E)-3,7,11-trimethyldodeca-2,6,10-trien-1-yl)-1,3-phenylene) disuccinate. (**PEG5-Grifolin, 11**)

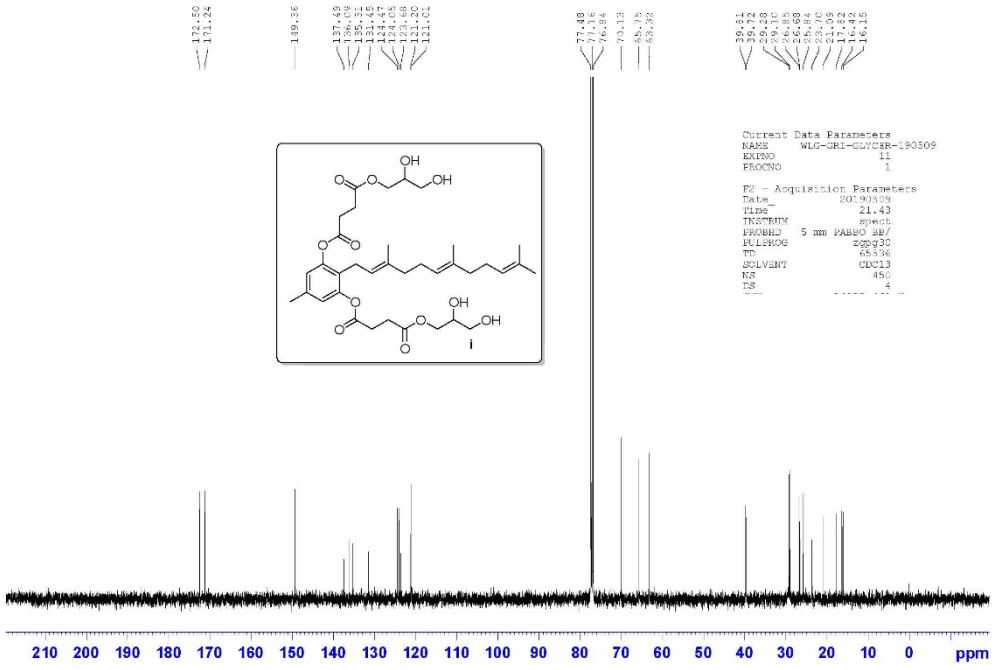
SMILES code:

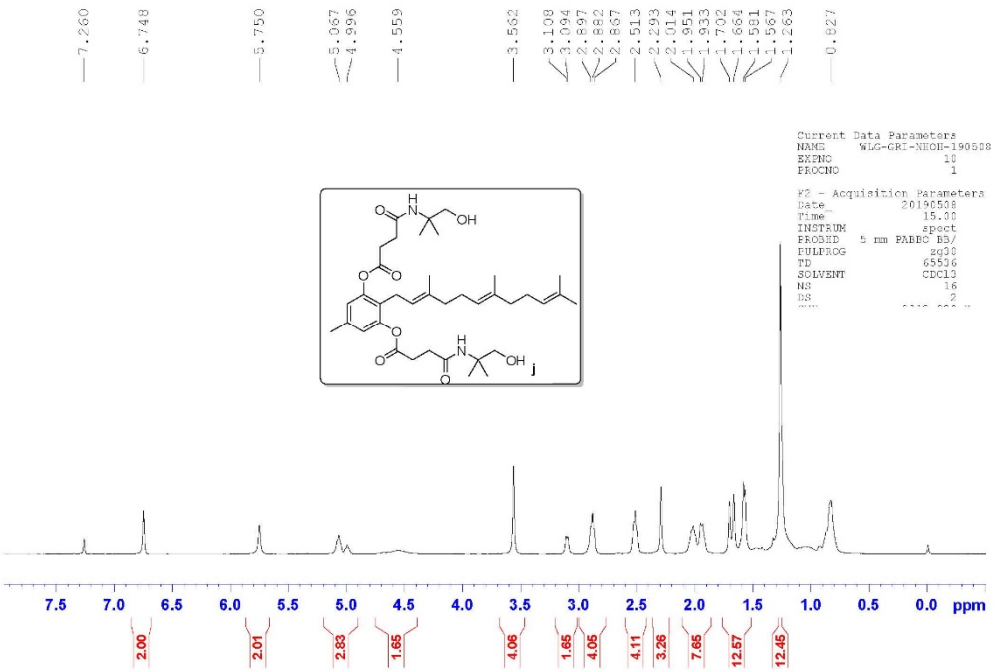
CC1=CC(OC(CCC(OCCOCCOCCOCCOCCO)=O)=O)=C(C/C=C(C)/CC/C=C(C)/CC/C=C(C)/C)C(OC(CCC(OCCOCCOCCOCCOCCO)=O)=O)=C1

Followed the general procedure, DCM was used as the solvent. 3,6,9,12-tetraoxatetradecane-1,14-diol was used as the corresponding substrate. Finally, 209 mg compound **11** was obtained with 43% isolated yield. ^{1}H-NMR(400MHz, $CDCl_3$, ppm): 6.76(s, 2H), 5.06(m, 3H) , 4.26(t, J = 4.12, 4H), 3.70-3.60(m, 36H), 3.12(d, J = 6.16, 2H), 2.87(t, J = 6.52, 4H), 2.76(m, 6H), 2.29(s, 3H), 2.03-1.93(m, 8H), 1.70(s, 3H), 1.66(s, 3H), 1.58(s, 3H), 1.56(s, 3H). ^{13}C-NMR (100MHz, $CDCl_3$, ppm): 172.20, 170.78, 149.44, 137.22, 135.80, 135.20, 131.38, 124.49, 124.11, 123.50, 121.37, 120.93, 72.66, 70.70, 70.67, 70.64, 70.39, 69.16, 64.07, 61.82, 39.80, 39.72, 29.18, 29.05, 26.84, 26.71, 25.84, 23.66, 21.10, 17.82, 16.43, 16.14. LC-MS: calculated for $C_{50}H_{81}O_{18}$ $[M+H]^+$: 969.54, found 970.11. HRMS: calculated for $C_{50}H_{79}O_{18}$ $[M-H]^-$:967.5272, found 967.5286

Copies of NMR Spectra



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4.559
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PROCNO 1
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PULPROG zg30
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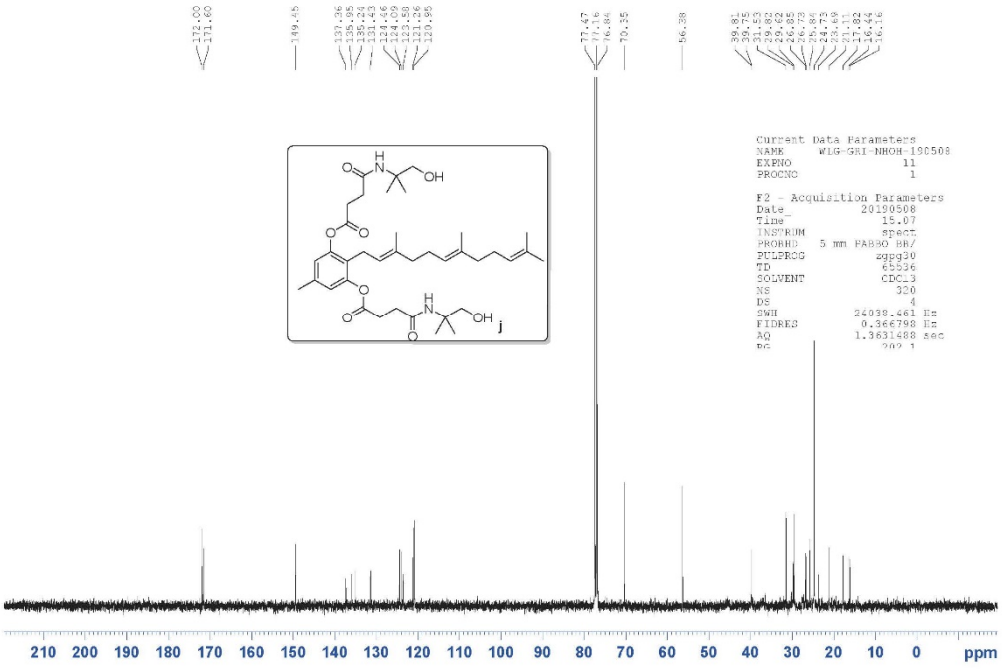
DS 2
OH
j
7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 ppm
2.00 2.01 2.83 1.65 4.06 1.65 4.05 4.11 3.28 7.85 12.57 12.45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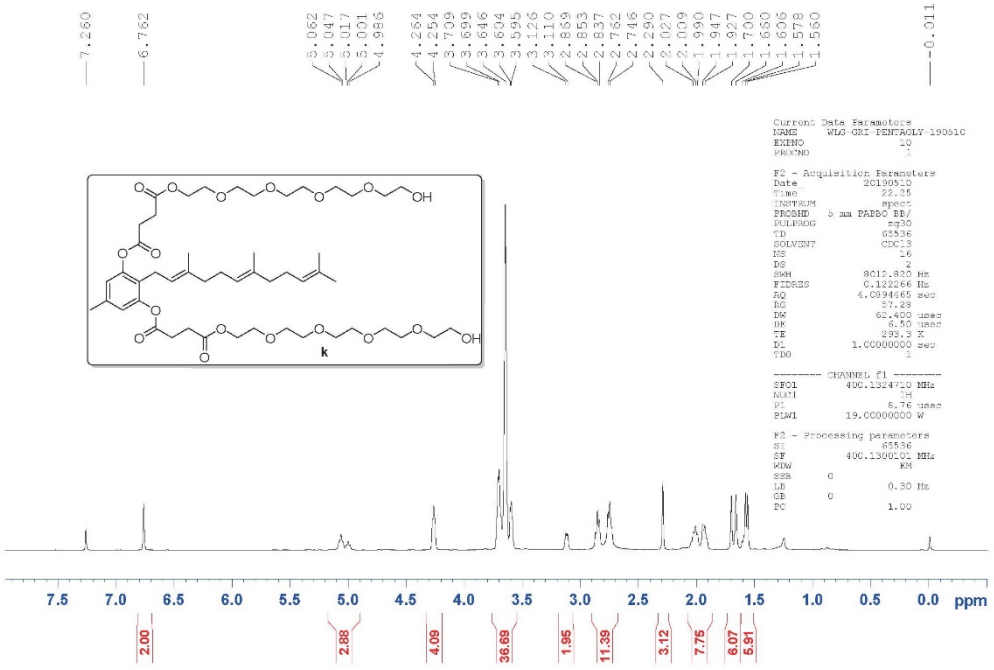
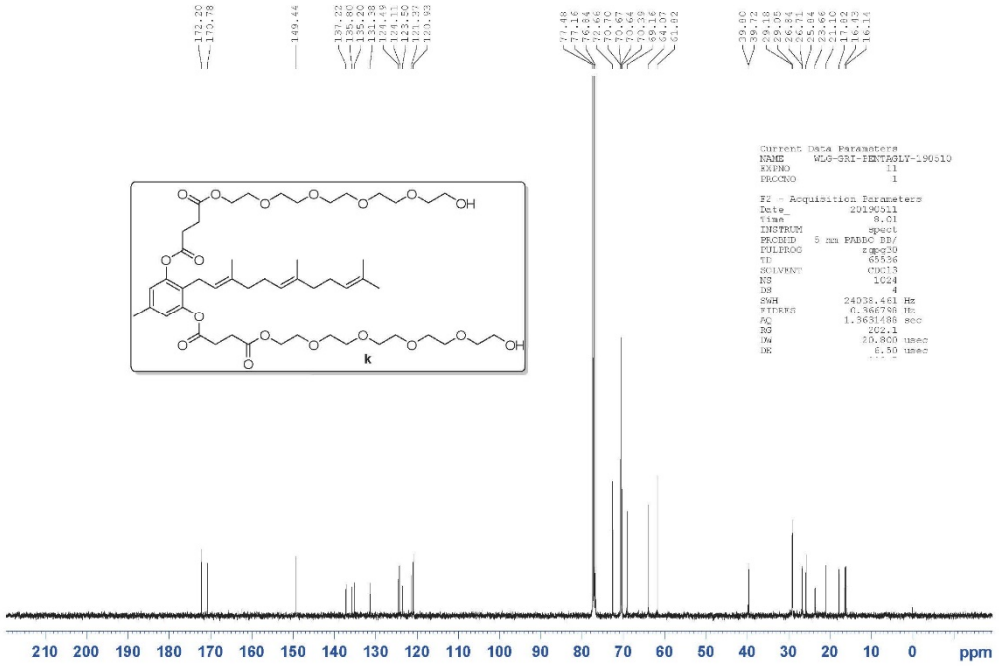
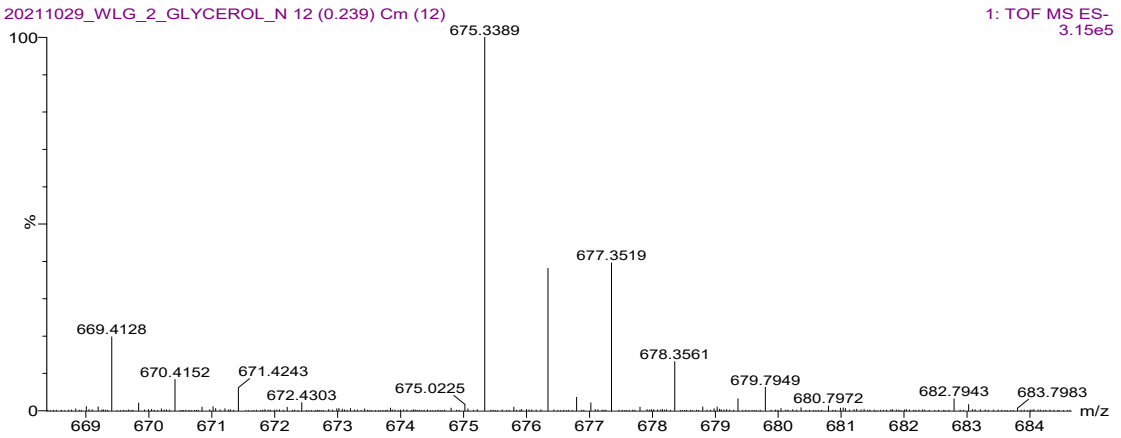


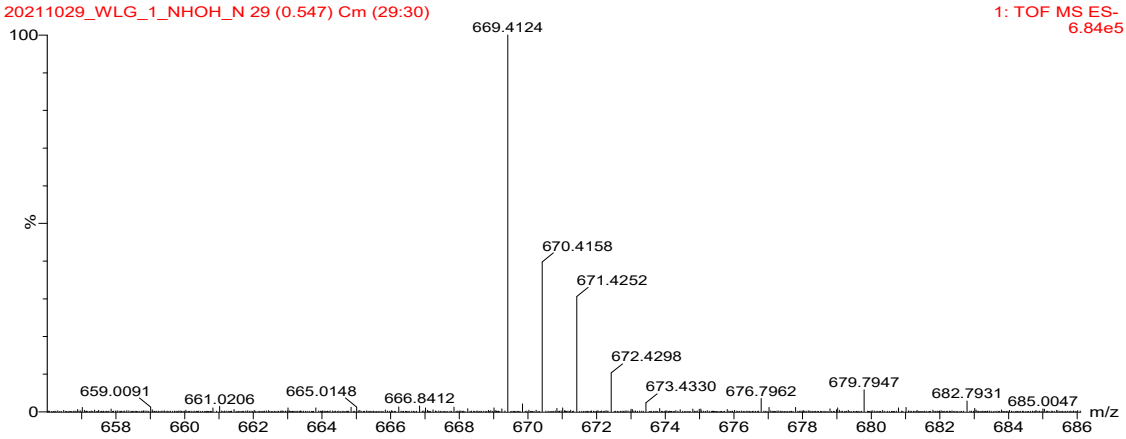


Copies of HRMS spectrum

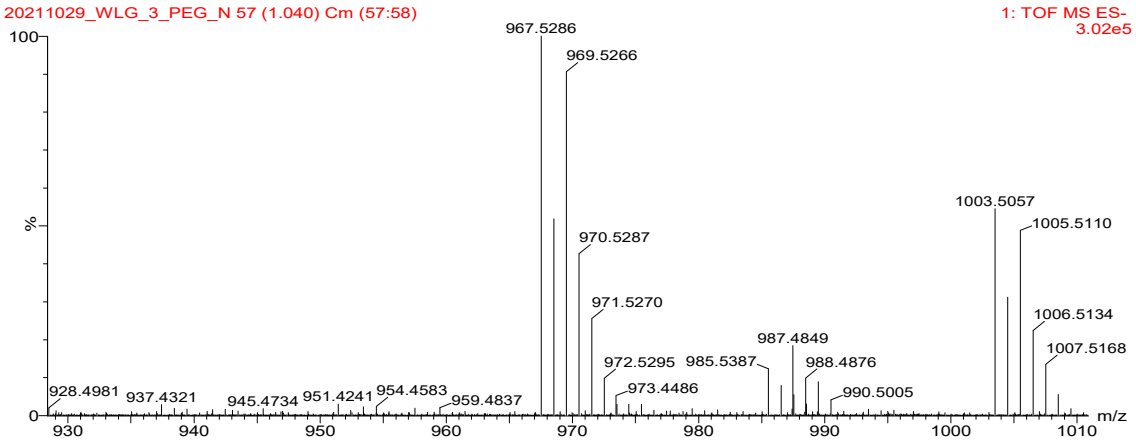
Compound 9



Compound 10



PEG5-Grifolin (Compound 11)



Antibody

The antibody for detecting P16 was from Proteintech (Illinois, NA, USA).The antibodies against PTEN and E-cadherin were purchased from Cell Signaling Technology(Danvers, MA, USA). The anti-DAPK1 was obtained Sigma-Aldrich (St. Louis, MO, USA). The antibody against DNMT1 was from Santa Cruz Biotechnology（Santa Cruz, CA, USA）.

HPLC protocol

The solubility and stability data were collected by SHIMADZU LC-20AT and SPD-20A. The workstation software was LabSolutions (Version 5.73). An Agilent Eclipse Plus C18 3.5μM column was used. The mobile phase consisted of MeCN (B) and water (A) was used. The linear gradient time-table is listed as follows. Detection was set at 214nm and 254 nm. Sulfanilamide was used as internal standard substrate for the stability experiment.

The linear gradient time-table parameters

A concentration	B concentration	Time(min)	Flow(ml/min)
99	1	0	1.00
95	5	2	1.00
1	99	15	1.00
1	99	25	1.00

Method of Solubility Test

The solubility test was conducted by HPLC method. First, a linear regression curve was established. Samples were weighted out in gradient and dissolved in acetonitrile. The concentration of each sample was calculated by the weight and the volume of acetonitrile. The peak area of each sample was measured by HPLC. Based on the concentrations and peak area, the linear regression curve was established.

Then, excess amount of sample was dissolved in PBS buffer (pH = 7.4). The solution was shaken vigorously, followed by sonicated for 15 min at 25℃. Then the solution was centrifuged under 6,000g for 3 min. Then, the supernatant solution was passed through ultrafiltration membrane (0.45 μM, HY-RMF05). Next, the filtrate was analyzed by HPLC. The peak area was substituted into the linear regression curve to get the concentration.

Method of Stability Test

PEG5-Grifolin was dissolved in PBS buffer (pH = 7.4) to get the 40 mM storage solution. This storage solution was separated in several small fraction packages in EP tubes (1.5 ml, ThermoFisher).

These tubes were kept at -78 ℃. Then, these EP tubes could warm to room temperature after indicated time. At every point of time, three tubes were taken out to room temperature. The EP tubes were marked with the take-out time. Finally, the new thawed storage solution and the solution in room temperature was analyzed by HPLC. The area of product peak $\mathbf{S_0}$ in new thawed storage solution was set to 1. The other PE tubes were analyzed by HPLC. S represent the area of the samples in room temperature. The figure was drawn with S/S_0 as y axis and time as x axis. S/S_0 determines the percentage of remaining percent of PEG5-Grifolin. The data was analyzed by GraphPad Prism 7, exponential model.

For the in vitro stability experiment in plasma, PEG5-Grifolin and internal standard substrate sulfanilamide was dissolved in new collected mice plasma. Then, the solutions were placed in 37 ^oC. After indicated time, the solutions were frozen with liquid nitrogen and stored at -80 ^oC until the HPLC analysis. For HPLC analysis, the plasma was injected into HPLC via a 20 μL needle and the peak area of sulfanilamide (A_0) and PEG5-Grifolin (A_1) were collected at each time point. The ratio A_1/A_0 represent the relative concentration of PEG5-Grifolin. The A_1/A_0 at the first time point was set to 100%.

Growth Inhibition assessment

The growth inhibition assessment was performed referred to reported method[2]. 5000 cells in 50μL corresponding culture medium were seeded into 96-wells plate. After two hours, 50μL full culture medium containing double indicated concentration drug was supplement into each well. Then, the plates were incubated in cell incubator for 3 days, followed by adding 10μL CCK-8 into each well. After 1~4h treatment, the OD value of each well of 96-well plates was detected at 490 nm by SpectraMax Plus Microplate Reader. The data were analysed by GraphPad Prism 7 nonlinear regression curve fit.

Cell line and Cell Culture

Thanks to the Department of Anatomy and Cellular Pathology, The Chinese University of Hong Kong for constructing and donating C666-1 cell. 293T and Beas2b cells were from National Collection of Authenticated Cell Cultures of China. The human nasopharyngeal carcinoma cell line

C666-1 was grown in RPMI-1640 media (Gibco) supplemented with 10% v/v heat-inactivated fetal bovine serum (FBS), 1% w/v glutamine and 1% w/v antibiotics and cultured at 37°C in a humidified incubator containing 5% CO_2. 293T and Beas2b cells were grown in DMEM media (Gibco) supplemented with 10% v/v heat-inactivated fetal bovine serum (FBS), 1% w/v glutamine and 1% w/v antibiotics and cultured at 37°C in a humidified incubator containing 5% CO_2. Tumor Xenograft Study

Immunohistochemical Analysis

The tumor tissue sections were deparaffinized in environmentally friendly dewaxing agent (solarbio, china) and rehydrated with an ethanol-aqueous solution of decreasing concentrations. For antigen re-trieval, tissue sections were incubated in 10 mM sodium citrate buffer (pH = 6.0) for 20 min in a microwave oven. The endogenous peroxidase activity was removed by incubating with 3% hydrogen per-oxide for 10 min and was blocked in normal donkey serum for 30 min. The primary antibodies (anti-DNMT1, anti-DAPK, anti-PTEN, anti-E-cadherin) were applied at 4 °C overnight. Chromogen was developed using DAB (Zsgbbio, China) and counterstained with hematoxylin staining kit. Immuno-histochemical staining of these sections was evaluated based on all of the available tumor cells or epithelial cells meeting the typical morphological criteria by 3 pathologists using the qualitative scale that is described in the literature.

DNA Bisulfite Sequencing PCR

Genomic DNA was extracted using a Tissue DNA Kit (QIAGEN, Inc., Hilden, Germany) and treated with bisulfate according to the EZ DNA Methylation-Gold™ Kit instruction manual (Zymo Research, USA). The promoter sequence was amplified from the isolated DNA using touchdown PCR, extracted from an agarose gel and loaded into the Puc18-T vector (Sangon Biotech Co., Ltd.,Shanghai, China) for TA cloning and sequencing by Shanghai Sangon Biotech Company. The PCR conditions were as follows: 95 °C for 5 min; 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 50 s at 72 °C; and a final extension at 72 °C for 8 min. The BSP primer sequences for CpG region of DAPK1 were as follows: for-ward, 5′- TTTTTTAAAAAGTAAATAGGTGAGGT -3′ and reverse, 5′- CACCTCCAAAATTCAAATAATTC -3′. The BSP primer sequences for CpG region of

p16 were as follows: for-ward, 5′- TTTGTAGTTAAGGGGGTAGGAGT-3′ and reverse, 5′-CTTTCCTACCTAATCTTCTAAAAAAC-3′. In detail, 10 individual clones from each group were selected and the number of detected methylated CpG sites was divided by the total 10 clones to evaluate the methylation percentage of each CpG site. The total methylation status of the core CpG region within the promoter from each group was calculated by averaging the methylation rate of each CpG site.

Genomic DNA Extraction

The tumor tissues were cut into small pieces (<25 mg) and placed in 1.5 ml centrifuged tubes. 20 μl proteinase K and 180 μl ATL buffer were added, followed by violent vortexing. The sample was incubated under 56℃ for 2 h. Then, 200 μl AL buffer was added to the tubes and mixed thoroughly by vortexing. After incubated under 70℃ for 10 min, 200 μl ethanol was added, followed by vortexing for 15 s and centrifuging. Then, the samples were transferred to QIAamp mini spin columns placed in a 2 ml collection tube. The samples were centrifuged at 6,000 g for 1 min. Discard flow-through and collection tube. Place the QIAamp mini spin column in a 2 ml tube. Add 500 μl AW1 and centrifuge at 6,000 g for 1 min. Discard flow-through and collection tube. Place the QIAamp mini spin column in a new 2 ml tube. Add 500 μl AW2 and centrifuge at 20,000 g for 3 min. Discard flow-through and collection tube. Place the QIAamp mini spin column in a 1.5 ml tube. Add 200 μl AE buffer and incubate for 1 min. Centrifuge at 6,000 g for 1 min to provide DNA samples for BSP analysis.

Reference

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