Electronic Supplementary Information for

Highly GSH-sensitive SN-38 prodrug with "OFF-to-ON" fluorescence switch as a bifunctional anticancer agent

Chang-Hee Whang,[a] Eunsoo Yoo,[b] Seong Kwon Hur,[c] Kyeong Soo Kim,[a] Dongin Kim[b] and Seongbong Jo*[a]

[a] *Department of Pharmaceutics and Drug Delivery, School of Pharmacy, The University of Mississippi, University, MS 38655, USA.*
**E-mail: seongjo@olemiss.edu*
[b] *Department of Pharmaceutical Sciences, Irma Lerma Rangel College of Pharmacy, Texas A&M Health Science Center, College Station, TX 77843, USA.*
[c] *Department of Molecular & Cellular Medicine, College of Medicine, Texas A&M Health Science Center, College Station, TX 77843, USA*

Materials

SN-38 (7-ethyl-10-hydroxyl-camptothecin) was purchased from Carbosynth, LLC (San Diego, CA). 2,4-dinitrobenzene sulfonyl chloride (DNS) and triethylamine (TEA) was purchased from Alfa Aesar (Ward Hill, MA). All other chemicals and solvents were purchased from Fisher Scientific (Pittsburgh, PA).

1. Synthesis of DNS-SN38

SN-38 (0.255 mmol, 1 eq.) was first dissolved in 10 mL of anhydrous DCM and excess TEA was added (0.765 mmol, 3.0 eq.). The mixture was then lowered to 0^oC and stirred for 15 minutes. Next, 2,4-dinitrobenzene sulfonyl chloride (0.3 mmol, 1.2 eq.) dissolved in 5 mL of anhydrous DCM was added to the mixture slowly in drop-wise fashion over 30 minutes. Once addition was finished, the reaction mixture was left to stir for additional 1.5 hours at 0^oC. After monitoring the reaction progress via TLC, the organic phase was washed with saturated $NaHCO_3$ solution and brine. The collected phase was dried using sodium sulfate. The organic phase was then concentrated under vacuum and crude product was recrystallized with acetone to yield slightly-yellow solid powder as the final product. (%Yield = 55%)

2. ^{1}H NMR and HPLC Characterization of DNS-SN38

^{1}H NMR (Bruker Avance, 400 MHz, DMSO-d_6; ppm): 9.14 (d, 1H), 8.59 (dd, 1H), 8.35 (d, 1H), 8.23 (d, 1H), 8.05 (d, 1H), 7.66 (dd, 1H), 7.34 (s, 1H), 5.44 (s, 2H), 5.34 (s, 2H), 3.13 (q, 2H), 1.87 (m, 2H), 1.17 (tr, 3H), 0.87 (tr, 3H).

High performance liquid chromatography (HPLC) was utilized to characterize DNS-SN38 in comparison to SN-38. Waters (Milford, MA) HPLC system ($\lambda_{Detection}$ = 365 nm) with Waters 1525 binary pump, Waters 717plus auto sampler, Waters 2487 UV detector, and Phenomenex Luna C18 column (150x4 60 mm, 5 micron). The mobile phase was 25 mM NaH_2PO_4 (pH 3.1):ACN = 50:50 (*v/v*) eluting at a flow rate of 1.0 ml/min. All samples were filtered with 0.45 µM filters before injection into HPLC system.

3. UV-Vis Spectroscopy

The UV-Vis spectroscopy was performed for both DNS-SN38 and SN-38 using Genesys 10S UV-Vis Spectrophotometer from Thermo Fisher Scientific (Waltham, MA) in DMSO:PBS (pH 7.4) = 1:2 (*v*/*v*) solution.

4. GSH-Activation of DNS-SN38 via Fluorescence Activation

LC500 Fluorescence Spectrophotometer from Perkin-Elmer (Waltham, MA) was used for fluorescence measurements. The excitation wavelength was set at 365 nm. The solution used to prepare SN-38 and DNS-SN38 stock samples was DMSO:PBS (pH 7.4) = 1:2 (*v*/*v*) solution. Appropriate dilutions of these stock samples were carried out with the same solution. For time-dependent studies, FL WinLab software's kinetic study mode was utilized

with consistent delay of 60 seconds between the scans. The first scan was made 30 seconds after GSH injection into sample solution.

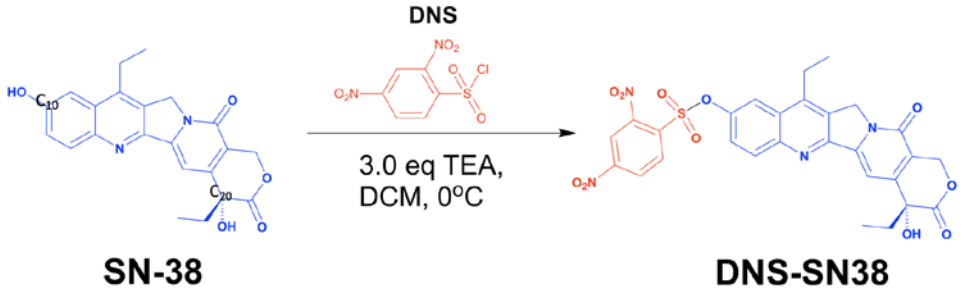
5. Confocal Microscopy of DNS-SN38 in B16-F10 cells

For tracking of internalization of SN-38 in B16F10 cells, confocal images were obtained on a Nikon TiE confocal microscope using the NIS-Elements software. B16F10 at a density of 1 X 10^5 cells per well were seeded on the coverslips in 24 well plate under the standard conditions at 37 ºC with 5 % CO_2 and 100 % humidity. When the cell confluency was around 90 %, 10 μM DNS SN-38 was treated four wells and another well without DNS SN-38 was used for control. Then, cells were incubated for 1, 5, 15, and 30 mins. For cell fixation, all media were aspirated at different time points and washed with 1 mL of PBS for 5 min. Then, 500 μL of 4 % PFA was treated at room temperature for 10 minutes. All wells were rinsed with 1 mL of PBS for three times. Then, 500 μL of DAPI was added to all wells and incubated at room temperature for 10 min. All DAPI solution was removed and washed with 1 mL of PBS for 5 min. Then, the coverslip was mounted on the slide glass for confocal microscopy.
The reported percentage values were normalized DNS SN-38 fluorescence intensity compared to the DAPI intensity. ImageJ software was used to measure the mean gray values of DAPI and SN-38 from four different regions. The normalized DNS SN-38 fluorescence intensity is given by the following equation:

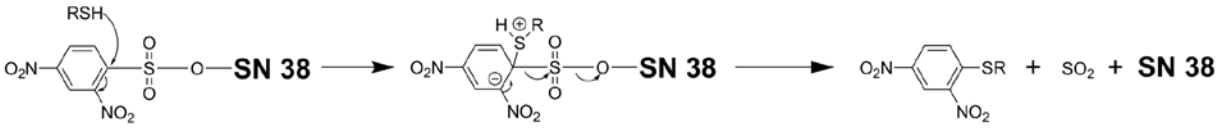
$$\text{Normalized DNS SN 38 (\%)} = \left(\frac{DNS\ SN\ 38\ fluorescence\ intensity}{DAPI\ intensity}\right) \times 100$$

6. Cell Viability Tests: A2780 & mCherry + OCSC1-F2

A2780 cell at 5 x 10^4 cells/cm^2 were seed in a 96 well plate and cultured until 100 % confluent under standard conditions at 37 ºC with 5 % CO_2 and 100 % humidity. The stock solutions of natural product samples were diluted to 0.01, 0.1, 1, 10, 100, 1000 and 10,000 nM in cell medium (RPMI-1640). Cells were exposed to natural products at 0.001, 0.01, 0.1, 1, 10, 100, and 1000 μg/mL for 24 hours. After 24 hours of incubation, 10 μL of the cell counting kit-8 (CCK-8; Enzo Life Science, Lausen, Switzerland) solution was added into appropriate wells. After 1 hour of incubation, the absorbance was measured at 450 nm using a SpectraMax M3 microplate reader (Molecular Devices Inc., Sunnyvale, CA). The positive control was treated with fresh RPMI-1640 (Lonza, Walkersville, MD). The exactly same condition without CCK-8 solution was used to subtract background signal that is absorbed by cell medium or natural product. The results were normalized as percentage based on the control. The mCherry-labeled ovarian cancer cell line OCSC1-F2 was generated and propagated as previously described.



Scheme S1. Synthetic scheme for DNS-SN38.



Scheme S2. GSH-triggered activation scheme for DNS-SN38

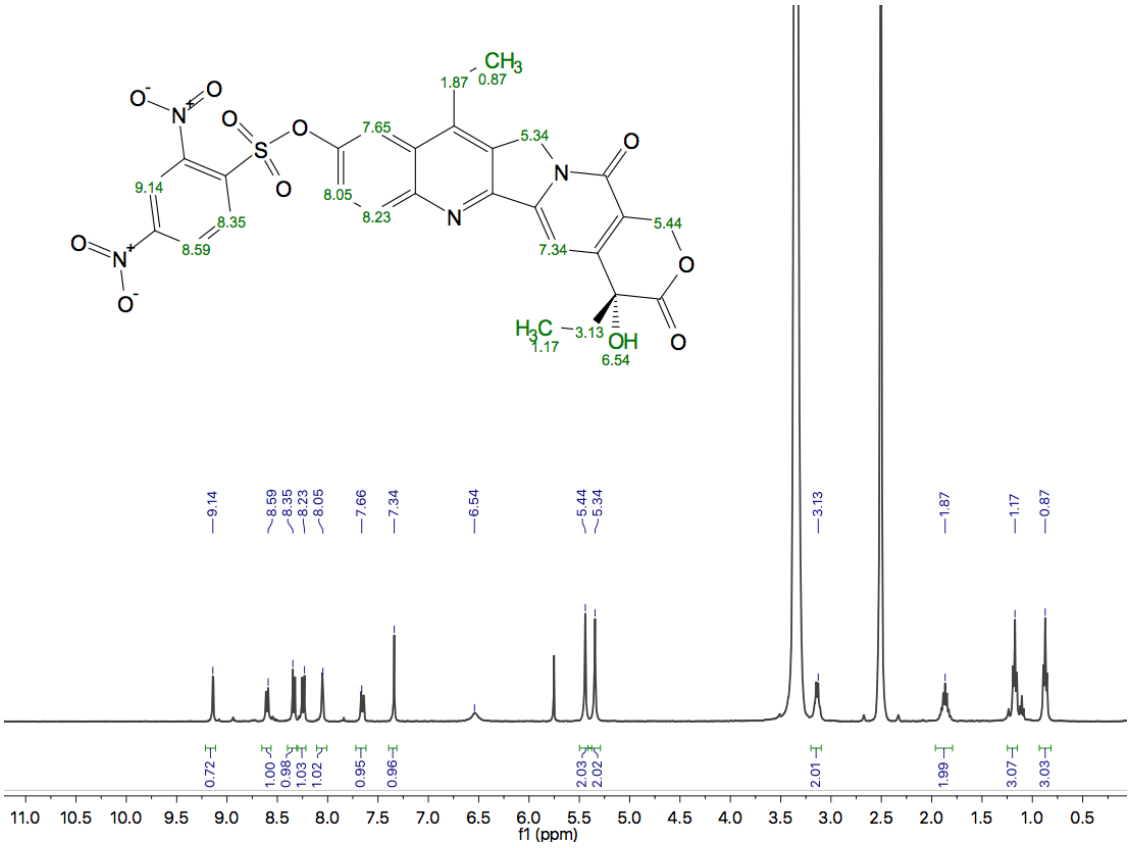


Figure S1. ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of DNS-SN38.

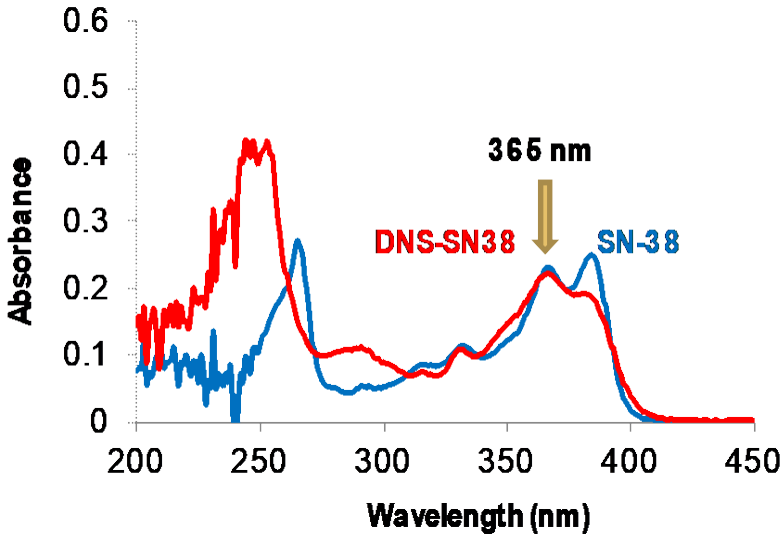


Figure S2. UV-vis absorbance spectra of DNS-SN38 and SN-38 in DMSO:PBS (pH 7.4) = 1:2 (v/v).

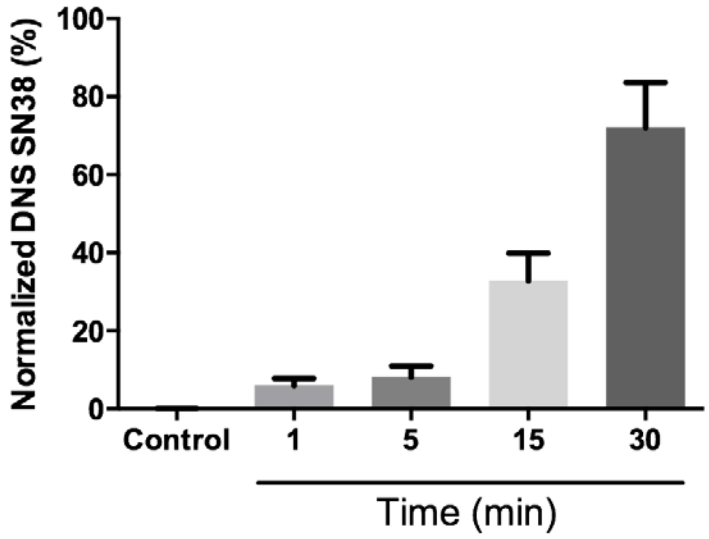


Figure S3. Normalized DNS SN-38 fluorescence intensities were calculated by ImageJ software from different regions (n=4).