Supplementary Information

Furin-Mediated Intracellular Self-Assembly of Olsalazine Nanoparticles For Enhanced MR Imaging and Tumor Therapy

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Supplementary movie captions

Supplementary Movie 1: 3D-SIM super-resolution fluorescence imaging of HCT116 cells incubated with 8 μM Alexa-RVRR. The video is representative for experiments repeated independently three times with similar results.

Supplementary Movie 2: 3D-SIM super-resolution fluorescence imaging of LoVo cells incubated with 8 μM Alexa-RVRR. The video is representative for experiments repeated independently three times with similar results.

Supplementary Movie 3: 3D-SIM super-resolution fluorescence imaging of HCT116 cells incubated with 8 μM Alexa 488. The video is representative for experiments repeated independently three times.

Supplementary Movie 4: 3D-SIM super-resolution fluorescence imaging of LoVo cells incubated with 8 μM Alexa 488. The video is representative for experiments repeated independently three times.

Supplementary Movie 5: 3D-SIM super-resolution fluorescence imaging of HCT116 tumors after i.v. injection of 50 nmol Alexa-RVRR. The video is representative for experiments repeated independently three times.

Supplementary Movie 6: 3D-SIM super-resolution fluorescence imaging of LoVo tumors after i.v. injection of 50 nmol Alexa-RVRR. The video is representative for experiments repeated independently three times.

Supplementary Movie 7: 3D-SIM super-resolution fluorescence imaging of HCT116 tumors after i.v. injection of 50 nmol Alexa 488. The video is representative for experiments repeated independently three times.

Supplementary Movie 8: 3D-SIM super-resolution fluorescence imaging of LoVo tumors after i.v. injection of 50 nmol Alexa 488. The video is representative for experiments repeated independently three times.

1. General methods

All starting materials were obtained from Sigma or Thermo Fisher. Commercially available reagents were used without further purification, unless otherwise specified. All chemicals were reagent grade or better. Olsalazine sodium was purchased from Santa Cruz Biotechnology. Furin was purchased from Biolabs (2,000 U mL^{-1}). Milli-Q water (18.2 MΩcm) was used throughout all experiments. Mass spectra were performed on a Voyager DE-STR MALDI-TOF mass spectrometer. ^{1}H-NMR spectra were obtained on a 500 MHz Bruker Avance 500 or 400 MHz Bruker Avance 400. HPLC analysis was performed on a Bio-Rad (Hercules, CA) Biologic DuoFlow system equipped with QuadTec UV detector and BioFrac fraction collector using a Phenomenex (Torrance, CA) 00G-4350-N0 C12 column with CH_3CN (0.1% of trifluoroacetic acid (TFA)) and water (0.1% of TFA) as the eluent. UV-vis absorption spectra were

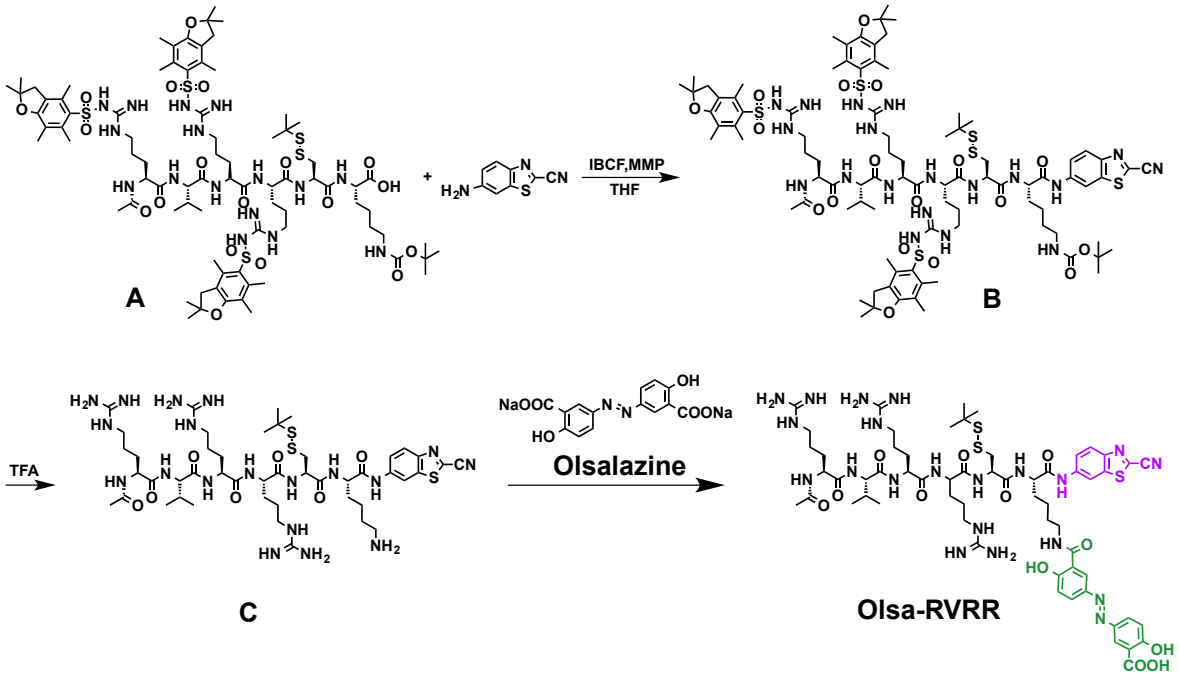
recorded with a JASCO V-630 UV-Vis spectrophotometer. Dynamic light scattering (DLS) was measured on a Zeta Sizer Nano Series (Malvern Instruments). Cell images were obtained with a Zeiss AX10 fluorescence microscope. Transmission electron micrograph (TEM) images were obtained using a Libra-120 transmission electron microscope (Carl Zeiss, Jena, Germany) operating at 120 kV. HCT116 cells, LoVo cells, and CCD-18Co cells were obtained from ATCC and routinely cultured at 37 °C, 5% CO_2, and a humidified atmosphere in McCoy's 5A (modified) medium, F-12K medium, or Eagle's Minimum Essential Medium (EMEM), respectively, supplemented with 10% fetal bovine serum.

2. Synthesis and characterization of compounds

Preparation of *Ac-Arg-Val-Arg-Arg-Cys(StBu)-Lys(Olsalazine)-CBT* (Olsa-RVRR):

2-cyano-6-aminobenzothiazole (CBT) was synthesized as described previously (White, E. H., Worther, H., Seliger, H. H., McElroy, W. D. Amino analogs of firefly luciferin and biological activity thereof. *J. Am. Chem. Soc.* **1966**, *88*, 2015-2019).

Supplementary Scheme 1. **Synthetic route for Olsa-RVRR.**



Synthesis of B: Compound A was synthesized with solid phase peptide synthesis (SPPS). Isobutyl chloroformate (IBCF, 63 mg, 0.46 mmol) was added to a mixture of compound A (830 mg, 0.46 mmol) and 4-methylmorpholine (MMP, 93 mg, 0.92 mmol) in THF (10.0 mL) at 0 °C under N_2. The reaction mixture

was stirred for 40 min. A solution of 2-cyano-6-aminobenzothiazole (CBT, 90 mg, 0.51 mmol) and additional IBCF (21 mg, 0.15 mmol) was added to the reaction mixture and further stirred for 1 h at 0 °C, then stirred overnight at room temperature (RT). Compound B (730 mg, yield: 81 %) was purified with HPLC using water-methanol added with 0.1% TFA as the eluent (from 20:80 to 0:100). MS: calculated for B [$(M+H)^+$]: m/z 1960.85; observed (obsvd.) MALDI-TOF/MS: m/z 1961.04.

Synthesis of C: The Boc and Pbf protecting groups of compound B were removed with dichloromethane (DCM, 1 mL) and triisopropylsilane (TIPS, 200 µL) in TFA (19 mL) for 3 h. Compound C (352 mg, yield: 86 %) was obtained after HPLC purification using water-methanol added with 0.1% TFA as the eluent (from 5:5 to 5:95). MS: calculated for C [$(M+H)^+$]: m/z 1104.55; obsvd. MALDI-TOF/MS: m/z 1104.38.

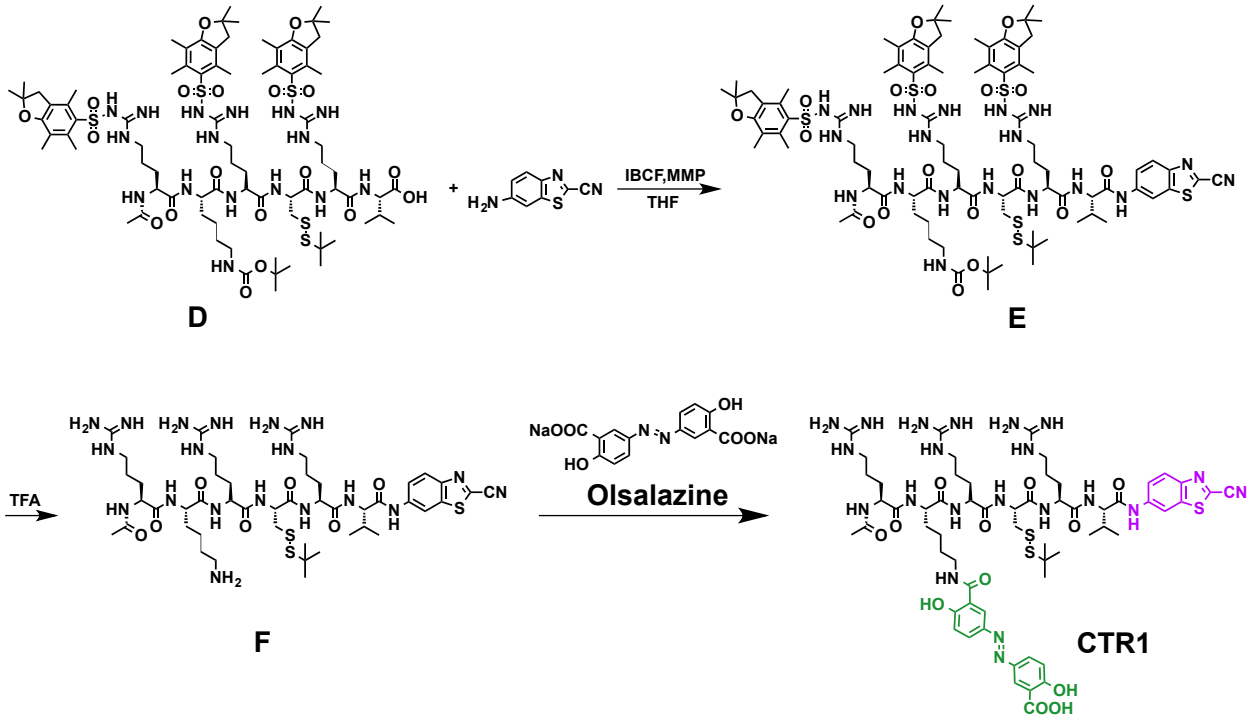
Synthesis of Olsa-RVRR: A mixture of olsalazine sodium (100 mg, 0.29 mmol), 2-(1H-benzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate (HBTU, 110 mg, 0.29 mmol), and 1-hydroxybenzotriazole (HOBT, 39 mg, 0.29 mmol) in DMF (20 mL) was stirred for 30 min in the presence of DIPEA (57 mg, 0.44 mmol). Compound C (250 mg, 0.23 mmol, dissolved in 4 mL of DMF) was added dropwise to the mixture. After 6 h stirring at RT, compound Olsa-RVRR (230 mg, yield: 72%) was purified with HPLC using water-acetonitrile added with 0.1% TFA as eluent (from 65:35 to 15:85). MS: calculated for Olsa-RVRR $C_{60}H_{86}N_{21}O_{12}S_3^+$ [$(M+H)^+$]: m/z 1388.59215; obsvd. MALDI-TOF/MS: m/z 1388.59483 (**Supplementary Fig. 1**).

^{1}H-NMR of Olsa-RVRR ($C_{60}H_{85}N_{21}O_{12}S_3$, d_6-DMSO, 500 MHz, **Supplementary Fig. 2**): 13.39 (d, J = 20 Hz, 1 H), 10.55 (s, 1H), 10.46 (s, 1 H), 9.10 (q, J = 5 Hz, 1 H), 8.71 (dd, J1 = 2 Hz, J2 = 18 Hz, 1 H), 8.61 (d, J = 7 Hz, 1 H), 8.47 (dd, J_1 = 2 Hz, J_2 = 12 Hz, 1 H), 8.35 (d, J = 7 Hz, 1 H), 8.30 (dd, J_1 = 3 Hz, J_2 = 4 Hz, 2 H), 8.26 (d, J = 7 Hz, 1 H), 8.15 (m, 3 H), 8.02 (m, 2 H), 7.96 (s, 1 H), 7.91 (m, 1 H), 7.78 (dd, J_1 = 2 Hz, J_2 = 9 Hz, 1 H), 7.71 (dd, J_1 = 2 Hz, J_2 = 9 Hz, 1 H), 7.65 (d, J = 8 Hz, 2 H), 7.58 (m, 1 H), 7.53 (m, 1 H), 7.42 (t, J = 8 Hz, 1 H), 7.15 (dd, J_1 = 3 Hz, J_2 = 9 Hz, 2 H), 7.05 (dd, J_1 = 4 Hz, J_2 = 9 Hz, 2 H), 4.54 (m, 1 H), 4.42 (q, J = 7 Hz, 1 H). 4.29 (m, 3 H), 4.18 (m, 1 H), 3.34 (m, 2 H), 3.05 (m, 9 H), 2.89 (s, 1 H), 2.74 (s, 1 H),

2.08 (s, 1 H), 1.96 (m, 1 H), 1.87 (d, J = 3 Hz, 3 H), 1.68 (m, 6 H), 1.49 (m, 11 H), 1.26 (d, J = 7 Hz, 9 H), 0.82 (m, 6 H).

Preparation of *Ac-Arg-Lys(Olsalazine)-Arg-Cys(StBu)-Arg-Val-CBT* (CTR1):

Supplementary Scheme 2. **Synthetic route for CTR1.**



Synthesis of E: Compound D was synthesized with solid phase peptide synthesis (SPPS). Isobutyl chloroformate (IBCF, 41 mg, 0.3 mmol) was added to a mixture of compound D (540 mg, 0.3 mmol) and 4-methylmorpholine (MMP, 61 mg, 0.6 mmol) in THF (7.0 mL) at 0 °C under N_2. The reaction mixture was stirred for 40 min. A solution of 2-cyano-6-aminobenzothiazole (CBT, 62 mg, 0.35 mmol) and additional IBCF (14 mg, 0.1 mmol) was added to the reaction mixture and further stirred for 1 h at 0 °C, then stirred overnight at room temperature (RT). Compound B (445 mg, yield: 76 %) was purified with HPLC using water-methanol added with 0.1% TFA as the eluent (from 20:80 to 0:100). MS: calculated for B [$(M+H)^+$]: m/z 1960.85; observed MALDI-TOF/MS: m/z 1961.13.

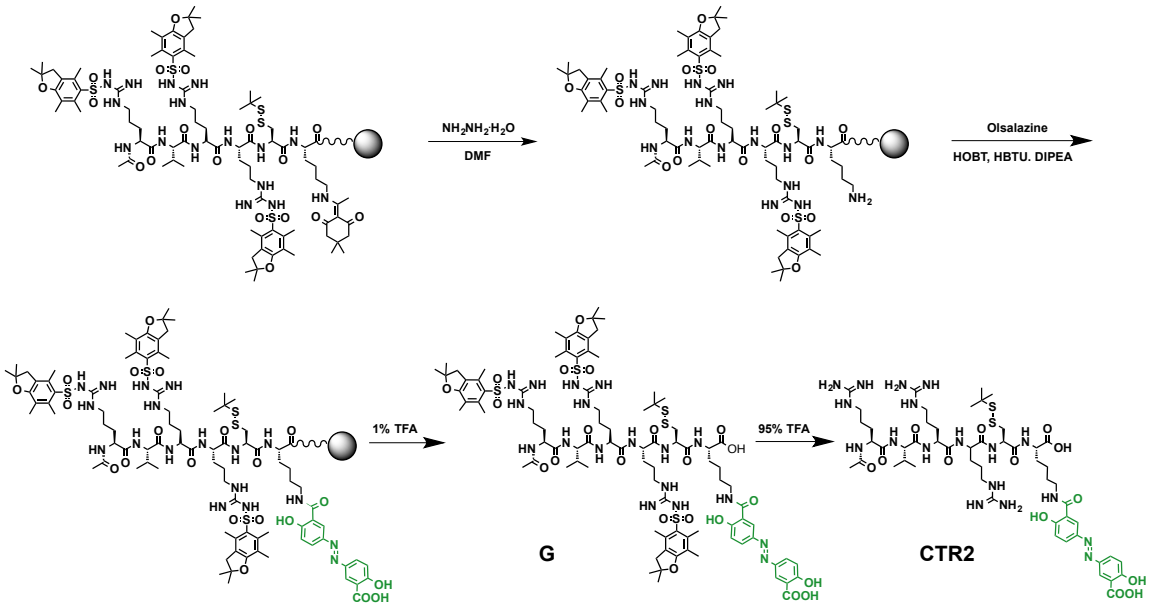
Synthesis of F: The Boc and Pbf protecting groups of compound B were removed with dichloromethane (DCM, 1 mL) and triisopropylsilane (TIPS, 200 µL) in TFA (19 mL) for 3 h. Compound C (210 mg, yield: 84 %) was obtained after HPLC purification using water-methanol added with 0.1% TFA as the eluent (from 5:5 to 5:95). MS: calculated for C [$(M+H)^+$]: m/z 1104.55; observed MALDI-TOF/MS: m/z 1104.89.

Synthesis of CTR1: A mixture of olsalazine sodium (62 mg, 0.18 mmol), 2-(1H-benzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate (HBTU, 68 mg, 0.18 mmol), and 1-hydroxybenzotriazole (HOBT, 24 mg, 0.29 mmol) in DMF (15 mL) was stirred for 30 min in the presence of DIPEA (35 mg, 0.27 mmol). Compound C (150 mg, 0.14 mmol, dissolved in 4 mL of DMF) was added dropwise to the mixture. After 6 h stirring at RT, compound CTR2 (129 mg, yield: 68%) was purified with HPLC using water-acetonitrile added with 0.1% TFA as eluent (from 65:35 to 15:85). MS: calculated for *CTR1* $C_{60}H_{86}N_{21}O_{12}S_3^+$ [$(M+H)^+$]: m/z 1388.59215; observed MALDI-TOF/MS: m/z 1388.58924 (**Supplementary Fig. 6**).

^{1}H-NMR of CTR1 ($C_{60}H_{85}N_{21}O_{12}S_3$, d_6-DMSO, 400 MHz, **Supplementary Fig. 7**): 13.43 (d, J = 20 Hz, 1 H), 10.65 (s, 1H), 9.16 (t, J = 5 Hz, 1 H), 8.70 (d, J = 2 Hz, 1 H), 8.53 (d, J = 2 Hz, 1 H), 8.32 (d, J = 3 Hz, 1 H), 8.28 (d, J = 8 Hz, 1 H), 8.20 (d, J = 9 Hz, 1 H), 8.12 (m, 3 H), 8.00 (m, 3 H), 7.94 (dd, J_1 = 2 Hz, J_2 = 9 Hz, 2 H), 7.77 (dd, J_1 = 2 Hz, J_2 = 9 Hz, 1 H), 7.62 (q, J = 5 Hz, 2 H), 7.08 (m, 3 H), 5.32 (t, J = 5 Hz, 1 H), 4.53 (m, 1 H), 4.36 (m, 2 H). 4.25 (m, 3 H), 3.32 (m, 2 H), 3.11 (m, 8 H), 2.96 (dd, J_1 = 9 Hz, J_2 = 13 Hz, 1 H), 2.90 (s, 1 H), 2.73 (s, 1 H), 2.02 (m, 2 H), 1.85 (s, 3 H), 1.56 (m, 20 H), 1.27 (s, 9 H), 1.23 (s, 3 H), 0.91 (dd, J_1 = 1 Hz, J_2 = 7 Hz, 6 H).

Preparation of *Ac-Arg-Val-Arg-Arg-Cys(StBu)-Lys(olsalazine)* (CTR2):

Supplementary Scheme 3. **Synthetic route for CTR2.**



Synthesis of compound G: The peptide Ac-Arg(Pbf)-Val-Arg(Pbf)-Arg(Pbf)-Cys(StBu)-Lys(Dde)-COOH was synthesized with 2-chlorotrityl chloride resins using the SPPS method. After shaking the resins with 2.5% hydrazine hydrate in DMF (twice, 5 min each), Dde was removed from Lys, and the resins were rinsed four times with DMF. A mixture of olsalazine sodium (104 mg, 0.3 mmol), 2-(1H-benzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate (HBTU, 114 mg, 0.3 mmol), and 1-hydroxybenzotriazole (HOBT, 40 mg, 0.3 mmol) in DMF (6 mL) was shaken for 6 h in the presence of DIPEA (58 mg, 0.45 mmol). After sequential washing with DMF and dichloromethane (DCM), 1% TFA/DCM was used to treat the resins until they turned red. 275 mg Compound G was produced after the solvent was removed using a rotary evaporator followed by recrystallization with diethyl ether. MS: calculated for G [$(M+H)^+$]: m/z 1987.83; observed MALDI-TOF/MS: m/z 1988.24.

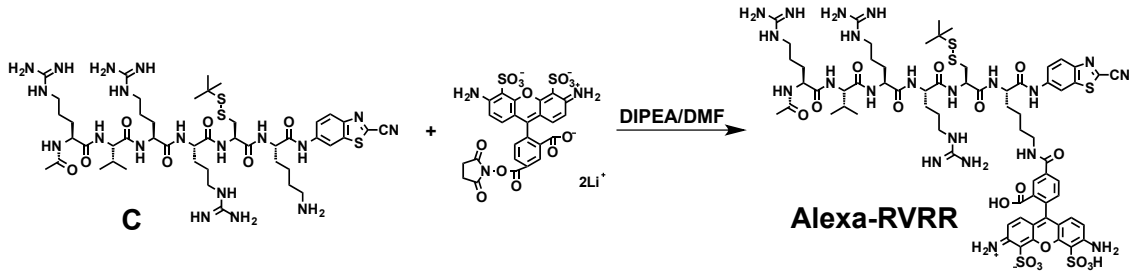
Synthesis of CTR2: The Boc and Pbf protecting groups of compound G were removed with dichloromethane (DCM, 1 mL) and triisopropylsilane (TIPS, 200 μL) in TFA (19 mL) for 3 h. Compound *CTR2* (118 mg, yield: 69 %) was obtained after HPLC purification using water-acetonitrile added with 0.1%

TFA as eluent (65:35 to 15:85). MS: calculated for *CTR2* $C_{52}H_{83}N_{18}O_{13}S_2^+$ [$(M+H)^+$]: m/z 1231.58229; observed MALDI-TOF/MS: m/z 1231.57830 (**Supplementary Fig. 8**).

^{1}H-NMR of CTR2 ($C_{52}H_{82}N_{18}O_{13}S_2$, d_6-DMSO, 400 MHz, **Supplementary Fig. 9**): 13.47 (s, 1 H), 9.15 (t, J = 5 Hz, 1 H), 8.54 (d, J = 2 Hz, 1 H), 8.33 (d, J = 3 Hz, 1 H), 8.30 (s, 1 H), 8.23 (d, J = 8 Hz, 1 H), 8.16 (d, J = 8 Hz, 2 H), 8.05 (dd, J_1 = 3 Hz, J_2 = 9 Hz, 1 H), 8.01 (s, 1 H), 7.95 (dd, J_1 = 2 Hz, J_2 = 9 Hz, 1 H), 7.76 (m, 1 H), 7.66 (m, 4 H), 7.16 (d, J = 9 Hz, 3 H), 7.08 (d, J = 9 Hz, 3 H), 4.53 (m, 1 H), 4.30 (m, 3 H). 4.17 (m, 2 H), 4.04 (q, J = 7 Hz, 1 H), 3.33 (q, J = 6 Hz, 2 H), 3.11 (m, 8 H), 2.94 (dd, J_1 = 2 Hz, J_2 = 9 Hz, 1 H), 1.96 (m, 2 H), 1.86 (s, 3 H), 1.57 (m, 20 H), 1.27 (s, 10 H), 1.17 (t, J = 7 Hz, 1 H), 0.82 (q, J = 7 Hz, 6 H).

Preparation of *Ac-Arg-Val-Arg-Arg-Cys(StBu)-Lys(Alexa 488)-CBT* (Alexa-RVRR):

Supplementary Scheme 4. **Synthetic route for Alexa-RVRR.**

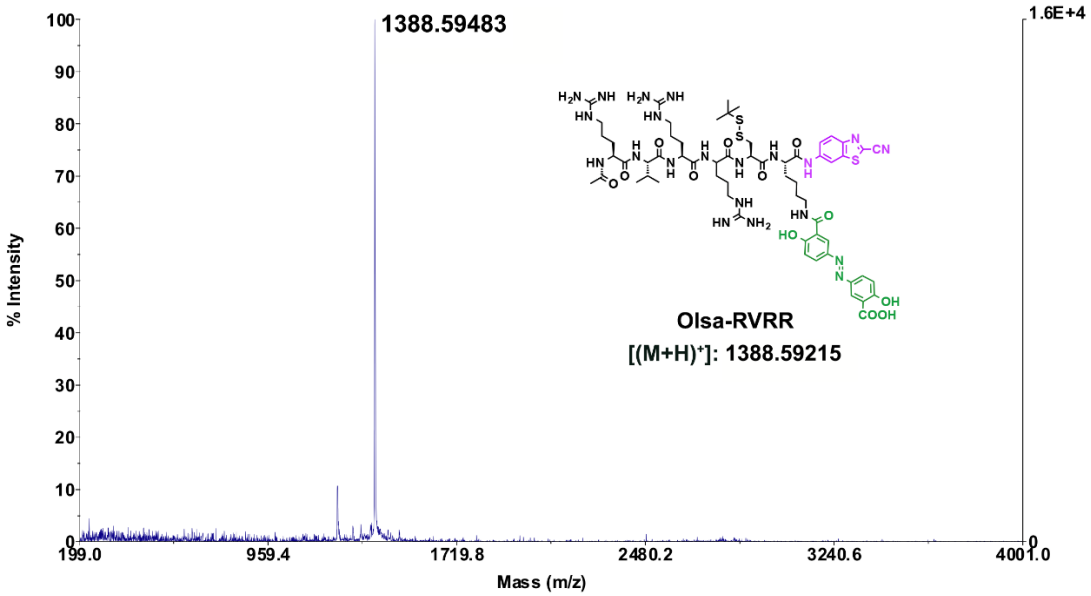


Alexa Fluor® 488 NHS ester (1 mg) and DIPEA (10 µL) were added to a solution of compound C (10 mg) in dry DMF, and the mixture was stirred at RT for 2 h. Alexa-RVRR was purified from the mixture with HPLC in 61% yield. MS: calculated for Alexa-RVRR $C_{67}H_{90}N_{21}O_{17}S_5^+$ [$(M+H)^+$]: m/z 1620.54216; obsvd. MALDI-TOF/MS: m/z 1620.54744 (**Supplementary Fig. 28**). The HPLC chromatogram of Alexa-RVRR and the corresponding UV-Vis spectra are shown in **Supplementary Fig. 29.**

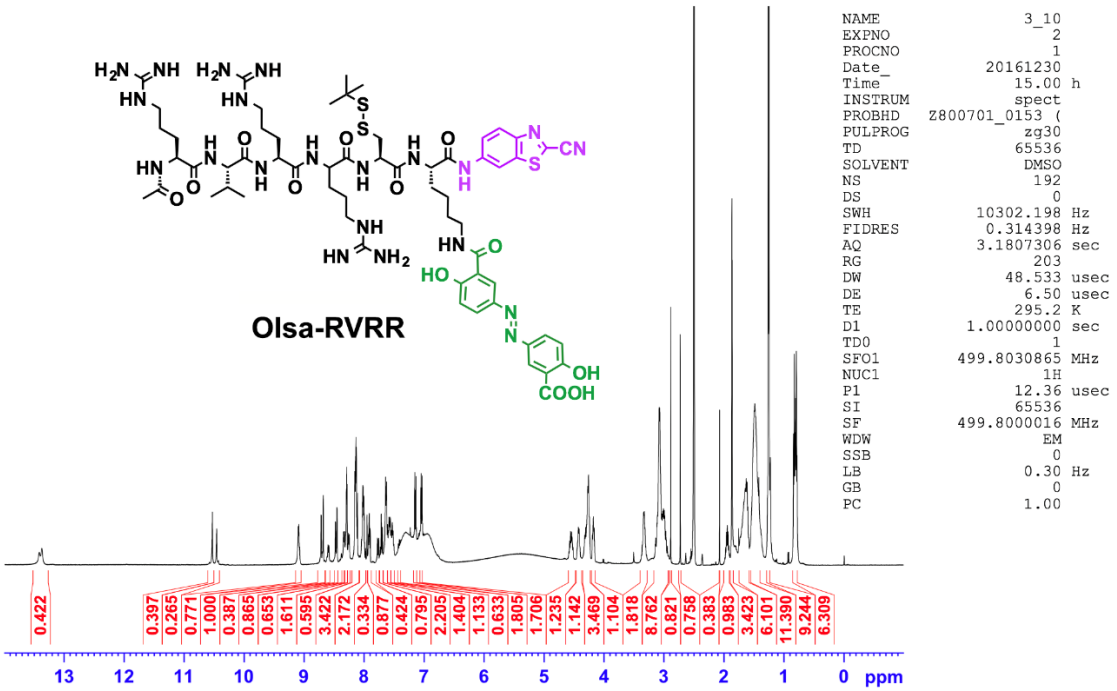
^{1}H-NMR of Alexa-RVRR ($C_{67}H_{89}N_{21}O_{17}S_5$, d_6-DMSO, 500 MHz, **Supplementary Fig. 30**): 10.52 (s, 1H), 8.87 (s, 1 H), 8.67 (m, 2 H), 8.21 (m, 8 H), 7.94 (s, 1 H), 7.77 (d, J = 9 Hz, 1 H), 7.65 (d, J = 8 Hz, 1 H), 7.54 (s, 1 H), 7.44 (s, 2 H), 7.34 (d, J = 7 Hz, 1 H), 6.89 (m, 7 H), 4.56 (dd, J_1 = 8 Hz, J_2 = 13 Hz, 1 H), 4.43 (dd, J_1 = 8 Hz, J_2 = 13 Hz, 1 H), 4.30 (m, 3 H), 4.17 (t, J = 8 Hz, 1 H), 3.08 (m, 7 H), 2.90 (s, 2 H), 2.74 (s, 2 H),

2.57 (t, J = 6 Hz, 7 H), 2.37 (s, 1 H), 1.98 (m, 1 H), 1.87 (s, 4 H). 1.75 (m, 1 H), 1.65 (m, 4 H), 1.50 (m, 8 H), 1.47 (s, 3 H), 1.26 (s, 9 H), 1.06 (t, J = 7 Hz, 1 H), 0.94 (d, J = 7 Hz, 1 H), 0.81 (m, 6 H).

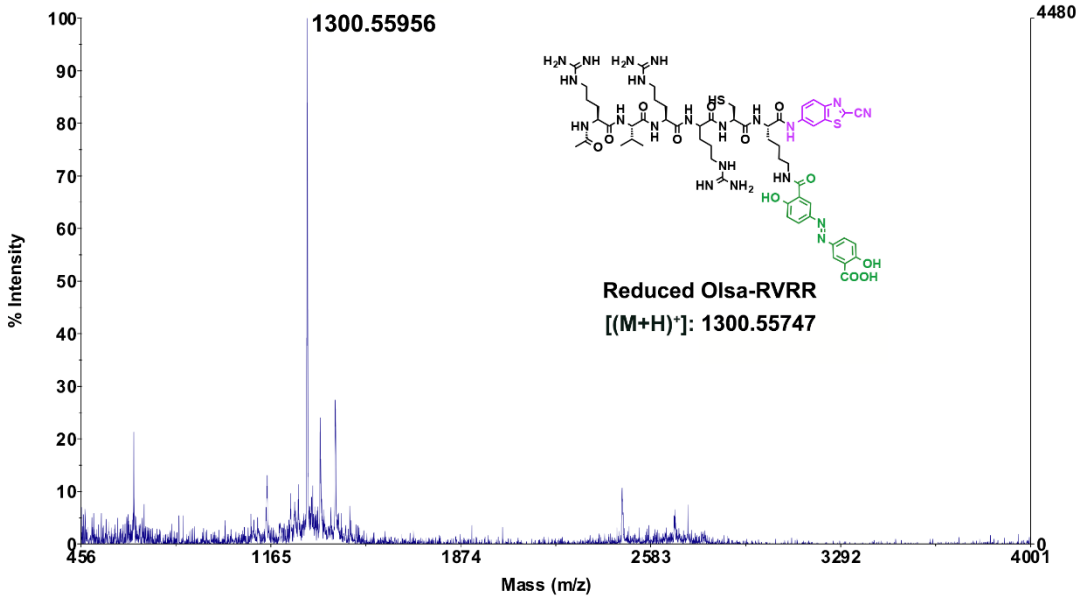
3. Supplementary figures



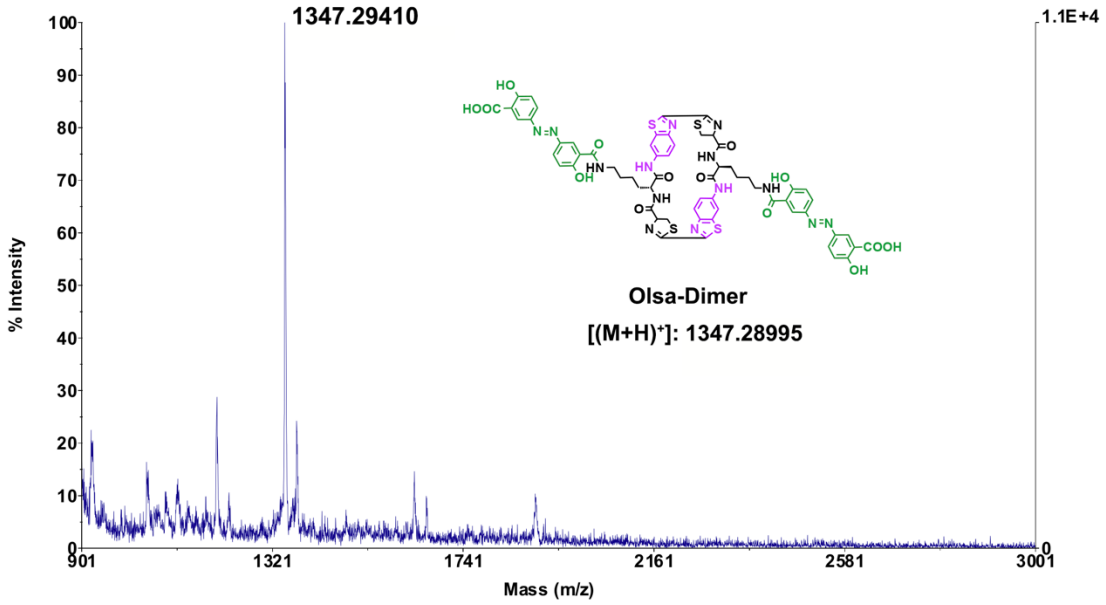
Supplementary Figure 1. **HR-MALDI-TOF/MS spectrum of Olsa-RVRR.** The spectrum reflects representative data from *in vitro* experiments repeated three times.



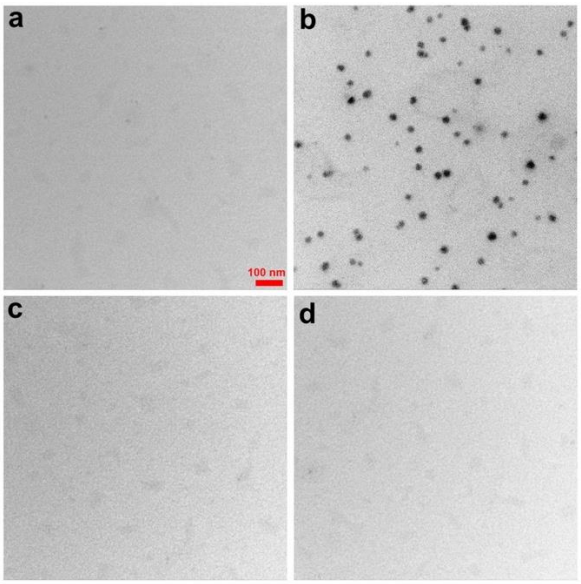
Supplementary Figure 2. **^{1}H NMR spectrum of Olsa-RVRR.** The spectrum reflects representative data from *in vitro* experiments repeated two times.



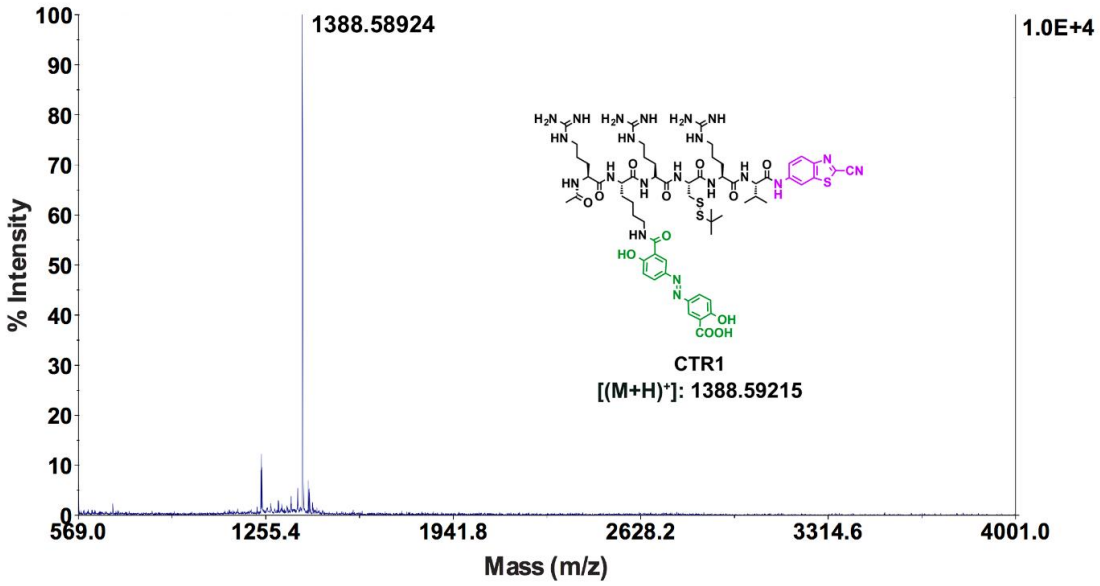
Supplementary Figure 3. **HR-MALDI-TOF/MS spectrum of reduced Olsa-RVRR.** The spectrum reflects representative data from *in vitro* experiments repeated three times.



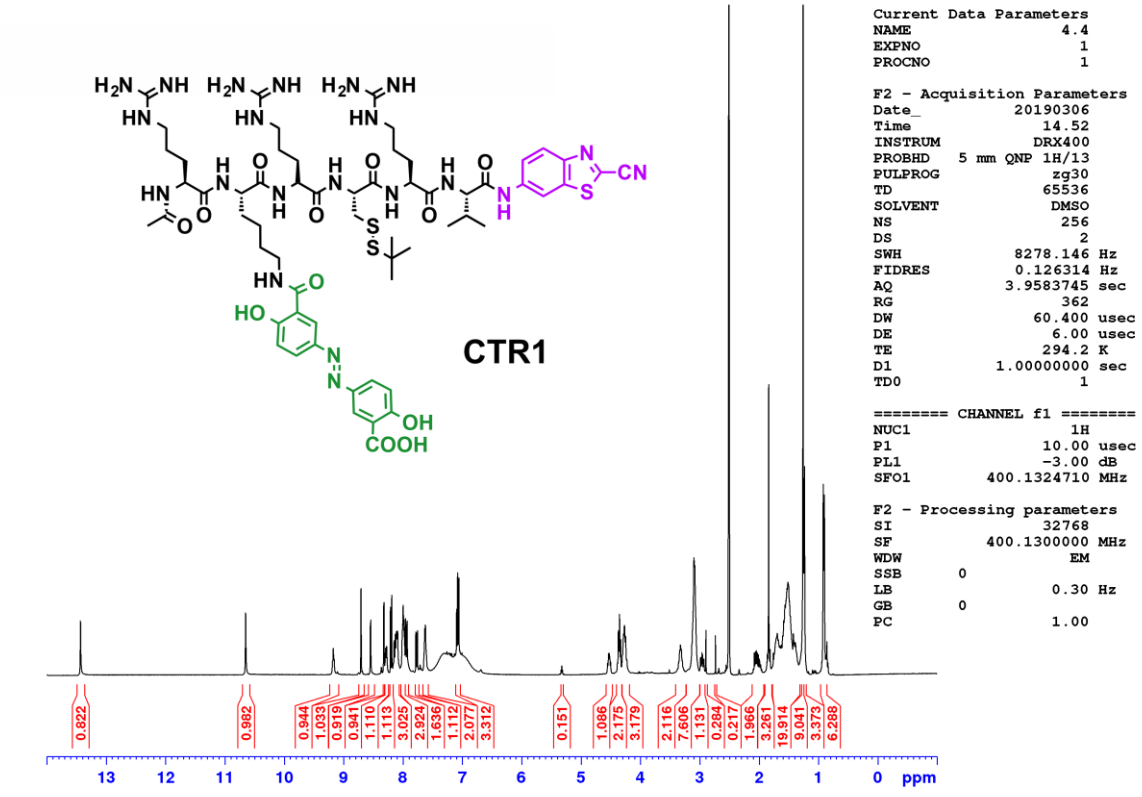
Supplementary Figure 4. **HR-MALDI-TOF/MS spectrum of Olsa-Dimer.** The spectrum reflects representative data from *in vitro* experiments repeated three times.



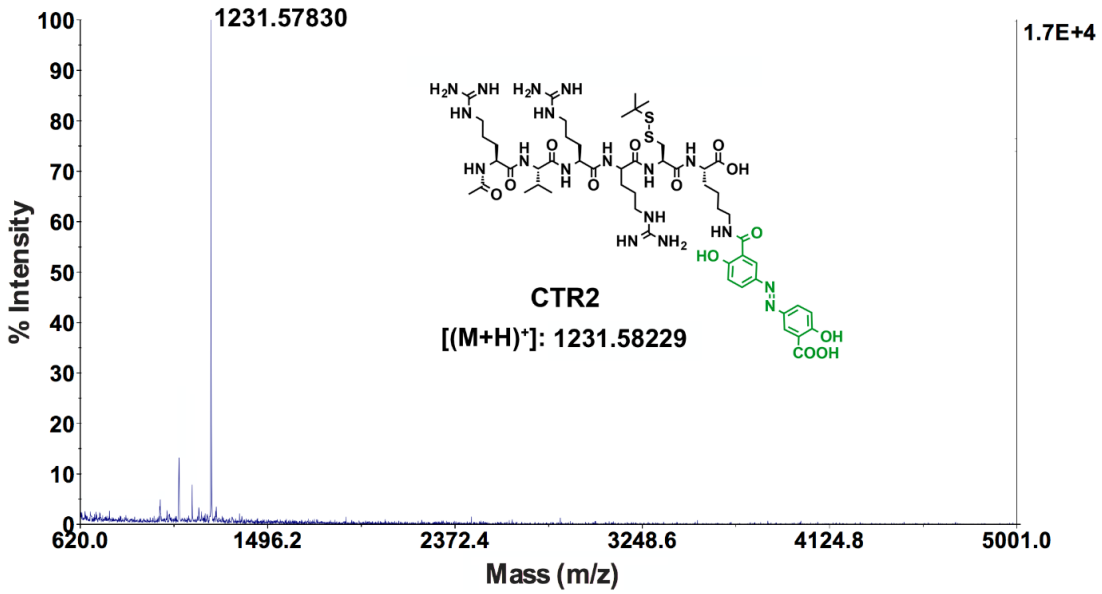
Supplementary Figure 5. **TEM imaging of (lack of) nanoparticle formation.** Shown are Olsa-RVRR samples (a) before and (b) after incubation with 250 µM GSH and 0.5 nmol U^{-1} furin for 12 h in furin buffer, and samples of (**c**) CTR1 and (**d**) CTR2 after incubation with 250 µM GSH and 0.5 nmol U^{-1} furin for 12 h in furin buffer. Shown are representative data from *in vitro* experiments repeated three times.



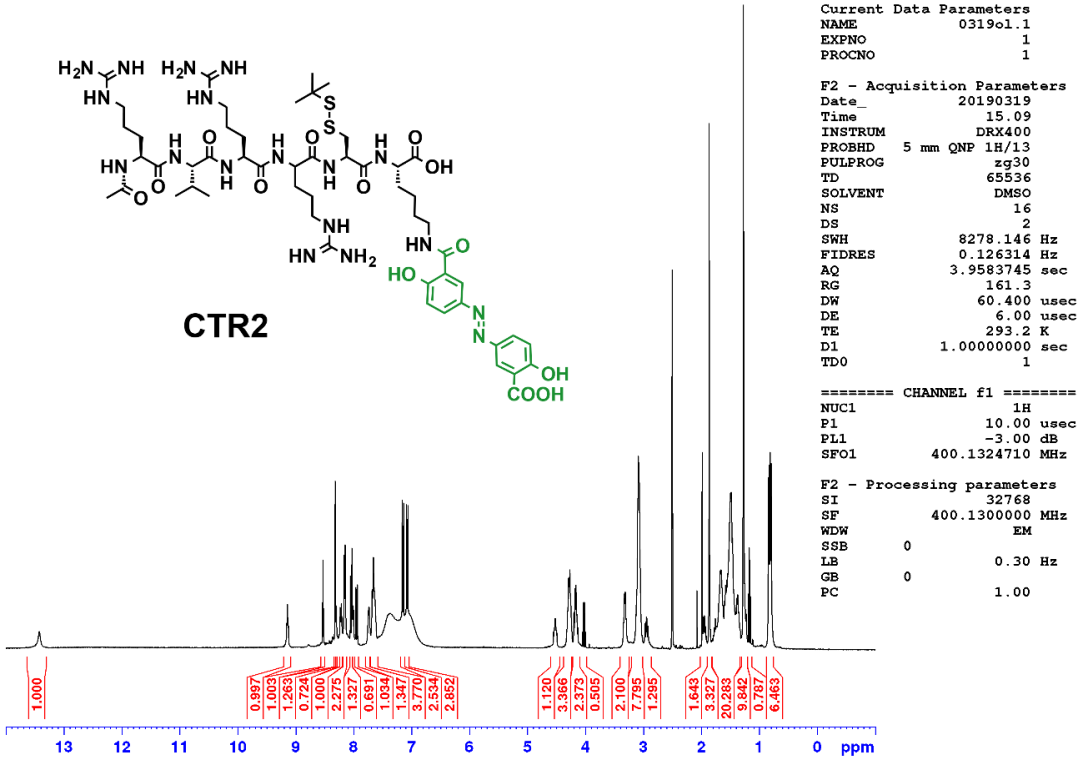
Supplementary Figure 6. **HR-MALDI-TOF/MS spectrum of CTR1.** The spectrum reflects representative data from *in vitro* experiments repeated three times.



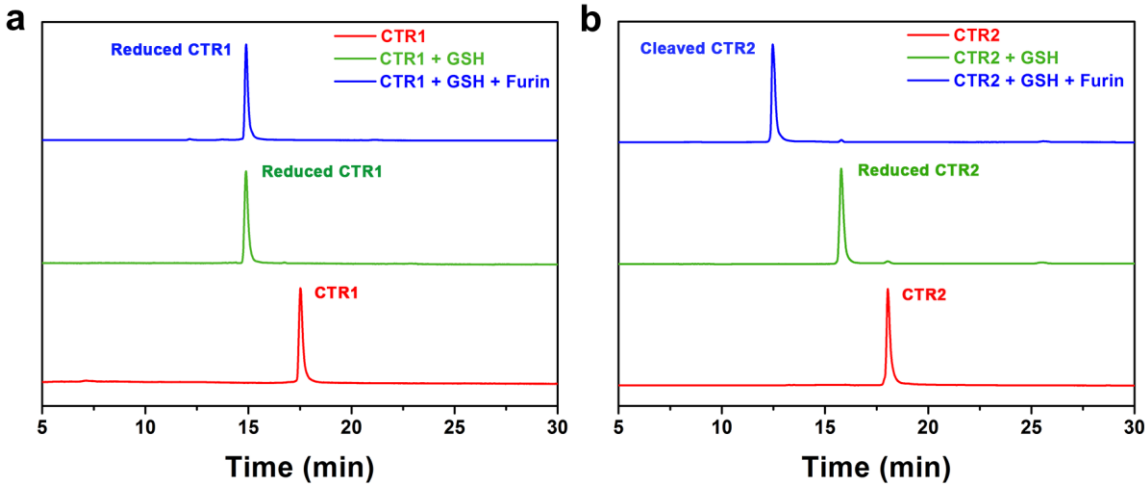
Supplementary Figure 7. **^{1}H NMR spectrum of CTR1.** The spectrum reflects representative data from *in vitro* experiments repeated two times.



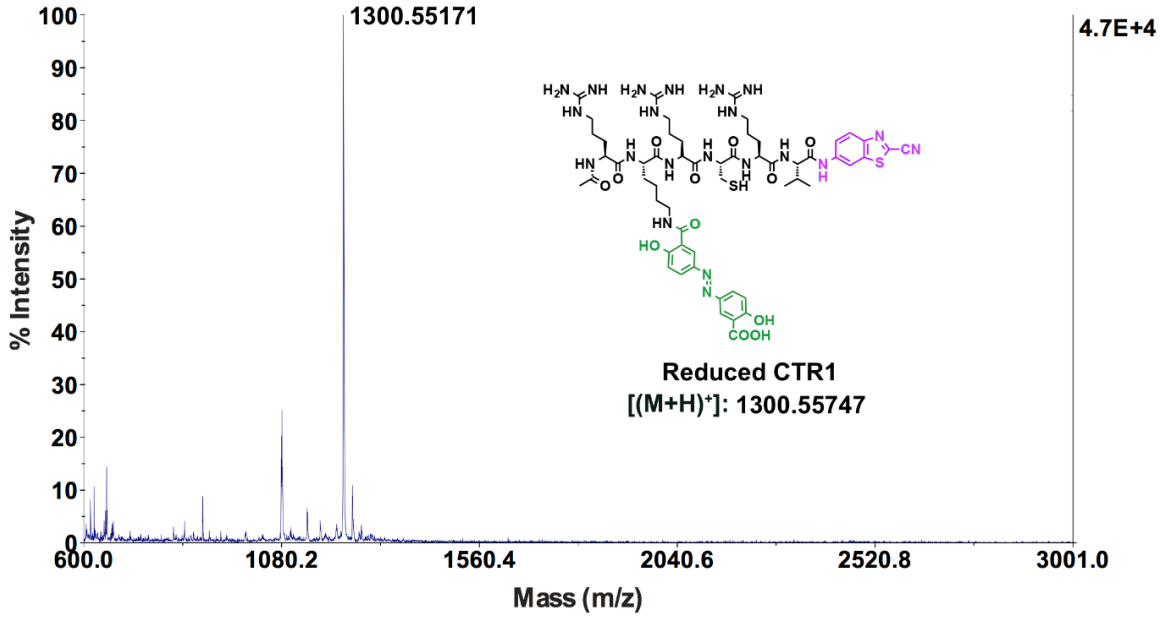
Supplementary Figure 8. **HR-MALDI-TOF/MS spectrum of CTR2.** The spectrum reflects representative data from *in vitro* experiments repeated three times.



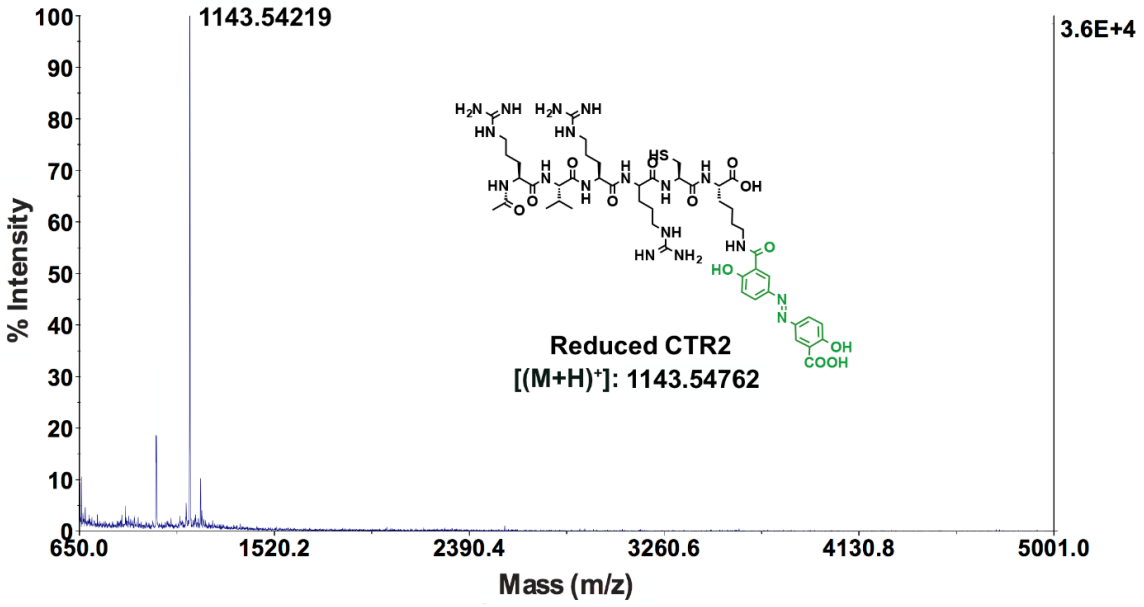
Supplementary Figure 9. **^{1}H NMR spectrum of CTR2.** The spectrum reflects representative data from *in vitro* experiments repeated two times.



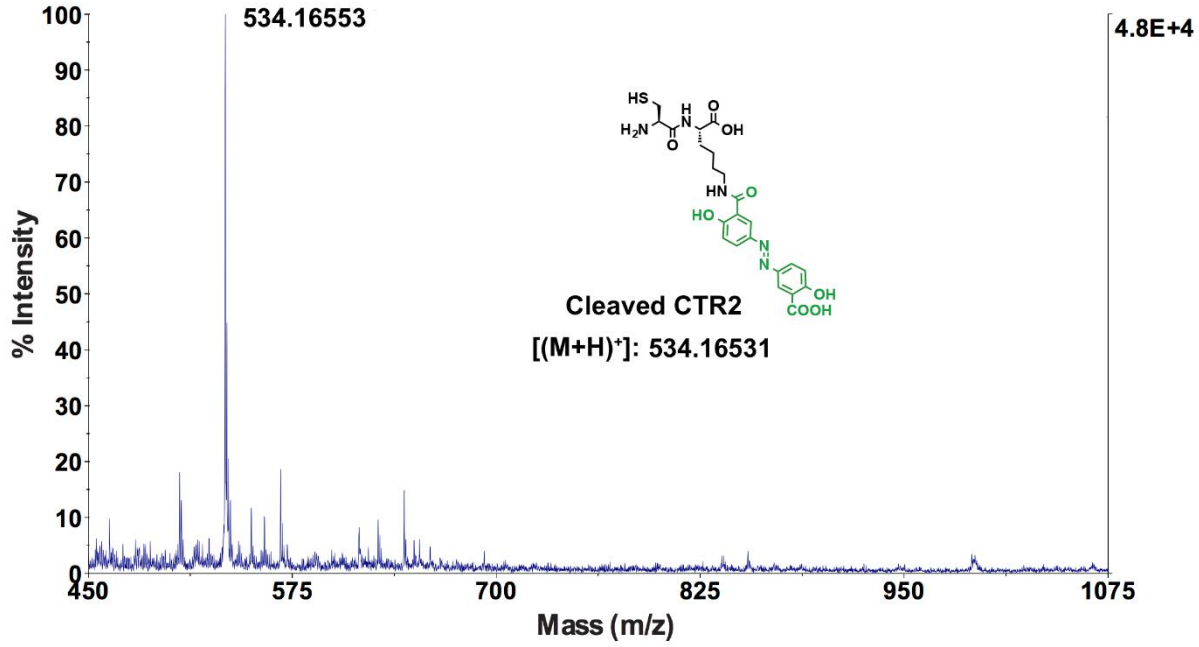
Supplementary Figure 10. **HPLC chromatograms of control compounds before and after GSH reduction and furin cleavage.** (**a**) HPLC chromatogram of 25 μM CTR1 (red), 25 μM CTR1 + 250 μM GSH incubated for 2 h (green), and 25 μM CTR1 + 250 μM GSH + 0.5 nmol U^{-1} furin incubated for 12 h (blue). (**b**) HPLC chromatogram of 25 μM CTR2 (red), 25 μM CTR2 + 250 μM GSH incubated for 2 h (green), and 25 μM CTR2 + 250 μM GSH + 0.5 nmol U^{-1} furin incubated for 12 h (blue). All chromatograms are representative data from *in vitro* experiments repeated three times.



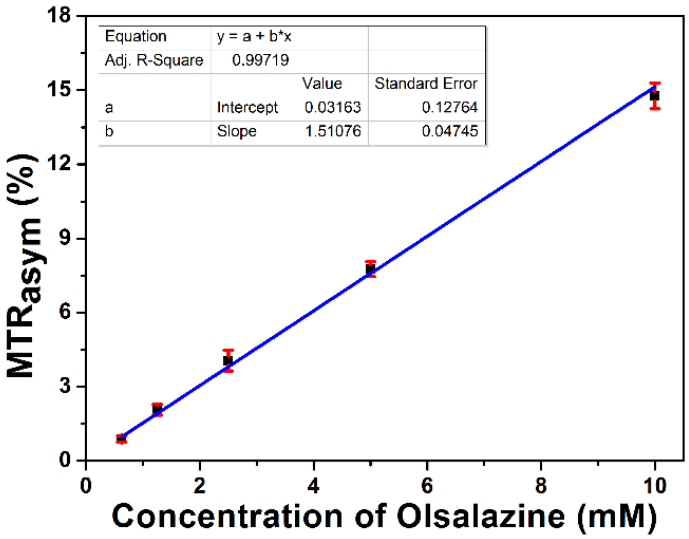
Supplementary Figure 11. **HR-MALDI-TOF/MS spectrum of reduced CTR1.** The spectrum reflects representative data from *in vitro* experiments repeated three times.



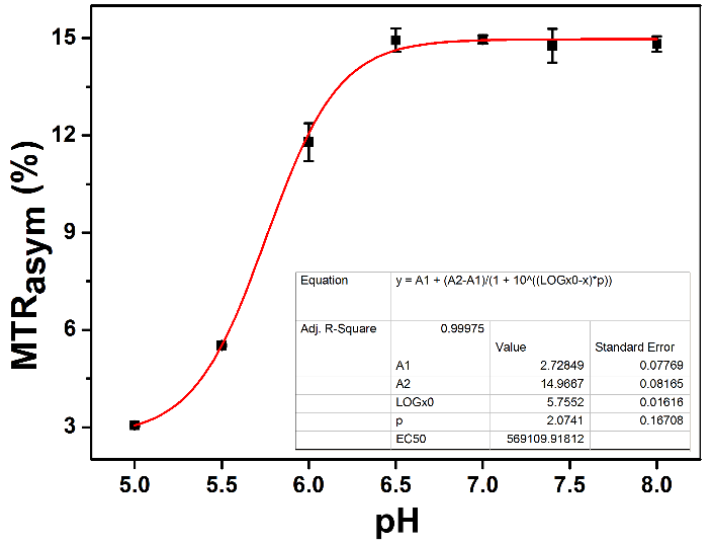
Supplementary Figure 12. **HR-MALDI-TOF/MS spectrum of reduced CTR2.** The spectrum reflects representative data from *in vitro* experiments repeated three times.



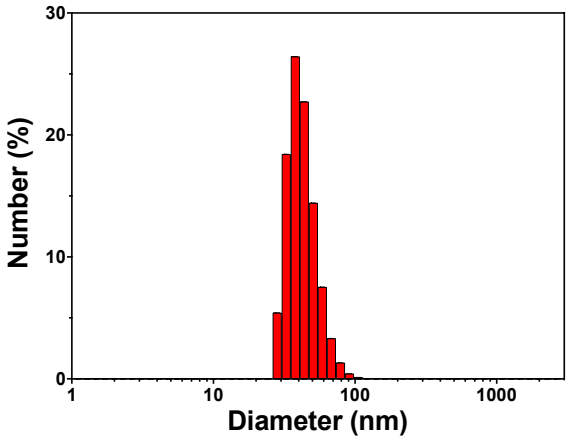
Supplementary Figure 13. **HR-MALDI-TOF/MS spectrum of cleaved CTR2.** The spectrum reflects representative data from *in vitro* experiments repeated three times.



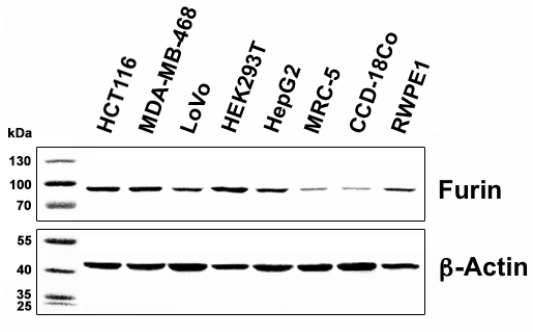
Supplementary Figure 14. **Correlation between MTR_{asym} values (9.8 ppm) and olsalazine at different concentrations in PBS.** Data are shown as mean±SD (n=3 independent *in vitro* experiments).



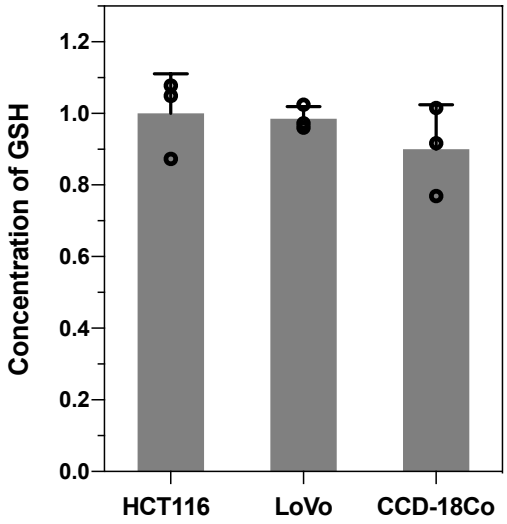
Supplementary Figure 15. **MTR_{asym} values at 9.8 ppm for 10 mM olsalazine at different pH values.** Data are shown as mean±SD (n=3 independent *in vitro* experiments).



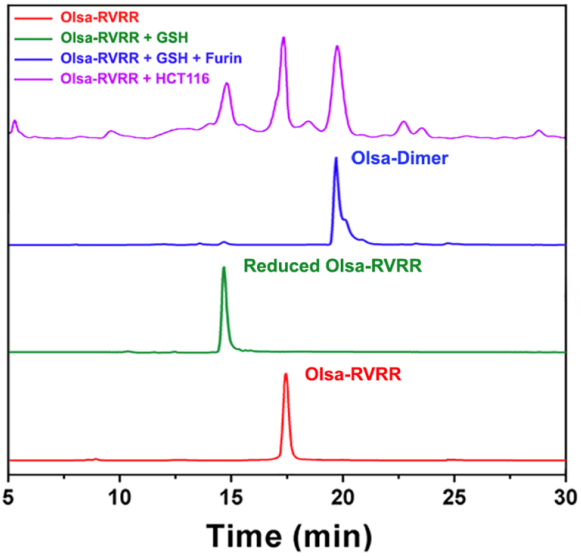
Supplementary Figure 16. **DLS size distribution of Olsa-NPs after incubation of 5 mM Olsa-RVRR with 10 mM GSH and 2 nmol U^{-1} furin.** The figure reflects representative data from *in vitro* experiments repeated three times.



Supplementary Figure 17. **Western blot analysis of furin in different cell lines**. The figure reflects representative data from *in vitro* experiments repeated three times.

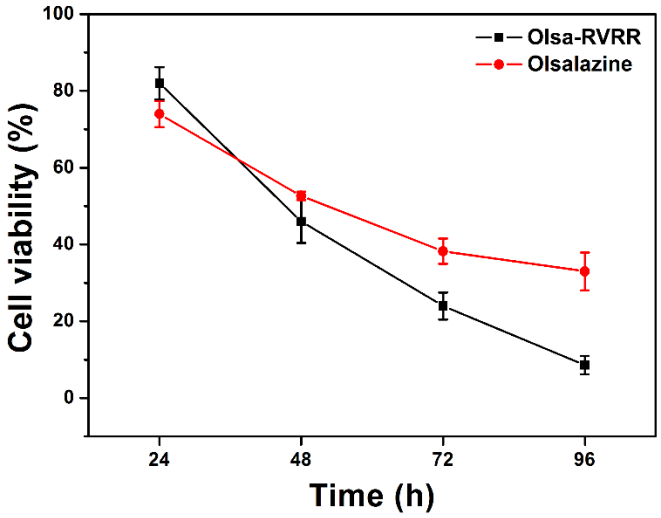


Supplementary Figure 18. **Normalized GSH levels of furin-overexpressing HCT116 cells, furin-lowexpressing LoVo cells, and furin-deficient CCD-18Co cells.** Data are shown as mean±SD (n=3 independent *in vitro* experiments). *P*>0.05 vs. all other groups (one-way ANOVA with Tukey's HSD multiple comparison post-hoc test).

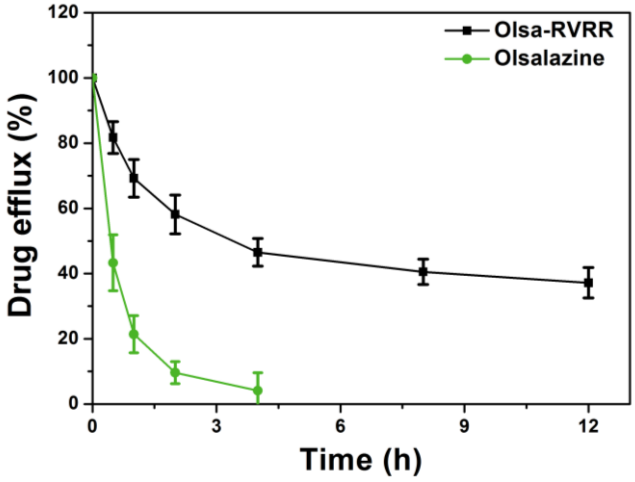


Supplementary Figure 19. **HPLC analysis of cell lysate from Olsa-RVRR treated HCT116 cells.** Shown are the HPLC chromatograms for 25 µM Olsa-RVRR (red), 25 µM Olsa-RVRR + 250 µM GSH incubated for 2 h (green), 25 µM Olsa-RVRR + 250 µM GSH + 0.5 nmol U^{-1} furin incubated for 12 h (blue), and cell lysate of HCT116 cells incubated with 100 µM Olsa-RVRR for 3 h (purple). All incubations were

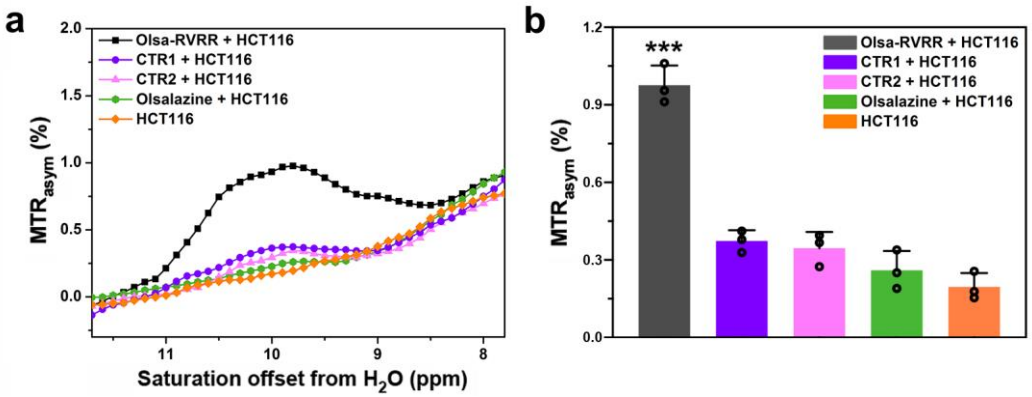
done at 37 °C. All chromatograms reflect representative data from *in vitro* experiments repeated three times.



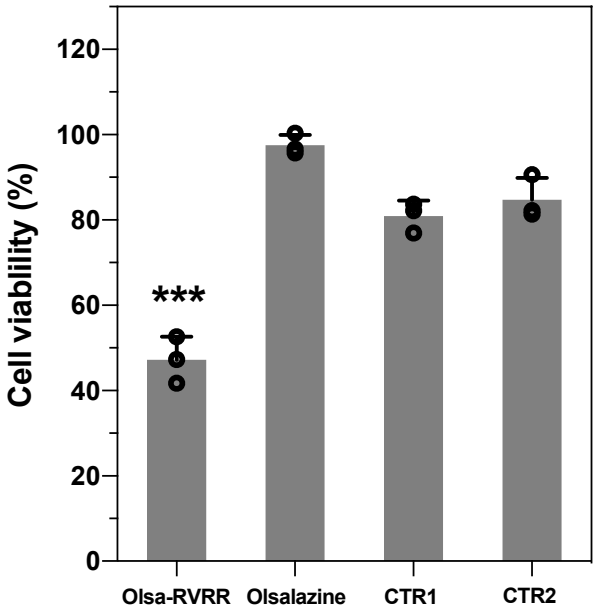
Supplementary Figure 20. **Time-dependent cell viability.** Cell viability of HCT116 cells treated with 250 μM Olsa-RVRR or 5 mM olsalazine for 24 h, 48 h, 72 h, and 96 h. Data are shown as mean±SD (n=3 independent *in vitro* experiments).



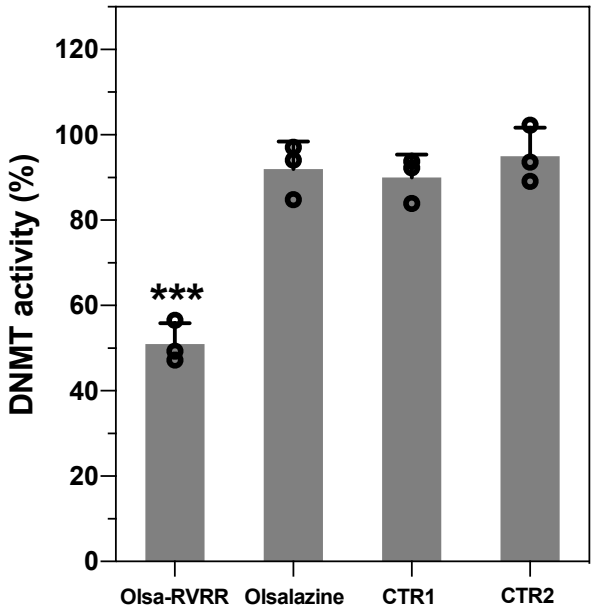
Supplementary Figure 21. **Efflux kinetics of Olsa-RVRR or olsalazine in HCT116 cells.** Data are shown as mean±SD (n=3 independent experiments).



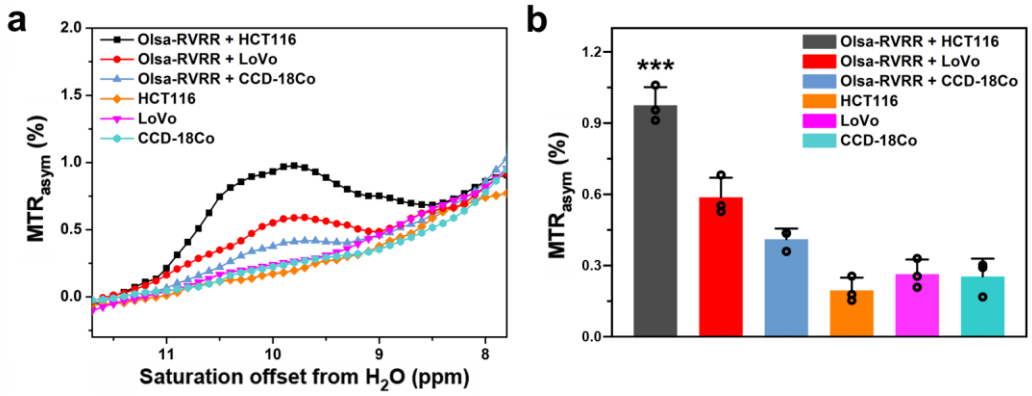
Supplementary Figure 22. **(a) MTR_{asym} spectra and (b) OlsaCEST signal at 9.8 ppm for HCT116 cells incubated with CTR1 and CTR2**. All incubations were performed with 500 μM compound for 3 hours. Data are shown as mean±SD; n=3 independent *in vitro* experiments; one-way ANOVA, followed by Dunnett's post-hoc test; ****P*<0.001 vs. all other groups.



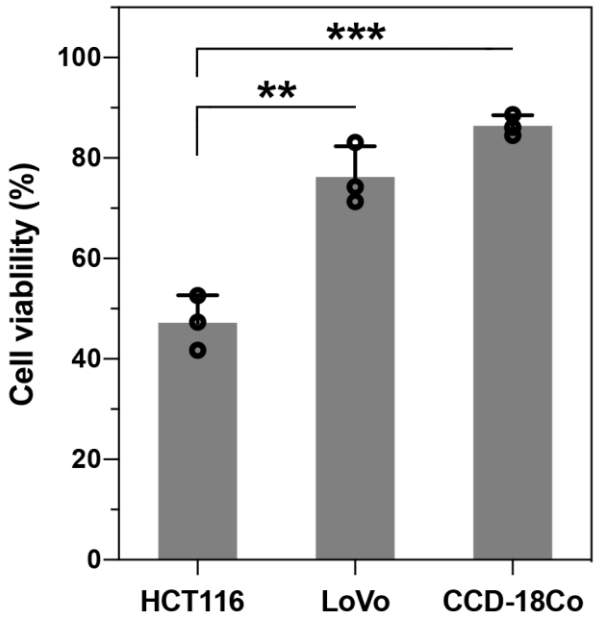
Supplementary Figure 23. **Cell viability of HCT116 cells incubated with 250 μM Olsa-RVRR, olsalazine, CTR1, or CTR2 for 48h.** Data are shown as mean±SD; n=3 independent *in vitro* experiments; one-way ANOVA, followed by Dunnett's post-hoc test; ****P*<0.001 vs. all other groups.



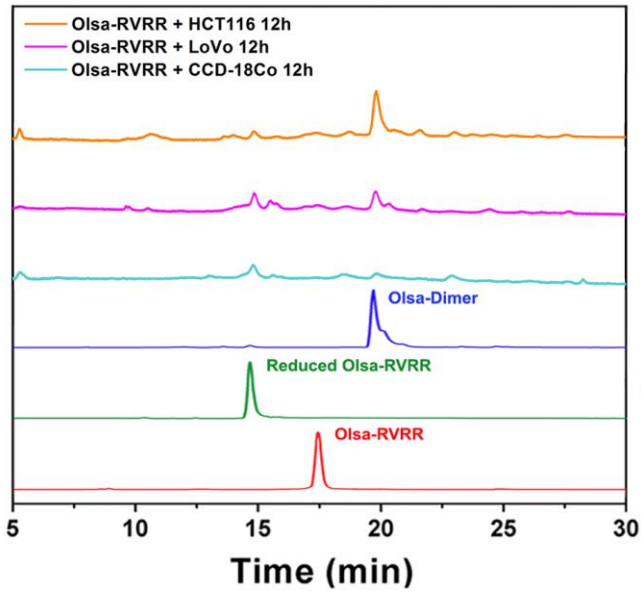
Supplementary Figure 24. **DNMT activity of HCT116 cells was detected 48 hours after incubation with 500 μM Olsa-RVRR, olsalazine, CTR1, or CTR2 for 3 h.** Data are shown as mean±SD; n=3 independent *in vitro* experiments; one-way ANOVA, followed by Dunnett's post-hoc test; ****P*<0.001 vs. all other groups.



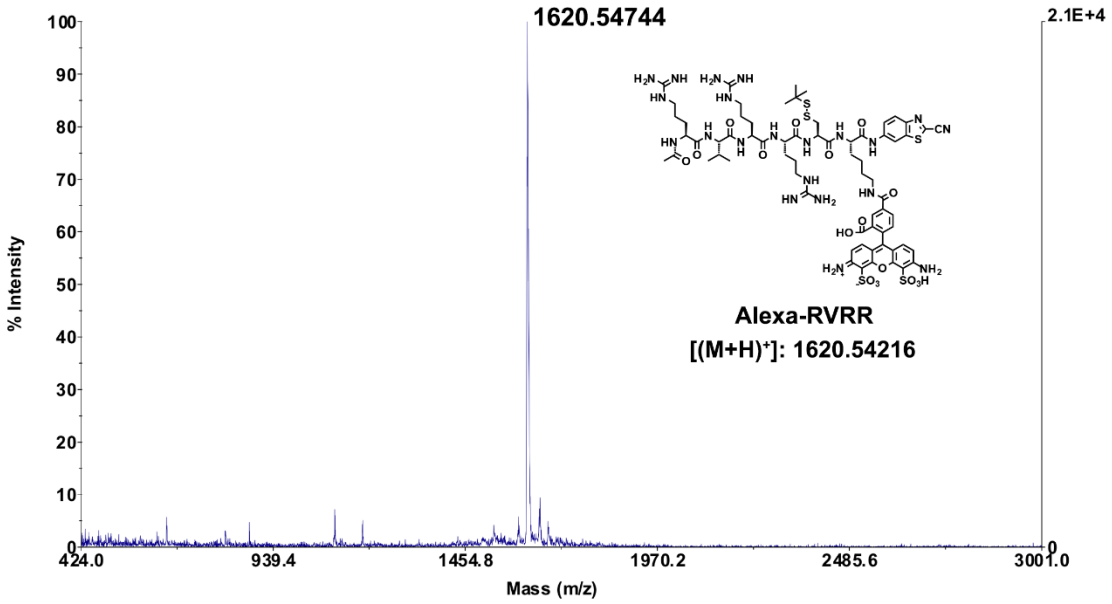
Supplementary Figure 25. **(a) MTR_{asym} spectra and (b) OlsaCEST signal at 9.8 ppm for HCT116, LoVo, and CCD-18Co cells incubated with and without 500 μM Olsa-RVVR.** Data are shown as mean±SD; n=3 independent *in vitro* experiments; one-way ANOVA, followed by Dunnett's post-hoc test; ****P*<0.001 vs. all other groups.



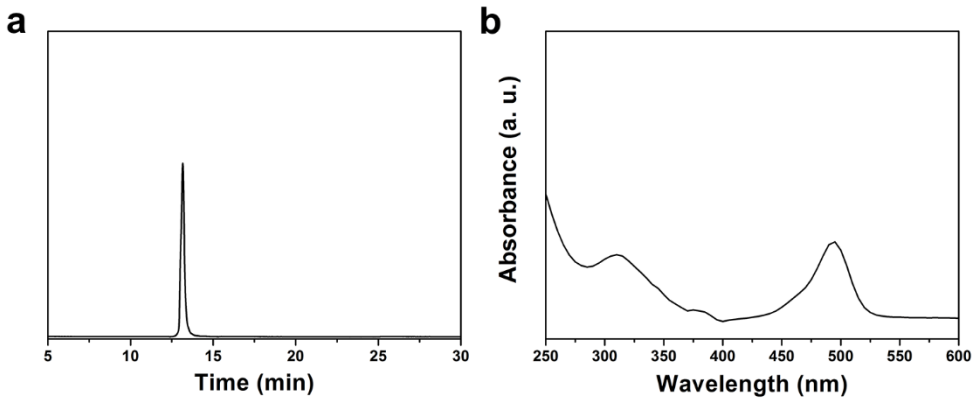
Supplementary Figure 26. **Cell viability (acridine orange/propidium iodide staining) of HCT116, LoVo, and CCD-18Co cells incubated with 250 μM Olsa-RVRR for 48 hours.** Data are shown as mean±SD; n=3 independent *in vitro* experiments; two-tailed Student's t-test; ***P*=0.0036; ****P*=0.0003.



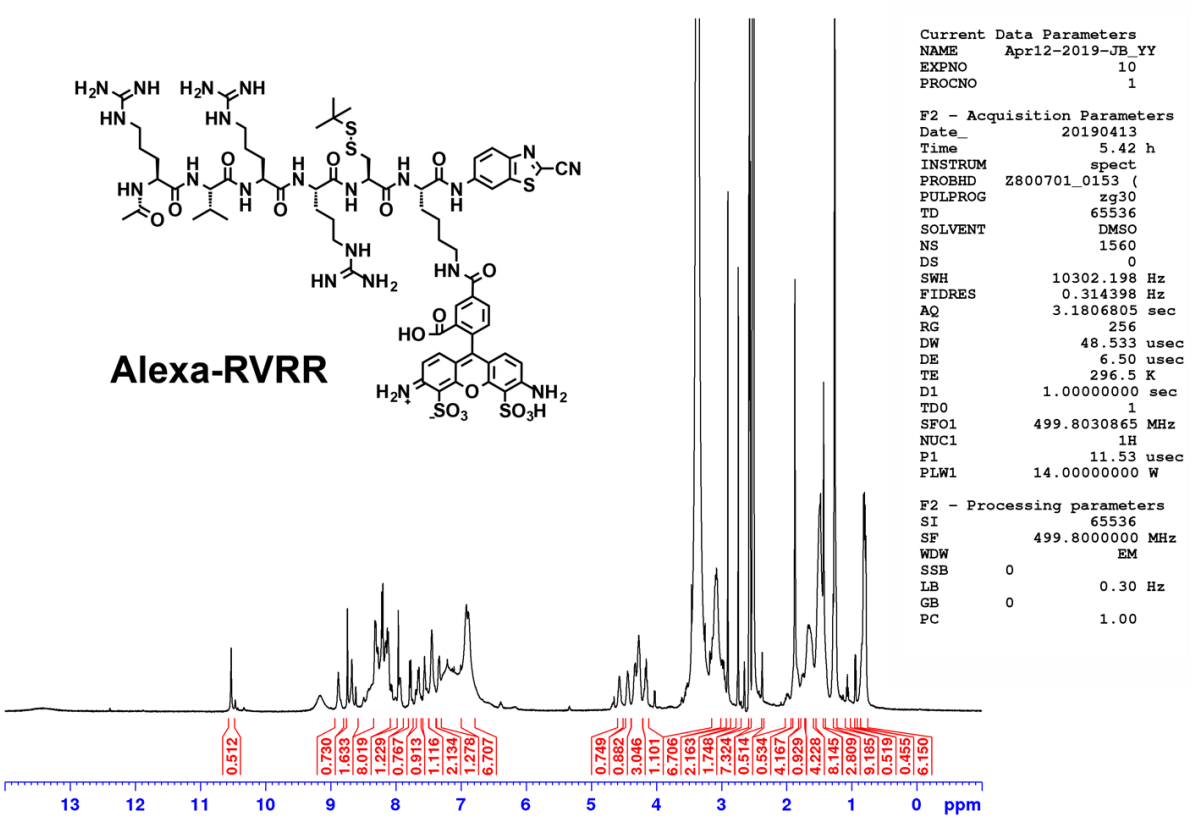
Supplementary Figure 27. **HPLC analysis of HCT116, LoVo, and CCD-18Co cell lysates after incubation with 100 μM Olsa-RVRR at 37 °C for 12 h.** All chromatograms reflect representative data from *in vitro* experiments repeated three times.



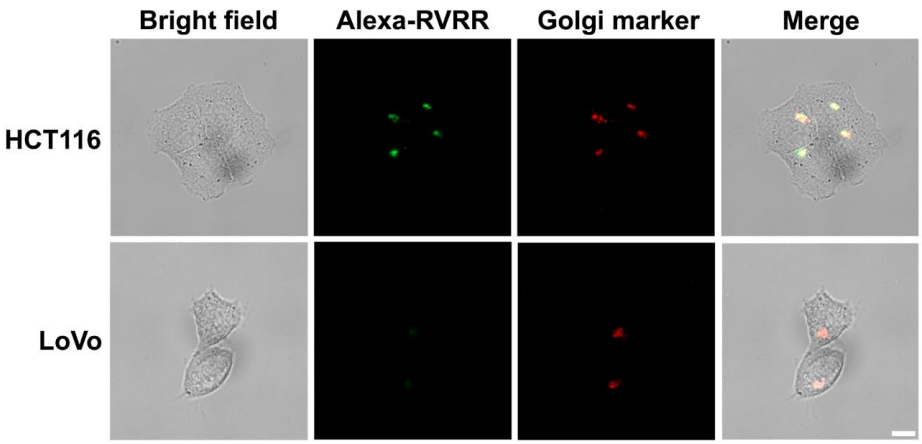
Supplementary Figure 28. **HR-MALDI-TOF/MS spectrum of Alexa-RVRR.** The spectrum reflects representative data from *in vitro* experiments repeated three times.



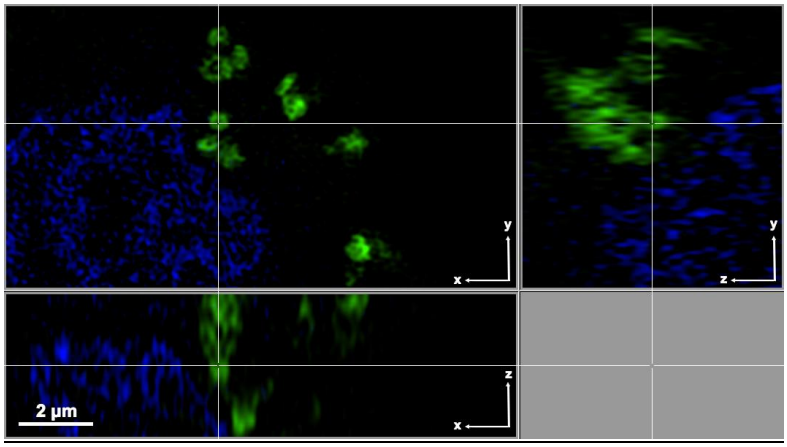
Supplementary Figure 29. **(a) HPLC chromatogram of Alexa-RVRR and (b) corresponding absorption spectra.** Both subpanels reflect representative data from *in vitro* experiments repeated three times.



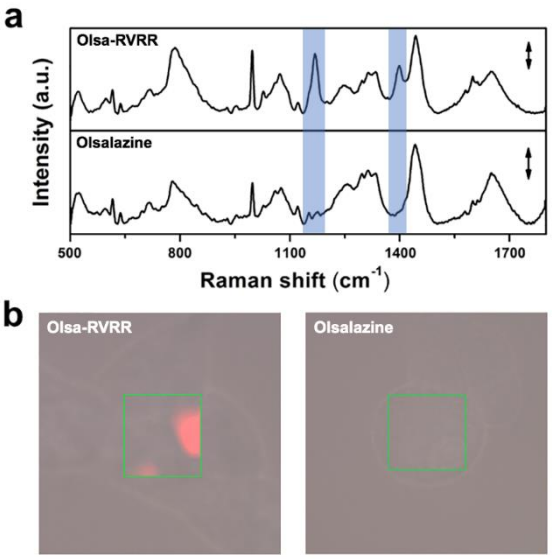
Supplementary Figure 30. **^{1}H NMR spectrum of Alexa-RVRR.** The spectrum reflects representative data from *in vitro* experiments repeated two times.



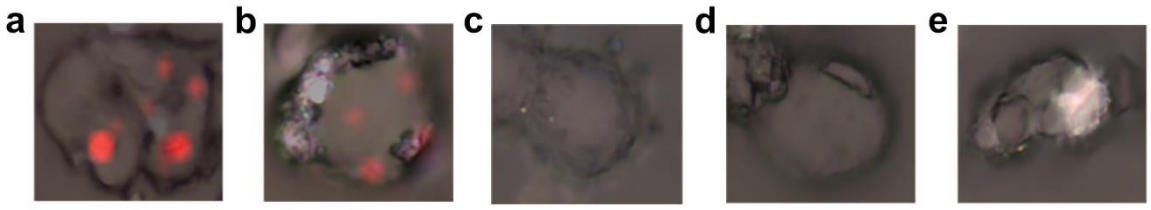
Supplementary Figure 31. **Imaging of furin-induced localized accumulation of nanoparticles in live cells.** HCT116 or LoVo cells were incubated with 5 µL Golgi marker (Red, Golgi-RFP, BacMam 2.0, Invitrogen) for 24 h. After washing three times with PBS, cells were further incubated with Alexa-RVRR (Green, 8 µM) for 3 h in culture medium. Green and red fluorescence mages were obtained with 488 and 555 nm excitation channels, respectively. Scale bar=10 µm. All subpanels reflect representative data from *in vitro* experiments repeated three times.



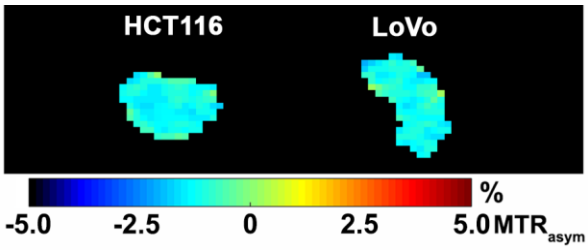
Supplementary Figure 32. **3D high-magnification 3D-SIM images of Alexa-RVRR-incubated HCT116 cells shown in Fig. 3h of the main manuscript.** Upper left panel shows *xy* slices, upper right and lower left panels show orthogonal *yz* and *xz* views of the processed *z* stack, respectively. As can be seen in the *yz* and *xz* views, the larger intracellular fluorescent dots represent clusters of individual Alexa488 nanoparticles, with a diameter of ~100 nm in the *x* or *y* dimension, which is the resolution limit of 3D-SIM. This figure reflects representative data from *in vitro* experiments repeated three times.



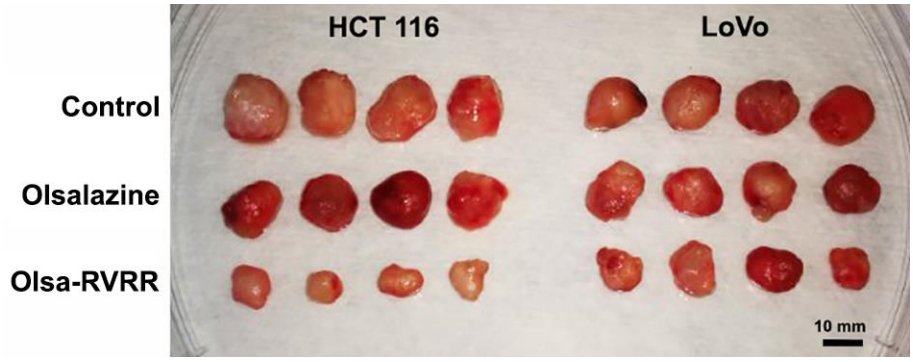
Supplementary Figure 33. **Raman imaging of Olsa-RVRR or olsalazine-treated live cells.** (a) Raman spectra from HCT116 cells after incubation of 100 μM Olsa-RVRR or olsalazine for 3 h. The modes arising from the nanoparticles are marked in blue. Arrows correspond to an intensity of 75 adu*$mW^{-1}s^{-1}$. (b) Bright-field images superimposed on Raman images (red color corresponds to the nanoparticle peak intensity at 1168 cm^{-1}) of 100 μM Olsa-RVRR or olsalazine-treated cells. All subpanels reflect representative data from *in vitro* experiments repeated three times.



Supplementary Figure 34. **Raman imaging of Olsa-RVRR or olsalazine-treated fixed cells.** Shown are bright-field images superimposed on Raman images (red color corresponds to the nanoparticle peak intensity at 1168 cm^{-1}) of (a) 100 μM Olsa-RVRR-incubated HCT116 cells, (b) 100 μM Olsa-RVRR-incubated LoVo cells, (c) 100 μM olsalazine-incubated HCT116 cells, (d) untreated HCT116 cells, and (e) untreated LoVo cells. The incubation time for each group was 3 hours. All subpanels reflect representative data from *in vitro* experiments repeated three times.



***Supplementary Figure 35. Ex vivo* OlsaCEST MRI of HCT116 and LoVo tumors 0.5 h after i.v. injection of 0.2 mmol/kg olsalazine.** This figure reflects representative *ex vivo* imaging data from experiments repeated four times.



***Supplementary Figure 36.* Tumor size measurements.** *Ex vivo* macroscopic images of tumors for each treatment group 33 days after i.v. injection of PBS alone (control), 0.1 mmol/kg olsalazine, or 0.1 mmol/kg Olsa-RVRR (Q3D × 8 doses). All tumors for n=12 (4 cohorts, 3 treatment groups) mice are shown.