Sequential metabolic probes illuminate nuclear DNA for discrimination of cancerous and normal cells

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1. Supplementary methods

Materials and general methods

Unless specially stated, all solvents and chemicals were purchased from commercial suppliers in analytical grade and used without further purification. The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AM 400 spectrometer, using TMS as an internal standard. High-resolution mass spectrometry (HRMS) data were obtained with a Waters LCT Premier XE spectrometer and a Thermo Fisher (Q EXACTIVE Plus). Absorption spectra were collected on a Varian Cary 500 spectrophotometer, and fluorescence spectra measurements were performed on a Varian Cary Eclipse fluorescence spectrophotometer. Confocal fluorescence images were taken on a confocal laser scanning microscope (Leica confocal microscope STELLARIS 8 FALCON). The super-resolution fluorescence images were taken on a structured illumination microscopy (Elyra 7 with Lattice SIM). *In vivo* fluorescence images were acquired by using PerkinElmer IVIS Lumina Kinetic Series III imaging system.

HPLC analysis of VdU-Lys reaction with HDAC1 and Cathepsin L protease.

Human recombinant histone deacetylase 1 was used as a model HDAC enzyme (Sigma-Aldrich; SRP5265). Human recombinant Cathepsin L protease was purchased from Sigma-Aldrich (SRP0291). First, VdU-Lys (200 μg) and HDAC1 (0.1 μg) were incubated in buffer containing protein in reaction buffer including 25 mM Tris-HCl pH 8.0, 137 mM NaCl, 2.7 mM $MgCl_2$, 1 mM KCl and 0.1 mg/mL BSA for 30 min at 37 °C for three hours.

The solution was then filtered over a size-exclusion column and the supernatant lyophilized to dryness. To the dried pellet was added buffer solution for Cathepsin L protease (50 mM MES, 5 mM DTT, 1 mM EDTA, 0.005% (w/v) Brij-35, pH 6.0) and 0.001 μg of Cathepsin L protease was added and incubated at 37 °C for 3 hours. The solution was spun down and decanted for HPLC loading.

Reactions were analyzed by analytical HPLC chromatography with the methanol/water (4/1, v/v) buffer for 30 minutes. Product identities were confirmed by comparing retention times with controls and mass spectrometry.

Spectral measurements

Et-PT-Tz were respectively dissolved in DMSO to obtain stock solutions (1.0 mM) and diluted to 10 μM for all spectral studies. 1.0 cm square quartz cell was used for all optical measurements. Absorption spectra were collected on a Varian Cary 500 spectrophotometer, and fluorescence spectra measurements were performed on a Varian Cary Eclipse fluorescence spectrophotometer.

Kinetic measurements with the VdU and Et-PT-Tz

Second order reaction rates between Et-PT-Tz and VdU were determined under pseudo first order conditions with a large excess of the dienophile over the tetrazine. The increase of emission at 550 nm (λ_{ex} = 450 nm) was used to monitor the progress of the reaction. Measurements were performed in 96 well plates covered with 20 μL spectroscopic grade silicon oil to avoid evaporation. Solutions contained final concentrations of 100 μM Et-PT-Tz with 0-5 mM VdU (0-50 eq) in PBS 9:1 DMSO. Samples were measured over 1300 min in intervals of 10 min. Control conditions (0 mM VdU) indicate full stability and negligible background signal of Et-PT-Tz under these conditions. Reactions rates were

calculated using pseudo first order approximations fitting the measurements to mono exponential equations.

Cell culture and treatment

Human hepatoma cell line HepG2, adenocarcinomic human alveolar basal epithelial cell line A549, human epithelioid cervical carcinoma cell line HeLa, human colorectal carcinoma cell line HCT116, human embryonic kidney 293T cell lines, human kidney cell line HK2, human keratinocyte cell line HaCaT, and simian vacuolating virus 40-transformed human urothelial cell line-1 SV-Huc-1 were purchased from the Institute of Cell Biology. HepG2, A549, HCT116, HeLa, HEK 293T, HaCaT, and HK2 cells were propagated in T-25 flasks cultured at 37 ^{o}C under a humidified 5% CO_2 atmosphere in DMEM medium (GIBCO/Invitrogen, Camarillo, CA, USA), which were supplemented with 10% fetal bovine serum (FBS, Biological Industry, Kibbutz Beit Haemek, Israel) and 1% penicillin-streptomycin (10,000 U mL^{-1} penicillin and 10 mg mL^{-1} streptomycin, Solarbio life science, Beijing, China). SV-Huc-1 cells were propagated in T-25 flasks cultured at 37 ^{o}C under a humidified 5% CO_2 atmosphere in F-12K medium (GIBCO/Invitrogen, Camarillo, CA, USA), which were supplemented with 10% fetal bovine serum (FBS, Biological Industry, Kibbutz Beit Haemek, Israel) and 1% penicillin-streptomycin (10,000 U mL^{-1} penicillin and 10 mg mL^{-1} streptomycin, Solarbio life science, Beijing, China).

MTT assay

The cell cytotoxicity of VdU, VdU-Lys, and Et-PT-Tz to two cancerous cell lines, including HepG2 and HeLa cells, and one noncancerous cell lines HK2 cell was measured by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were plated in 96-well plates in 0.1 mL volume of DMEM medium without 10 % FBS, at a density of 1×10^4 cells/well and added with desired concentrations of VdU, VdU-Lys, or Et-PT-Tz. After incubation for 24 h, change the culture solution to DMSO and continue for 3-4 hours. Absorbance was measured at 450 nm for VdU and VdU-Lys, and 590 nm for Et-PT-Tz with a Tecan GENios Pro multifunction reader (Tecan Group Ltd., Maennedorf, Switzerland). Each concentration was measured in triplicate and used in three independent experiments. The relative cell viability was calculated by the equation: cell viability (%) = $(OD_{treated}/OD_{control}) \times 100\%$.

Confocal fluorescence imaging

The cells at 1×10^5 cells/well were seeded onto glass-bottom petri dishes with complete medium (1.0 mL) for 12 h. Then the cells were exposed to desired concentrations (100 μM) of VdU or VdU-Lys for 24 h. PBS (pH 7.4) was used to wash cells three times to clean the background. Cells were fixed in 4% paraformaldehyde and washed with 0.2% Triton X-100 in PBS for 5 min, then denatured using 2 M HCl for 30 min at room temperature. Cells were then washed once with PBS, neutralized with 0.1 M aq. Borax ($Na_2B_4O_7\bullet10H_2O$) solution for 10 min and washed twice with PBS. Then the cells were exposed to desired concentrations (10 μM) of Et-PT-Tz for 5 h. PBS (pH 7.4) was used to wash cells three times to clean the background. PBS was added to culture cells. The fluorescence images were photographed by using a confocal laser scanning microscope (CLSM, Leica confocal microscope STELLARIS 8 FALCON). λ_{ex} = 450 nm, λ_{em} = 500-650 nm.

Colocalization experiments

Cells were treated by method of "Confocal fluorescence imaging", and then the medium was replaced with fresh medium in the presence of Hoechst 33342 (1 μM) or propidium iodine (PI, 10 μg/mL) for 30 min. PBS (pH 7.4) was used to wash cells three times to clean the background. Then cells were the confocal fluorescence imaged. Hoechst 33342, λ_{ex} = 405 nm, λ_{em} = 440-500 nm; PI, λ_{ex} = 435 nm; λ_{em} = 590-630 nm; Et-PT-Tz, λ_{ex} = 450 nm, λ_{em} = 500-650 nm.

SIM imaging

Cells were treated by method of "Confocal fluorescence imaging", and then the medium was replaced with fresh medium in the presence of Hoechst 33342 (1 μM) for 30 min. PBS (pH 7.4) was used to wash cells three times to clean the background. The super-resolution fluorescence images were photographed by using a structured illumination microscopy (Elyra 7 with Lattice SIM). Hoechst, λ_{ex} = 405 nm; λ_{em} < 560 nm; Et-PT-Tz. λ_{ex} = 488 nm; λ_{em} > 560 nm.

IP injection experiment

The nude mice were injected with HepG2 cells into mammary pads once on each side. Once tumors reached appreciable size, the mice received direct peritoneal cavity injections to the lower quadrant of the abdomen, lateral to the animal's midline. Injections of 0.2 mg either VdU, or VdU-Lys once per 24 h over three days. After three days, the mice were sacrificed, and the tumors and organs were collected and placed in a solution of 4% paraformaldehyde. The tumors and organs were fixed for 1 h at 4 °C with gentle shaking. After 1 hour, the PFA was removed, and the samples were washed in PBS buffer for 10 minutes at 4 °C with gentle shaking. The wash was repeated twice. After the third wash, the PBS was removed and the samples were placed in a 30% sucrose solution and left at 4 °C with gentle shaking overnight. The samples were then removed from the sucrose solution and placed in Tissue Tek-OCT (VWR 25608-930) and frozen. The frozen blocks were sectioned on a ThermoScientific Cryostar NX50 Cryostat. Sections were cut at 20 μM thickness and placed onto VWR Superfrost Plus Micro slides (VWR 48311-703). Slides were placed at 80 °C until further needed. All animal care and experimental procedures were reviewed and approved by the East China University of Science and Technology Animal Studies Committee (ECUST-2021-07001).

Tumor and organ slice imaging

Slides were removed from -80 °C and place in 60 °C incubator for 10 minutes and then washed for 5 minutes each with DPBS to remove excess OCT. The slides were then dried, and a hydrophobic barrier was drawn around each section with a PAP Pen. From this point on, all washes were done at room temperature with no shaking unless otherwise stated. The sections were then washed with 0.1% Triton X-100 in PBS to permeabilize for 30 minutes. Then the sections were washed 2× with PBS for 10 minutes each, followed by one wash of PBS for 5 minutes. The sections were then washed with a 1 mg/mL solution of BSA in PBS for 35 minutes, followed by two washes of PBS for 5 minutes each. Et-PT-Tz was prepared to a final concentration of 10 μM and stained at 37 °C for 5 h. The sections were washed 3× with 0.1% Triton PBS for 5 minutes each followed by one wash with PBS for 5 minutes each. And sections were stained with DAPI (10 μM) for 30 minutes in PBS. The sections were then washed 3× with PBS for 5 minutes each. The fluorescence images were photographed by using a confocal laser scanning microscope (CLSM, Leica confocal microscope STELLARIS 8 FALCON). Et-PT-Tz, λ_{ex} = 450 nm, λ_{em} = 500-650 nm; DAPI, λ_{ex} = 405 nm, λ_{em} = 450-500 nm.

In vivo fluorescence imaging in tumor-bearing mice

The nude mice were injected with HeLa cells into mammary pads once on each side. Once tumors reached appreciable size, the mice received direct peritoneal cavity injections to the lower quadrant of the abdomen, lateral to the animal's midline. Injections of 0.2 mg either VdU, or VdU-Lys once per 24 h over three days. After three days, FL images were acquired as control group. Then, 100 μL Et-PT-Tz (100 μM) in PBS solution was injected into the HeLa cell tumor-bearing nude mice. After 5 hours, *in vivo* fluorescence images of tumor were acquired by using PerkinElmer IVIS Lumina Kinetic Series III imaging system. And, the mice were sacrificed, and the tumors and organs were collected for *ex vivo* imaging. λ_{ex} = 460 nm, λ_{em} = 550, 600, and 650 nm. All animal care and experimental procedures were reviewed and approved by the East China University of Science and Technology Animal Studies Committee (ECUST-2021-07001).

Statistics and reproducibility

Leica Application Suite X and ImageJ 1.49k were used to process imaging data. GraphPad Prism 8.4.3 and OriginPro 2024b were used for data analysis. Statistical significance is represented in the figures. The experiments were repeated independently at least twice with similar results. Blinding was not conducted, and all data collection and analysis were objective in nature. No data were excluded from the analysis. Data distribution was assumed to be normal, but this was not formally tested.

2. Synthesis of VdU-Lys and Et-PT-Tz

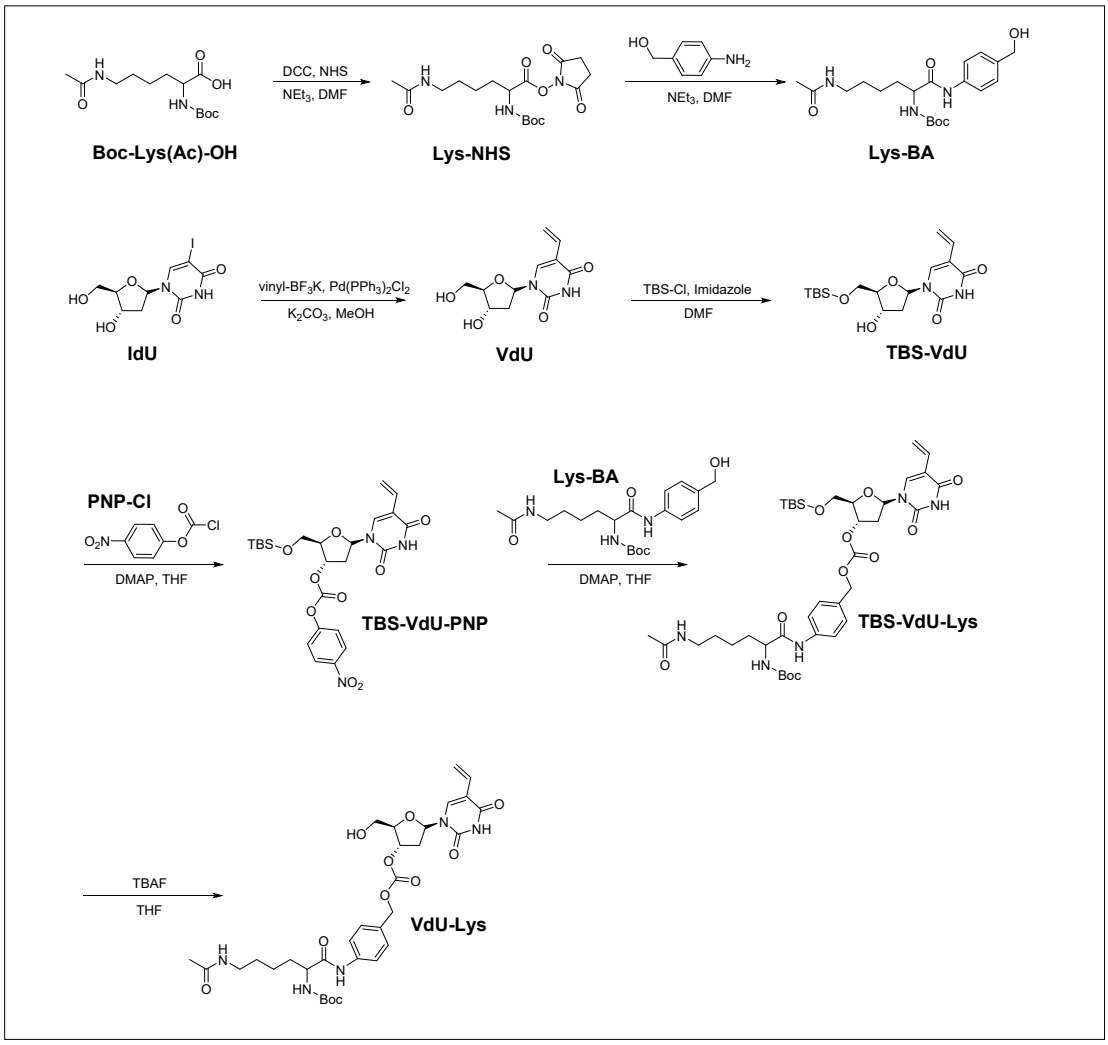


Fig. S1 Synthetic route of VdU-Lys

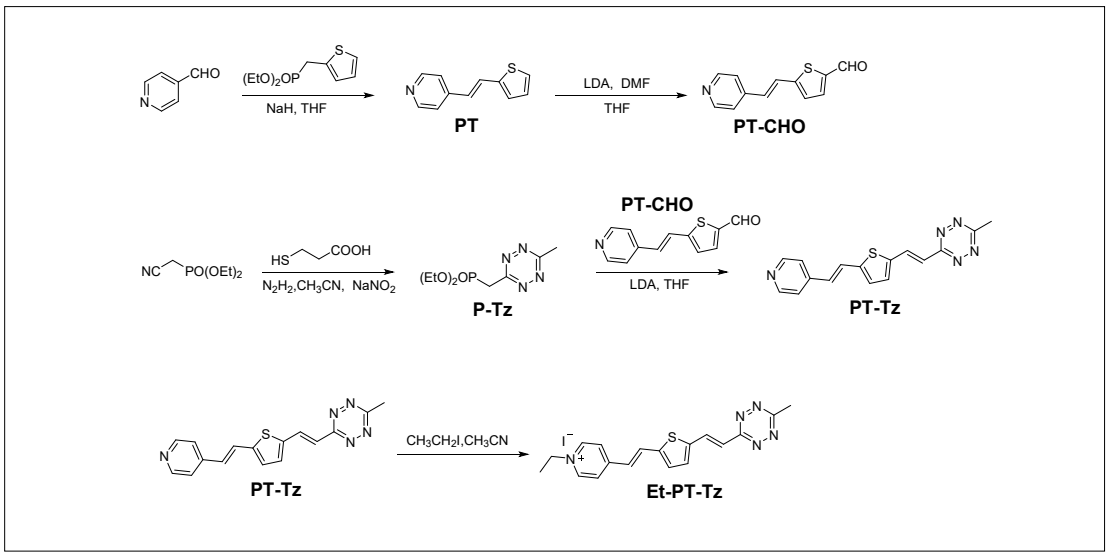
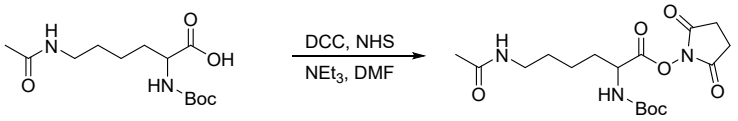


Fig. S2 Synthetic route of Et-PT-Tz

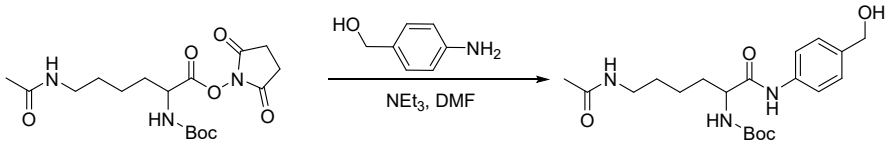
Synthesis of Lys-NHS



Boc-Lys(Ac)-OH **Lys-NHS**

Boc-Lys(Ac)-OH (577 mg, 2.0 mmol) and triethylamine (213 mg, 2.1 mmol) was dissolved in anhydrous 5 mL DMF, followed by the addition of DCC (433 mg, 2.1 mmol) and NHS (242 mg, 2.1 mmol). The reaction mixture was stirred at room temperature for 24 h. The precipitate was filtered off, and the solvent was removed under reduced pressure. The crude product was directly used without further purification.

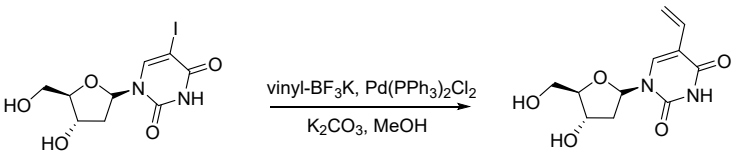
Synthesis of Lys-BA



Lys-NHS **Lys-BA**

Lys-NHS (270 mg, 0.7 mmol) and (4-aminophenyl)methanol (86 mg, 0.7 mmol) were dissolved in anhydrous 10 mL DMF, followed by the addition of triethylamine (71 mg, 0.7 mmol). The mixture was stirred at 40 ℃ for 24 h. After removal of the solvent, the crude product was purified by silica gel column chromatography using ethyl acetate to ethyl acetate/methanol (20/1, v/v) as the eluent to yield a light yellow solid (200 mg): Yield 73%. ^{1}H NMR (400 MHz, CD_3OD, ppm): δ 1.39 (s, 2H, -C***H***$_2$-), 1.44 (s, 9H, (C***H***$_3$)$_3$C-), 1.50-1.55 (m, 1H, -C***H***-), 1.63-1.78 (m, 1H, -C***H***-), 1.90 (s, 3H, C***H***$_3$CO-), 3.16 (t, J = 8.0 Hz, 2H, -C***H***$_2$-), 4.07-4.14 (m, 1H, -C***H***$_2$-), 4.55 (s, 2H, -C***H***$_2$OH), 7.29 (d, J = 8.0 Hz, 2H, phenyl-H), 7.52 (d, J = 8.0 Hz, 2H, phenyl-H); ^{13}C NMR (100 MHz, CD_3OD, ppm) δ: 22.56, 24.34, 28.74, 29.91, 30.04, 32.49, 33.22, 40.19, 54.84, 56.67, 64.85, 80.65, 121.36, 128.62, 138.77, 157.96, 173.25, 173.61; Mass spectrometry (ESI-MS, m/z): $[M+Na]^+$ calcd. for $[C_{20}H_{31}O_5N_3Na]^+$ 416.2156: found 416.2156.

Synthesis of VdU

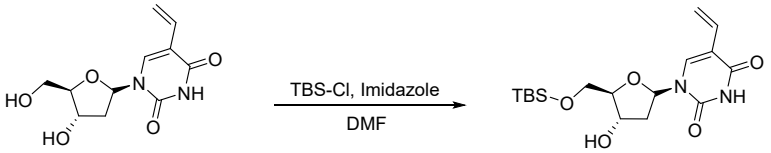


IdU **VdU**

A solution of 5-iodo-2'-deoxyuridine (IdU, 2.0 g, 5.7 mmol), potassium trifluoro vinyl borate (vinyl-BF_3K, 1.1 g, 8.5 mmol) and K_2CO_3 (1.6 g, 11.9 mmol) were dissolved in 75 mL MeOH were sparged with argon for 1 h. $Pb(PPh_3)_2Cl_2$ (396 mg, 0.6 mmol) were added and the solution was freeze-pumped-thawed × 3, and the reaction mixture was heated to 70 ℃ for 21 h. After removal of the solvent, the crude product was purified by silica gel column chromatography using dichloromethane/methanol (10/1, v/v) as the eluent to yield a white solid (665 mg): Yield 46%. ^{1}H NMR (400 MHz, CD_3OD, ppm): δ 2.21-2.33 (m, 2H, -C***H***$_2$-), 3.73-3.85 (m, 2H, -C***H***$_2$-), 3.93 (t, J = 4.0 Hz, 1H, -C***H***-), 4.40-4.44 (m, 1H, -C***H***-), 5.14 (dd, J_1 = 12.0 Hz, J_2 = 2.0 Hz, 1H, alkene-H), 5.91 (dd, J_1 = 12.0 Hz, J_2 = 2.0 Hz, 1H, alkene-H), 6.23 (t, J = 8.0 Hz, 1H, alkene-H), 6.40-6.48 (m, 1H, -C***H***-), 8.22 (s, 1H, uracil-H); ^{13}C NMR (600 MHz, DMSO-d_6,

ppm) δ: 61.44, 70.51, 84.87, 87.93, 111.27, 114.41, 129.26, 138.36, 150.05, 162.57; Mass spectrometry (ESI-MS, m/z): $[M]^-$ calcd. for $[C_{11}H_{13}O_5N_2]^-$ 253.0819: found 253.0817.

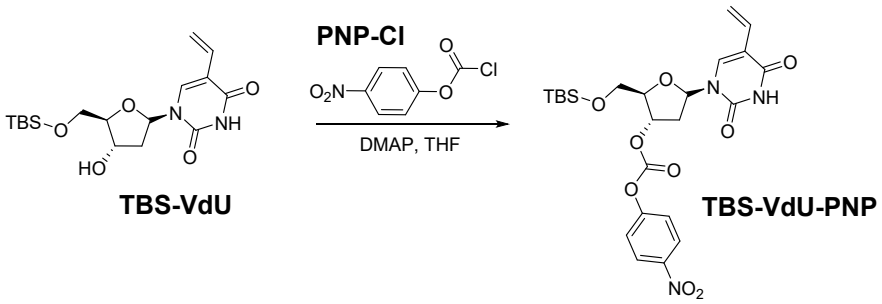
Synthesis of TBS-VdU



VdU **TBS-VdU**

VdU (200 mg, 0.8 mmol) and imidazole (118 mg, 1.7 mmol) were dissolved in anhydrous 10 mL DMF. The solution was cooled to 0 °C and TBS-Cl (119 mg, 0.8 mmol, in 3 mL THF) was added dropwise. The reaction was warmed to rt and stirred 24 h. The reaction was extracted with ethyl acetate after adding water, quickly washed with brine, dried with Na_2SO_4, filtered, and evaporated. The crude product was purified by silica gel column chromatography using dichloromethane/methanol (10/1, v/v) as the eluent to yield a white solid (64 mg): Yield 22%. ^{1}H NMR (400 MHz, CD_3OD, ppm): δ 0.16 (s, 6H, $(CH_3)_2$Si-), 0.92 (s, 9H, $(CH_3)_3$C-), 2.13-2.20 (m, 1H, -C***H***-), 2.31-2.37 (m, 1H, -C***H***-), 3.83-3.94 (m, 2H, -CH_2-), 4.40 (t, J = 4.0 Hz, 1H, -C***H***-), 4.35-4.38 (m, 1H, -C***H***-), 5.17 (dd, J_1 = 12.0 Hz, J_2 = 2.0 Hz, 1H, alkene-H), 5.94 (dd, J_1 = 12.0 Hz, J_2 = 2.0 Hz, 1H, alkene-H), 6.24 (t, J = 8.0 Hz, 1H, alkene-H), 6.36-6.43 (m, 1H, -C***H***-), 7.83 (s, 1H, uracil-H); Mass spectrometry (ESI-MS, m/z): $[M]^-$ calcd. for $[C_{17}H_{27}O_5N_2Si]^-$ 367.1695: found 367.1690.

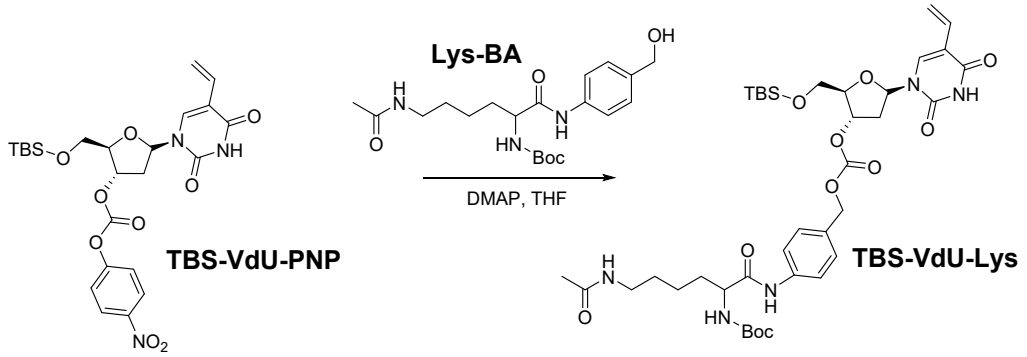
Synthesis of TBS-VdU-PNP



TBS-VdU **TBS-VdU-PNP**

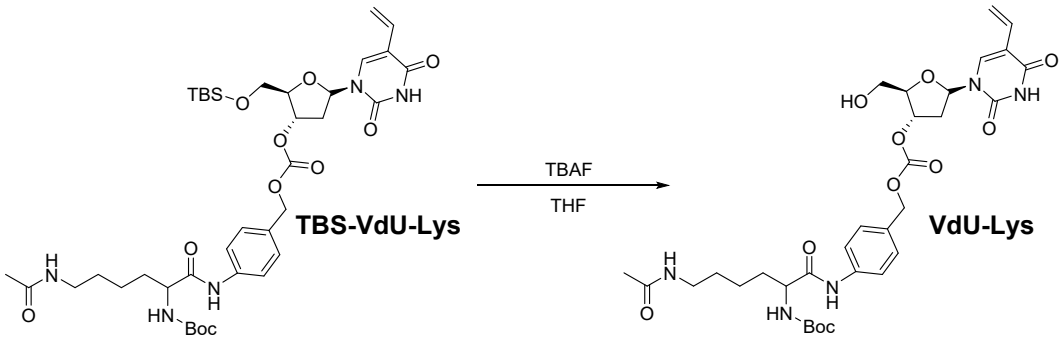
TBS-VdU (790 mg, 2.1 mmol) and PNP-Cl (410 mg, 2.0 mmol) was dissolved in anhydrous 10 mL THF. And DMAP (374 mg, 3.1 mmol, in 5 mL THF) was added dropwise. The mixture was stirred at rt for 4 h. The reaction was extracted with ethyl acetate after adding NH_4Cl aq, quickly washed with brine, dried with Na_2SO_4, filtered, and evaporated. The crude product was purified by silica gel column petroleum ether using petroleum ether/ethyl acetate (1/1, v/v) as the eluent to yield a white solid (230 mg): Yield 22%. ^{1}H NMR (400 MHz, CD_3OD, ppm): δ 0.14 (s, 6H, $(CH_3)_2$Si-), 0.93 (s, 9H, $(CH_3)_3$C-), 2.23-2.34 (m, 1H, -C***H***-), 2.66-2.71 (m, 1H, -C***H***-), 3.98 (t, J = 4.0 Hz, 2H, -CH_2-), 4.39 (s, 1H, -C***H***-), 5.19 (dd, J_1 = 12.0 Hz, J_2 = 2.0 Hz, 1H, alkene-H), 5.34 (d, J = 4.0 Hz, 1H, -C***H***-), 5.95 (dd, J_1 = 12.0 Hz, J_2 = 2.0 Hz, 1H, alkene-H), 6.27 (t, J = 8.0 Hz, 1H, alkene-H), 6.37-6.44 (m, 1H, -C***H***-), 7.51 (d, J = 8.0 Hz, 2H, phenyl-H), 7.84 (s, 1H, uracil-H), 8.32 (d, J = 8.0 Hz, 2H, phenyl-H); Mass spectrometry (ESI-MS, m/z): $[M+Na]^+$ calcd. for $[C_{24}H_{31}O_9N_3SiNa]^+$ 556.1727: found 556.1725.

Synthesis of TBS-VdU-Lys



TBS-VdU-PNP (50 mg, 0.1 mmol) and DMAP (28 mg, 0.23 mmol) were dissolved in anhydrous 3 mL THF. And Lys-BA (53 mg, 0.14 mmol, in 2 mL THF) was added dropwise. The mixture was stirred at rt for 24 h. The crude product was purified by silica gel column chromatography using dichloromethane/methanol (10/1, v/v) as the eluent to yield a white solid (40 mg): Yield 51%. ^{1}H NMR (400 MHz, CD_3OD, ppm): δ 0.11 (s, 6H, (C***H***$_3$)$_2$Si-), 0.91 (s, 9H, (C***H***$_3$)$_3$C-), 1.40 (s, 2H, -C***H***$_2$-), 1.45 (s, 9H, (C***H***$_3$)$_3$C-), 1.52-1.55 (m, 2H, -C***H***$_2$-), 1.67-1.73 (m, 1H, -C***H***-), 1.77-1.80 (m, 1H, -C***H***-), 1.90 (s, 3H, C***H***$_3$CO-), 2.23-2.30 (m, 1H, -C***H***-), 2.52 (dd, J_1 = 12.0 Hz, J_2 = 4.0 Hz, 1H, -C***H***-), 3.16 (t, J = 8.0 Hz, 2H, -C***H***$_2$-), 3.90-3.97 (m, 2H, -C***H***$_2$-), 4.07-4.12 (m, 1H, -C***H***-), 4.20 (d, J = 4.0 Hz, 1H, -C***H***-), 5.14 (s, 2H, -C***H***$_2$OH), 5.17-5.21 (m, 2H, alkene-H and -C***H***-), 5.94 (dd, J_1 = 12.0 Hz, J_2 = 2.0 Hz, 1H, alkene-H), 6.19 (t, J = 4.0 Hz, 1H, alkene-H), 6.36-6.43 (m, 1H, -C***H***-), 7.35 (d, J = 8.0 Hz, 2H, phenyl-H), 7.59 (d, J = 8.0 Hz, 2H, phenyl-H), 7.81 (s, 1H, uracil-H); ^{13}C NMR (400 MHz, CD_3OD, ppm) δ: 5.22, 24.50, 27.80, 29.57, 31.72, 33.98, 38.43, 44.55, 45.42, 61.93, 69.99, 75.91, 85.26, 85.90, 92.11, 92.24, 118.70, 121.38, 126.53, 134.78, 135.56, 137.79, 143.43, 145.25, 156.62, 161.13, 163.21, 169.52, 178.49, 178.95; Mass spectrometry (ESI-MS, m/z): $[M]^-$ calcd. for $[C_{38}H_{56}O_{11}N_5Si]^-$ 786.3751: found 786.3743.

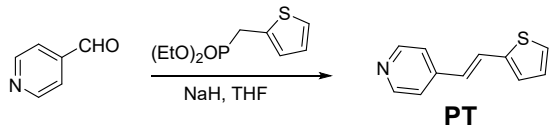
Synthesis of VdU-Lys



TBS-VdU-Lys (40 mg, 0.05 mmol) was dissolved in anhydrous 2 mL THF. The solution was cooled to 0 °C and TBAF (1 M in 3 mL THF, 75 μL, 0.075 mmol) was added dropwise. The reaction was warmed to rt and stirred for 2 h. The reaction was added ethyl acetate and wash with 1 M HCl aq, quickly washed with brine, dried with Na_2SO_4, filtered, and evaporated. The crude product was purified by silica gel column chromatography using dichloromethane/methanol (10/1, v/v) as the eluent to yield a white solid (13 mg): Yield 39%. ^{1}H NMR (400 MHz, CD_3OD, ppm): δ 1.34 (s, 2H, -C***H***$_2$-), 1.45 (s, 9H, (C***H***$_3$)$_3$C-), 1.50-1.55 (m, 2H, -C***H***$_2$-), 1.63-1.74 (m, 1H, -C***H***-), 1.77-1.80 (m, 1H, -C***H***-), 1.90 (s, 3H, C***H***$_3$CO-), 2.35-2.50 (m, 2H, -C***H***$_2$-), 3.12-3.19 (m, 2H, -C***H***$_2$-), 3.79-3.86 (m, 2H, -C***H***$_2$-), 4.06-4.13 (m, 1H, -

C***H***-), 4.15 (d, *J* = 4.0 Hz, 1H, -C***H***-), 5.14 (s, 2H, -C***H***$_2$OH), 5.16 (dd, J_1 = 12.0 Hz, J_2 = 2.0 Hz, 1H, alkene-H), 5.25 (d, *J* = 4.0 Hz, 1H, -C***H***-), 5.91 (dd, J_1 = 12.0 Hz, J_2 = 2.0 Hz, 1H, alkene-H), 6.27 (t, *J* = 4.0 Hz, 1H, alkene-H), 6.40-6.47 (m, 1H, -C***H***-), 7.35 (d, *J* = 8.0 Hz, 2H, phenyl-H), 7.58 (d, *J* = 8.0 Hz, 2H, phenyl-H), 8.21 (s, 1H, uracil-H); ^{13}C NMR (600 MHz, DMSO-d_6, ppm) δ: 22.53, 22.57, 23.10, 28.17, 28.86, 36.93, 38.26, 61.21, 69.08, 78.00, 78.48, 84.24, 84.59, 111.15, 114.23, 119.02, 128.63, 129.18, 129.75, 137.55, 139.24, 149.55, 153.71, 162.00, 168.86, 171.53; Mass spectrometry (ESI-MS, m/z): $[M+Na]^+$ calcd. for $[C_{32}H_{43}O_{11}N_5Na]^+$ 696.2851: found 696.2825.

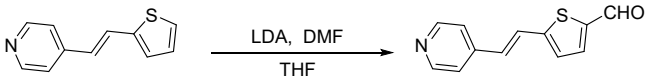
Synthesis of PT



PT

60% NaH (280 mg, 7.0 mmol) was dissolved in anhydrous 15 mL THF, and thiophene methyl phosphonate (1.0 g, 4.3 mmol, in 10 mL THF) was added dropwise. The reaction was stirred in rt for 10 min, and 4-pyridinecarboxaldehyde (500 mg, 4.7 mmol, in 10 mL THF) was added dropwise. The reaction was heated to 60 ℃ for 2 h. The reaction was extracted with dichloromethane after adding NH_4Cl aq, dried with Na_2SO_4, filtered, and evaporated. The crude product was purified by silica gel column petroleum ether using petroleum ether/ethyl acetate (1/1, v/v) as the eluent to yield a white solid (385 mg): Yield 48%.^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 6.80 (d, *J* = 16.0 Hz, 1H, alkene-H), 7.04 (t, *J* = 4.0 Hz, 1H, thiophene-H), 7.16 (d, *J* = 4.0 Hz, 1H, thiophene-H), 7.28 (d, *J* = 4.0 Hz, 1H, thiophene-H), 7.31 (d, *J* = 4.0 Hz, 2H, pyridine-H), 7.41 (d, *J* = 16.0 Hz, 1H, alkene-H), 8.55 (d, *J* = 4.0 Hz, 2H, pyridine-H); Mass spectrometry (DART-MS, m/z): $[M+H]^+$ calcd. for $[C_{11}H_{10}NS]^+$ 188.0528: found 188.0514.

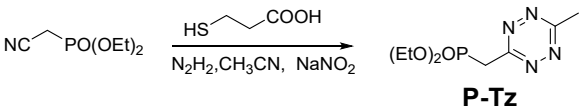
Synthesis of PT-CHO



PT **PT-CHO**

PT (200 mg, 1.1 mmol) was dissolved in 10 mL THF, and the solution was freeze–pumped–thawed × 3. The solution was cooled to 0 °C and LDA (2.0 M in THF, 0.8 mL, 1.6 mmol) was added dropwise. The reaction was warmed to rt and stirred 1 h. DMF (164 mg, 2.3 mmol, in 3 mL THF) was added dropwise and the reaction was stirred in rt for 3 h. The reaction was extracted with dichloromethane after adding NH_4Cl aq, dried with Na_2SO_4, filtered, and evaporated. The crude product was purified by silica gel column petroleum ether using petroleum ether/ethyl acetate (1/1, v/v) as the eluent to yield a light yellow solid (50 mg): Yield 21%. ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 7.03 (d, *J* = 16.0 Hz, 1H, alkene-H), 7.24 (t, *J* = 4.0 Hz, 1H, thiophene-H), 7.35 (d, *J* = 8.0 Hz, 2H, pyridine-H), 7.39 (d, *J* = 16.0 Hz, 1H, alkene-H), 7.70 (d, *J* = 4.0 Hz, 1H, thiophene-H), 8.61 (d, *J* = 8.0 Hz, 2H, pyridine-H), 9.90 (s, 1H, C***H***O-); Mass spectrometry (ESI-MS, m/z): $[M+H]^+$ calcd. for $[C_{12}H_{10}ONS]^+$ 216.0478: found 216.0465.

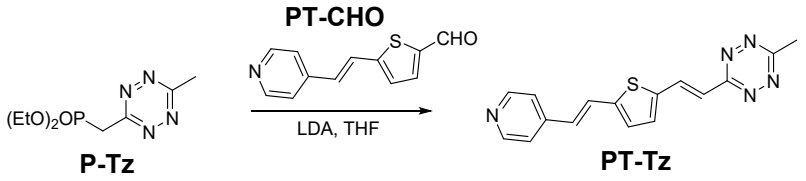
Synthesis of P-Tz



P-Tz

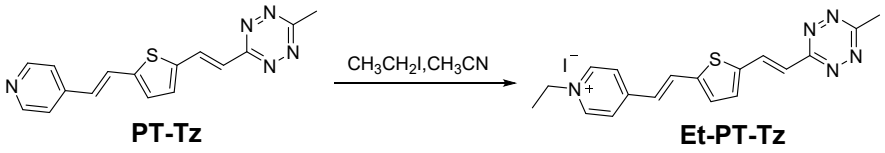
Diethylcyanomethylphosphonate (1.0 g, 5.6 mmol), acetonitrile (4.6 g, 113.0 mmol), and 3-mercaptopropionic acid (598 mg, 5.6 mmol) were dissolved in 2 mL EtOH. The solution was cooled to 0 °C and hydrazine hydrate (4.5 g, 90 mmol) was added dropwise. The reaction was warmed to rt and stirred 18 h. Upon completion, the reaction solution was cooled with ice water, and sodium nitrite (5.8 g, 85 mmol) dissolved in ice water (100 mL) was slowly added, followed by slow addition of 1 M HCl under ice bath during which the solution turned bright red, and gas evolved. Addition of 1M HCl was continued until gas evolution ceased and the pH value was 3-4. The reaction was extracted with dichloromethane, dried with Na_2SO_4, filtered, and evaporated. The crude product was purified by silica gel column petroleum ether using petroleum ether/ethyl acetate (1/2, v/v) as the eluent to yield a pink oil (440 mg): Yield 32%. ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 1.33 (t, J = 8.0 Hz, 6H, C***H***$_3$-), 3.07 (s, 3H, C***H***$_3$-), 3.90 (d, J = 24.0 Hz, 2H, -C***H***$_2$-), 4.15-4.26 (m, 4H, -C***H***$_2$-); Mass spectrometry (ESI-MS, m/z): $[M+Na]^+$ calcd. for $[C_8H_{15}O_3N_4Na]^+$ 269.0774: found 269.0766.

Synthesis of PT-Tz



P-Tz (86 mg, 0.35 mmol), and PT-CHO (50 mg, 0.23 mmol) were dissolved in 5 mL THF, and the solution was freeze-pumped-thawed × 3. The solution was cooled to -20 °C and LDA (1.0 M in THF, 0.23 mL, 0.23 mmol) was added dropwise, and the reaction was stirred at -20 °C for 3 h. The reaction was extracted with dichloromethane after adding NH_4Cl aq, dried with Na_2SO_4, filtered, and evaporated. The crude product was purified by silica gel column petroleum ether using dichloromethane/ethyl acetate (1/1, v/v) as the eluent to yield red solid (50 mg): Yield 71%. ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 3.05 (s, 3H, C***H***$_3$-), 6.91 (d, J = 16.0 Hz, 1H, alkene-H), 7.15 (d, J = 4.0 Hz, 1H, thiophene-H), 7.24 (d, J = 16.0 Hz, 1H, alkene-H), 7.28 (d, J = 4.0 Hz, 1H, thiophene-H), 7.35 (d, J = 4.0 Hz, 2H, pyridine-H), 7.41 (d, J = 16.0 Hz, 1H, alkene-H), 8.35 (d, J = 16.0 Hz, 1H, alkene-H), 8.59 (d, J = 8.0 Hz, 2H, pyridine-H); ^{13}C NMR (400 MHz, DMSO-d_6, ppm) δ: 16.05, 16.11, 20.72, 31.91, 33.22, 62.11, 62.17, 120.78, 150.07, 164.03, 164.12, 167.22; Mass spectrometry (ESI-MS, m/z): $[M+H]^+$ calcd. for $[C_{16}H_{14}N_5S]^+$ 308.0964: found 308.0966.

Synthesis of Et-PT-Tz



PT-Tz (50 mg, 0.16 mmol) and ethyl iodide (254 mg, 1.6 mmol) was dissolved in 5 mL acetonitrile, and the reaction was stirred at 80 °C for 8 h. After removal of the solvent, the crude product was purified by silica gel column chromatography using ethyl acetate to dichloromethane/methanol (10/1, v/v) as the eluent to yield a red solid (20 mg): Yield 27%. ^{1}H NMR (400 MHz, DMSO-d_6, ppm): δ 1.54 (t, J = 8.0 Hz, 3H, C***H***$_3$-), 2.97 (s, 3H, C***H***$_3$-), 4.52 (q, J = 8.0 Hz, 2H, -C***H***$_2$-), 7.33 (d, J = 16.0 Hz, 2H, alkene-H), 7.57 (d, J = 4.0 Hz, 1H, thiophene-H), 7.75 (d, J = 4.0 Hz, 1H, thiophene-H), 8.24-8.28 (m ,3H, pyridine-H and alkene-H), 8.41 (d, J = 16.0 Hz, 2H, alkene-H), 8.96 (d, J = 8.0 Hz,

2H, pyridine-H); ^{13}C NMR (400 MHz, DMSO-d_6, ppm) δ: 16.13, 20.88, 55.26, 121.34, 123.75, 131.92, 132.72, 132.75, 133.11, 142.82, 142.95, 143.99, 152.02, 163.85, 165.96; Mass spectrometry (ESI-MS, m/z): $[M]^+$ calcd. for $[C_{18}H_{18}N_5S]^+$ 336.1277: found 336.1281.

3. Analysis of sequential digestion-reaction of VdU-Lys

(a)

CTSL	HDAC	VdU-Lys	VdU	MS
-	+	+	-	b
-	+	-	+	c
+	-	+	-	d
+	+	+	-	e

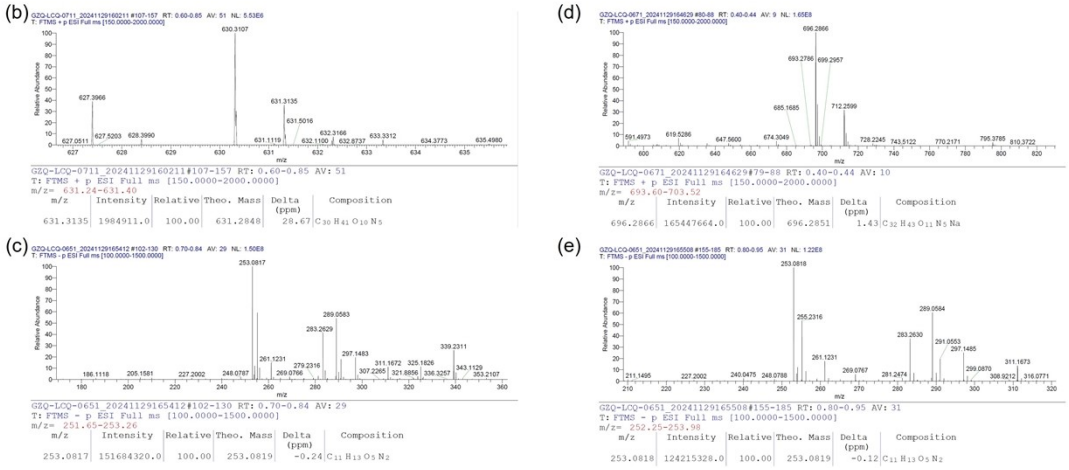


Fig. S3 (a-e) Mass spectrometry (MS) analysis of reaction of VdU-Lys with HDAC1 and CTSL protease.

4. The kinetics of IEDDA reaction of Et-PT-Tz with VdU

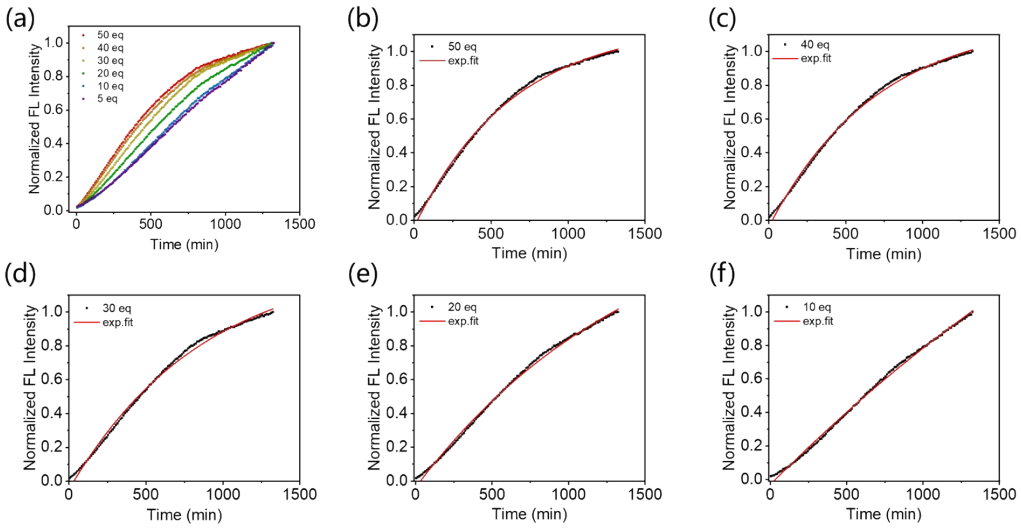


Fig. S4 Kinetic analysis of Et-PT-Tz (100 μM) reacting with VdU (0-5 mM, 0-50 eq). Fluorescence increases versus time upon mixing Et-PT-Tz with VdU.

5. Biocompatibility of the probes

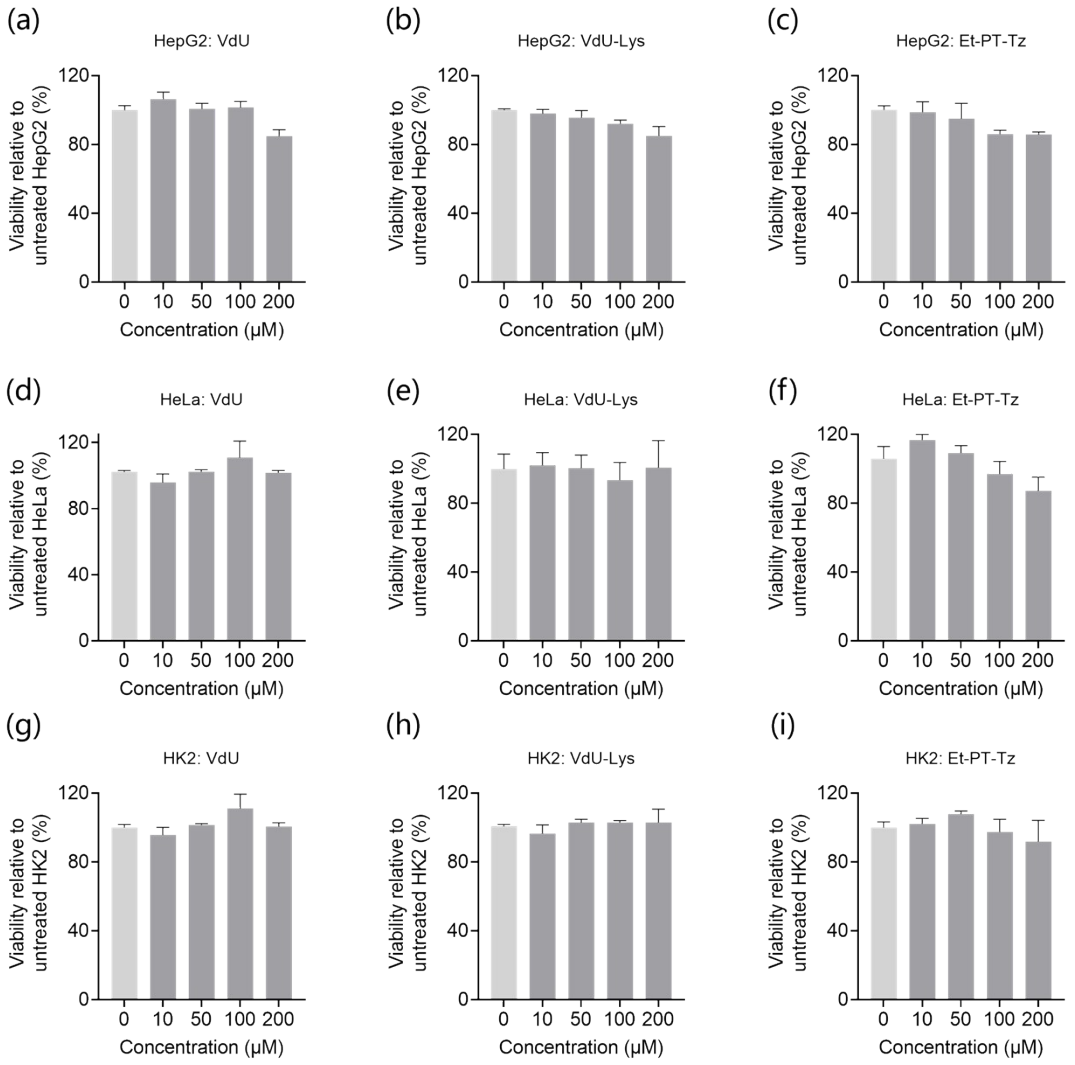


Fig. S5 MTT assays were performed in 96-well plates with two cancerous cell lines, including HepG2 (a-c) and HeLa (d-f), and one noncancerous cell line HK2 (g-i) cells seeded at densities of 10,000 cells per well. Cells were grown for 24 hours and then treated with different concentrations of VdU (a, d, and g), VdU-Lys (b, e, and h) or Et-PT-Tz (c, f, and i) for 24 hours, and then cell viability was measured by absorbance at 450 nm (VdU and VdU-Lys) and 590 nm (Et-PT-Tz) using a microplate reader. n = 3 replicate MTT assays.

6. Colocalization images of Et-PT-Tz and commercial DNA binder

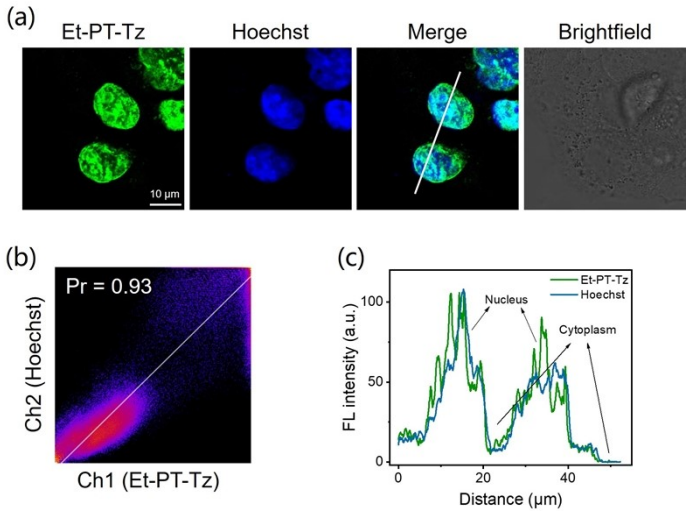


Fig. S6 (a-c) Colocalization images of VdU-induced HepG2 cells with Et-PT-Tz (10 μM; λ_{ex} = 450 nm; λ_{em} = 500-650 nm) and Hoechst 33342 (commercial DNA staining dye; 1 μM; λ_{ex} = 405 nm; λ_{em} = 440-500 nm). Et-PT-Tz demonstrates a significant colocalization (Pr = 0.93) effect with Hoechst in the nucleus, providing compelling evidence for the effective lighting-up of vinyl-modified DNA in nucleus by using Et-PT-Tz.

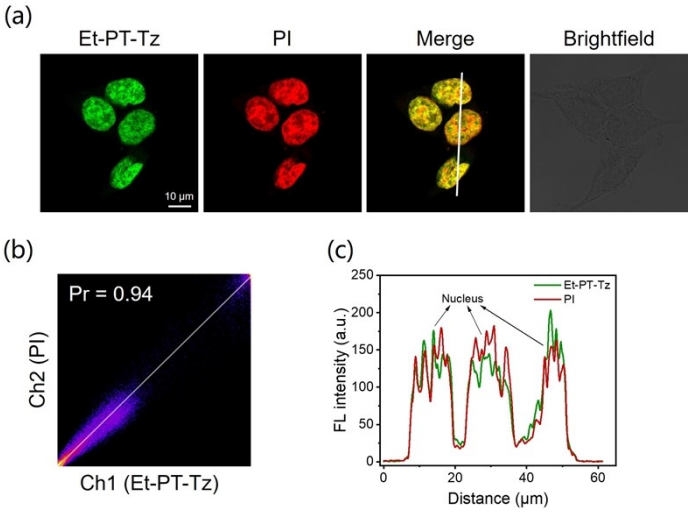


Fig. S7 (a-c) Colocalization images of VdU-induced HepG2 cells with Et-PT-Tz (10 μM; λ_{ex} = 450 nm; λ_{em} = 500-650 nm) and propidium iodine (PI, commercial DNA staining dye; 10 μg/mL; λ_{ex} = 535 nm; λ_{em} = 590-630 nm). Et-PT-Tz demonstrates a significant colocalization (Pr = 0.94) effect with PI in the nucleus, providing compelling evidence for the effective lighting-up of vinyl-modified DNA in nucleus by using Et-PT-Tz.

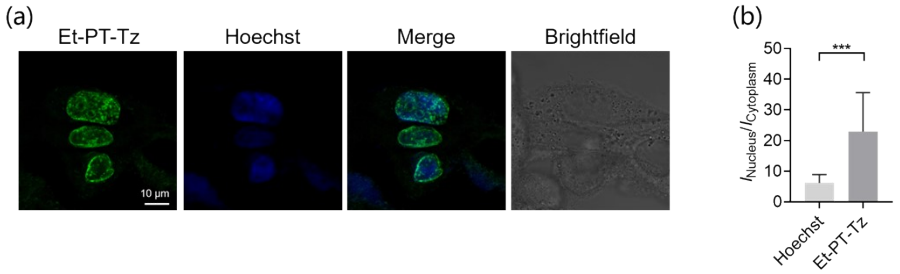


Fig. S8. (a) Confocal fluorescence microscopic images of VdU-induced HepG2 cells with Et-PT-Tz and Hoechst. (b) The signal-to-noise ratio of nucleus imaging (by calculating the ratio of the average fluorescent gray value of nuclear region to cytoplasm region) with Et-PT-Tz and Hoechst. Statistical significance was calculated using two tailed *t*-test. ***P<0.001.

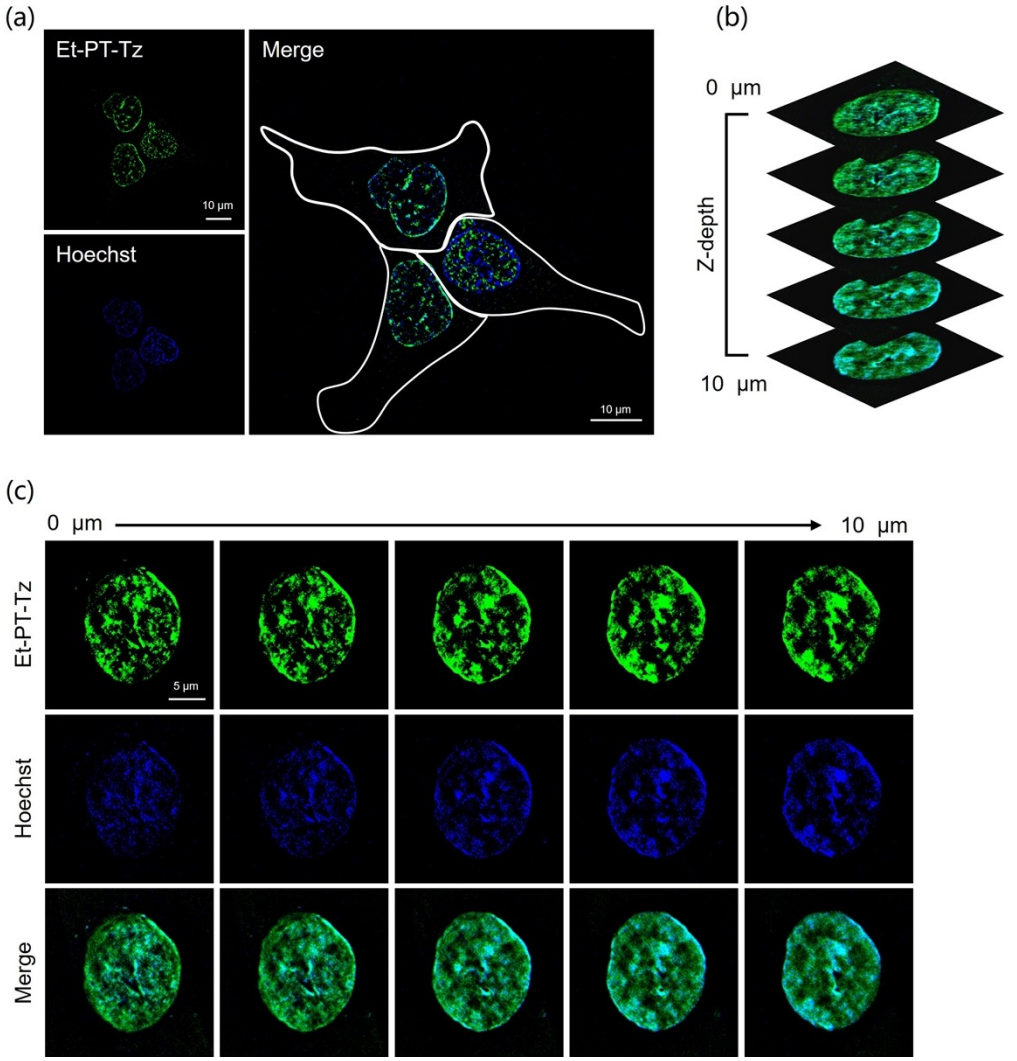


Fig. S9 (a) Super-resolution fluorescence images (by using structured illumination microscopy, SIM) of VdU-induced HCT116 cells and stained with Et-PT-Tz (10 μM treatment for 5 h) and Hoechst 33342 (1 μM treatment for 0.5 h). (b and c) Three-dimensional images of HCT116 cell nucleus obtained along the z-axis direction at different depths. Hoechst, λ_{ex} = 405 nm, λ_{em} < 560 nm; Et-PT-Tz, λ_{ex} = 488 nm, λ_{em} > 560 nm.

7. Cancer-cell-specific fluorescence labeling of DNA

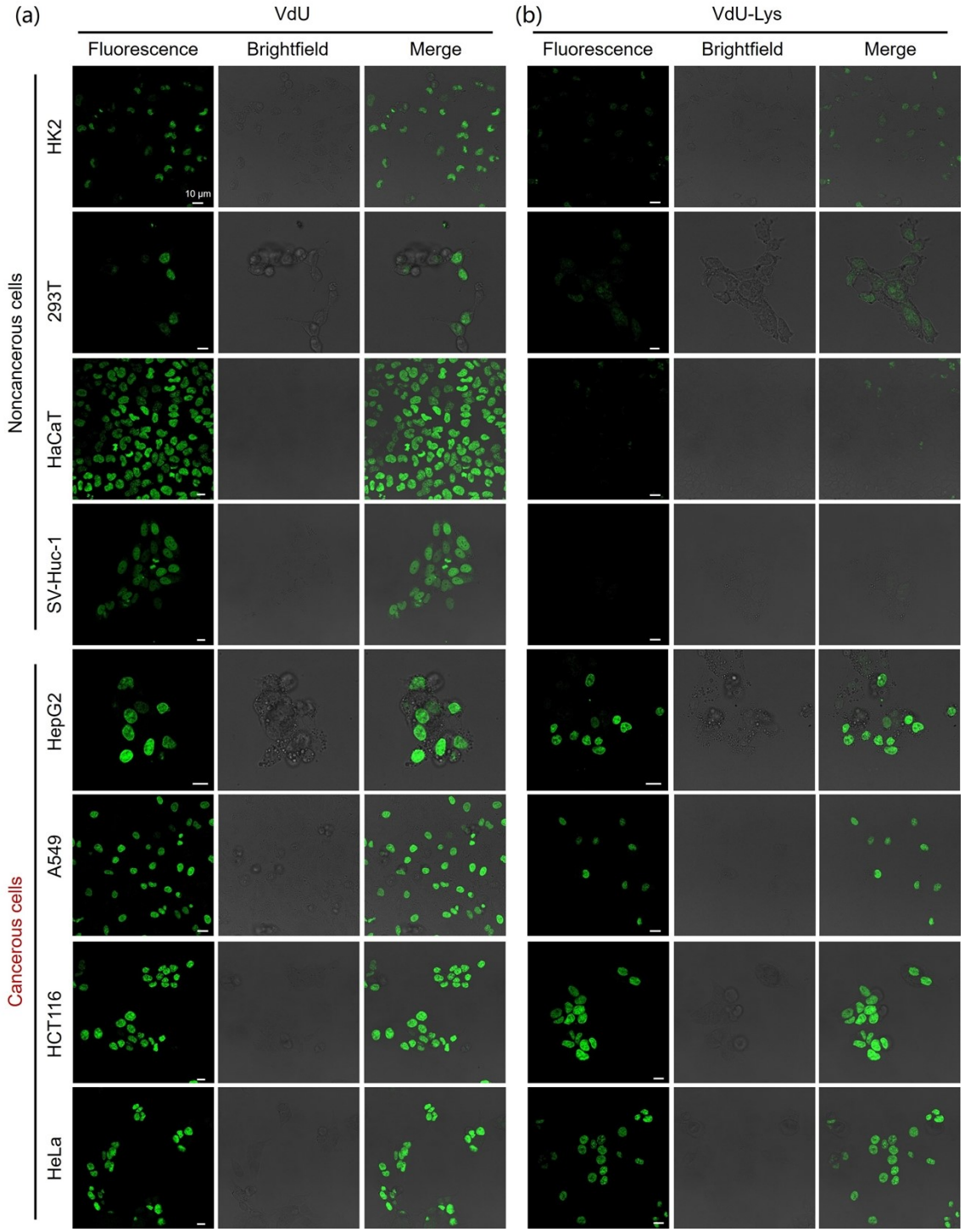


Fig. S10 Cancer-cell-specific fluorescence imaging of cancerous cell DNA. Cells were incubated with 100 μM VdU or VdU-Lys for 24 h, after fixation subsequently stained with 10 μM Et-PT-Tz for 5 h. (a and b) Representative fluorescence images of VdU-induced (a) or VdU-Lys-induced (b) cells including noncancerous cells (human kidney HK2 cell, human embryonic kidney 293T cell, human keratinocyte HaCaT cell, and simian vacuolating virus 40-transformed human urothelial SV-Huc-1 cell) and cancerous cells (human hepatoma HepG2 cell, human non-small-cell lung cancer A549 cell, human colorectal carcinoma HCT116 cell, and human epithelioid cervical carcinoma HeLa cell). λ_{ex} = 450 nm; λ_{em} = 500-650 nm.

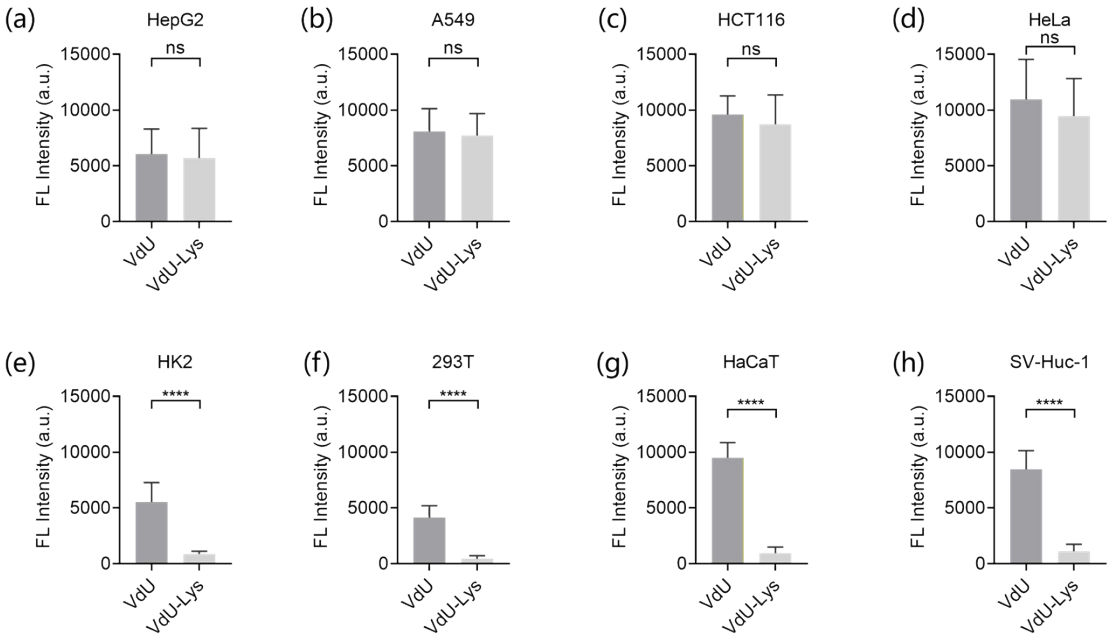


Fig. S11 Cells were incubated with 100 μM VdU or VdU-Lys for 24 h, after fixation subsequently stained with 10 μM Et-PT-Tz for 5 h. (a-h) Quantitative analysis of fluorescence intensity of HK2, 293T, HaCaT, SV-Huc-1, HepG2, A549, HCT116, and HeLa cells. n > 13 biologically independent cells for each group. Statistical significance was calculated using two tailed *t*-test. ns=non-significant, ****P<0.0001.

8. Cancer-specific DNA labeling *in vivo*

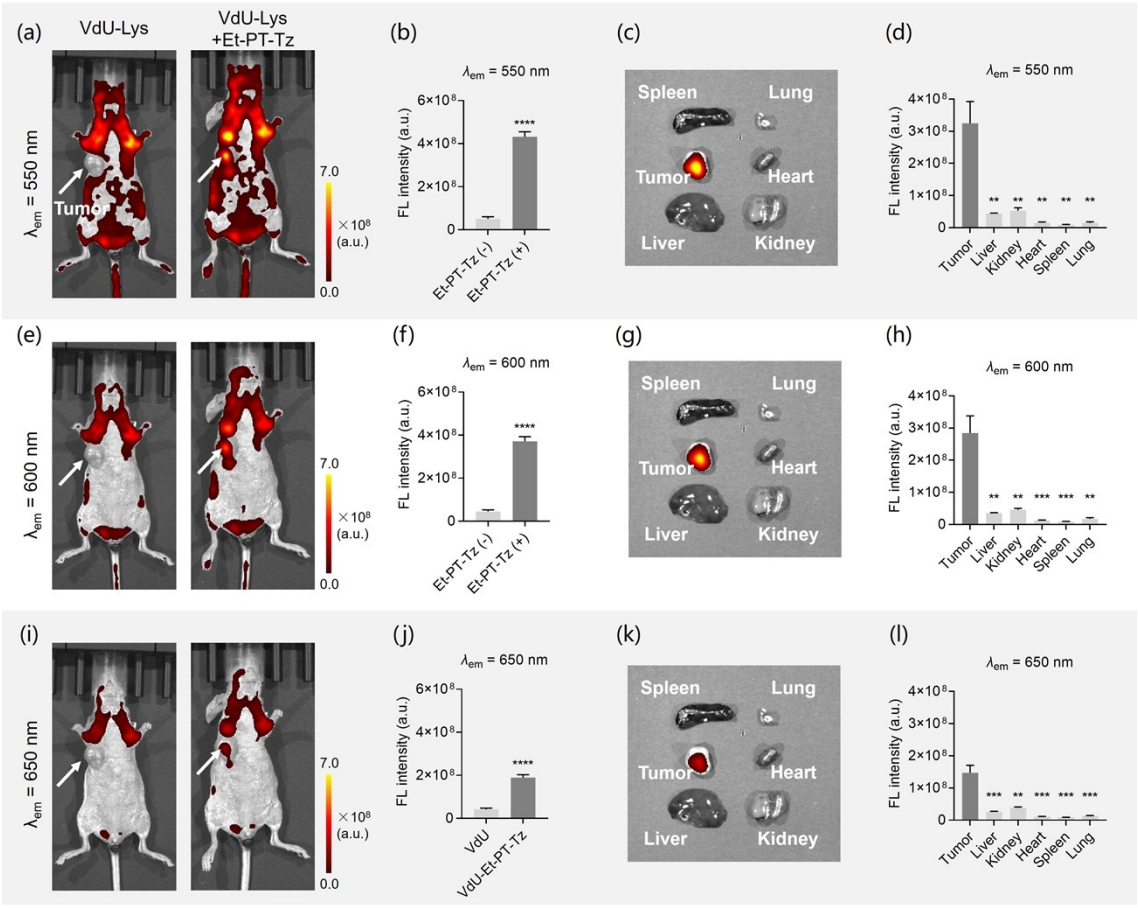


Fig. S12 (a-l) Fluorescence imaging of tumor using VdU-Lys and Et-PT-Tz *in vivo*. (a, e, and i) Representative whole-body fluorescence imaging of xenograft tumor model mice injected with only VdU-Lys and both of VdU-Lys and Et-PT-Tz in 550 nm (a), 600 nm (e), and 650 nm (i). (b, f, and j) Quantitative analysis of the fluorescence imaging of tumor in 550 nm (b), 600 nm (f), and 650 nm (j). (c, g, and k) Representative images of *ex vivo* imaging of tumor tissue and five major organs (liver, kidney, heart, spleen, and lung) form morse injected with VdU-Lys and Et-PT-Tz in 550 nm (c), 600 nm (g), and 650 nm (k). (d, h, and l) Quantitative analysis of the fluorescence imaging of tumor tissue and major organs in 550 nm (d), 600 nm (h), and 650 nm (l). n = 3 biologically independent ROI for each group. λ_{ex} = 450 nm. Statistical significance was calculated using two tailed *t*-test. ***P*<0.01, ****P*<0.001, *****P*<0.0001.

Specifically, we labeled tumor tissue with our strategy and conduct *in vivo* fluorescence imaging. The results clearly showed tumor-specific fluorescence signals, with a significant 8-fold increase in fluorescence intensity at 550 nm following injection of Et-PT-Tz. In addition, fluorescence imaging of excised organs revealed that the tumor tissue exhibited significantly higher fluorescence intensity compared to other organs, confirming the tumor-specificity of our probe. These new *in vivo* results demonstrate that our strategy is not only effective at the cellular level but also highly compatible with applications at the whole-organism scale for cancer-specific fluorescent labelling.

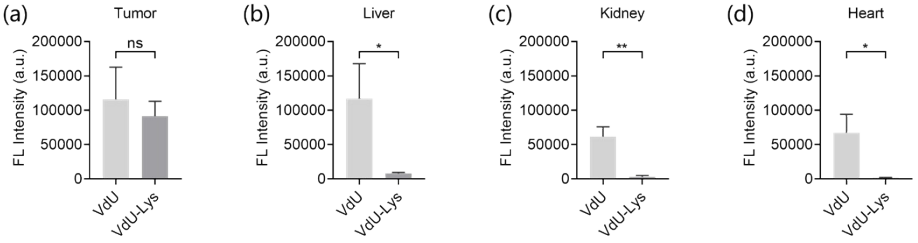


Fig. S13 (a-d) Quantitative analysis of fluorescence intensity of organs and tumor slices from mice exposed to VdU and VdU-Lys. n = 3 biologically independent sections for each group. λ_{ex} = 450 nm; λ_{em} = 500-650 nm. Statistical significance was calculated using two tailed *t*-test. ns=non-significant, **P*<0.05, ***P*<0.01.

9. Characterization of intermediates and VdU-Lys and Et-PT-Tz

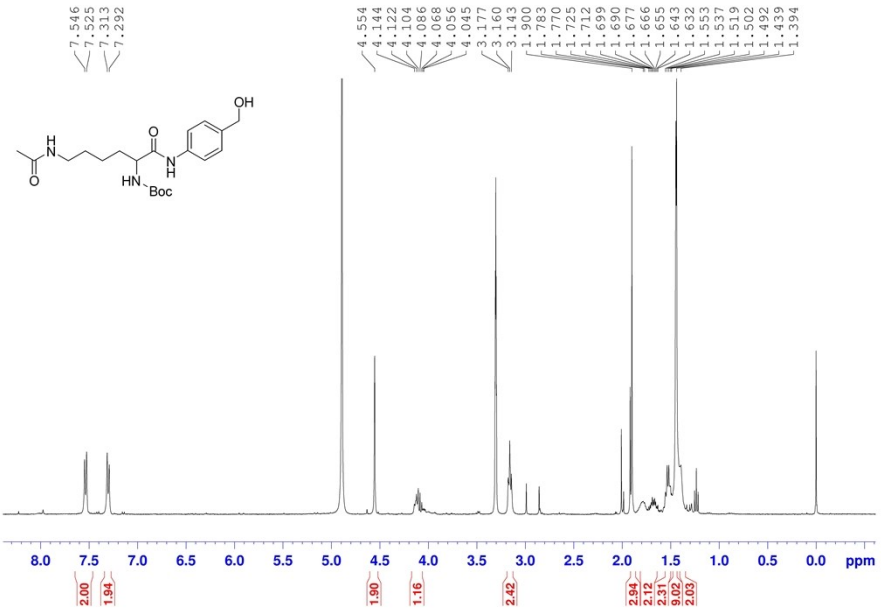


Fig. S14 ^{1}H NMR spectrum of Lys-BA in CD_3OD

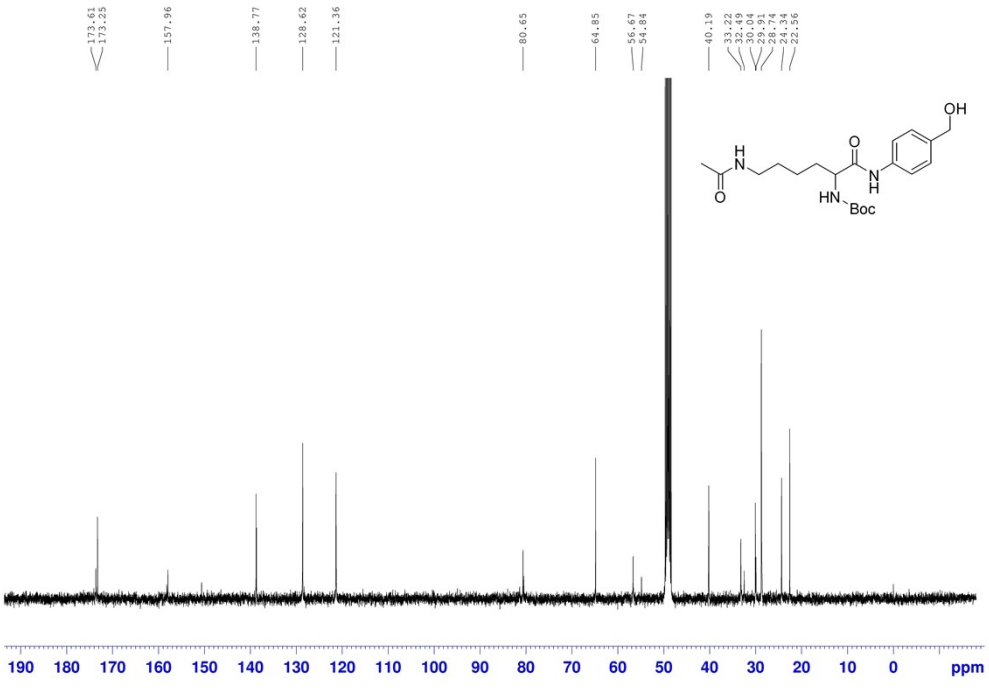
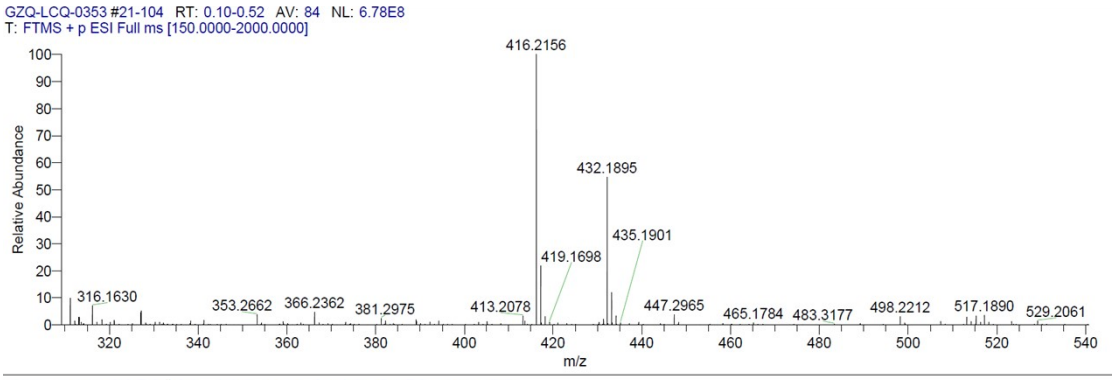


Fig. S15 ^{13}C NMR spectrum of Lys-BA in CD_3OD



GZQ-LCQ-0353#21-104 RT: 0.10-0.52 AV: 84
T: FTMS + p ESI Full ms [150.0000-2000.0000]
m/z= 413.40-419.34

m/z	Intensity	Relative	Theo. Mass	Delta (ppm)	Composition
416.2156	686068864.0	100.00	416.2156	0.03	$C_{20}H_{31}O_5N_3Na$

Fig. S16 HRMS spectrum of Lys-BA

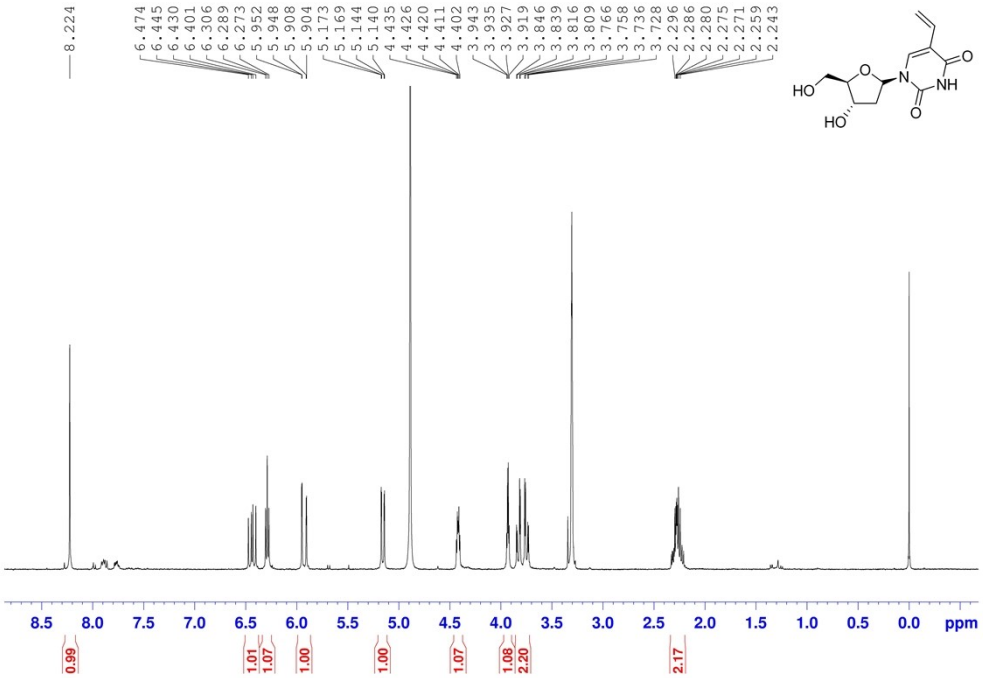


Fig. S17 ^{1}H NMR spectrum of VdU in CD_3OD

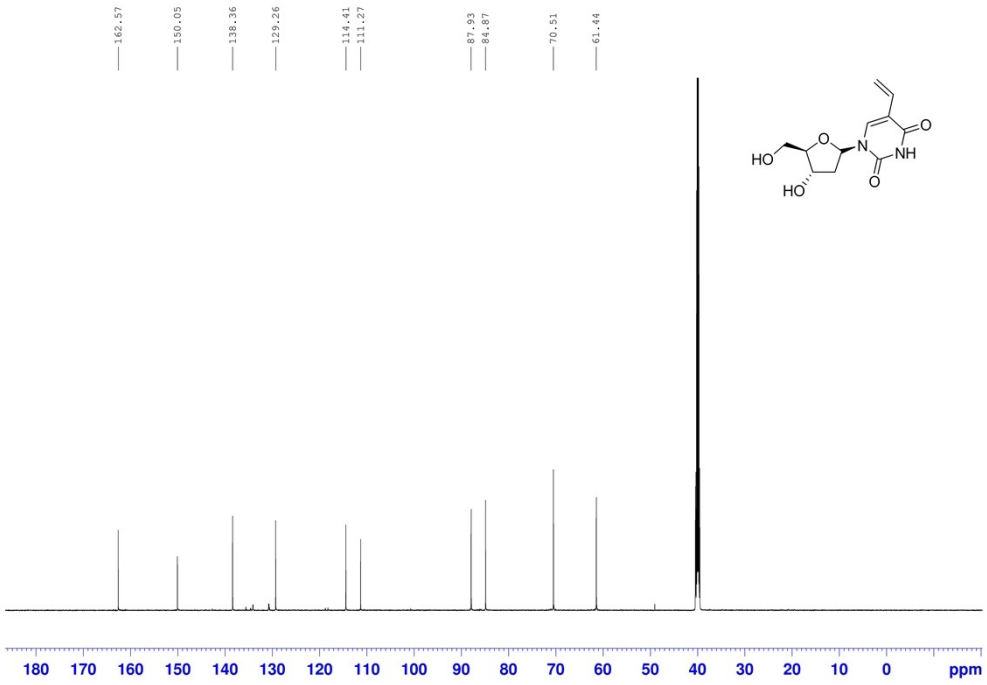
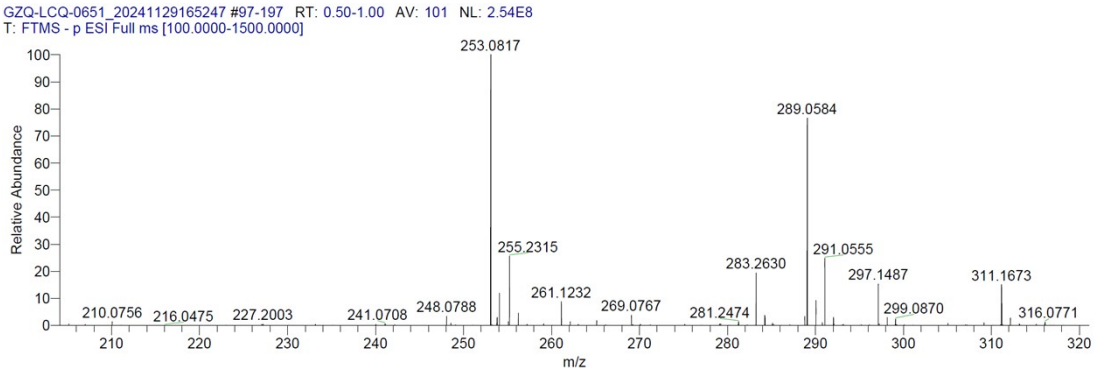


Fig. S18 ^{13}C NMR spectrum of VdU in DMSO-d_6



GZQ-LCQ-0651_20241129165247#97-197 RT: 0.50-1.00 AV: 101
T: FTMS - p ESI Full ms [100.0000-1500.0000]
m/z= 252.28-253.85

m/z	Intensity	Relative	Theo. Mass	Delta (ppm)	Composition
253.0817	254569600.0	100.00	253.0819	-0.19	$C_{11}H_{13}O_5N_2$

Fig. S19 HRMS spectrum of VdU

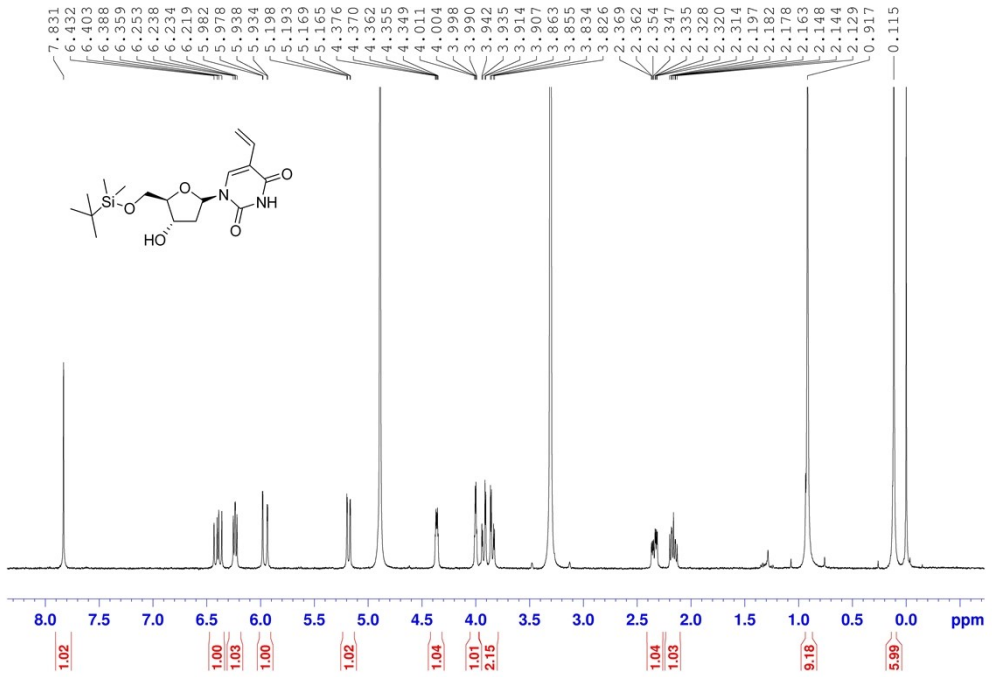


Fig. S20 ^{1}H NMR spectrum of TBS-VdU in CD_3OD

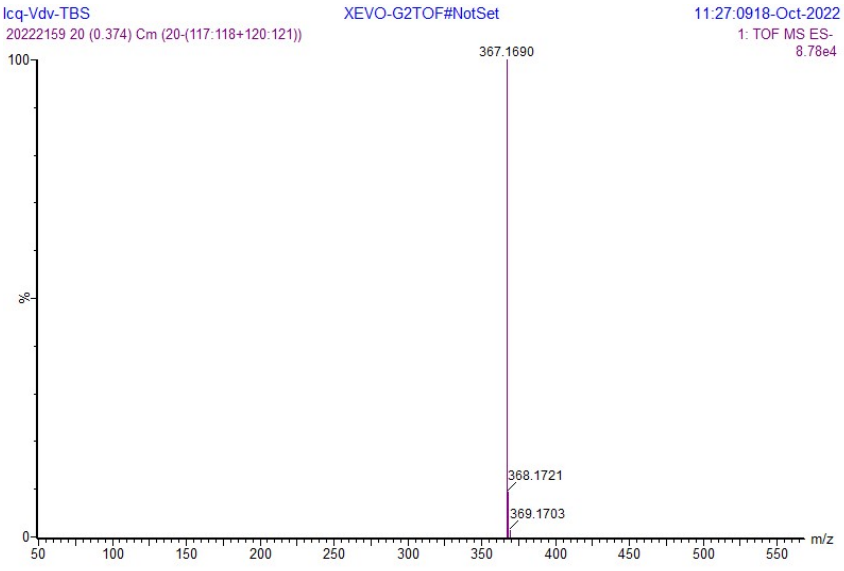


Fig. S21 HRMS spectrum of TBS-VdU

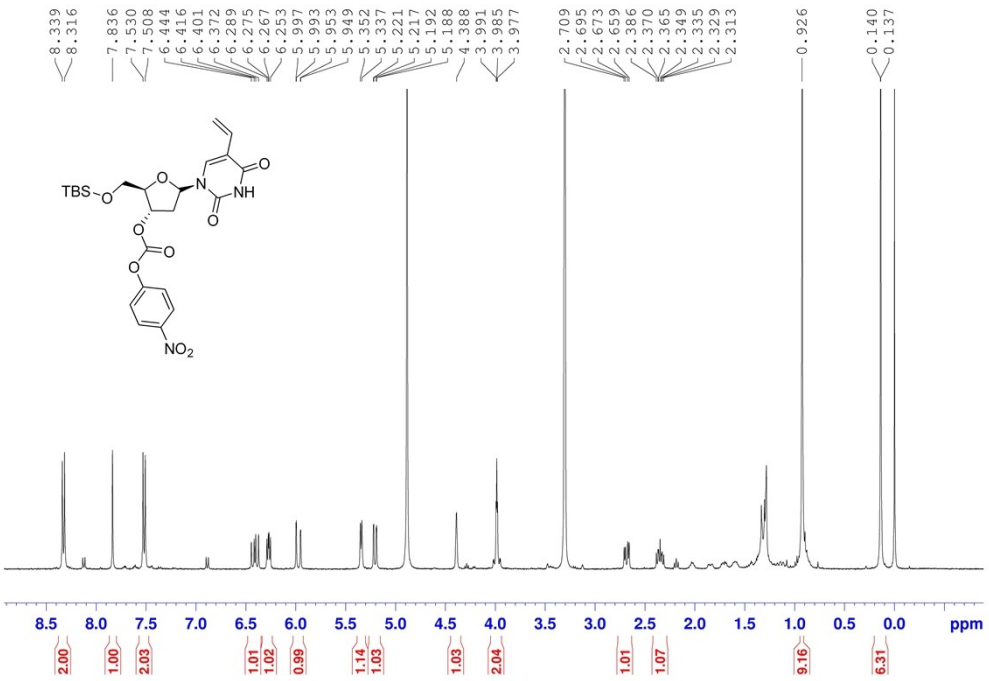


Fig. S22 ^{1}H NMR spectrum of TBS-VdU-PNP in CD_3OD

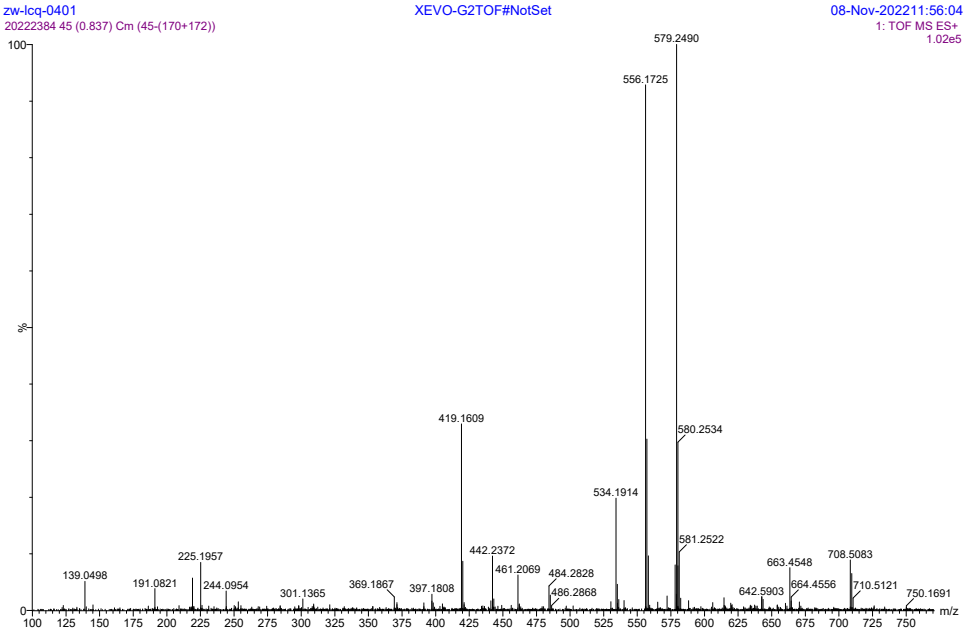


Fig. S23 HRMS spectrum of TBS-VdU-PNP

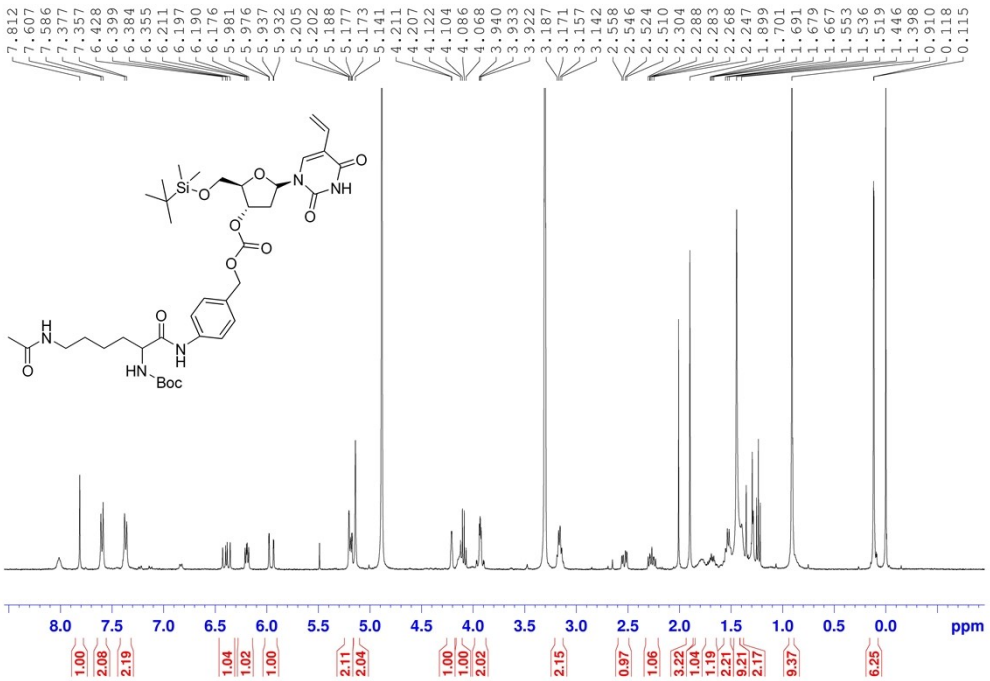


Fig. S24 ^{1}H NMR spectrum of TBS-VdU-Lys in CD_3OD

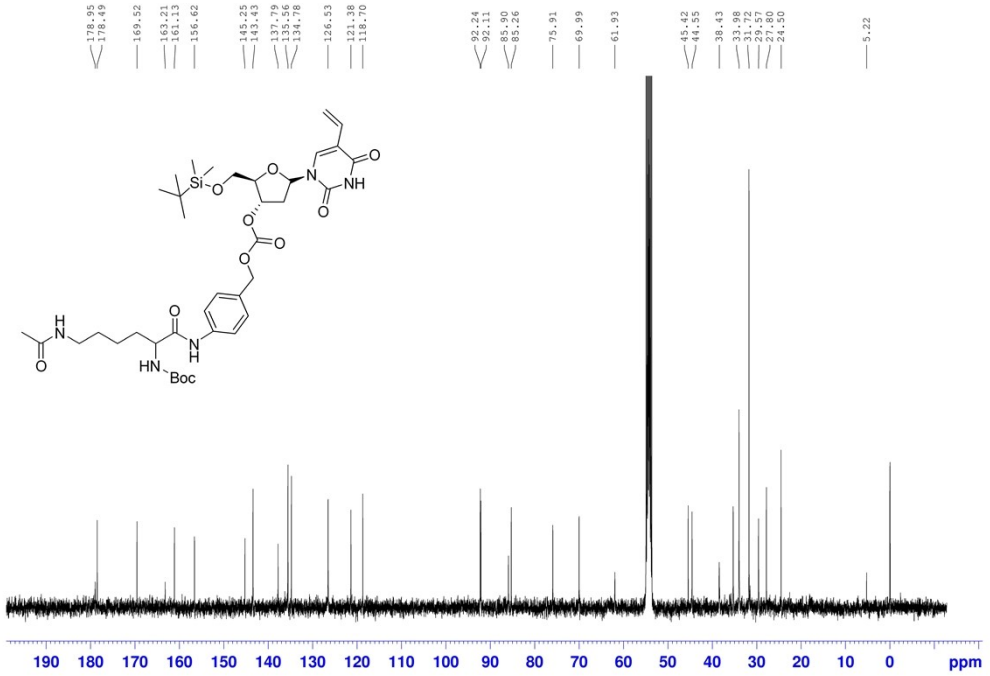


Fig. S25 ^{13}C NMR spectrum of TBS-VdU-Lys in CD_3OD

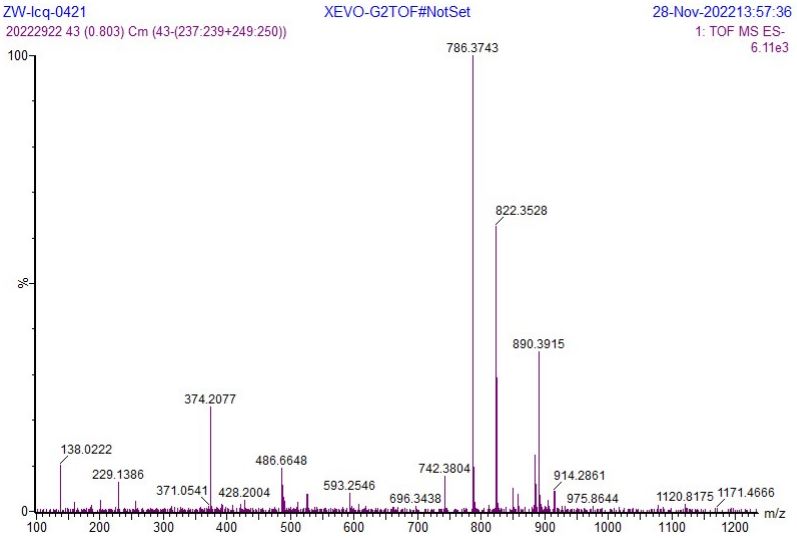


Fig. S26 HRMS spectrum of TBS-VdU-Lys

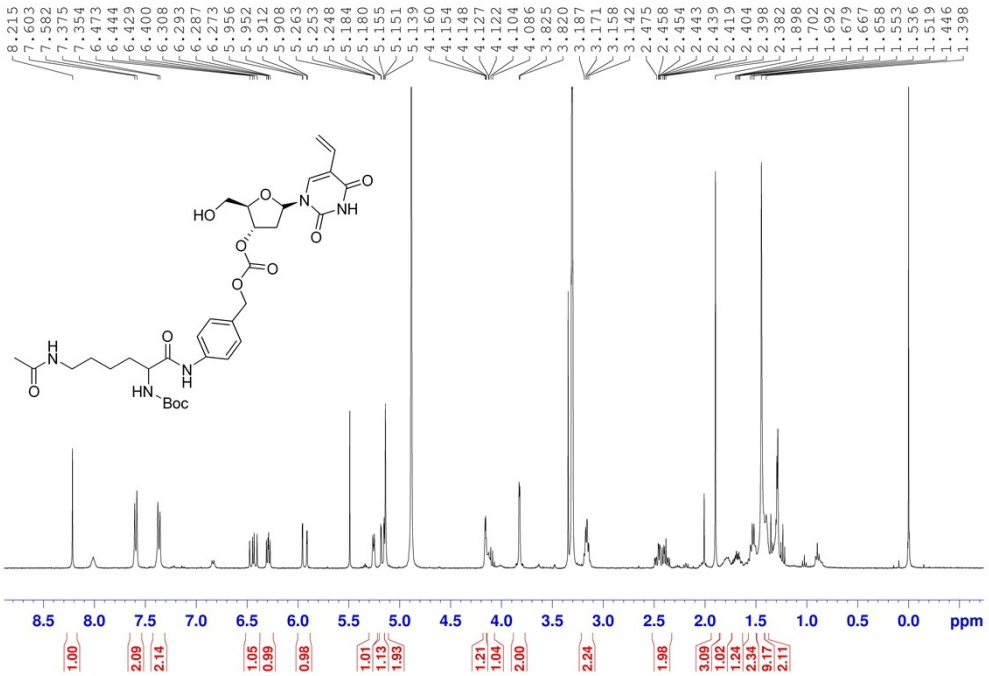


Fig. S27 ^{1}H NMR spectrum of VdU-Lys in CD_3OD

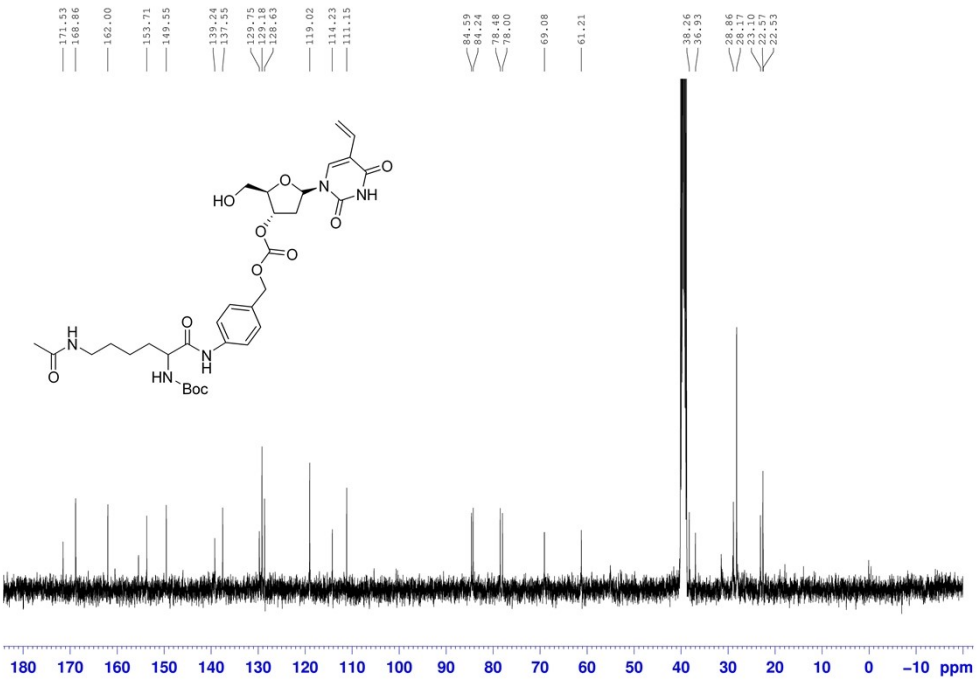
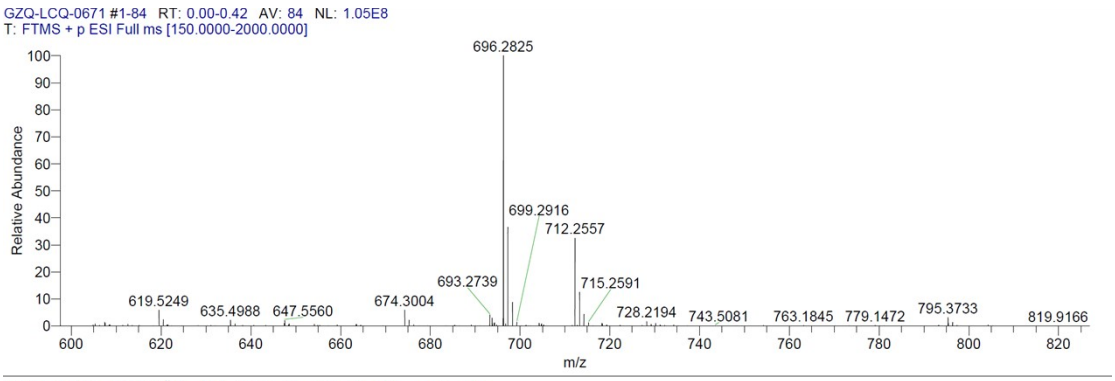


Fig. S28 ^{13}C NMR spectrum of VdU-Lys in DMSO-d_6



GZQ-LCQ-0671#1-84 RT: 0.00-0.42 AV: 84
T: FTMS + p ESI Full ms [150.0000-2000.0000]
m/z= 694.03-700.94

m/z	Intensity	Relative	Theo. Mass	Delta (ppm)	Composition
696.2825	106804112.0	100.00	696.2851	-2.62	$C_{32}H_{43}O_{11}N_5Na$

Fig. S29 HRMS spectrum of VdU-Lys

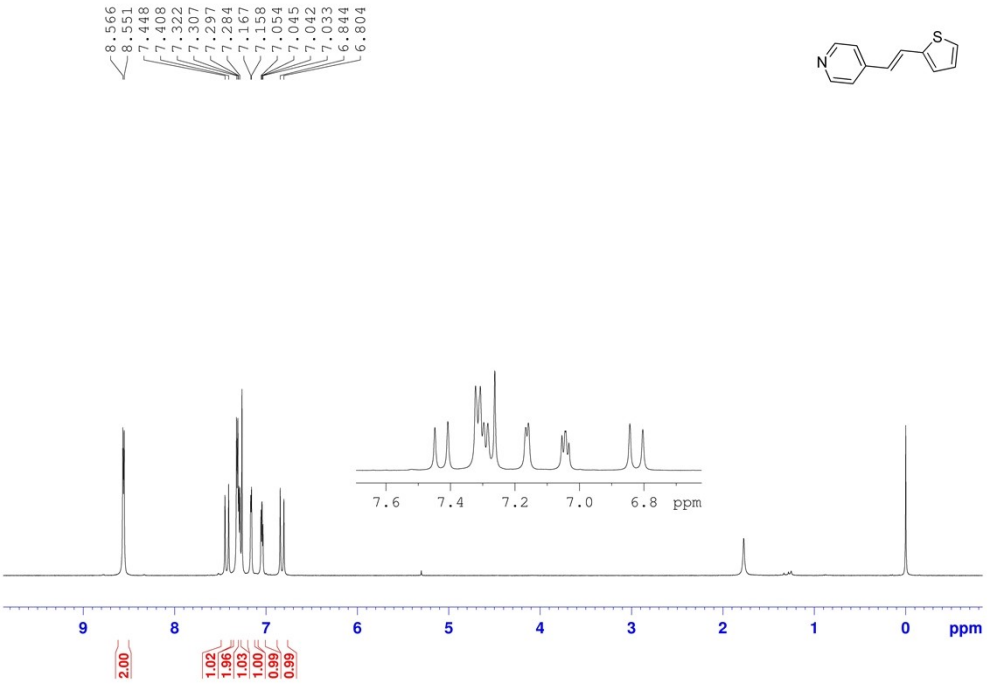
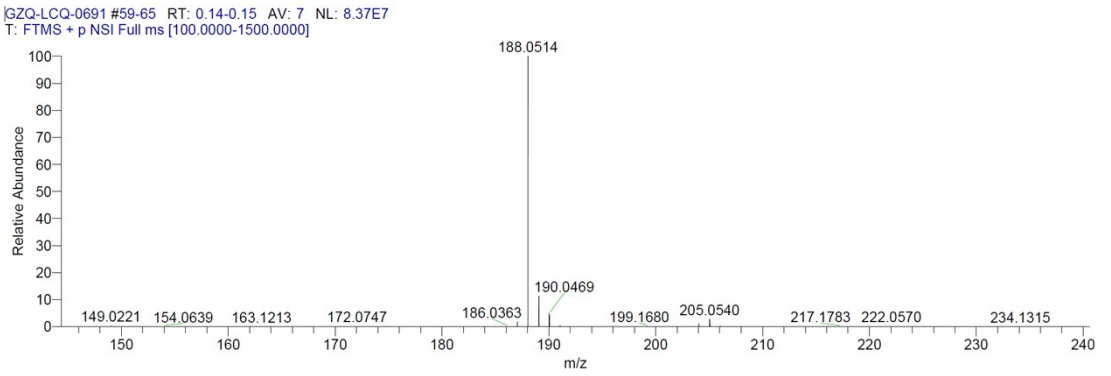


Fig. S30 ^{1}H NMR spectrum of PT in $CDCl_3$



GZQ-LCQ-0691#58-65 RT: 0.13-0.15 AV: 8
T: FTMS + p NSI Full ms [100.0000-1500.0000]
m/z= 187.38-189.17

m/z	Intensity	Relative	Theo. Mass	Delta (ppm)	Composition
188.0514	75411128.0	100.00	188.0528	-1.41	$C_{11}H_{10}NS$

Fig. S31 HRMS spectrum of PT

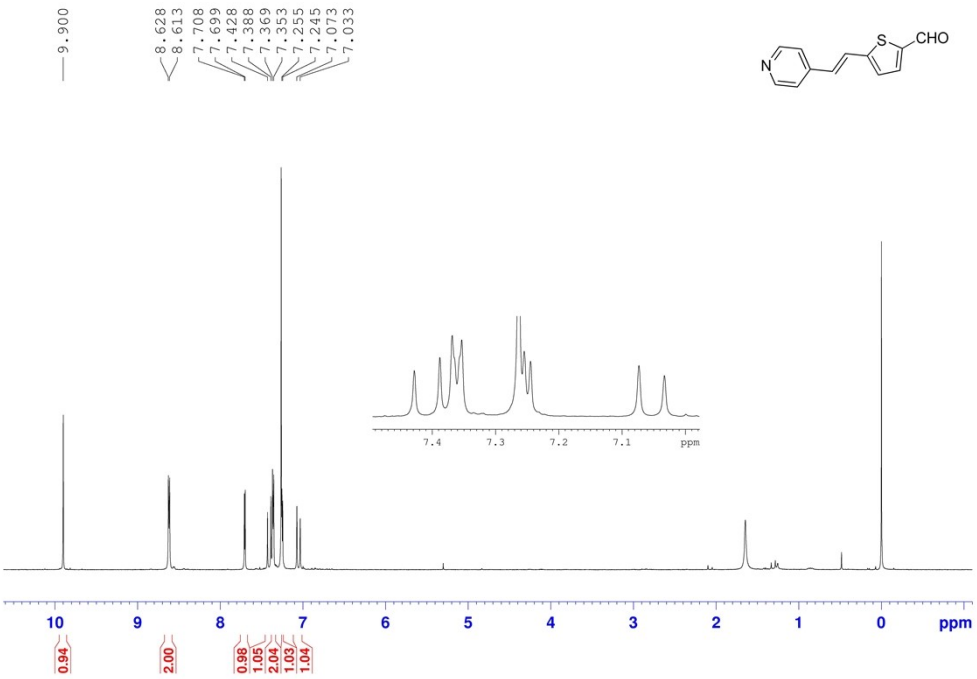
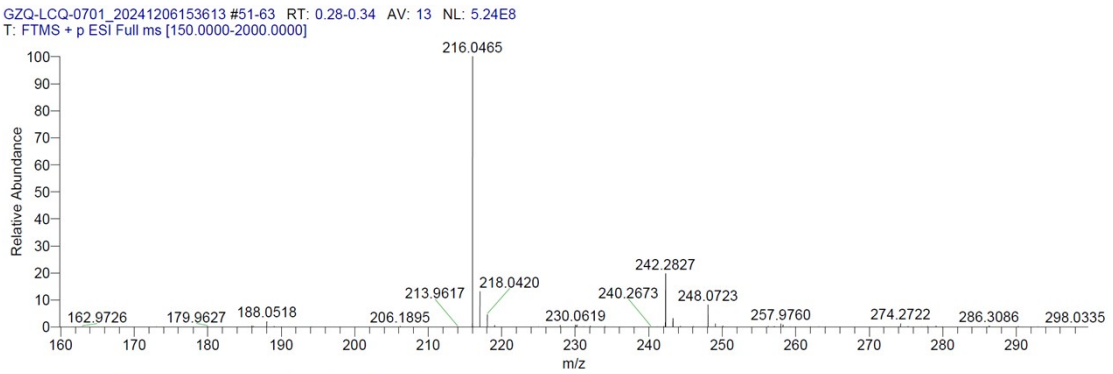


Fig. S32 ^{1}H NMR spectrum of PT-CHO in $CDCl_3$



GZQ-LCQ-0701_20241206153613#51-63 RT: 0.28-0.34 AV: 13
T: FTMS + p ESI Full ms [150.0000-2000.0000]
m/z= 215.67-216.75

m/z	Intensity	Relative	Theo. Mass	Delta (ppm)	Composition
216.0465	534888064.0	100.00	216.0478	-1.23	$C_{12}H_{10}ONS$

Fig. S33 HRMS spectrum of PT-CHO

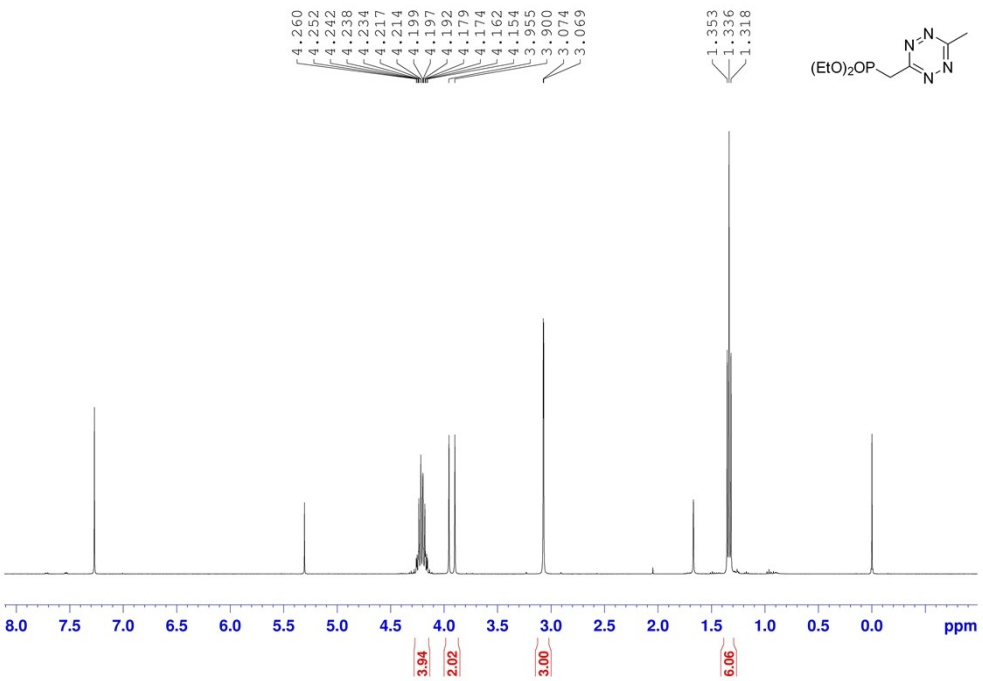
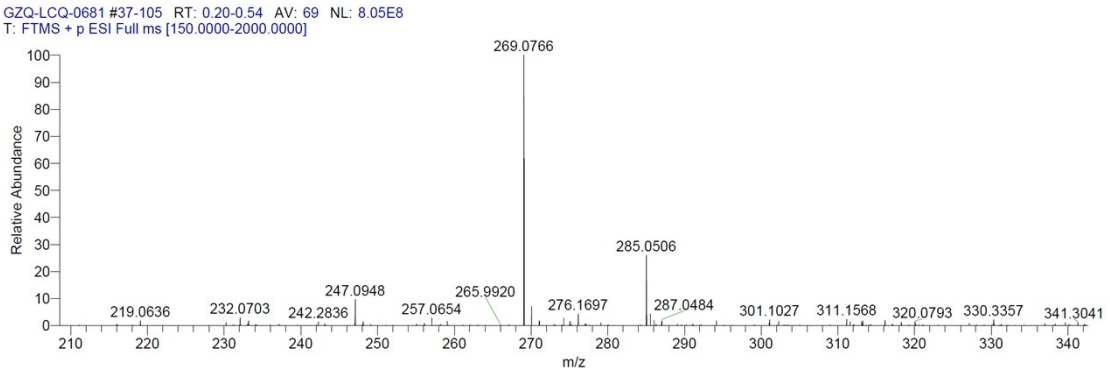


Fig. S34 ^{1}H NMR spectrum of P-Tz in $CDCl_3$



GZQ-LCQ-0681#37-105 RT: 0.20-0.54 AV: 69
T: FTMS + p ESI Full ms [150.0000-2000.0000]
m/z= 267.71-270.20

m/z	Intensity	Relative	Theo. Mass	Delta (ppm)	Composition
269.0766	821263296.0	100.00	269.0774	-0.77	$C_8H_{15}O_3N_4NaP$

Fig. S35 HRMS spectrum of P-Tz

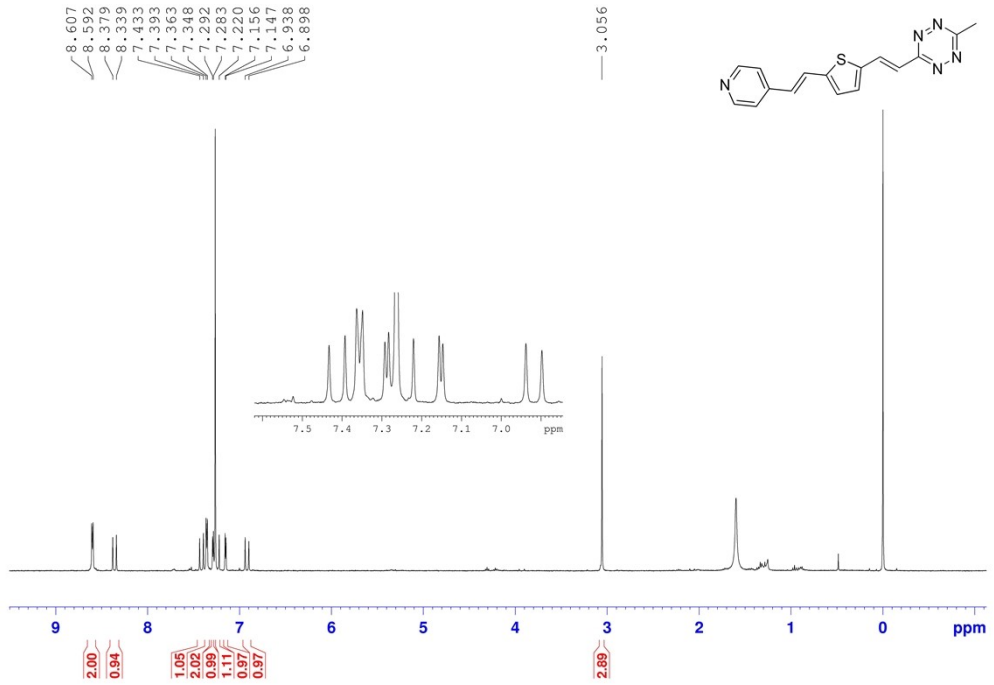


Fig. S36 ^{1}H NMR spectrum of PT-Tz in $CDCl_3$

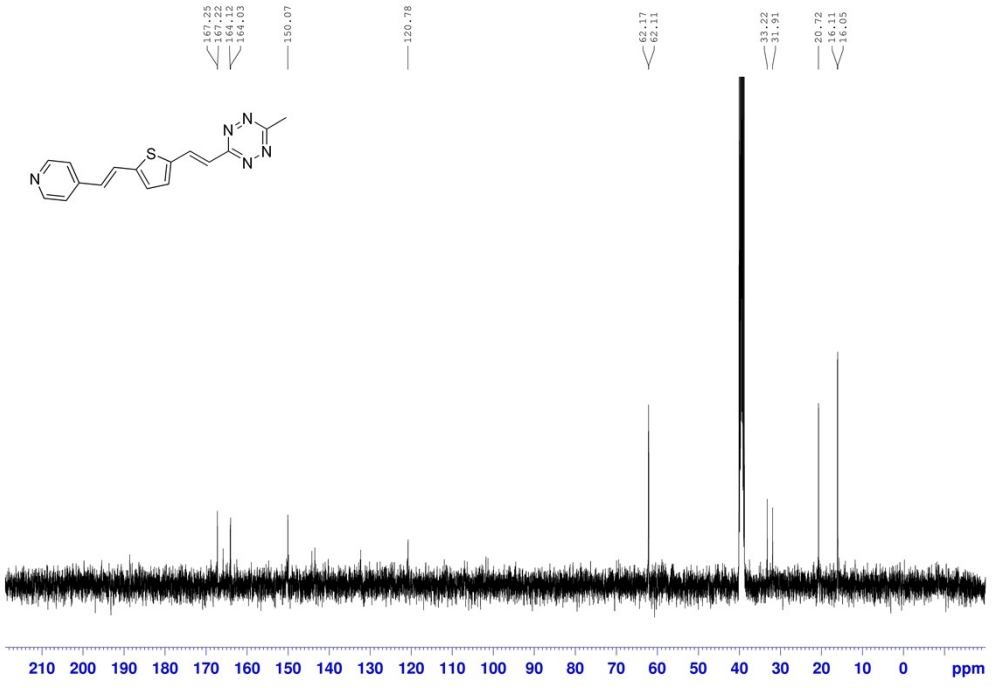
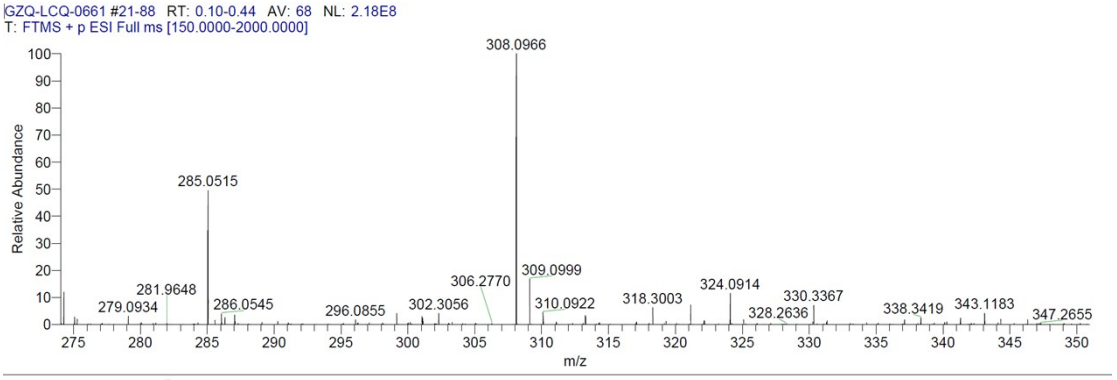


Fig. S37 ^{13}C NMR spectrum of PT-Tz in DMSO-d_6



GZQ-LCQ-0661#21-88 RT: 0.10-0.44 AV: 68
T: FTMS + p ESI Full ms [150.0000-2000.0000]
m/z= 307.73-308.57

m/z	Intensity	Relative	Theo. Mass	Delta (ppm)	Composition
308.0966	222325568.0	100.00	308.0964	0.13	$C_{16}H_{14}N_5S$

Fig. S38 HRMS spectrum of PT-Tz

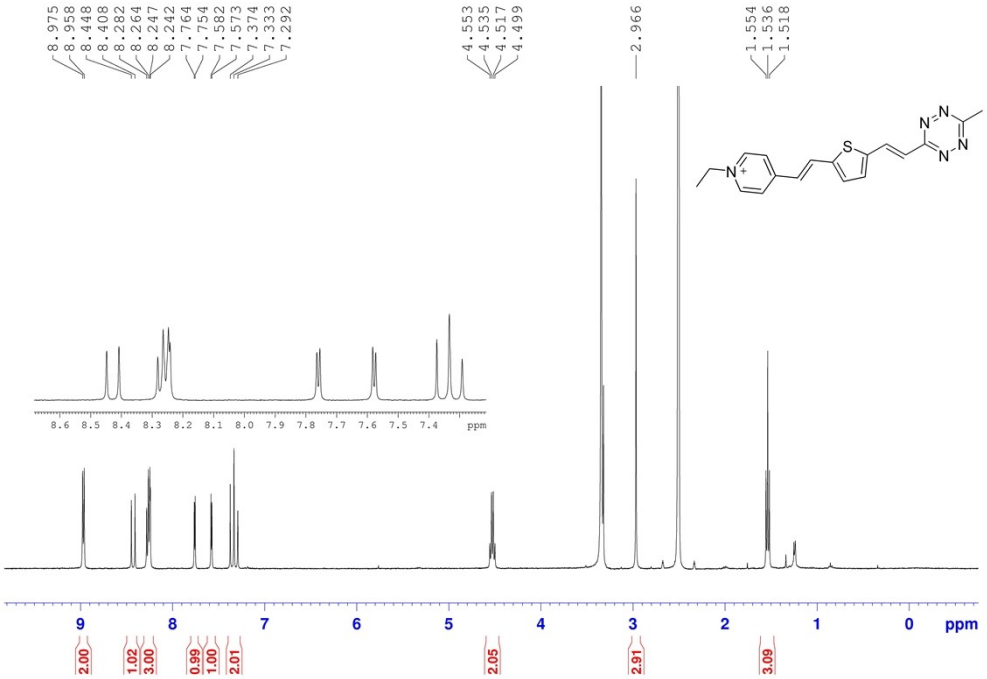


Fig. S39 ^{1}H NMR spectrum of Et-PT-Tz in DMSO-d_6

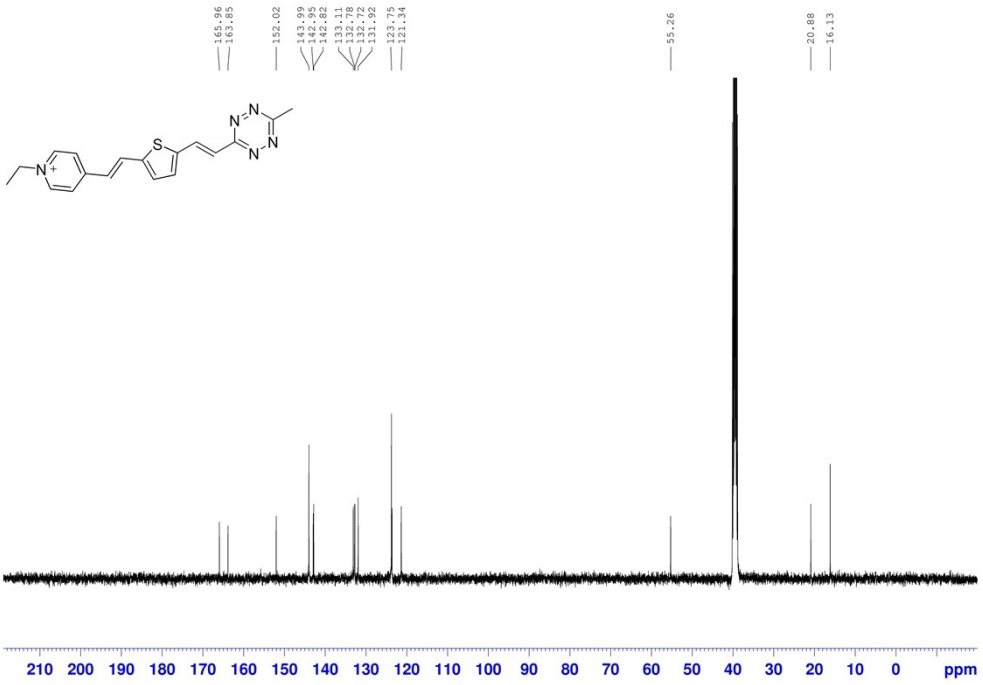


Fig. S40 ^{13}C NMR spectrum of Et-PT-Tz in DMSO-d_6

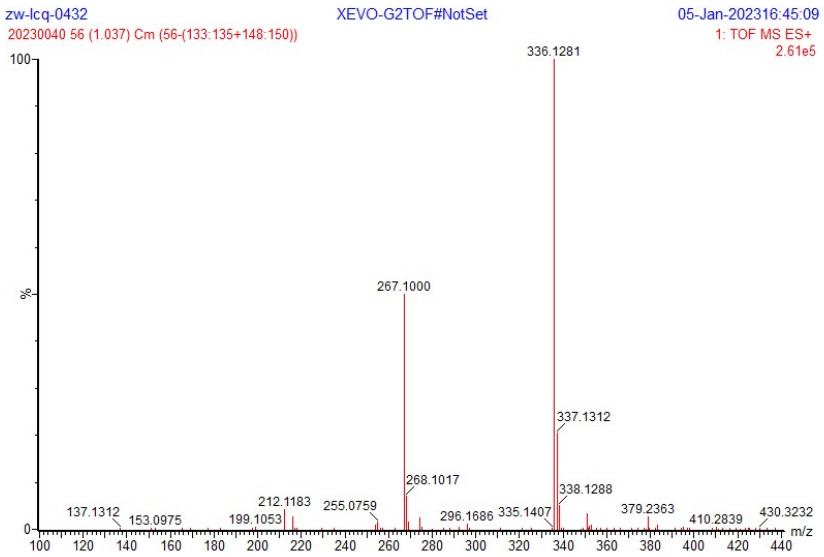


Fig. S41 HRMS spectrum of Et-PT-Tz