Supporting Information

Photo-triggered Fluorescent Theranostic Prodrug for DNA Alkylation Agent Mechlorethamine Releasing and Spatiotemporal Monitoring

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CONTENTS

General Information
Synthesis of prodrug 1
References
HPLC monitoring of drug release
Protocol of study of UV irradiation reaction of prodrug 1
Spectroscopic materials and methods
Preparation of cell cultures
Interstrand DNA cross-link formation study
Cell permeability test
Cell viability test
Fluorescence imaging of photoactivated prodrug 1
Fluorescence subcellular imaging of photoactivated prodrug 1
$^1$H and $^{13}$C NMR Spectra
General Information

All reactions were carried out in dried flasks. The reactions were monitored by TLC for completion. Commercially available reagents were used as received without further purification unless otherwise specified. Merck 60 silica gel was used for column chromatography, and Whatman silica gel plates with fluorescence F254 were used for thin-layer chromatography (TLC) analysis. $^1$H and $^{13}$C NMR spectra were recorded on Bruker Avance 500 or 300. Data for $^1$H are reported as follows: chemical shift (ppm), and multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet). Data for $^{13}$C NMR are reported as ppm. Mass Spectra were obtained from University of New Mexico Mass Spectral facility.

7-Hydroxy-8-methyl-2H-chromen-2-one (3)

A mixture of equimolar amount of 2-methylbenzene-1,3-diol (2.48 g, 20.0 mmol) and 2-hydroxysuccinic acid (2.48 g, 20.0 mmol) with 3.0 equiv. of concentrated sulfuric acid (5.88 g, 3.2 mL, 60 mmol) was exposed to microwave irradiation (240W, 120 °C) for 4 min. The reaction mixture was extracted with ethyl acetate and water. After solvent evaporation, the crude product was purified by silica gel column chromatography to give brown yellow solid as product (2.53 g, 72% yield). $^1$H NMR (CD$_3$OD, 300 MHz): δ 7.75 (d, $J = 9.3$ Hz, 1H), 7.22 (d, $J = 8.4$ Hz, 1H), 6.75 (d, $J = 8.4$ Hz, 1H), 6.11 (d, $J = 9.3$ Hz, 1H), 2.20 (s, 3H).

8-Methyl-2-oxo-2H-chromen-7-yl acetate (4)

7-Hydroxy-8-methyl-2H-chromen-2-one (1.76 g, 10 mmol) was dissolved in acetic anhydride (20.4 g, 200 mmol) and immersed in an ice water bath. After the addition of pyridine (948 mg, 12 mmol), the reaction mixture was stirred at rt for 12 h. Excess acetic anhydride was removed under reduced pressure. Residue was dissolved in dichloromethane, washed with saturated sodium bicarbonate, water, dried over sodium sulfate. The crude product was further purified by silica gel column chromatography to give (2.18 g, quantitative yield) white solids as product. $^1$H NMR (CDCl$_3$, 300 MHz): δ 7.66 (d, $J = 9.6$ Hz, 1H), 7.31 (d, $J = 8.4$ Hz, 1H), 6.97 (d, $J = 8.4$ Hz, 1H), 6.35 (d, $J = 9.6$ Hz, 1H), 2.34 (s, 3H), 2.25 (s, 3H). $^{13}$C NMR (CDCl$_3$, 75 MHz): δ 169.21, 161.05, 153.63, 152.26, 143.90, 126.06, 119.92, 119.04, 117.16, 116.15, 21.24, 9.48. ESI-MS: calcd for C$_{12}$H$_{11}$O$_4$ (M$^+$ + H): 219.0657, found 219.0644.

8-(Bromomethyl)-2-oxo-2H-chromen-7-yl acetate (5)

8-Methyl-2-oxo-2H-chromen-7-yl acetate (2.18 g, 10.0 mmol) was dissolved in 15 mL of carbon tetrachloride, then NBS (2.136 g, 12.0 mmol) was added to the reaction mixture followed by AIBN (49 mg, 0.2 mmol). The reaction mixture was stirred under reflux for 6 hours. After solvent removal under reduced pressure, the crude product was further purified by silica gel column chromatography to give light yellow solid as product (2.25 g, 76% yield).
7-Hydroxy-8-(hydroxymethyl)-2H-chromen-2-one (2)
A mixture of calcium carbonate (2.6 g, 26 mmol) in 12 mL of water was added to a solution of 8-(bromomethyl)-2-oxo-2H-chromen-7-yl acetate (1.468 g, 5.0 mmol) in 12 mL of dioxane. The mixture was stirred at 50 °C for 24 h. Dioxane was removed under reduced pressure to give a white solid. After extraction using ethyl acetate and 2M HCl, the crude product was further purified by silica gel column chromatography to give white solids as product (968 mg, 80% yield). 1H NMR (acetone-2, 300 MHz): δ 8.22 (d, J = 8.1 Hz, 1H), 7.90 (d, J = 7.8 Hz, 1H), 7.65 (m, 2H), 7.56 (m, 1H), 7.42 (d, J = 8.7 Hz, 1H), 6.94 (d, J = 8.7 Hz, 1H), 6.31 (d, J = 9.6 Hz, 1H), 5.64 (s, 2H), 5.07 (d, J = 6.0 Hz, 2H), 2.35 (t, J = 9.6 Hz, 1H), 1.13 C NMR (DMSO-2, 75 MHz): δ 160.20, 158.99, 152.91, 144.62, 134.25, 132.48, 129.12, 129.02, 128.84, 124.95, 116.83, 113.03, 112.80, 109.38, 67.06, 50.85. ESI-MS: calcd for C10H9O4 [M+H]+: 193.0501, found 193.0492.

8-(Hydroxymethyl)-7-(2-nitrobenzoyloxy)-2H-chromen-2-one (6).
Potassium carbonate (662 mg, 4.8 mmol) was dissolved in 25 mL of acetonitrile, followed by the addition of 7-hydroxy-8-(hydroxymethyl)-2H-chromen-2-one (936 mg, 4 mmol) and 1-(bromomethyl)-2-nitrobenzene (1046 mg, 4.8 mmol). The reaction mixture was stirred in dark at 50 °C for 5 h. After solvent removal under reduced pressure, the crude product was further purified by silica column chromatography to give white solids as product (968 mg, 74% yield). 1H NMR (CDCl3, 300 MHz): δ 8.22 (d, J = 8.1 Hz, 1H), 7.90 (d, J = 7.8 Hz, 1H), 7.65 (m, 2H), 7.56 (m, 1H), 7.42 (d, J = 8.7 Hz, 1H), 6.94 (d, J = 8.7 Hz, 1H), 6.31 (d, J = 9.6 Hz, 1H), 5.64 (s, 2H), 5.07 (d, J = 6.0 Hz, 2H), 2.35 (t, J = 9.6 Hz, 1H), 13C NMR (DMSO-d6, 75 MHz): δ 160.20, 158.99, 152.91, 144.62, 134.25, 132.48, 129.12, 129.02, 128.84, 124.95, 116.83, 113.03, 112.80, 109.38, 67.06, 50.85. ESI-MS: calcd for C17H13NO6 (M+): 327.0743, found 327.0751.

Synthesis of 8-(bromomethyl)-7-(2-nitrobenzoyloxy)-2H-chromen-2-one (7)
A solution of tribromophosphine (643 mg, 226 μL, 2.4 mmol) in 2.0 mL of anhydrous dichloromethane was added dropwise to a solution of 8-(hydroxymethyl)-7-(2-nitrobenzoyloxy)-2H-chromen-2-one (654 mg, 2 mmol) in 10 mL of anhydrous dichloromethane over 30 min with the temperature between -5 °C and -3 °C. The reaction mixture was stirred in dark at rt for 40 min. After the reaction is complete, it was washed with brine, concentrated and run column to give white solids as product (599 mg, 77% yield). 1H NMR (CDCl3, 300 MHz): δ 8.23 (d, J = 2.5 Hz, 1H), 8.04 (d, J = 7.8 Hz, 1H), 7.76 (d, J =
7.2 Hz, 1H), 7.65 (d, J = 9.6 Hz, 1H), 7.55 (d, J = 7.8 Hz, 1H), 7.43 (d, J = 8.7 Hz, 1H), 6.92 (d, J = 8.7 Hz, 1H), 6.32 (d, J = 9.6 Hz, 1H), 5.69 (s, 2H), 4.88 (s, 2H). $^{13}$C NMR (CDCl$_3$, 75 MHz): δ 160.15, 158.64, 152.91, 146.71, 143.25, 134.50, 132.59, 129.23, 128.78, 128.32, 125.21, 114.58, 114.07, 113.51, 108.60, 67.57, 20.22. ESI-MS: calcld for C$_{17}$H$_{12}$BrNO$_3$ (M$^+$): 388.9899, found 388.9881.

![Structure of 2-Hydroxy-N-(2-hydroxyethyl)-N-methyl-N-((7-(2-nitrobenzyloxy)-2-oxo-2H-chromen-8-yl)methyl)ethanaminium bromide (8)](image)

2-Hydroxy-N-(2-hydroxyethyl)-N-methyl-N-((7-(2-nitrobenzyloxy)-2-oxo-2H-chromen-8-yl)methyl)ethanaminium bromide (8)

A solution of 8-(bromomethyl)-7-(2-nitrobenzyloxy)-2H-chromen-2-one (389 mg, 1.0 mmol) and 2,2′-(methylazanediyldiethanol (357 mg, 3.0 mmol) in 10 mL of anhydrous acetonitrile was stirred in dark at rt for 12 h. After filtration, white solids were obtained as product (309mg, 61% yield). $^1$H NMR (DMSO-$d_6$, 300 MHz): δ 8.21 (d, J = 8.1 Hz, 1H), 8.07 (d, J = 9.6 Hz, 1H), 7.89 (d, J = 8.7 Hz, 1H), 7.81 (m, 2H), 7.68 (m, 1H), 7.29 (d, J = 9.0 Hz, 1H), 6.42 (d, J = 9.6 Hz, 1H), 5.71 (s, 1H), 5.25 (t, J = 4.8 Hz, 2H), 4.84 (s, 2H), 3.89 (m, 4H), 3.64 (m, 2H), 3.46 (m, 2H), 3.02 (s, 3H). $^{13}$C NMR (DMSO-$d_6$, 75 MHz): 160.31, 159.16, 154.58, 147.20, 144.34, 134.42, 132.50, 131.46, 129.51, 129.31, 125.16, 113.56, 113.41, 109.87, 104.30, 68.08, 64.08, 55.64, 55.23, 54.92, 48.48. ESI-MS: calcld for C$_{22}$H$_{25}$N$_2$O$_7$ (M$^+$) 429.1656, found 429.1638.

![Structure of 2-Chloro-N-(2-chloroethyl)-N-methyl-N-((7-(2-nitrobenzyloxy)-2-oxo-2H-chromen-8-yl)methyl)ethanaminium bromide (1)](image)

2-Chloro-N-(2-chloroethyl)-N-methyl-N-((7-(2-nitrobenzyloxy)-2-oxo-2H-chromen-8-yl)methyl)ethanaminium bromide (1)

2-Hydroxy-N-(2-hydroxyethyl)-N-methyl-N-((7-(2-nitrobenzyloxy)-2-oxo-2H-chromen-8-yl)methyl)ethanaminium bromide (254 mg, 0.5 mmol) was dissolved in 10 mL of thionyl chloride. The reaction mixture was stirred at rt for 3 d. After solvent evaporation under reduced pressure, white solid was obtained as product (272 mg, quantitative yield). $^1$H NMR (DMSO-$d_6$, 300 MHz): δ 8.21 (d, J = 8.1 Hz, 1H), 8.08 (d, J = 9.3 Hz, 1H), 7.90 (d, J = 8.7 Hz, 1H), 7.78 (m, 2H), 7.67 (m, 1H), 7.28 (d, J = 8.7 Hz, 1H), 6.43 (d, J = 9.3 Hz, 1H), 5.74 (s, 2H), 4.88 (s, 2H), 4.17 (m, 4H), 3.86 (m, 4H), 3.15(s, 3H). $^{13}$C NMR (DMSO-$d_6$, 75 MHz): 160.33, 158.95, 154.73, 147.23, 144.39, 134.32, 132.85, 131.31, 129.54, 129.45, 125.14, 113.56, 113.37, 109.98, 103.54, 68.21, 61.64, 55.03, 48.07, 36.51. ESI-MS: calcld for C$_{22}$H$_{23}$Cl$_2$N$_2$O$_3$ (M$^+$) 465.0984, found 465.0973.

References
Figure S1. HPLC monitoring of drug release of prodrug 1. (a) 0 min; (b) 28 min; (c) 85 min. Photolytic activation of 0.1 mM fluorescent prodrug 1 in pH = 7.4 HEPES buffer, irradiated by a hand-held UV lamp (λ = 365 nm). Prodrug 1 (2.0 mM) was dissolved in an aqueous solution of MeCN/H$_2$O (20:80), a hand-held UV lamp (365 nm, 2W) was positioned 10 cm away from the reaction vial as light source. After designated reaction time, an aliquot of 20 μL solution was taken and submitted to RP-HPLC to be analyzed by a 254 nm UV detector, the amount was integrated by the area under each peak, and calculated from the standard curves under the same HPLC condition.

HPLC condition: Eclipse XDB-C8 column, flow rate is 0.8 mL/min, 0-20 min: MeCN/H$_2$O (10:90), 20-40 min: MeCN/H$_2$O (30:70).

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Figure S2. UV-vis spectra in the absence of UV and after 15 min UV exposure of prodrug 1. Photolytic activation of 0.1 mM fluorescent prodrug 1 in pH = 7.4 HEPES buffer, irradiated by a hand-held UV lamp (λ = 365 nm, 2W, 8.9 mW/cm$^2$).
Protocol of study of UV irradiation reaction of prodrug 1

Prodrug 1 (13 mg) was dissolved in 239.2 µL of DMSO and 2152.8 µL of PBS buffer to make a solution of 10 mM. A hand-held UV lamp (365 nm, 2W, 8.9 mW/cm²) was positioned 10 cm away from the reaction vial as light source. Reaction was complete in 90 min, extracted with ethyl acetate and water, after solvent removal, the crude mixture was submitted to column chromatograph to obtain 1.5 mg white solids as product (isolated yield of 71%). ¹H NMR (acetone-d₆, 300 MHz): δ 9.62 (s, 1H), 7.84 (d, J = 9.6 Hz, 1H), 7.43 (d, J = 8.7 Hz, 1H), 6.82 (d, J = 8.4 Hz, 1H), 6.15 (d, J = 9.6 Hz, 1H), 5.02 (s, 2H), 2.02 (d, J = 6.0 Hz, 1H). ¹³C NMR (acetone-d₆, 300 MHz): δ 161.11, 160.85, 153.87, 145.08, 129.13, 114.57, 113.95, 112.62, 112.55, 56.05. ESI-MS: [M+H] calculated for C₁₀H₉O₄: 193.05; found: 193.05.

Spectroscopic materials and methods

Millipore water was used to prepare all aqueous solutions. The pH was recorded by a Beckman ΦTM 240 pH meter. UV absorption spectra were recorded on a Shimadzu UV-1800 spectrophotometer. Fluorescence emission spectra were obtained on a Shimadzu RF-5301PC spectrofluorophotometer. Prodrug 1 in DMSO (10 mM) was prepared as the stock solution, 50 µL of the stock solution was added to 4.950 mL of 20 mM pH 7.4 HEPES buffer to make the final concentration of 0.1 mM. A hand-held UV lamp (365 nm, 2W) was positioned 10 cm away from the reaction vial as light source. Change in fluorescence emission (λₜ₉ = 375 nm, λₜ₉ = 448 nm) and UV-vis spectra were measured at designated time interval.

Preparation of cell cultures

HeLa cell line was purchased from American Type Culture Collection. The cells were cultured in DMEM containing 10% FBS and 1% (v/v) antibiotic-antimycotic solution at 37 °C with 95% air/5% CO₂ in an incubator.

Human Epidermal Keratinocytes (HEKn) cells were obtained from Lifeline Cell Technology. The cells were cultured in DermaLife Basal Medium (DermaLife K Medium Complete Kit) at 37 °C with 95% air/5% CO₂ in an incubator.

Interstrad DNA cross-links formation study

General Procedure for Linearization of Plasmid pBR322 by EcoRI.

pBR322 Vector (New England Biolabs) (28µL, 28 µg) was incubated with EcoRI-HF (New England Biolabs) (20 µL), EcoR1 buffer (10 × 20 µL), and 132 µL of H₂O (sterile) for 3 h at 37 °C. NaOAc (20 µL, 3M) and ethanol (750 µL) were added, and the solution was cooled at -20 °C overnight. The mixture was centrifuged for 15 min at 16000 rpm, and the ethanol was decanted off. The remainder of the ethanol was evaporated off in vacuo at -20 °C, and the remaining linearized DNA was suspended in 100 µL of sterile H₂O. The amount of linearized pBR322 was quantitated by NANODROP 2000 spectrophotometer (Thermo Scientific).

Photoreaction of DNA with prodrug 1, control compound 4-14, or mechloethamine.

Solutions contained 1 µg linearized plasmid DNA in 0.15 M NaCl, 0.01 M Tris-HCl, and 0.001 M EDTA, pH 7.4. 10mM stock solution of prodrug 1 or control compound 8 or mechloethamine was added to a final concentration of 1mM, obtaining a final volume of 50 µL. The Interstrad DNA cross-link formation reactions were conducted 75 ± 5 cm away from UV light (30 W g30t8 bubble from General Electronic Company, 5.3 mW/cm²). After 1 hour incubation, the reaction solution was submitted to alkaline agarose gel electrophoresis.
General Protocol for Alkaline Agarose Gel Electrophoresis.
The agarose gels were prepared by adding 150 mL of a 50 mM NaCl/2 mM EDTA (at pH = 8.0) to 1.8 g of agarose. The suspension was heated in a microwave oven until all of the agarose was dissolved (3 min). The gel was allowed to cool until 50 °C and poured and solidified for 1 h at room temperature. The gel was soaked in an alkaline running buffer (25 mL of 2 N NaOH, 4 mL of 0.25 M EDTA in 1 L of H2O). Agarose loading dye (New England Biolabs) (6 × 10 µL) was added to the samples (50 µL), and the samples were loaded into the wells. The gel was run for 3 h at 200 mA/30 V. The gel was then neutralized for 45 min in a 1 M Tris pH = 7/1.5 M NaCl solution, which was refreshed every 15 min. The gel was subsequently stained in an ethidium bromide solution (100 µL of a 10 mg/mL ethidium bromide solution in 1 L of 1 M Tris /1.5 M NaCl buffer at pH = 7.5) for 1 h. Gels were visualized by UV and photographed using Gel Doc™ XR+ System (BIO-RAD).

Cell permeability test
HeLa cell line was obtained from American Type Culture Collection. The cells were seeded in the 35mm dishes at a density of 3 × 105 cells/dish. The cells were cultured in DMEM (Life technology, USA) containing 10% (v/v) FBS (Life technology, USA) and 1% (v/v) Anti-anti solution (Life technology, USA) at 37 °C with 95% air/5% CO2 in an incubator. After the cells reach confluence (1.2 × 106 cells/dish), 0.8 mM prodrug 1 was incubated with HeLa cells for 2 h, then RIPA buffer (Sigma, USA) with 1% (v/v) protease inhibitor cocktail (Life technology, USA) was added, cell lysate was collected and injected into RP-HPLC, prodrug 1 peak was detected and quantified.

Cell viability test
Cell viability was measured by using cell counting kit-8 (CCK-8), which quantitatively measures activities of dehydrogenases in cells. Cells (5 × 10^3 cells/well) were seeded into 96-well microtiter plates. Following treatment with prodrug 4-1 and different reaction conditions (in the presence or absence of 365 nm UV light (30 W g30t8 bubble from General Electronic Company, 8.9 mW/cm²)), 10 µL of CCK-8 solution was added to each well of the plate. The plate was placed into the incubator at 37 °C with 95% air/5% CO2. After one hour incubation, the absorbance was measured at 450 nm in a Bio-Rad 3350 microplate reader. Cells without any treatment were used as 100% cell viability. The cell viability was calculated by using the formula: Cell viability = (Experimental absorbance value – culture medium absorbance value)/(without treatment absorbance value – culture medium absorbance value).

Fluorescence imaging of photoactivated prodrug 1
HeLa cells were plated onto polylysine-coated glass coverslips. After reaching 70% confluence, 350 µM prodrug 1 was added to Hela cells, incubated for 2 h before 30 min UV exposure (30 W g30t8 bubble from General Electronic Company). Next, cells were washed thoroughly by DMEM. The coverslips were mounted on a glass slide. Images were acquired using an inverted microscope with a DAPI dichroic mirror.

Fluorescence subcellular imaging of photoactivated prodrug 1
HeLa cells were plated onto polylysine-coated glass coverslips. After reaching 70% confluence, cells were incubated with prodrug 1 for 30 min, followed by 30 min UV exposure (30 W g30t8 bubble from General Electronic Company). Cells were then washed 3 times with warmed DMEM medium, fixed with 4% paraformaldehyde for 15 min at room temperature.
temperature (rt) and washed with PBS again. For counter staining, cells were permeabilized with 0.1% Triton-X100 for 10 min and rinsed with PBS again. Nuclei were labeled using propidium iodide (10 mg/mL) and cover glasses were mounted by AntifadeH mounting media. All slides were kept in dark until fluorescence images were acquired using an inverted microscope with a DAPI dichroic mirror for prodrug imaging and DSRED dichroic mirror for nuclei stain image.