Supplementary Material

Targeted methotrexate prodrug conjugated with heptamethine cyanine dye improving chemotherapy and monitoring itself activating by dual-modal imaging

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**Supplementary Figure 1:** Synthetic route of Cy-SS-MTX/Cy-CC-MTX

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**Supplementary Figure 2:** **(A)** Normalized UV-Vis absorption of Cy-SS-MTX (5 μM) in the DMSO/PBS (V/V=4:6) solution with or without 250 μM GSH. **(B)** Normalized UV-Vis absorption of Cy-CC-MTX (5 μM) in the DMSO/PBS (V/V=4:6) solution with or without 250 μM GSH. All spectrums were measured after incubation for 15 min at 37℃ temperature (Cy: IR780 and MTX: Methotrexate).

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**Supplementary Figure 3: (A)** Normalized UV-Vis absorption of Cy-CC-MTX (5 μM) in the DMSO/PBS (V/V=4:6) solution with various concentration GSH. **(B)** The fluorescence spectra of Cy-CC-MTX excited by 640 nm (5 μM) in the DMSO/PBS (V/V=4:6) solution with various concentration GSH Cy-SS-MTX (5 μM). **(C)** Cy-CC-MTX excited by 745 nm.

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**Supplementary Figure 4:** **(A)** The fluorescence spectra of Cy-SS-MTX (5 μM) excited by 640 nm with thiol-containing structure (750 μM GSH/DTT) or other amino acids (750 μM). **(B)** Cy-SS-MTX (5 μM) excited by 745 nm. **(C)** The time dependence of fluorescence intensity at 808 nm of Cy-SS-MTX/Cy-CC-MTX in the presence of GSH (750 μM).

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**Supplementary Figure 5:** The photoacoustic imaging of Cy-SS-MTX **(A)**/Cy-CC-MTX **(B)** excited by 680 nm and 808 nm respectively in the presence of various concentration of GSH. The value of concentration of Cy-SS-MTX/Cy-CC-MTX was 15 μg/ml. The samples were tested after exposure to GSH for 1h at 37℃.

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**Supplementary Figure 6:** The PA intensity responses of Cy-SS-MTX/Cy-CC-MTX to GSH according to **Supplemental figure 4**.

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**Supplementary Figure 7**: Time dependence of photoacoustic imaging of Cy-SS-MTX **(A)**/Cy-CC-MTX **(B)** excited by 680 nm and 808 nm respectively in the presence of GSH (1500 μg/ml). The value of concentration of Cy-SS-MTX/Cy-CC-MTX was 15 μg/ml.

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**Supplementary Figure 8:** The PA intensity responses of Cy-SS-MTX/Cy-CC-MTX to time according to Supplemental figure 6.

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**Supplementary Figure 9:** Western Blot to study the cell cycle arrest and cytotoxic assay induced by Cy-SS-MTX in MCF-7 cells.

**Supplementary Figure 10:** TOF Mass Spectrometer of Cy-SS/Cy-SS-MTX/Cy-CC/Cy-CC-MTX.

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**Supplementary Figure 11:** 1H NMR and 13C NHR (Bruker 400 MHz) of below materials

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1H NMR (400 MHz, HDMSO): δ 8.33 (s, 1H), 7.70 (t, J = 11.4 Hz, 1H), 7.46 (dd, J = 12.0, 7.5 Hz, 1H), 7.30 (t, J = 7.7 Hz, 1H), 7.17 (d, J = 7.9 Hz, 1H), 7.07 (t, J = 7.3 Hz, 1H), 5.82 (d, J = 13.0 Hz, 1H), 3.97 (dd, J = 19.2, 12.5 Hz, 2H), 3.36 (s, 11H), 3.19 (dd, J = 10.8, 5.2 Hz, 1H), 3.13 – 3.01 (m, 1H), 3.04 – 2.90 (m, 1H), 2.58 – 2.38 (m, 3H), 1.72 (d, J = 7.1 Hz, 3H), 1.58 (d, J = 39.1 Hz, 6H), 1.24 (s, 1H), 1.20 (d, J = 4.8 Hz, 1H), 1.04 – 0.84 (m, 3H).

13C NMR(101 MHz, DMSO): δ 169.45, 167.67, 143.53, 140.36, 138.74, 128.56, 122.91, 122.53, 120.35, 109.70, 95.06, 49.05, 48.12, 47.74, 44.24, 40.62, 40.41, 40.20, 39.99, 39.78, 39.57, 39.36, 38.19, 37.98, 34.53, 34.15, 28.85, 25.14, 21.82, 20.14, 19.34, 11.72.

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1H NMR (400 MHz, DMSO): δ 8.55 (d, *J* = 6.5 Hz, 1H), 8.33 (s, 1H), 8.10 (d, *J* = 6.3 Hz, 1H), 7.82 (d, *J* = 6.5 Hz, 1H), 7.74 (d, *J* = 8.8 Hz, 1H), 7.67 – 7.52 (m, 1H), 7.57 – 7.43 (m, 1H), 7.41 (d, *J* = 7.1 Hz, 1H), 7.26 (t, *J* = 7.6 Hz, 1H), 7.10 (d, *J* = 7.9 Hz, 1H), 7.01 (t, *J* = 7.4 Hz, 1H), 6.91 – 6.72 (m, 1H), 6.59 (dd, *J* = 8.9, 3.9 Hz, 1H), 5.75 (d, *J* = 12.2 Hz, 1H), 4.77 (s, 1H), 3.93 (d, *J* = 29.0 Hz, 3H), 3.36 (d, *J* = 5.7 Hz, 8H), 3.29 – 3.05 (m, 5H), 2.95 (s, 1H), 2.86 – 2.69 (m, 1H), 2.55 – 2.39 (m, 4H), 2.39 – 2.14 (m, 1H), 2.13 (t, *J* = 7.6 Hz, 1H), 2.06 – 1.82 (m, 1H), 1.70 (dd, *J* = 14.3, 7.1 Hz, 2H), 1.59 (s, 4H), 1.24 (s, 1H), 0.93 (t, *J* = 7.1 Hz, 2H).

13C NMR(101 MHz, DMSO): δ 173.98, 172.99, 166.57, 165.62, 163.32, 163.17, 155.67, 154.42, 151.30, 151.24, 149.75, 149.66, 146.41, 144.51, 139.23, 132.37, 129.46, 128.73, 128.44, 122.44, 122.22, 121.88, 111.73, 111.50, 109.08, 107.17, 92.62, 71.62, 55.31, 54.00, 51.78, 51.57, 47.22, 46.36, 43.83, 40.62, 40.41, 40.20, 39.99, 39.79, 39.58, 39.37, 39.06, 38.27, 30.53, 29.24, 28.81, 28.72, 28.04, 25.51, 21.84, 20.03, 19.72, 19.34, 11.80, 11.73.

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1H NMR (400 MHz, HDMSO): δ 8.54 (s, 1H), 7.83 (s, 3H), 7.56 (d, J = 12.9 Hz, 2H), 7.43 (d, J = 7.2 Hz, 2H), 7.30 – 7.18 (m, 2H), 7.21 – 7.04 (m, 3H), 7.04 (d, J = 7.4 Hz, 2H), 5.76 (d, J = 12.8 Hz, 2H), 3.90 (t, J = 6.8 Hz, 4H), 3.67 (s, 1H), 3.65 (d, J = 3.9 Hz, 2H), 2.73 (s, 2H), 2.47 (dt, J = 8.8, 4.4 Hz, 7H), 1.68 (dd, J = 14.2, 7.1 Hz, 8H), 1.66 – 1.44 (m, 15H), 1.32 (s, 5H), 1.20 (s, 1H), 0.91 (t, J = 7.4 Hz, 6H).

13C NMR(101 MHz, DMSO): δ 169.41, 167.23, 143.56, 140.16, 138.41, 128.59, 122.75, 122.54, 120.19, 109.60, 94.71, 71.61, 50.18, 49.05, 47.57, 44.11, 30.91, 28.82, 27.67, 27.39, 26.36, 26.21, 25.23, 21.82, 20.10, 19.34, 11.73.

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1H NMR (400 MHz, DMSO) δ 8.65 – 8.42 (m, 1H), 8.13 (s, 1H), 7.83 (s, 1H), 7.69 (ddd, *J* = 39.3, 24.7, 8.9 Hz, 2H), 7.49 (d, *J* = 8.9 Hz, 1H), 7.41 (d, *J* = 7.3 Hz, 1H), 7.25 (t, *J* = 7.6 Hz, 1H), 7.07 (d, *J* = 7.7 Hz, 1H), 7.00 (d, *J* = 7.4 Hz, 1H), 6.90 – 6.71 (m, 2H), 6.61 (s, 2H), 5.70 (d, *J* = 11.4 Hz, 1H), 4.77 (d, *J* = 5.7 Hz, 2H), 3.87 (s, 2H), 3.66 (s, 1H), 3.35 (s, 26H), 3.18 (s, 6H), 2.51 (dt, *J* = 3.5, 1.7 Hz, 8H), 2.14 (dd, *J* = 23.9, 17.0 Hz, 2H), 1.87 (d, *J* = 6.7 Hz, 1H), 1.69 (d, *J* = 7.1 Hz, 3H), 1.57 (s, 4H), 1.36 (s, 4H), 0.93 (t, *J* = 6.8 Hz, 3H).

13C NMR (101 MHz, DMSO) δ 167.40, 163.30, 163.15, 155.65, 149.64, 146.51, 132.16, 132.04, 129.46, 129.15, 128.88, 121.86, 111.67, 111.48, 71.61, 56.49, 55.31, 40.89, 40.60, 40.39, 40.18, 39.97, 39.77, 39.56, 39.35, 30.26, 29.48, 28.75, 27.67, 22.86, 22.55, 19.34, 19.02, 14.36, 11.73, 11.27.